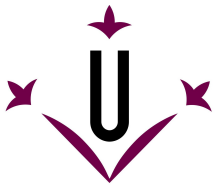

Universitat de Lleida

Risk analysis of ochratoxin A in the frame of food safety: Exposure assessment

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Risk analysis of ochratoxin A in the frame of food safety:

Exposure assessment

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INDEX

Summary/Resumen/Resum 15

List of abbreviations 21

I. INTRODUCTION 23

1. Food safety 25

2. Risk analysis 26

3. Risk assessment of ochratoxin A 28

4. Risk management 61

5. Risk communication 65

References 65

II. OBJECTIVES AND WORK PLAN 85

III. RESEARCH WORK 91

PART I: Biomarkers of exposure

Study I: Assessment of the exposure to ochratoxin A in the province of Lleida, Spain 95

Study II: Ochratoxin A in adult population of Lleida, Spain: Presence in blood plasma and consumption in different regions and seasons 113

Study III: Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain 139

PART II: Contamination of foodstuffs

Study IV: Ochratoxin A in Spanish retail ground roasted coffee: Occurrence and assessment of the exposure in Catalonia 161

Study V: Assessment of the exposure to ochratoxin A by its detection in certain foodstuffs sampled in Catalonia, Spain 177

IV. GENERAL DISCUSSION 205

V. CONCLUSIONS AND FUTURE RESEARCH 225

VI. ANNEXES 229

Annex 1: Food frequency questionnaire 231

Annex 2: Review: Ocratoxin A: Presence in human plasma and intake estimation 235

SUMMARY

Ochratoxin A (OTA) is a toxic secondary metabolite produced by certain species of the fungal genera *Aspergillus* and *Penicillium* that may contaminate foods and feeds. This mycotoxin has nephrotoxic, hepatotoxic, neurotoxic, immunogenic, teratogenic, and carcinogenic properties in animals. In humans, chronic exposure to OTA has been related to the development of tumours in the urinary tract, and to the Balkan endemic nephropathy. The International Agency for Research on Cancer classified OTA as possibly carcinogenic to humans. OTA can be found in several foods of vegetal origin, such as cereals and derivatives, beer, coffee, wines and grape juices, nuts and dried fruits, cocoa and derivatives, spices, and in certain animal by-products. Such ubiquity may lead to a chronic exposure by humans.

The assessment of the exposure of a population to a food contaminant, in this case, OTA, can be done through two main procedures. In the first case, through the detection of this compound in the foodstuffs that are supposed to be contaminated by OTA and the study of the consumption habits of the assessed population regarding the mentioned foodstuffs. In the second case, through the use of biomarkers of the exposure, which implies the detection of OTA or its metabolites in biological fluids of individuals of the selected population.

The general objective of this work was the evaluation of the exposure to OTA of certain groups of people who live in the Spanish region of Catalonia, through the estimation of the daily intake of the toxin. For this, the procedures mentioned above were followed: possibly contaminated food products were purchased in this region, and consumption data by the inhabitants were collected. Biomarkers of exposure were also studied, and the collected fluids were blood plasma and urine.

Occurrence in certain foodstuffs and in body fluids confirmed the exposure of the studied population to OTA. Contamination levels in the analysed foodstuffs were below the limits set by the European Commission, and the occurrence in most cases was below the 50%. In the case of biomarkers, occurrence was almost 100% in plasma, lower in urine (12.5%), and it was observed that the metabolite ochratoxin alpha had a higher incidence (60.6%) in urine than OTA. No correlation was observed between OTA levels in plasma and the consumption of possibly contaminated foodstuffs, but significant correlations were observed between OTA and its metabolite ochratoxin alpha in urine and food consumption. Differences in the results could be observed when the population was classified by gender and age, although a general tendency among the studies of this work could not be established. Regional or seasonal variations of the exposure were not statistically significant.

The daily intake of OTA was estimated by deterministic and probabilistic methodologies, by modelling food consumption and contamination data. It was also estimated by considering the levels of OTA in plasma, by means of the

Klaassen equation. Mean and median results of daily intake obtained through both methodologies were below the suggested by the European Food Safety Authority (17 ng/kg bw/day): up to 3% of that value, in the first case, and up to 10% in the second. However, outliers were observed when the estimation was done from OTA levels in plasma: such values ranged from 14 to 43 ng/kg bw/day. Therefore, exposure to OTA will not produce adverse health effects to the general assessed population, but further efforts should be invested in order to minimize the exposure, as extreme cases of exposure were observed.

RESUMEN

La ocratoxina A (OTA) es un metabolito secundario tóxico producido por algunas especies fúngicas de los géneros *Aspergillus* y *Penicillium,* que pueden contaminar alimentos y piensos. Esta micotoxina es neurotóxica, hepatotóxica, inmunogénica, teratogénica y carcinogénica en animales. En humanos, la exposición crónica a la OTA se ha relacionado con el desarrollo de tumores en el tracto urinario, y con la nefropatía endémica de los Balcanes. La Agencia Internacional para la Investigación en Cáncer (IARC) ha clasificado la OTA como posiblemente carcinógena para los humanos. La OTA puede estar presente en varios alimentos de origen vegetal, como cereales y derivados, cerveza, café, vinos y zumos de uva, frutos secos y fruta deshidratada, cacao y derivados, especias, y en algunos productos de origen animal. Esta ubicuidad puede causar en las personas una exposición crónica.

La evaluación de la exposición de una población a un contaminante presente en alimentos, en este caso la OTA, puede llevarse a cabo mediante dos procedimientos principales. En primer lugar, a través de la detección de este compuesto en los alimentos que se supone puedan estar contaminados por OTA, junto con el estudio de los hábitos de consumo de la población evaluada con respecto a los alimentos mencionados. En segundo lugar, mediante el uso de biomarcadores de la exposición, lo cual implica la detección de la OTA o sus metabolitos en fluidos biológicos de individuos de la población estudiada.

El objetivo general de este trabajo fue la evaluación de la exposición a OTA por parte de ciertos grupos de personas que habitan en la Comunidad Autónoma de Cataluña, a través del cálculo de la ingesta diaria de la toxina. Para ello se tuvieron en cuenta los procedimientos antes mencionados: se adquirieron en la zona ciertos alimentos posiblemente contaminados, y se obtuvieron datos de consumo de los habitantes. También se estudiaron los biomarcadores de la exposición, y los fluidos recolectados fueron plasma sanguíneo y orina.

La incidencia observada en los alimentos y fluidos biológicos estudiados confirmó la exposición de la población evaluada a la OTA. Los niveles de contaminación en los alimentos analizados fueron menores que los valores límite definidos por la Comisión Europea, y la incidencia en la mayoría de los casos fue menor que el 50%. En el caso de los biomarcadores, la incidencia fue de casi el 100% en plasma, menor en orina (12.5%), y se observó que el metabolito ocratoxina alfa presentó una mayor incidencia (60.6%) en orina que la OTA. No se observó correlación entre los niveles de OTA en plasma y el consumo de alimentos posiblemente contaminados, pero se observaron correlaciones significativas entre la OTA y su metabolito ocratoxina alfa en orina y el consumo de alimentos. Se observaron diferencias en los resultados al clasificar la población de acuerdo al sexo y la edad, pero no se pudo establecer una tendencia general entre los estudios de este trabajo.

La ingesta diaria de OTA se estimó mediante métodos determinísticos y probabilísticos, utilizando los datos de contaminación y consumo de alimentos. También se estimó teniendo en cuenta los niveles de OTA en plasma, a través de la ecuación de Klaassen. Las ingestas diarias medias y medianas de OTA obtenidas mediante ambas metodologías fueron menores que el valor sugerido por la Autoridad Europea en Seguridad Alimentaria (17 ng/kg peso corporal/día): hasta un 3% de ese valor en el primer caso, y hasta el 10 % en el segundo caso. Sin embargo, se observaron casos atípicos cuando se estimó la ingesta de OTA teniendo en cuenta los niveles de OTA en plasma: esos valores estuvieron en el rango de 14 a 43 ng/kg peso corporal/día. Por lo tanto, la exposición a la OTA no producirá efectos adversos para la salud a la población general evaluada, pero se deberían extremar las medidas para minimizar la exposición, ya que se observaron casos extremos de exposición.

RESUM

L'ocratoxina A (OTA) és un metabòlit secundari tòxic produït per algunes especies fúngiques dels gèneres *Aspergillus* i *Penicillium* que poden contaminar aliments i pinsos. Aquesta micotoxina és neurotòxica, hepatotòxica, immunogènica, teratogènica i carcinogènica en animals. En humans, l'exposició crònica a OTA s'ha relacionat amb el desenvolupament de tumors en el tracte urinari i amb la nefropatia endèmica dels Balcans. L'Agència Internacional per a la Investigació en Càncer (IARC) ha classificat l'OTA com a "possiblement carcinògena per als humans". L'OTA pot estar present en diferents aliments d'origen vegetal com per exemple cereals i derivats, cervesa, cafè, vi i sucs de fruita, fruits secs i fruita deshidratada, cacau i derivats, espècies, i en alguns productes d'origen animal. Aquesta ubiqüitat pot causar en les persones una exposició crònica.

L'avaluació de l'exposició d'una població a un determinat contaminant alimentari, en aquesta cas particular l'OTA, pot portar-se a terme mitjançant dos procediments. En primer lloc mitjançant la detecció d'aquest compost en els aliments que suposadament poden estar contaminats per OTA, incloent un estudi dels hàbits de consum de la població avaluada referent als aliments esmentats. En segon lloc, mitjançant l'ús de biomarcardors d'exposició, la qual cosa implica la detecció d'OTA o els seus metabòlits en fluids biològics pertanyents a individus de la població en estudi.

L'objectiu general d'aquest treball va ser l'avaluació de la exposició a OTA de determinats grups de persones que habiten a la Comunitat Autònoma de Catalunya mitjançant el càlcul de la ingesta diària d'aquesta toxina. Per a fer-ho es van tenir en compte els procediments anteriorment esmentats: es van adquirir aliments possiblement contaminats procedents d'aquesta zona alhora que es van obtenir les seves dades de consum dels habitants. També es van estudiar els biomarcadors d'exposició, essent els fluids recol·lectats plasma sanguini i orina.

La incidència observada en els aliments i fluids biològics estudiats va confirmar l'exposició de la població avaluada a l'OTA. Els nivells de contaminació en els aliments analitzats van ser menors que els valors límit definits per la Comissió Europea, i la seva incidència en la majoria dels casos va ser menor al 50%. Pel que respecta als biomarcadors, la incidència va ser de gairebé el 100% en plasma, menor en orina (12.5%) i es va observar que el metabòlit ocratoxina alfa presentava una major incidència (60.6%) en orina que l'OTA. No es va observar correlació entre els nivells d'OTA en plasma i el consum d'aliments possiblement contaminats però sí que es van observar correlacions significatives entre l'OTA i el seu metabòlit ocratoxina alfa en orina i el consum dels esmentats aliments. Es van observar diferències en els resultats al classificar la població en funció al sexe i l'edat però no es va poder establir una tendència general entre els estudis d'aquest treball.

La ingesta diària d'OTA es va estimar mitjançant mètodes determinístics i probabilístics en els quals es van utilitzar les dades de contaminació i consum d'aliments. També es va estimar tenint en compte els nivells d'OTA en plasma, a través de l'equació de Klaassen. Les ingestes diàries mitjanes i medianes d'OTA obtingudes mitjançant ambdues metodologies van ser menors al valor suggerit per l'Autoritat Europea en Seguretat Alimentaria (17 ng/kg pes corporal/dia): fins a un 3% d'aquest valor en el primer cas i fins a un màxim del 10% en el segon cas. No obstant, es van observar casos atípics quan es va estimar la ingesta d'OTA tenint en compte els nivells d'OTA en plasma: aquests valors van estar en el rang de 14-43 ng/kg de pes corporal/dia. Per tant, l'exposició a OTA no produirà efectes negatius per a la salut a la població general avaluada però s'haurien d'extremar les mesures per tal de minimitzar l'exposició ja que es van observar casos d'exposició extrema.

LIST OF ABBREVIATIONS

3DR: three-day food consumption record

DI: daily intake

FFQ: food frequency questionnaire

OTA: ochratoxin A

OTα: ochtaroxin α

PTDI: provisional tolerable daily intake

TDI: tolerable daily intake

I. INTRODUCTION

1. FOOD SAFETY

Food safety is a discipline that encompasses actions aimed at ensuring that all food is as safe as possible. Food safety policies and actions need to cover the entire food chain, from production to consumption (World Health Organization, 2011a).

The United Nations Organization is involved in this matter through the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), organizations that promoted Joint FAO/WHO discussions and activities concerning food safety. A result of such activities was the creation in 1957 of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), whose aim is to utilise the services of internationally recognised scientists in expert meetings to evaluate available data on food additives, animal drug residues in food, and other food contaminants, such as mycotoxins, heavy metals, and industrial chemicals (Lupien, 2000).

Later, in the 1960's, the Codex Alimentarius Commission was created in order to support the development of food standards and guidelines, as well as the regulation of the fair trade, under the Joint FAO/WHO Food Standards Programme. The formulated food standards should then be published in the document named "Codex Alimentarius", and kept up to date by the Commission. Nowadays, the Codex Alimentarius provides a basis for national regulations in order to improve the quality and safety of domestic and imported foodstuffs, and promotes export possibilities (Lupien, 2000). Unlike JECFA, the members of Codex Alimentarius Commission are governments, and at present over 180 countries are represented.

In Europe, the Commission's guiding principle on food safety was primarily set out in its "White Paper on Food Safety" (Commission of the European Communities, 2000), and its main objective was to guarantee that the EU had a high level of food safety, and thus set guidelines for a new food policy, such as:

- Principles of food safety, including the definition of an integrated food safety policy throughout the food chain ("farm to table"), traceability, and risk analysis, among others.
- Establishment of an European Food Safety Authority, which would be in charge of a series of tasks integrating independent scientific advice relating to food safety, operation of rapid alert systems, communication and dialogue with consumers on food safety and health issues as well as networking with national agencies and scientific bodies.
- Regulatory aspects: the White Paper indicates the need of the creation, together with the Food Safety Authority, of a set of rules necessary to improve food safety standards and the update of the existing European legislation on food and feed.

- Development and operation of national control systems, taking into account all parts of the food production chain.
- International dimension: the key principle for imported foodstuffs and animal feed is that they must meet health requirements at least equivalent to those set by the Community for its own production.

In 2002, following the expressed on the White Paper on Food Safety, the European Parliament and the Council adopted Regulation (EC) 178/2002 laying down the General Principles and requirements of Food Law (European Commission, 2002). This regulation, known as "General Food Law", "provides the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, whilst ensuring the effective functioning of the internal market" (Article 1.1). It establishes the European Food Safety Authority and states that food law shall be based on risk analysis. Finally, it sets up a rapid alert system and the principles for crisis management.

The European Food Safety Authority (EFSA) was created to "provide scientific advice and scientific and technical support for the Community's legislation and policies in all fields which have a direct or indirect impact on food and feed safety. It shall provide independent information on all matters within these fields and communicate on risks" (Article 22.2). The role of EFSA was defined in the context of risk analysis, which comprises risk assessment, risk management and risk communication; thus, the cited article defines EFSA as the authority in charge of risk assessment and risk communication.

In Spain, the national authority whose functions are equivalent to EFSA is the Spanish Agency of Food Safety and Nutrition (Agencia Española de Seguridad Alimentaria y Nutrición, AESAN); and in a regional level, for what concerns to this Thesis document, the Catalan Agency of Food Safety (Agència Catalana de Seguretat Alimentària, ACSA) in the Autonomous Community of Catalonia.

2. RISK ANALYSIS

Risk analysis is the process chosen by the European Commission to be the frame for food law, becoming an important tool for the management of food safety. It is employed to make an estimate of the risks to human health and safety, to identify and implement appropriate measures to control the risks, and to communicate with the participants of the process (food producers, consumers, risk managers, risk assessors, governments, and media) about the risks and measures applied (FAO/WHO, 2006). Risk analysis consists of three components: risk assessment, risk management, and risk communication. All of them are complementary, and interactions occur among them (Figure 1).

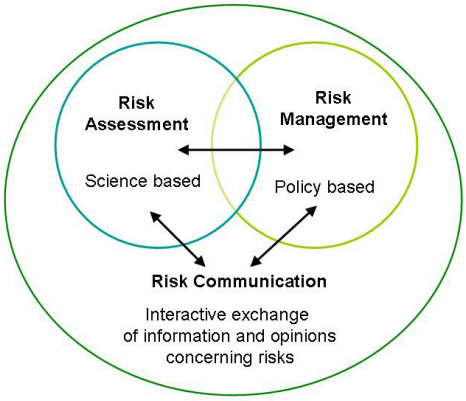

Figure 1. Risk analysis framework
(World Health Organization, 2011b).

The following are some of the definitions regarding risk analysis stated in the updated Procedural Manual (19th edition, 2010) published by the Codex Alimentarius Commission:

Hazard: A biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect.

Risk: A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food.

Risk Assessment: A scientifically based process consisting of the following steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization:

Hazard Identification: The identification of biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods.

Hazard Characterization: The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food.

Exposure Assessment: The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant.

Risk Characterization: The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment.

Risk Management: The process, distinct from risk assessment, of weighing policy alternatives, in the light of the results of risk assessment, in consultation with all interested parties, considering factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control options.

Risk Communication: The interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions.

3. RISK ASSESSMENT OF OCHRATOXIN A

The present section deals with the risk assessment of the mycotoxin ochratoxin A, and focusses specially on the exposure assessment step in a certain population.

3.1. Hazard identification

3.1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by certain filamentous fungi and cause a toxic effect in animals and humans. Most of the known mycotoxins are produced by some species of the fungal genera *Aspergillus, Penicillium,* and *Fusarium.* A certain mycotoxin can be produced by different species, and a certain strain can produce different mycotoxins. If the conditions are favourable, these moulds can develop and produce mycotoxins in growing crops for human and animal consumption, and also during their harvest, drying and storage. Ambient conditions (chemical, physical, and biological) affect fungal growth and mycotoxin production, and therefore it is expected to find heterogeneous distributions of mycotoxins in foodstuffs from year to year and according to the production process they undergo. The presence of mycotoxins in food commodities is of concern for trade, as it may lead to important economic losses.

Exposure of humans to mycotoxins occurs mainly by the ingestion of contaminated foodstuffs from vegetal or animal origin. Contamination in the last case takes place when animals are fed with contaminated feed. An alternative way of exposure is the inhalation of contaminated dusts.

The diseases caused by mycotoxins in humans or animals are called “mycotoxicoses”. While most animal mycotoxicoses have been experimentally confirmed, human mycotoxicoses are less well understood and not as clearly defined (Smith et al., 1995). The toxicity of a certain mycotoxin in an organism

can be classified according to the exposure dose in acute or chronic. Acute toxicity refers to the ability of the compound to cause adverse effects within a short time of exposure, especially at high doses. Chronic toxicity stands for the effects of a prolonged exposure to small quantities of toxin (CAST, 2003). This last case is of special concern for human health, considering the fact that several foodstuffs of frequent consumption are susceptible to be contaminated by one or more mycotoxins.

Several mycotoxins have been identified up to the present, but those of special interest in food and feed safety are: aflatoxins (B1, B2, G1, G2, M1), fumonisins (B1, B2), ochratoxin A, patulin, trichothecenes (deoxynivalenol, T-2 toxin, HT-2 toxin, …), and zearalenone. International agencies have studied the problem of mycotoxins in food in order to obtain guidelines regarding the limits of contamination in food and the tolerable intakes of the toxins. Thus, the International Agency for Research on Cancer (IARC, 1993) has investigated the carcinogenic potential of most of these toxins (Table 1), and the Codex Alimentarius Commission (1995) has set the maximum levels in foodstuffs for aflatoxins, ochratoxin A, and patulin. In Europe, the European Commission (2006a) has set the maximum levels in foodstuffs for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, and T-2 and HT-2 toxins; and stated a recommendation on the presence of some mycotoxins in such products. Opinions regarding tolerable intakes of mycotoxins have been published by JECFA and EFSA as values of Provisional Tolerable Weekly Intake (PTWI) or Provisional Tolerable Daily Intake (PTDI).

Table 1. Evaluation of carcinogenicity of some mycotoxins by IARC (1993 and 1998*).

Mycotoxin	Degree of evidence of carcinogenicity		Overal evaluation of carcinogenicity to humans
	Human	Animal	
Aflatoxins, naturally occurring mixtures of	S	S	1
Aflatoxin B1	S	S	1
Aflatoxin B2		L	
Aflatoxin G1		S	
Aflatoxin G2		I	
Aflatoxin M1	I	S	2B
Ochratoxin A	I	S	2B
Toxins derived from *Fusarium graminearum, F. culmorum and F. crookwellense:*	I		3
Zearalenone		L	
Deoxynivalenol		I	
Nivalenol		I	
Fusarenone X		I	
Toxins derived from *Fusarium moniliforme:*	I	S	2B
Fumonisins*, naturally occurring mixtures of	I		
Fumonisin B1*		S	2B
Fumonisin B2		I	
Fusarin C		L	
Toxins derived from *Fusarium sporotrichioides:*	I[a]		3
T-2 toxin		L	

Degrees of evidence: S = sufficient; L = limited; I = inadequate; N = negative.

Evaluation of carcinogenicity: Group 1: Carcinogenic to humans, Group 2A: Probably carcinogenic to humans, Group 2B: Possibly carcinogenic to humans, Group 3: Not classifiable as to its carcinogenicity to humans, Group 4: Probably not carcinogenic to humans.

[a] No data available.

3.1.2. Ochratoxins

Ochratoxins are a group of mycotoxins produced by fungi of the genera *Aspergillus* and *Penicillium.* Their chemical structure consists of a dihydroisocoumarin moiety coupled to L-β-phenylalanine by a peptide bond (Figure 2 and Table 2). The group comprises ochratoxin A, its dechloro analogue ochratoxin B, its ethyl ester ochratoxin C, and its hydroxylated forms 4-*R*-hydroxyochratoxin A and 4-*S*-hydroxyochratoxin A. Ochratoxin α and ochratoxin β are products of the

hydrolysis of the peptide bound of ochratoxins A and B, respectively, and lack the phenylalanine moiety. OTA is the most important ochratoxin due to its incidence and toxicity.

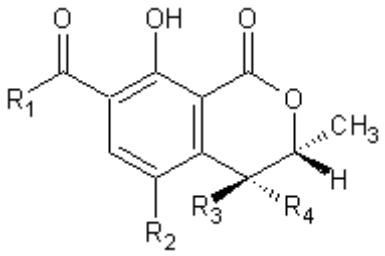

Figure 2. Chemical structures of ochratoxins and major derivatives (Li et al., 1997).

Table 2. R groups in the structure of ocratoxins (adapted from Li et al., 1997).

Common name	Abbreviation	R_1	R_2	R_3	R_4
Ochratoxin A	OTA	Phenylalanyl	Cl	H	H
Ochratoxin B	OTB	Phenylalanyl	H	H	H
Ochratoxin C	OTC	Phenylalanyl, ethyl ester	Cl	H	H
4-*R*-hydroxyochratoxin A	4-*R*-OTA-OH	Phenylalanyl	Cl	H	OH
4-*S*-hydroxyochratoxin A	4-*S*-OTA-OH	Phenylalanyl	Cl	OH	H
Ochratoxin α	OTα	OH	Cl	H	H
Ochratoxin β	OTβ	OH	H	H	H

3.1.3. Ochratoxin A: Chemical and physical properties

The systematic chemical nomenclature (IUPAC) of OTA is (*R*)-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-bemzopyran-7-yl)-carbonyl]-L-β-phenylalanine (Figure 3). Its empirical formula is $C_{20}H_{18}O_6NCl$ and its molecular weight is 403.82.

OTA is a colourless, crystalline compound with a melting point of about 90 ºC when crystallised from benzene and 169 ºC when crystallised from xylene (Kuiper-Goodman and Scott, 1989). It is soluble in polar organic solvents, slightly soluble in water and soluble in diluted aqueous bicarbonate solutions (Valenta, 1998). The UV absorption spectrum varies with pH and solvent polarity (Kuiper-Goodman and Scott, 1989).

OTA is unstable to air and light, though ethanol solutions are stable for longer than one year if kept refrigerated and in the dark (U.S. Department of Health and Human Services, 2005). Thermal stability of OTA varies according to the matrix where it can be found, and it seems to be stable in some food matrices up to 180º C (Raters and Matissek, 2008). Furthermore, Tsubouchi et al. (1987) stated that the mode of contamination also influenced on the strength of the heat resistance.

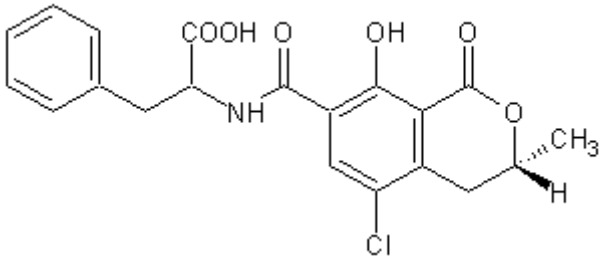

Figure 3. Chemical structure of ochratoxin A.

3.1.4. Toxicity of ochratoxin A

Adverse effects of OTA in animals can vary according to several factors, such as the dose administered, the form of administered OTA (crystalline or naturally occurring in feed, oral ingestion or intravenous administration), the diet composition, the animal species and health status, as well as sex and age.

Acute toxicity

Acute doses of OTA caused multifocal haemorrhages, intravascular coagulation as well as necrosis in the kidney, liver and lymphoid tissues (Galtier et al., 1979a and 1981; Mortensen et al., 1983; Ballinger et al., 1986; Fukui et al., 1987; Hagelberg et al., 1989; JECFA, 2001). Oral LD_{50} (lethal dose, 50%, the dose needed to kill the 50% of the observed population, measured per body weight) varied in the mentioned studies according to the tested species, from 0.2-1 mg/kg bw in pigs, cats, rabbits, and dogs, to 20-30 mg/kg bw in rats and 46-58 mg/kg bw in mice.

In humans, a single case probably produced by OTA was described by Di Paolo et al. (1994). They reported an acute renal failure suffered by a farmer and his wife after they had worked for eight hours in a wheat granary closed for several months. They also suffered respiratory distress. Although OTA was not analysed in blood, a biopsy revealed tubulonecrosis, and the toxin and a strain of *Aspergillus ochraceus* were detected in the wheat.

Short-term toxicity

The most frequent adverse effect of OTA observed in animals is the nephrotoxicity. In short-term studies (up to 90 days), development of nephropathy was observed in rats, dogs, and pigs (Kuiper-Goodman and Scott, 1989). OTA also induced hematologic changes in rat and mice (Galtier et al., 1979b, Gupta et al., 1979 and 1983) and changes in liver glycogen in several species (Harwig et al., 1983). Degenerative changes in immune and reproductive organs were also observed (Kuiper-Goodman and Scott, 1989).

Chronic toxicity

Many studies and reviews on the chronic toxic effects of OTA in animals have been published (Kuiper-Goodman and Scott, 1989; Marquardt and Frohlich, 1992; Pohland et al., 1992; O'Brien and Dietrich, 2005; EFSA, 2006; Pfohl-Leszkowicz and Manderville, 2007; among others). These have characterised OTA as nephrotoxic, hepatotoxic, neurotoxic, immunotoxic, teratogenic, and carcinogenic for animals, with the kidney being the main target in most cases.

In humans, chronic exposure to OTA has been related to the Balkan Endemic Nephropathy (BEN) and the development of urinary tract tumours (UTT). BEN was described in the 1950's by a series of publications from Bulgaria, Romania, and the former Yugoslavia, as a chronic progressive kidney disease occurring in those areas, particularly to individuals from farming households (Pfohl-Leszkowicz and Manderville, 2007). In addition, it was shown that tumours of the upper urinary tract often accompanied BEN (Vukelic et al., 1991; Sostaric and Vukelic, 1991). On the basis of a series of epidemiological observations, Akhmeteli (1972) suggested that fungal toxins were involved in the aetiology of BEN, and Krogh (1972), in view of the similarities between BEN and OTA-induced porcine nephropathy, suggested that OTA might be involved in the aetiology of BEN (Pfohl-Leszkowicz and Manderville, 2007), but up to the present the relation between OTA, BEN and UTT has not been clearly established. However, epidemiological data correlate a moderate increase in serum OTA levels with a significantly higher incidence of nephropathy and UTT in humans (O'Brien and Dietrich, 2005). Moreover, according to O'Brien and Dietrich (2005), studies carried out in Tunisia (Maaroufi et al., 1995) or Egypt (Abdelhamid, 1990), where climatic conditions and/or suboptimal storage of grain and grain products promote OTA production by fungal species, have also indicated a link between dietary intake of OTA and the development of renal and urothelial tumours. Based on the available data in animals and humans, IARC (1993) has classified OTA as "possibly carcinogenic to humans" (Group 2B), having sufficient evidence in experimental animals for its carcinogenicity, but inadequate evidence in humans.

3.1.5. Toxicodynamics

Toxicodynamics are understood to encompass all mechanisms through which the concentration/amount at the site of action elicits the toxic effect (Heinrich-Hirsch et al., 2001).

Several hypotheses on the mechanism of interaction of OTA and its metabolites with endogenous molecules have been postulated to explain its toxicity. These mechanisms are related to specific binding onto specific sites of a target molecule, and to nonspecific interactions, based on the chemical reactivity of OTA and metabolites and their vicinity to the target molecule (Ringot et al., 2006). Some publications on this subject and on the toxicokinetics of OTA are those of Kuiper-Goodman and Scott (1989), O'Brien and Dietrich (2005), Ringot et al. (2006), and Pfohl-Leszkowicz and Manderville (2007).

The changes suffered by OTA due to the metabolism have not been completely defined yet. However, it is worth to mention the biotransformation pathways that are common to the metabolism of xenobiotics, which consists of a series of reactions that transform the initial toxic molecule in other less toxic compounds and facilitate its elimination from the organism.

Phase I reactions: These reactions consist of detoxification or bioactivation reactions, by the modification of the OTA molecule. The major metabolic pathway of OTA consists of the hydrolysis of its peptide bound, resulting in ochratoxin α (OTα), a much less toxic compound (Ringot et al., 2006). In rodents, it occurs mainly by the action of the large intestine bacteria, although it also occurs in a small fraction in the stomach and in the small intestine (Madhyasta et al., 1992). In the case of ruminants, their resistance to the toxicity of OTA has been attributed to the ability of the rumen microbiota to degrade the toxin (Ringot et al., 2006). Additionally, a small percentage of absorbed OTA is hydroxylated, principally in the liver, into 4-(R)-hydroxy-OTA (in human and rat), and into 4-(S)-hydroxy-OTA (in pig) (Oster et al., 1991; Størmer and Pedersen, 1980; Størmer et al., 1981). Bioactivation reactions are supposed to be the responsible of the formation of the OTA analogues that cause its toxic effects, such as ochratoxin-derived free radicals (Hoehler et al., 1996, 1997), or OTA-derived quinones (Gillman et al., 1999; Dai et al., 2002).

Phase II reactions: They consist of the formation of conjugates through reactions of glucuronidation and sulphate conjugation, mainly, and in a minor frequency the formation of *O*-labile ester conjugates and glutathione conjugation. The objective of these reactions is to change the structure of the initial compound to facilitate its excretion; unchanged OTA and phase I OTA-derivatives can also suffer these phase II transformations. These reactions occur mainly in the liver: conjugates are secreted with the bile, and then circulate to the small intestine and back to the liver in the enterohepatic circulation. In the intestine, the conjugated compounds are

hydrolysed by the intestinal microbiota. These compounds are excreted by renal (urine) or biliary (faeces) routes.

3.1.6. Toxicokinetics

Toxicokinetics refer to the modelling and mathematical description of the time course of disposition (absorption, distribution, biotransformation, and excretion) of xenobiotics in the whole organism.

Toxicokinetic studies of OTA have been done in different animal species: pigs, rabbits and chickens (Galtier et al., 1981); fish, quail, mouse, monkey (Hagelberg et al., 1989), rat (Hagelberg et al., 1989; Li et al., 1997; Mantle, 2008; Vetorazzi et al., 2009, 2010 and 2011) and vervet monkeys (Stander et al., 2001). Concerning humans, the toxicokinetic profile of OTA was studied in one volunteer, and intra-individual fluctuations of OTA plasma levels were studied in eight persons (Studer-Rohr et al., 2000).

The course of OTA in the organism is briefly as follows: once ingested, OTA is absorbed in the stomach and the intestines and bound to the blood, due to its high affinity to the blood proteins. Afterwards, it is distributed to the organs and tissues by the bloodstream. The toxin is reabsorbed in the kidneys as well as in the liver due to the enterohepatic circulation, which is the circulation of bile, carrying the metabolism compounds from the liver to the small intestine, and from there back to the liver via the hepatic portal system. Excretion of OTA can occur via urine, faeces or milk in lactating mammals.

Absorption

After ingestion, OTA is rapidly absorbed from stomach and the small intestine in rats and mice (Pfohl-Leszkowicz and Manderville, 2007). The percentage of OTA absorbed differs among species: 66% in pigs, 56% in rats and rabbits and 40 % in chicken (Galtier et al., 1981). Moreover, bioavailability may vary in the presence of food components (Pfohl-Leszkowicz and Manderville, 2007). Once OTA reaches the bloodstream, it is bound to serum proteins (Galtier et al., 1981, Hult and Fuchs, 1986, Hagelberg et al., 1989), mainly albumin (Chu, 1971; Chu, 1974) and other macromolecules (Stojkovic et al., 1984; Hagelberg et al., 1989), in a proportion that varies depending on the species. In the study done by Hagelberg et al. (1989), the fraction of unbound toxin ranged from 0.02% in rat and man to 0.2 % in quail, and in fish it was a 22%. Erytrocites contain only traces of OTA (Ringot et al., 2006). Stojkovic et al. (1984) found that human and porcine plasma macromolecules had higher affinity than albumin, though the concentrations of those high-affinity molecules were lower than the concentrations of the low-affinity molecules (i.e., low molecular weight plasma proteins and albumin).

Circulating plasma levels

In animals, the concentration of the toxin and its metabolites in tissues and plasma depends on the animal species and sex (Kumagai, 1985; Heussner et al., 2002), the dose administered, the way of administration (crystalline or naturally occurring in feed, oral ingestion or intravenous administration), the duration of administration, the diet composition as well as the health status of the animal (Ringot et al., 2006). Species-specific factors such as half-life and the degree of serum binding also influence the levels of OTA in the organism (Hagelberg et al., 1989; Kuiper-Goodman and Scott, 1989). The species difference in serum half-life of OTA was reviewed by Petzinger and Ziegler (2000). Humans' serum half-life of OTA was determined to be 35.5 days (Studer-Rohr et al., 2000). Generally, the elimination half-life of OTA is longer in blood than in tissues, which may be related, in part, to the higher binding affinity of the toxin to blood proteins (Chang et al., 1979; Hagelberg et al., 1989; Fuchs and Hult, 1992; Marquardt and Frohlich, 1992; Li et al., 1997; Studer-Rohr et al., 2000).

Fuchs and Hult (1992) considered that OTA bound to serum albumin and other serum macromolecules is a mobile storage of toxin in the body, readily available to several tissues over a prolonged period of time. According to these authors, a process such as the enterohepatic circulation of OTA in the human body could maintain the saturation of the specific binding macromolecules for a long time. This process involving OTA has been studied in rodents (Kumagai and Aibara, 1982, Fuchs et al., 1988b, Roth et al., 1988) and prerumiant calves (Sreemannarayana et al., 1988). In these cases the toxin was secreted together with the bile and afterwards reabsorbed in the intestine. OTA can also be reabsorbed in kidney, and it was suggested that this process facilitates the residual persistence of the toxin, and then the renal toxicity in rodents (Stein et al., 1985, Albassam et al., 1987). Stojkovic et al. (1984) stated that OTA binding to low molecular weight plasma constituents would facilitate the accumulation of the toxin into the kidney, as these small molecules can pass through the glomerular membrane. If food containing very low concentrations of OTA is consumed over a long period of time or even daily, exposure of the kidney to such very low doses may become toxicologically significant (Fuchs and Hult, 1992).

Ichiyama and Saito (1987), in an *in vitro* study, found that OTA had a high affinity to serum albumin but little affinity to soluble tissue proteins of liver and kidney of rats. The authors suggested that OTA in vivo may be present in kidney or liver mainly in the form of serum albumin-bound OTA, and that this argumentation can be applied to the explanation of the distribution of OTA in meat products.

Tissue distribution

As mentioned, OTA levels in tissues and blood of animals depend on several factors. Tissue distribution of OTA was studied in trout, quail and mice using whole-body autoradiography with [^{14}C] labelled OTA. The presence of the labelled

toxin was observed in the liver, bile and intestinal content (Fuchs and Hult, 1992) and there was a large difference in the accumulation of the toxin for other organs. In birds, labelled OTA disappeared rapidly from the body and did not accumulate in the organs, but the deposition of the toxin was observed in the egg yolk (Fuchs and Hult, 1992). Other studies showed that OTA accumulated in organs such as kidneys, liver, muscle, lung, heart, intestine, testicles, spleen, brain, skin; and also in the fat, in different proportions, according to the dose and frequency of administration (Madsen et al., 1982; Harwig et al., 1983; Mortensen et al., 1983; Kane et al., 1986, Fuchs et al., 1988a; Hald, 1991; Breitholtz-Emanuelsson et al., 1992; Ferrufino-Guardia et al., 2000; Canadas et al., 2005a; Canadas et al., 2005b; Gaou et al., 2005; Canadas, 2006) with the kidneys and liver as the principal targets, followed by the muscle, lungs and heart. Auodia et al. (2008) observed a protective effect of micronized wheat fibres in terms of OTA concentrations in plasma, kidney and liver of rats when these fibres were included in their OTA naturally contaminated diet.

Canadas et al. (2005a, 2005b, 2005c, 2006) and Gaou et al. (2005) demonstrated that OTA tissue distribution was also sex-dependent in rats. They observed that except in brain, male rats accumulated higher amounts of OTA in liver, kidney and lung than female rats (Gaou et al., 2005; Canadas, 2006). Other studies showed transference of OTA in utero of mammals such as mice, rat and swine (Kuiper-Goodman and Scott, 1989; Hallen et al., 1998), and also transference via milk (Ferrufino-Guardia et al., 2000). In humans, OTA levels of foetal serum (Zimmerli and Dick, 1995) and placenta (Miraglia et al., 1998) doubled the maternal serum concentration.

Elimination

Elimination of OTA can take place through renal, biliary or faecal, and milk routes, being the first two the principal ones.

In humans and monkeys, the major route of excretion is renal elimination (Studer-Rohr et al., 2000; Stander et al., 2001), whereas the biliary excretion is the main way for rats and mice (Storen et al., 1982; Moroi et al., 1985; Kuiper-Goodman and Scott, 1989; Li et al., 1997) and also for fish and quail (Fuchs and Hult, 1992). The relative contribution of each excretory route is influenced by the route of administration, the dose, the degree of binding with plasma proteins and the enterohepatic circulation of OTA (Pfohl-Leszkowicz and Manderville, 2007). Differences in effectiveness of enterohepatic circulation of OTA might partially be responsible for the differences in retention of the toxin in plasma among species (Fuchs and Hult, 1992).

Concerning filtration through the kidneys, renal excretion ability can be measured by the clearance. Clearance is done mainly by glomerular filtration, the process in which the fluids are filtered in the glomerular capillaries of the kidney. Fuchs and Hult (1992) considered that the reduced filtration of the toxin through the kidney

agrees with the fact that glomerular impermeability for large proteins (such as albumin) is also restricted. Then, in the case of albumin deficiency, OTA is eliminated from plasma in a very short time (Fuchs and Hult, 1992; Hagelberg et al., 1989; Pfohl-Leszkowicz and Manderville, 2007). After its excretion, OTA is reabsorbed in the nephrons (Gekle et al., 2005). This process retards its excretion and may lead to the accumulation of the toxin in the kidneys (Ringot et al., 2006).

OTA metabolites can be cleared in the kidneys at a faster rate than OTA. Li et al. (1997) studied in rats the pharmacokinetics of OTA and some analogues as ochratoxin B, ochratoxin C, as well as some metabolites as ochratoxin α, hydroxylated ochratoxin A, and the open form of ochratoxin A. The study included the determination of biliary, renal and estimated metabolic clearances of these compounds. Clearance data suggested significant differences among the various forms of OTA with regard to the preferred routes of elimination (renal, biliary, and/or metabolism). The results of the complete study demonstrated that OTA was very slowly cleared from the body and that its metabolites were cleared at a much faster rate, and via the bile and kidney. OTA and its metabolite OTα are also excreted in faeces.

Concerning milk excretion, several studies evaluated OTA presence in milk of many species: rabbit (Galtier et al., 1977; Ferrufino-Guardia et al., 2000), rats (Breitholtz-Emanuelsson et al., 1993b; Hallen et al., 1998), sows (Mortensen et al., 1983) and cows (Ribelin et al., 1978; Breitholtz-Emanuelsson et al., 1993a). Presence of OTA in human milk has also been reported (see section 3.3.2.4).

The above mentioned data indicate the biological fluids of the different stages of the metabolism in which OTA can be detected in order to determine the exposure of an individual or a certain population to the mycotoxin.

3.2. Hazard characterization

Toxicology studies carried out in animals aim to characterise the effects of the evaluated chemical. Thus, the "no observed adverse effect level" (NOAEL, mg/kg bw/day) is generally determined. This level is the highest dose for a specific endpoint (i.e., the sign that constitutes one of the target outcomes of the toxicology study) at which no adverse effects are observed. Another value, as the "lowest observed adverse effect level" (LOAEL, mg/kg body weight/day), is also determined. However, it is necessary to extrapolate to humans the results of the studies in animals.

It has been presumed that for many of the non-carcinogenic adverse effects observed in animals or humans there is a threshold dose below which these effects are not observed. With regard to carcinogens, it is generally presumed that there is no threshold dose below which there is no induction or cancer initiation and that there will always be some risk. In the extrapolation to safe intake estimates, effects on which a threshold is presumed (non-carcinogenic

toxins) are treated differently from effects from which no threshold is presumed (carcinogenic toxins) (Kuiper-Goodman, 2004). To characterize the hazard to humans of mycotoxins that have a threshold dose, a "safe dose" such as a provisional tolerable daily intake (PTDI) is estimated. TDI is the dose that can be safely consumed daily over a lifetime without incurring appreciable adverse health effects, and involves a biologically insignificant risk (WHO, 1987 and 1999; Edler et al., 2002). The PTDI is calculated as the NOAEL (obtained in toxicological studies) divided by a safety factor.

As concerns to OTA, it has been classified by the IARC as "possibly carcinogenic to humans". Thus, as evidence for carcinogenicity is insufficient, for the purpose of risk assessment this mycotoxin is considered as a non-carcinogen.

EFSA (2006) based the hazard characterization of OTA on its nephrotoxic effect, and on data of studies in rats and pigs. From these studies, the LOAEL of 8 μg/kg bw/day in female pigs was considered as an early marker of renal toxicity, and likely to be close to a NOAEL. The safety factor was calculated taking into account the following issues:

- For the extrapolation from pigs to humans (interspecies differences): A factor of 2.5 was used for toxicodynamic differences (WHO-IPCS, 1999) and a factor of 6 for the kinetic differences (half-life).
- For the extrapolation from average human to potentially sensitive human sub-populations, a factor of 10 was used (WHO-IPCS, 1999).
- An additional factor of 3 was applied to take into account the use of a LOAEL instead of a NOAEL, in agreement with WHO-IPCS (1999) recommendations, which state that when a NOAEL has not been achieved but the LOAEL is of sufficient quality, this LOAEL should form the basis of the risk assessment.
- The resulting safety factor was 450.

Thereafter, the safety factor was applied to the LOAEL of 8000 ng OTA/kg bw/day, resulting in a tolerable daily intake of OTA by humans of approximately 18 ng OTA/kg bw/day. Finally, a provisional tolerable weekly intake (PTWI) of 120 ng OTA/kg bw/week was established.

In a similar way, JECFA (1991) considered also the LOAEL of 8000 ng OTA/kg bw/day, applied a safety factor of 500, and established a PTWI of 112 ng OTA/kg bw/week. In the following JECFA evaluation of 1996, that value was rounded to 100 ng OTA/kg bw/week, and maintained in the 2001 and 2007 evaluations. Table 3 shows the PTDIs of OTA suggested by different organizations.

Table 3. Provisional tolerable intakes of OTA.

PTDI (ng OTA/kg body weight/day)	PTWI (ng OTA/kg body weight/week)	Reference
5		The Nordic Working Group on Food Toxicology and Risk Evaluation (1991)
4		Health Canada (Kuiper-Goodman and Scott, 1989; Kuiper Goodman, 1996)
17*	120	EFSA (2006)
14*	100	JECFA (1996, 2001, 2007)

PTDI, Provisional Tolerable Daily Intake; PTWI, Provisional Tolerable Weekly Intake.
*Calculated values on the basis of the proposed weekly intake.

3.3. Exposure assessment

As mentioned in section 2, the assessment of the exposure, in this case of OTA, consists mainly of the estimation of its intake via food, although the inhalation of contaminated dusts has been proven to be another source of exposure in some workplaces. In addition, biomarkers of exposure (presence of the toxin in biological fluids) are another important tool for the exposure assessment.

3.3.1. Assessment of the exposure through food contamination and consumption data

Intake of foodstuffs contaminated with mycotoxins and the level of contamination in these foodstuffs are the factors on which exposure to mycotoxins depend (Kroes et al., 2002), and therefore assessment of the exposure can be done by the estimation of both factors. An example of this is the "Assessment of dietary intake of OTA by the population of EU Member States" presented in 2002 by the European Commission Scientific Cooperation (SCOOP) Task Report (from now on the "SCOOP Report", European Commission - Directorate-General Health and Consumer Protection, 2002), which included a compilation of the toxin's occurrence in several foodstuffs sampled in EU countries, as well as inhabitants' consumption data. The studied foodstuffs were those considered to be possibly contaminated by OTA.

3.3.1.1. Occurrence of OTA in food

OTA has been detected worldwide in several foodstuffs from vegetal origin, such as cereals (barley, bran, maize, millet, oat, rice, rye, sorghum, and wheat) and derivatives (recently reviewed by Duarte et al., 2010), nuts (Jiménez et al., 1991; Overy et al., 2003), dried fruits (Trucksess and Scott, 2008; Bircan,

2009), coffee (Jørgensen, 1998; Trucksess et al., 1999), cocoa (De Magalhães et al., 2011), beverages as beer, grape juices, and wine (Bellí, 2006; Mateo et al., 2007; Valero Rello, 2007), spices (Tavares dos Santos, 2010; Zaied et al., 2010), as well as in olive oil (Papachristou and Markaki, 2004) (the cited references are examples taken from the extended bibliography on OTA in foodstuffs).

The ingestion of contaminated feed by animals may also lead to the presence of the toxin in animal by-products (Bauer and Gareis, 1987). Occurrence of OTA has been reported in organs from monogastric animals for human consumption, especially in the kidneys and serum of pig (Golinski et al., 1985; Marquardt et al., 1988; Jørgensen and Petersen, 2002) and hence in derived goods such as sausages or black pudding, as well as in pig meat or ham (Dall'Asta et al., 2010). It can also be found in eggs (Tangni et al., 2009), cow milk (Coffey et al., 2009; Pattono et al., 2011), and cheese (Chapman et al., 1983; Norton et al., 1982), although in these last two cases occurrence might be very low due to the detoxifying metabolism of ruminants (Mobashar et al., 2010). The ubiquity of OTA makes possible its chronic ingestion by humans.

3.3.1.2. Food contamination data

Sampling

With regard to food contamination, the distribution of mycotoxins in foodstuffs is non-homogeneous, then appropriate sampling is necessary (Gilbert, 1996) to have a reasonable estimation of the contamination of foodstuffs. The European Commission in its Regulation (EC) 401/2006, amended by the Regulation (EC) 178/2010, set the methods of sampling and analysis of mycotoxins in foodstuffs to be applied in the official controls (European Commission, 2006b and 2010a). However, in the case of the exposure assessment, sampling of large quantities of foodstuffs according to the legislation is not practical and does not necessarily match with the objectives of the assessment. For that case, a more adequate option is to carry out the sampling in retails of different points of a certain geographic area (EFSA, 2006) where the assessed population lives. With this procedure, the obtained data should better describe the occurrence of the studied mycotoxins in a certain frame of time in relation to a selected population. In relation to this, FAO/WHO recommends the use of "Total Diet Studies", in which representative samples of widely consumed foods are collected and analysed for the constituents of interest. Three approaches can be distinguished (Kroes et al., 2002):

Market basket (food groups): This approach is based on the dietary intake of a defined population group. All food items, which are part of the average diet, are purchased, prepared according to standard household procedures and aggregated

into a number of food groups. Then each food group is analysed for the chemical of interest.

Individual food items: A list of foodstuffs representing the products most commonly consumed is composed based on national food consumption surveys for several age-sex groups. The selected food items are then analysed.

Duplicate portion or duplicate diet: The individual daily diet as consumed is analysed.

In the Spanish Community of Catalonia, the Catalan Agency of Food Safety (ACSA) has carried out total diet studies to evaluate the exposure to certain chemical contaminants (ACSA, 2005 and 2011) based on the guidelines published by the WHO (1985).

Occurrence data

The estimation of the daily intake of OTA can be done by taking into account data of contamination of foodstuffs classified in food categories or groups. This classification was made by JECFA (FAO/WHO, 2001) and by the SCOOP Report (2002), according to the degree of contribution of the foodstuffs to OTA exposure (Table 4). Average contamination data listed in Table 4 was summarised by EFSA (2006) and comes from the SCOOP Report (2002), which provided 18599 analytical results from 12 EU Member States (Denmark, Finland, France, Germany, Greece, Ireland, Italy, Portugal, Spain, Sweden, the Netherlands, United Kingdom) and Norway.

Table 4. OTA levels (μg/kg) in certain food groups (European Commission, Directorate-General Health and Consumer Protection, 2002).

Food group	***n***	**Mean OTA levels**
Cereals and products	5180	0.29
Beer	496	0.03
Wine	1470	0.36
Grape juice	146	0.55
Cocoa	547	0.24
Pork (edible offal)	1860	0.20
Roasted coffee	1148	0.72

3.3.1.3. Food consumption data

Different types of data can be used to assess food consumption. These data are obtained by different methods and correspond to a different stage of the food chain (Kroes et al., 2002):

Food supply data: These data are calculated in food balance sheets, which are accounts, on a national level, of annual production of food, changes in stocks, imports and exports, and agricultural and industrial use. The result is an estimate of the average value per head of the population, and does not take into account population characteristics such as age and gender.

Household surveys: Give information about the amount of food brought into the household, but not about the way food is prepared or the consumption by the family members.

Individual dietary surveys: Data from these surveys provide more accurate information on average food consumption at the individual level and thus are useful to evaluate the consumption of defined groups of individuals. The types of methods can be classified in record methods (food records), and recall methods (24-h recall, food frequency questionnaires, diet history):

Food records, dietary records or food diaries: The individual writes down the quantities (ideally the actual weight) of all the foodstuffs and beverages consumed.

24-hour recall method: The subject is asked by a trained interviewer to recall and describe the kinds and amounts of all foodstuffs and beverages ingested during the immediate past, mostly a 24- or 48-hour period. Food quantities are usually assessed by using household measures or photographs.

Food frequency method: A food frequency questionnaire (FFQ) consists of a list of individual foodstuffs or food groups, and its aim is to assess the frequency with which these items are consumed during a specific time period (daily, weekly, monthly, yearly).

Dietary history method: In this case, the respondent is asked to provide information about his/her pattern of eating over an extended period of time (often a "typical" week) and also to recall the foodstuffs eaten during the preceding 24 hours.

Both FFQs and dietary histories aim to reflect usual food consumption.

3.3.1.4. Estimation of the intake of OTA based on food consumption and contamination data: modelling methodologies

A model is a mathematical construct by which input data (in this case, consumption and contamination data) are combined to estimate quantitatively the outcome under study (in this case, the exposure to a food contaminant, OTA) (Counil et al., 2005).

The general model for the estimation of the intake or dietary exposure of a certain compound is:

Intake = Dietary exposure = Contamination x Consumption

Different modelling methods exist for processing the input data:

a- Deterministic modelling:

- Point estimations

- Simple distributions

b- Probabilistic modelling

In a point estimation, a fixed value of food consumption (such as the mean) is multiplied by a fixed value of concentration. Thus, no information can be obtained regarding the characteristics of the distributions of contamination and consumption, and the result implies the assumption that all the samples are contaminated in the average level, and that all the population has an average consumption (Kroes et al., 2002). This is a simple way to estimate the exposure, and has been employed in the SCOOP Report (2002) and in the EFSA Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to ochratoxin A in food (2006).

The analysis by simple distributions is a method that employs distributions of food consumption but uses a fixed value for the concentration variables (Kroes et al., 2002). In this way the information obtained is more complete than in the point estimations, as the variability of the consumption distribution is taken into account.

Probabilistic analysis or probabilistic modelling utilises distributions of both the food consumption data and the concentration data, and simulates dietary exposure by drawing random values from each input distribution (Kroes et al., 2002). It takes into account every possible value that each variable could take, and weighs each of them by its probability of occurrence (Counil et al., 2005). Briefly, this method consists on preparing (if needed) the input data, selecting a model, entering data to an appropriate simulation software, defining the number of simulations, and analysing the model to determine the range of probabilities of all possible outputs. Monte Carlo simulation is the most utilised tool to randomize the data in the modelling. An additional and important step is the quantification of the uncertainty of the exposure estimates by the construction of confidence intervals.

Each of these approaches may vary in their potential to over- or under-estimate exposure depending on the surveys used to provide the data and on the parameters used to represent the variables (Kroes et al., 2002).

3.3.2. Assessment of the exposure through biomarkers of exposure

A biomarker or biological marker is an indicator of changes or events in biological systems. Biomarkers of exposure refer to cellular, biochemical, analytical, or molecular measures that are obtained from biological media such as tissues, cells, or fluids, and are indicative of exposure to an agent (IPCS, 2004).

So far, the levels of OTA or some of its metabolism derivatives in biological fluids such as blood plasma, urine, or mother milk have been used as biomarkers of the human exposure to OTA. From these, the most used is the detection of OTA in blood plasma. In addition, the daily intake of OTA can be estimated from plasma levels taking into account pharmacokinetic relationships.

3.3.2.1. OTA in human plasma

Taking into account the binding properties of OTA to plasma proteins cited in section 3.1.6., the presence of OTA in blood plasma and in some occasions in serum, has been utilised in many studies as a biomarker of human exposure to OTA.

Table 5 shows OTA concentrations found in plasma or serum of apparently healthy volunteers in different countries all over the world. Surveys on the concentration of OTA in human plasma or serum have been carried out in several countries with two sorts of participants: apparently healthy people and people suffering nephropathic diseases (Hult et al., 1982; IARC, 1991; Breitholtz-Emanuelsson et al., 1994; Maaroufi et al., 1995; Radić et al., 1997; Jimenez et al., 1998; Malir et al., 2001; Grosso et al., 2003; Vrabcheva et al., 2004; Castegnaro et al., 2006; Sangare-Tigori et al., 2006; Dinis et al., 2007). Some of these studies were carried out in areas that were considered endemic for nephropathies (with healthy volunteers as well as patients), and some found that OTA levels in plasma were higher for the nephropathic patients.

The results in the literature were expressed as mean concentrations (ng OTA/mL plasma) and as occurrence (% of positive samples). However, samples were positive depending on the limit of detection of the method that was used in each assay. For this reason, some authors considered more appropriate to express occurrence in terms of concentration ranges (Jimenez et al., 1998; Pérez de Obanos et al., 2001). They expressed occurrence as a percentage of positive samples within certain concentration ranges. Thus, a better description of occurrence is obtained. For statistical purposes, when OTA was not detected by the analytical method, the assigned value of concentration used in most cases was half the limit of detection.

Taking into account the data of the studies listed on Table 5 (44 studies), OTA plasma levels were analysed in around 18,900 samples, from which a 70% was positive. Percentage of positive samples varied, as limits of detection of the methods were different in each case. Despite this, the average values for the minimum, maximum and mean of all the studies listed in the table were calculated, and resulted to be 0.18, 10.39 and 0.77 ng OTA/mL plasma, respectively.

Table 5. Occurrence of OTA in apparently healthy people from different countries, and estimation of the intake of OTA when done.

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Algeria	n.a.	0.1	346	66.9	n.a.-9.0 (2.8)	-	Khalef et al., 1993
Argentina	2004-2005	0.012	435	63	<0.012-74.8	-	Pacin et al., 2008
General Rodriguez			236	63.8	<0.012-47.6 (0.1537)	0.32[a]-0.47[b]	Pacin et al., 2008
Mar del Plata			199	62.3	<0.012-74.8 (0.4319)	0.15[a]-0.21[b]	Pacin et al., 2008
Bulgaria	1984, 1986, 1989, 1990	1-2	360	11	1-27.2	-	Petkova-Bocharova and Castegnaro, 1991
	n.a.	0.1	16	100	0.2-10.4 (1.59)	-	Petkova-Bocharova et al., 2003
Canada (15 localities)	1994	0.12	144	100	0.29-2.37 (0.88)	1.2[a]-1.7[b]	Scott et al., 1998
Chile	2004	0.1	88	70	0.07-2.75	-	Muñoz et al., 2006
Colbún			44	50	0.07-2.75	0.84[b]	Muñoz et al., 2006
San Vicente de Tagua			44	91	0.22-2.12	1.4[b]	Muñoz et al., 2006
Costa Rica	n.a.	0.025	149	95	0.01-1.906 (0.622)	-	Quintana Guzman et al., 2007
Croatia	1997-1998	0.2	198	44	<0.2-1.3 (0.19)	0.26[a]	Domijan et al., 1999
	1997	0.2	983	47.6	(0.30)	0.40[a]	Peraica et al., 2001
	March	0.2	242	53.7	(0.36)	0.48[a]	Peraica et al., 2001
	June	0.2	249	59.4	(0.39)	0.52[a]	Peraica et al., 2001
	September	0.2	242	41.7	(0.25)	0.34[a]	Peraica et al., 2001
	December	0.2	250	35.6	(0.19)	0.25[a]	Peraica et al., 2001
	1985-1993	2	2566	0-2.4	0-10	-	Radić et al., 1997

Former Czechoslovakia	n.a.	0.1	143	24.5	<0.1-1.26	-	Fukal and Reisnerova, 1990
Czech Republic	1991-1992	0.1 - 0.5	594	40	0.05-37 (0.625)	0.74[c]	Ruprich and Ostry, 1993
	1994	LOQ = 0.1	809	90.7	0.1-13.7 (0.23)	-	Ostry et al., 2005
	1995	LOQ = 0.1	413	97.8	0.1-1.9 (0.24)	-	Ostry et al., 2005
	1997	LOQ = 0.1	398	94.5	0.1-2.1 (0.33)	-	Ostry et al., 2005
	2000	LOQ = 0.1	400	96.3	0.1-2.0 (0.35)	-	Ostry et al., 2005
	2002	LOQ = 0.1	186	95.7	0.1-1.3 (0.36)	-	Ostry et al., 2005
Denmark	1986-1988	0.1	144	54.2	n.d.- 13.2 (1.8)	-	Hald, 1991
	1986				n.d.-9.7 (1.5)	-	Hald, 1991
	1987				n.d.-9.4 (2.3)	-	Hald, 1991
	1988				n.d.-13.2 (1.6)	-	Hald, 1991
Germany							
Oberschleissheim	1977	0.1	165	50.9	0.1-14.4 (0.79)	-	Bauer and Gareis, 1987
Munich	1985	0.1	141	63.1	0.1-1.8 (0.42)	-	Bauer and Gareis, 1987
	1988	0.1	208	68.3	n.a.-8.4 (1.1)	-	Hadlock and Wagner, 1993
	1996-1998	0.06	927	98.1	<0.06-2.03 (0.27)	0.46[d]	Rosner et al., 2000
	1990, 1991, 1995, 1997	0.05	102 (36 persons)	98	0.072-1.290 (0.368)	0.493[a]-0.725[b]	Märtlbauer et al., 2009
Hungary	n.a.	LOQ = 0.2	100	51	0.2-12.9	-	Kovács et al., 1995
	1995	0.2	355	81.8	<0.2-10.0	-	Solti et al., 1997
	1997	0.1	277	77	n.a.-1.4	-	Tápai et al., 1997

Table 5 *(Continued)*

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Italy	1994-1996	n.a.	138	97	0.12-2.84 (0.56)	0.77[a]	Palli et al., 1999
Ivory Coast	2001, 2004	n.a.	63	35	0.01-2.83 (0.83)	0.42[b]	Sangare-Tigori et al., 2006
	2001		42	47.6	0.01-2.83 (0.35)		Sangare-Tigori et al., 2006
	2004		21	9.5	1.23-2.32 (1.55)		Sangare-Tigori et al., 2006
Japan	1992, 1994, 1995, 1996	-	184	85	0.004-0.278 (0.068)	0.08[e]	Ueno et al., 1998
	1992	0.010	24	96	0.016-0.278 (0.093)		Ueno et al., 1998
	1994	0.004	34	38	0.004-0.263 (0.073)		Ueno et al., 1998
	1995	0.020	69	93	0.024-0.13 (0.062)		Ueno et al., 1998
	1996	0.010	57	98	0.019-0.151 (0.062)		Ueno et al., 1998
Lebanon	2001-2002	n.a.	250	33	0.1-0.87 (0.17)	0.23[a]	Assaf et al., 2004
Morocco	2000	n.a.	309	60	0.08-6.59 (0.29)	0.4[e]	Filali et al., 2002
Norway	1998	0.01	206	100	(0.18)	0.24[a] - 0.36[b]	Thuvander et al., 2001
Poland	1983-1984	1	1065	7.2	n.a.-40 (0.270)	0.4[f]	Golinski et al., 1991
	1983		397	6.3	(0.21)		Golinski et al., 1991
	1984		668	7.8	(0.31)		Golinski et al., 1991
Portugal	2001-2002	0.1	104	100	0.14-2.49	-	Lino et al., 2008
Coimbra	2002		29		0.19-0.96 (0.42)	0.56[a]	Lino et al., 2008
Verride	2001		31		0.25-2.49 (0.78)	0.59[a]	Lino et al., 2008
Ereira	2001		44		0.14-1.91 (0.44)	1.05[a]	Lino et al., 2008

Spain								
	Madrid	1997	0.02	168	100	0.12-5.58 (1.192)	0.26[a]	Burdaspal and Legarda, 1998
	Granada	1996-1997	0.22	83	86.8	<0.22-6.96 (0.63)	0.74[c]	Pérez de Obanos et al., 2001
	Navarra	1996, 1997, 1998	0.52	75	53.3	0.52-4.00 (0.71)	-	Jimenez et al., 1998
	Lleida	2008	0.075	279	98.6	0.11-8.68 (0.86)	1.69[b]	Coronel et al., 2009
	Valencia	2008	0.01	168	100	0.15-5.71 (1.09)	1.47[a]-2.16[b]	Medina et al., 2010
Sweden		1989	0.3	297	12.8	0.3- >0.8 (0.1)	0.13[a]	Breitholtz et al., 1991
	Östersund			99	29.3		0.04[a]	Breitholtz et al., 1991
	Uppsala			99	3		0.03[a]	Breitholtz et al., 1991
	Visby			99	6		0.35[a]	Breitholtz et al., 1991
	Visby	1997	0.01	200	100	(i0.21)	0.28[a]- 0.42[b]	Thuvander et al., 2001
Switzerland		1992-1993	0.002	368	100	0.06- 6.02 (mean around 0.30)	-	Zimmerli and Dick, 1995
		n.a.	0.05	79 (8 persons)	100	0.20-0.88	-	Studer-Rohr et al., 2000
Tunisia		n.a.	0.1	140	52	0.1-8.8 (0.7-2.2)	-	Maaroufi et al., 1995a
		1991	LOQ = 0.1	25	82	(3.35)		Abid et al., 2003
		1994	LOQ = 0.1	140	77	(2.25)		Abid et al., 2003
		1997	LOQ = 0.1	20	71	(2.6)		Abid et al., 2003
		2000	LOQ = 0.1	20	62	(1.22)		Abid et al., 2003

Table 5 *(Continued)*

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Tunisia	1996, 1998	LOQ = 0.1	62	100	0.12-8.06 (0.53)	-	Grosso et al., 2003
	n.a.	n.a.	20	71	0-7.5 (2.6)	-	Hassen et al., 2004
	n.a.	n.a.	20	62	0-3.2 (1.22)	-	Hassen et al., 2004
	n.a.	0.125	105	28	0.12-3.4 (0.49)	-	Hmaissia-Khlifa et al., 2008
	n.a.	0.1	44	52.3	0.11-6.1 (0.77)	-	Hmaissia-Khlifa et al, 2010
Turkey	2007-2008	0.025	239	85.1	-	-	Erkekoğlu et al., 2010
	Winter				0.0306-0.887 (0.137)	0.182[a]	Erkekoğlu et al., 2010
	Summer				0.0279-1.496 (0.312)	0.408[a]	Erkekoğlu et al., 2010
United Kingdom	2001	0.1	50	100	0.4-3.11 (1.09)	1.46[a]-2.15[b]	Gilbert et al., 2001
Former Yugoslavia	1981-1989	5	3378	0 - 3.7	n.a.-50	-	Fuchs et al., 1991

n.a.: not available
n.d.: not detected
LOD: limit of detection
LOQ: limit of quantification
[a] According to Breitholtz et al. (1991): $k_0 = 1.34 \times Cp$
[b] According to Miraglia et al. (1996): $k_0 = 1.97 \times Cp$
[c] $k_0 = 1.18 \times Cp$
[d] $k_0 = 1.98 \times Cp$
[e] According to Kuiper Goodman et al. (1993)
[f] According to Mortensen et al. (1983)

Factors influencing OTA concentrations in plasma

Some studies tried to establish differences on OTA plasma levels according to the regions where the population lived and the season when the samples were obtained, as well as those due to gender and age. Regional dietary habits of the population were considered in their discussions, although these assumptions were not always supported by experimental data. However, there are studies that established an experimentally-based correlation between the detected plasma levels and food consumption by two possible ways: determination of OTA on the food consumed by the study population, using duplicate diet samples (Gilbert et al., 2001; i.e., food samples provided by the participants, in the consumption way) and establishment of food consumption patterns by means of food questionnaires that were answered by the study population (Thuvander et al., 2001; Muñoz et al., 2006).

OTA and age

In order to analyse the correlation between the age of the participants and the levels of OTA in plasma, age groups were defined. No relation was found between OTA concentrations in plasma and the age of the participants in several studies (Jimenez et al., 1998; Scott et al., 1998; Ueno et al., 1998; Palli et al., 1999; Gareis et al., 2000; Grosso et al., 2003; Skaug, 2003; Assaf et al., 2004; Jonsyn-Ellis, 2007).

Four age groups (21-30, 31-40, 41-50 and 51-60 years) were compared in the Argentinean cities of Mar del Plata and General Rodriguez and significant differences were found among age groups in both cities. In the first city, the highest OTA levels were found in the 51 to 60 years group, and in the 31 to 40 years group in the second (Pacin et al., 2008). OTA plasma concentrations in the UK were higher in the age group from 30 to 44 (Gilbert et al., 2001). Lino et al. (2008) found the highest OTA concentrations in people aged over 40 in a rural area of Portugal. Filali et al. (2002) found no significant differences among age groups in Morocco, but analysed the frequency and the levels of exposure: the 40 to 50 years old group was the most exposed but the levels found were not high (0.23 ng OTA/mL plasma), whereas the 60.4 % of the 18 to 30 years old group was exposed to 0.42 ng OTA/mL plasma.

Taking into account the above mentioned studies, it can be observed that few studies have shown a relationship between age and OTA plasma levels. However, OTA-related age groups do not match from one study to another.

OTA and gender

Most of the studies on OTA levels in human plasma found no relation between OTA plasma levels and the sex of the participants (Breitholtz et al., 1991; Ueno et al., 1998; Thuvander et al., 2001).

In Switzerland, males living in the south of the Alps had higher OTA plasma concentrations than women from the same region and than the inhabitants of the

north of the Alps (Zimmerli and Dick, 1995). In Tuscany (Italy) men had a significantly higher mean value of OTA in plasma than women (Palli et al., 1999). In Morocco, female population was less exposed than the male population (Filali et al., 2002). In Portugal, OTA was detected in serum of the inhabitants of three populations, Verride and Ereira (rural areas) and Coimbra. In the rural populations, OTA levels in men were higher than in women (Lino et al., 2008). Pacin et al. (2008) studied OTA concentrations in the cities of Mar del Plata and General Rodriguez, and significant differences were found between men and women only in General Rodriguez, with men having the highest concentrations. Inhabitants of two agricultural zones in Chile were studied for OTA concentrations in plasma. No significant differences were found between men and women in the zone of Colbún, but in the zone of San Vicente de Tagua, the mean of OTA levels in women was significantly higher than that from men (Muñoz et al., 2006). Taking into account these data, it can be observed that when OTA levels in plasma were related to gender, in most of the cases male population presented the highest values.

OTA and geographic location

It has been observed that the distribution of OTA in plasma varies among regions within a country. Factors that have been considered to have influence on the regional variations of OTA are dietary habits and climate. In the first case, it is known that dietary habits are different on each zone in a country. For example, Zimmerli and Dick (1995) found that OTA plasma concentrations were higher in the south of the Alps than in the northern alpine region, which was related to the different food consumption habits of each region. Thereby, it was observed that in the south region consumption of rice, pasta, bread, biscuit, olive oil, cheese, grapes and tomatoes was higher than in the north, as well as a higher consumption of corn and alcoholic beverages, especially by men. Concerning the climate, Maaroufi et al. (1995) stated that the climate and the traditional way of food storage could influence the exposure of the population to OTA, as climatic conditions favour the proliferation of fungi during food storage. For example, when comparing OTA plasma levels of inhabitants of rural and urban areas in the same country, differences were observed. Peraica et al. (1999) found that the frequency of OTA positive samples was higher in rural than in urban populations, indicating that locally produced food may influence the exposure of OTA. Other authors also stated that rural populations are more exposed to OTA than the urban ones: Lino et al. (2008) studied OTA levels in serum of the inhabitants of two farming villages in Portugal (Verride and Ereira) and of the city of Coimbra. Significantly higher concentrations were found in Verride population. In Chile, OTA plasma levels were studied in two different agricultural zones: Colbún and San Vicente de Tagua. The levels in the later one were significantly higher than the levels found in Colbún (Muñoz et al., 2006).

Other studies compared levels of OTA in different cities. In Canada, Scott et al. (1998) compared the levels of the toxin in the population of inhabitants of fifteen

cities and found significant differences among some of them. Breitholtz et al. (1991) determined OTA concentrations in plasma of inhabitants of three districts of Sweden: Uppsala, Östersund and Visby. The highest frequency of positive samples and highest levels of OTA were found in Visby. It should be noted that Visby is situated on an island, unlike Uppsala and Östersund. The study was later repeated in the city of Visby, together with the city of Oslo (Norway) and in this case levels found in Visby were slightly higher than the levels found in Oslo (Thuvander et al., 2001). On another study, OTA plasma levels were significantly different in two cities of Argentina, Mar del Plata and General Rodriguez, with medians of 0.11 ng/mL and 0.24 ng/mL, respectively. Differently from General Rodriguez, Mar del Plata is a coast city (Pacin et al., 2008). Peraica et al. (2001) studied the plasma concentration of blood donors in five different cities from Croatia: Varadzin, Zagreb and Osijek, located in the inland of the country, and in Split and Rijeka, located on the Adriatic coast. The results showed that the higher frequency of positive samples and the highest mean concentration (0.56 ng/mL) occurred in Osijek, meanwhile the lowest frequency of positive samples and lowest mean concentration (0.13 ng/mL) was found in Rijeka. The authors related these differences to different dietary habits in each region: the coast and the inland. Bauer and Gareis (1987) detected OTA in human serum of inhabitants of the German cities of Oberschleissheim (0.42 ng/mL) and Munich (0.79 ng/mL) in 1977 and 1985, respectively. In later studies, OTA levels found in Jena were 0.34 ng/mL and 0.17 ng/mL in Munich (Gareis et al., 2000; Rosner et al., 2000). In Lebanon, OTA mean levels in Beiruth/Month Lebanon, Bekaa and in the North and South Lebanon were similar (0.17; 0.16; 0.16 and 0.18 ng/mL respectively), though a higher frequency of positives samples was found in the regions of Bekaa and in the South of Lebanon. This difference was related to the fact that in the first two regions, consumption of cereals and burghul is important, whereas in Beirut/Mont Lebanon, cereals consumption has been replaced by fat-containing foodstuffs (Assaf et al., 2004). In Spain, mean values varied in three different regions: 1.19 ng/mL in Navarra (Jimenez et al., 1998), 0.63 ng/mL in Madrid (Burdaspal and Legarda, 1998) and 0.71 ng/mL in Granada (Pérez de Obanos et al., 2001).

From the above mentioned it can be seen that most of the authors relate dietary habits of each particular region (rural, urban, inland or coast areas) to the differences found in levels of the toxin in plasma of their inhabitants.

OTA and season

There are studies in which samples were collected in different seasons of a year, and some other studies repeated measurements in the study population to determine differences in OTA levels from one year to another.

In Denmark, 144 blood samples were collected from January 1986 until December 1989. This study showed an inconsistent seasonal variability of OTA concentration: peak values were found in different months each year (September,

October and March) (Hald, 1991). In Granada, southern Spain, no significant differences were found between OTA plasma levels and season in two different years: 1996 and 1997 (Pérez de Obanos et al., 2001). In Madrid, central Spain, no relationship was found among the levels detected in the total study population (men and women) and the season (summer or winter). For men, OTA mean levels were lower in summer (1.119 ng/mL) than in winter (1.241 ng/mL), whereas levels of women were higher in summer (1.747 ng/mL), with a concentration of 0.974 ng/mL in winter (Burdaspal and Legarda, 1998). In Navarra, northern Spain, Jimenez et al. (1998) detected OTA in plasma of healthy people and nephropathy patients in the months of April, June, October and November 1996; January, February, March 1997 and January 1998. Levels found in the months of June and October 1996 were significantly lower than the obtained in the rest of the months.

Differences in plasma levels of OTA with season were found in a study made in five Croatian cities. Samples were taken four times in a year, in the months of June, September and December 1997, and March 1998. The highest frequency of the samples containing OTA in quantities above the limit of detection and the highest mean were found in June, whereas the month with the lowest frequency of positive samples was December (Peraica et al., 2001). The study done by Ruprich and Ostry (1993) in the district of Brno, Czech Republic, lasted 13 months, and showed an increase of OTA levels in plasma in the spring months. In Italy, a group of 138 participants provided blood samples in the months of July and October 1994. The samples collected in the summer period of the study year presented the highest OTA levels in plasma (Palli et al., 1999). Fuchs et al. (1991) also found higher values in the summer period in a non-endemic village in Croatia. Hence, summer was the season during which the highest levels of OTA have been found.

Repeated measurements

Variations through the years

Palli et al. (1999) studied the levels of OTA serum in healthy adults in Tuscany, Italy: 138 volunteers provided blood samples in the months of July and October 1994, and 69 of them agreed to provide an additional blood sample approximately one year later, in the months of October 1995 and January 1996. No correlation was

found between the first and second measurements, moreover, in the repeated collection period, OTA levels showed a high variation and presented a different seasonal pattern from the observed in the previous year. In Croatia, 6909 human sera samples were collected over a period of ten years, in endemic and control villages, and it was observed that OTA levels varied from year to year (Radić et al., 1997). OTA levels in blood plasma were studied in different years in Tunisia (Abid et al., 2003) and in the Ivory Coast (Sangare-Tigori et al., 2006). Although the authors did not test the variability among years, in Table 5 it can be observed that mean levels in the evaluated years were different.

In Denmark, OTA was analysed in samples taken from a blood bank weekly (Hald, 1991). Mean results were obtained for every month, and the mean value was calculated for each of the three years of the study (Table 5). It can be observed that mean levels of two years (non consecutive) were similar, and in the remaining year the mean was higher. A similar result was found in Germany: Märtlbauer et al. (2009) studied the levels of OTA in a group of 36 persons in different years (July, October, and December 1990; December 1991, December 1995 and January 1997). The mean and median contamination of positive samples were very constant throughout the years, at about 0.35-0.45 ng/mL, with the exception of a slightly lower mean (0.23 ng/mL) in December 1991. Ueno et al. (1998) studied OTA levels in plasma in Tokyo in 1992, 1994, 1995 and 1996, in a total of 184 healthy volunteers. No significant differences were observed between the means in the four years. Petkova-Bocharova and Castegnaro (1991) found little annual variation in the percentage of samples containing OTA in Bulgaria. In Table 5 it can be observed that mean levels in the different years did not vary largely in the Czech Republic (Malir et al., 2001; Ostry et al., 2005) and in Poland (Golinsky et al., 1991).

Intraindividual fluctuations

Ruprich and Ostry (1993) repeated the quantification of OTA in serum of four persons, twice for three people, and three times for the forth one. Repetitions were done from 14 until 62 days after the first extraction. OTA concentrations decreased from the first determinations, with a variation from 37 ng/mL to < 0.33 ng/mL in the most extreme case.

Variations were also found in repeated measurements in three Japanese men after more than three months of the first extraction (Kawamura et al., 1993). In Canada, serum levels of one person were 0.25 ng/mL in 1990 and 1.29 ng/mL in 1991 (Kuiper-Goodman et al., 1993). In the study presented by Zimmerli and Dick (1995), serum levels of one Swiss person were almost equal in the months of May and November 1993, and March and June 1994. Also in Switzerland, Studer-Rohr et al. (2000) studied the intraindividual fluctuation of OTA plasma levels in eight volunteers during two months. The levels of OTA through the time showed a different pattern for each person: only in two persons OTA levels were rather quite constant, whereas in the rest of the volunteers, a high variability was observed. The range of concentrations was 0.20-0.88 ng/mL. In Germany, Märtlbauer et al. (2009) studied the variation of OTA levels in plasma of nine persons over a period of almost seven years (July, October, and December 1990, December 1991, December 1995, and January 1997), and also found variations within persons and throughout the years.

A special case is that of the nephropathic patients. In the study presented by Jimenez et al. (1998), repetitions of samples taken in April 1996 from four haemodialysis patients were done in January 1998; levels of OTA in the repeated measurements were lower.

All these different results show no specific trend in the behaviour of OTA in the individual cases. Therefore toxicokinetics of OTA have to be considered and related to the intake of the mycotoxin as well as the dietary habits of the persons. Palli et al. (1999) state that OTA serum levels are a short-term biomarker with high within-subject variability; therefore they have limited use at the individual level but can be used to characterize populations or subgroups of subjects. Märtlbauer et al. (2009) consider that the levels measured in a single blood sample do not necessarily reflect the long-term OTA level of an individual.

3.3.2.2. Estimation of the daily intake of OTA based on its concentration in blood plasma

It is possible to estimate the daily intake of OTA from the levels of the toxin in blood plasma by means of the Klaassen equation (Klaassen, 1986), which also considers biological factors as plasma clearance and bioavailability of OTA.

Plasma clearance

Plasma clearance is determined by all the individual metabolizing/eliminating organ clearances and involves mainly liver and kidney clearances (Toutain and Bousquet-Mélou, 2004). From a pharmacological point of view, any clearance (total or for a given organ) should be defined as the ratio of two terms: the rate of drug elimination and the driving concentration of the drug in the system. For plasma clearance, the rate of drug elimination is the total rate of body elimination, and the driving concentration is the plasma concentration (Toutain and Bousquet-Mélou, 2004):

Plasma clearance = Total (body) rate of elimination /plasma concentration

The estimation of a plasma (total) clearance (Cl_{tot}) can be done if the drug is totally eliminated by a route which is experimentally measurable (e.g. urine, faeces). In most instances, total plasma clearance is the sum of organ clearances:

$$Cl_{tot} = Cl_{renal} + Cl_{liver} + Cl_{other}$$

Being Cl_{renal} the renal clearance, Cl_{liver} the hepatic clearance, and Cl_{other} all other clearance mechanisms. Generally, Cl_{other} is negligible and the plasma clearance is often the sum of renal and hepatic clearance. Moreover, if Cl_{liver} is negligible, Cl_{tot} reduces to Cl_{renal} (Toutain and Bousquet-Mélou, 2004).

Renal clearance

Seldin (2004) reviewed the development of the renal clearance concept. A preliminary definition of clearance in his work is the following: “The renal

clearance of a substance is the volume of plasma required to supply the amount of this substance excreted in the urine during a given period of time":

$$C_s = (U_s \times V) / P_s$$

Where *s* is any substance, C_s is the clearance of the substance (mL/min), U_s is the concentration of the substance in urine (mg/mL), V is the urine flow rate (mL/min), and P_s is the concentration of the substance in plasma (mg/mL). However, the author makes clear that the kidney does not completely remove a substance from the total renal plasma flow, and that the kidneys free a fraction (virtual volume) of each mL of total plasma flow of any substance. Taking this into account, the concept of clearance would be: "The clearance of any substance is the virtual volume of plasma flow required to supply the amount of a substance excreted in one minute".

Measure of renal clearance

Renal function can be assessed by different means: measures of the blood urea concentration, urea excretion, glomerular filtration rate, and renal plasma flow or filtration fraction. The most common way is to determine the glomerular filtration rate, which can be done by the determination of the clearance of markers such as inulin or creatinin, being the first the most appropriate (Seldin, 2004). To summarize, and taking into account all the above mentioned considerations, plasma clearance can be assessed by the inulin or creatinine clearance.

Estimation of OTA daily intake by means of Klaassen equation

Klaassen (1986) described the concentration of a chemical in plasma as well as in other tissues after repeated exposure. The "average" concentration (C^{av}_{∞}) could be described by the equation:

$$C^{av}_{\infty} = f \times D_{oral} / Cl_b \times \tau$$

Where f is the fraction absorbed, D_{oral} is the oral dose, Cl_b is the total body clearance, and τ is the constant time between administration or exposure. No units were stated in this equation.

Bretiholtz et al. (1991) adapted this equation to the estimation of the continuous mean daily intake of OTA (k_0, ng OTA/kg bw/day) as the following:

$$k_0 = Cl_p \times C_p / A$$

Where Cl_p is the plasma clearance (mL/kg bw/day), C_p is the plasma concentration of OTA (ng/mL), and A is the bioavailability of OTA. These authors utilised the values of Cl_p and A as 0.67 mL/kg bw/day and 50%, respectively, and the equation to estimate the daily intake of OTA from the plasma levels remained $k_0 = 1.34 \times C_p$. The values of the coefficients were derived taking into account the toxicokinetic data published by

Hagelberg et al. (1989). They assessed the toxicokinetics of OTA in five animal species, and, in addition, the fraction of toxin unbound to plasma proteins (principally albumin) in man, that resulted 0.02%. Clearance of OTA was estimated by the renal filtration, which in such case is restricted to the free fraction of the toxin, as OTA bound to plasma proteins cannot pass through the glomerular membrane. Therefore, renal clearance was assessed through the glomerular filtration rate. Values of inulin clearance found in the literature (Adolph, 1949; Brenner et al., 1986) were used as a measure of the glomerular filtration rate, which for man was 140 mL/kg/h. Taking this into account, and the data on free fractions of OTA experimentally obtained (0.02%), it can be calculated that in one hour 0.028 mL plasma/kg bw are cleared of OTA, and in one day, 0.67 mL/kg bw (Ruprich and Ostry, 1993). These values apply for a body weight of 70 kg.

Later on, Miraglia et al. (1996) took into account the value of 0.048 mL/min obtained by Studer-Rohr (1995) for the renal clearance of radioactivity during the elimination phase in an experiment with a human volunteer, and thus suggested the value of Cl_p as 0.99 mL/kg bw/day (if the body weight is 70 kg bw). Considering A = 50%, the proposed equation to estimate OTA daily intake is $k_0 = 1.97 \times C_p$.

In another study, Ruprich and Ostry (1993) and Pérez de Obanos et al. (2001) considered the biological availability of OTA in monkey (*Macaca mulata*) (57%, Hagelberg et al., 1989) and the value of 0.67 mL/kg bw/day for Cl_p. Then, $k_0 = 1.18 \times C_p$.

To our present knowledge, the above mentioned are the ways to estimate OTA daily intake when OTA plasma levels are available, and this estimation has been done mostly taking into account the coefficients proposed by Breitholtz et al. (1991) and Miraglia et al. (1996) (Table 5).

Additionally, Studer-Rohr et al. (2000) calculated the whole body clearance for radioactive OTA and metabolites/conjugates in an experiment with a human volunteer, and its value was 0.0935 mL/min. Taking this value into account, and the bioavailability of OTA in monkey (57%) as the best approximation to human, Klaassen equation adapted to the estimation of the OTA daily intake would be: $k_0 = 236.21 \times C_p/W$ (ng/kg bw/d), where W is the body weight (kg bw). Considering that the body weight of the volunteer in the experiment by Studer-Rohr (2000) was 62 kg, then $k_0 = 3.81 \times C_p$ (ng/kg bw/d).

3.3.2.3. OTA in urine

As mentioned, an important path for the excretion of OTA and its metabolites is the renal excretion. The occurrence of OTA in urine as a biomarker of the exposure to OTA has been studied (Table 6), though not as thoroughly as in plasma. Even though the occurrence and the levels detected were lower in comparison to those found in plasma, an advantage of the use of this biomarker is that the collection of the samples is non-invasive.

Taking into account the biotransformation pathways stated in section 3.2.2., previous studies have suggested the presence of OTA glucuronide or sulphate conjugates in rat urine and bile (Kühn et al., 1995; Li et al., 2000) and in human urine (Castegnaro et al., 1991; Kane et al. 1986; Pena et al., 2006). More recently, Muñoz et al. (2010a) studied the presence of OTA, its hydrolysis product, ochratoxin α, and their conjugates in human urine. With this, even though the detected OTA levels were low, additional information on the exposure to OTA can be achieved by the detection of its metabolite OTα.

With regard to the estimation of the intake of OTA, no relation between the levels of OTA (or its metabolites) in urine and the intake of the toxin has been established yet.

Table 6. Occurrence of OTA in urine samples in different countries according to the type of sampling[a] (modified from Duarte et al., 2009).

Country	City	*n*	% Positive	Mean ± SD (ng/mL)	Range (ng/mL)	Reference
			Point sampling			
Croatia		35	94	2.39 ± 1.29	0.99 - 5.22	Domijan et al., 2003
Germany	Dortmund	13	100	0.07 ± 0.05	0.02 - 0.14	Muñoz et al., 2010a
India		152[b]	40	n.a.	0.005 - 0.03	Castegnaro et al., 1990
Portugal	Coimbra	60	70	0.027 ± 0.004	0.021 - 0.105	Pena et al., 2006
	Lisbon	43	72.1	0.026 ± 0.017	ND - 0.071	Duarte et al., 2009
	Bragança	30	93.3	0.024 ± 0.014	ND - 0.069	Duarte et al., 2010
	Porto	30	73.3	0.021 ± 0.014	ND - 0.062	
	Coimbra	30	53.3	0.014 ± 0.007	ND - 0.034	
	Alentejo	40	90	0.023 ± 0.012	ND - 0.064	
	Algarve	25	80	0.024 ± 0.015	ND - 0.068	
Sierra Leone		190 rainy season	25	6.4 ± 3	0.6 - 72.2	Jonsyn-Ellis, 2000
		244 dry season	26	5.3 ± 5.6	0.05 - 148	

Table 6 *(Continued)*

Country	City	*n*	% Positive	Mean ± SD (ng/mL)	Range (ng/mL)	Reference
			24-hour sampling			
Bulgaria	Gorno-Peshtene	5	100	0.0508 ± 0.044	0.010 - 0.330	Petkova-Bocharova et al.,
	Beli Izvor	11	100	0.168 ± 0.111	0.010 - 1.910	2003
Hungary	Besenyotelek		73	0.022	0.006 - 0.065	Fazekas et al., 2005
	Füzesabony		57	0.018	0.007 - 0.029	
	Debrecen		56	0.008	0.006 - 0.011	
	Komadi		56	0.010	0.006 - 0.019	
	Kaposvar		59	0.008	0.006 - 0.012	
Italy		38 healthy	58	n.a.	0.012 - 0.046	Pascale and
		3 patients[c]				Visconti, 2000
UK		50	92	0.013	<0.01 - 0.058	Gilbert et al., 2001
			Morning and afternoon sampling			
Portugal	Coimbra	30 morning	43.3	0.019 ± 0.041	0.011 - 0.208	Manique et al., 2008
		30 afternoon	46.7	0.018 ± 0.027	0.008 - 0.110	
Spain	Valencia	31 morning	80.6	0.032 ± 0.031	0.007 - 0.124	Manique et al., 2008
		31 afternoon	83.9	0.028 ± 0.019	0.008 - 0.089	

n.a.: not available

ND: not detected

[a] Type of sampling that was performed in the studies: "point sampling" refers to the collection of a single sample at a certain moment of the day; "24-hour sampling" implies the collection of the urine during a period of 24 hours, followed by the pooling of all the samples; and "morning and afternoon sampling" means the collection of the samples at those two moments of the day.

[b] BEN (Balkan endemic nephropathy) and UTT (tumours of the urinary tract) patients and control families.

[c] Kariomegalic interstitial nepºhritis patients.

3.3.2.4. OTA in human milk

OTA presence in human milk is another possible biomarker of the exposure, as its presence has been already reported (Gareis et al., 1988; Breitholtz-Emanuelsson et al., 1993a; Jonsyn et al., 1995; Zimmerli and Dick, 1995; Micco et al., 1995; Skaug et al., 2001; El-Sayed et al., 2002; Turconi et al., 2004; Navas et al., 2005; Hassan et al., 2006; Postupolski et al., 2006; Biasucci et al., 2010; Dostal et al., 2008; Galvano et al., 2008; Muñoz et al., 2010b).

The use of this fluid as a biomarker has the advantage of the easiness in the sample collection. Moreover, if mother milk is the only food ingested by the suckling, it would give an accurate measure of its OTA intake.

As happens also in urine, the relation between the levels of the toxin in milk and its intake has not been established yet. Likewise, the levels detected were lower than the detected in plasma of rabbit (Galtier et al., 1977) and human plasma (Breitholtz-Emanuelsson et al., 1993a), and the metabolite OTα was also found (Muñoz et al., 2010a).

Even though the use of this biomarker is restricted to lactating women and their babies, it may be used as an alternative to the previuosly mentioned fluids of plasma and urine.

3.4. Risk characterization

The estimation of the probability of occurrence of adverse health effects in a given population due to its exposure to OTA can be done by the comparison of the intake estimations with the provisional tolerable intakes. For example, taking into account the estimated average daily intakes listed in Table 5, it can be observed that such values are far lower than the latest PTDIs suggested by EFSA and JECFA (Table 3). It is worth to note that these values were obtained from the mean levels of OTA in plasma, and that therefore the highly exposed population (maximum values) is not contemplated in that assessment. Other population groups to be considered are those exposed to higher amounts of OTA than the average adult consumers, such as newborns, children, vegetarians, or people exposed in their work places.

4. RISK MANAGEMENT

The primary goal of the management of risks associated with food is to protect public health by controlling such risks as effectively as possible through the selection and implementation of appropriate measures (FAO/WHO, 1997).

4.1. Prevention and remediation measures

Prevention and/or remediation measures are those implemented in order to minimise the mycotoxin contamination of foodstuffs, at different stages of their production process (pre-harvest, harvest, post-harvest, storage, and processing), by inhibiting the growth of fungi in the commodities, or by removing the mycotoxins from contaminated material, respectively.

A tool to implement the preventive measures is the application of management systems that were designed to prevent food safety problems in the different stages of the food chain: HACCP (Hazard Analysis and Critical Control Points), and its prerequisite, the employment of Good Practices (Good Agricultural Practice, Good Storage Practice, Good Manufacturing Practice, and Good Hygienic Practice).

Remediation options for the removal of mycotoxins can be physical (separation, milling, irradiation), chemical (extraction, adsorption), or biological (biodegradation by microorganisms) However, efforts should be focused in the preventive measures rather than in the treatment of the contaminated products, as procedures suitable for general use on an industrial scale for the destruction of multiple mycotoxins during processing are still lacking (Shapira and Paster, 2004).

The Food and Agriculture Organization in collaboration with the World Health Organization works on the application of risk management to food safety matters (FAO/WHO, 1997). In particular, regarding mycotoxins, it is worth to mention the papers presented in the Third Joint FAO/WHO/UNEP International Conference on Mycotoxins (FAO, 1999), and the "Manual on the application of the HACCP system in mycotoxin prevention and control" (FAO, 2001). The Codex Alimentarius Commission (2003) published a "Code of practice for the prevention and reduction of mycotoxin contamination in cereals", which included annexes specific for OTA, zearalenone, fumonisins and trichothecenes.

4.2. Control measures: Legislation for OTA in food

The Official Journal of the European Union published in 2006 the Regulation (EC) 1881/2006 to set the maximum levels for certain contaminants in foodstuffs (European Commission, 2006a). Regarding OTA, these levels varied from 0.5 μg/kg in baby foods to 10 μg/kg in soluble coffee and dried vine fruits. This Regulation was amended by Regulation (EC) 105/2010, which included maximum levels for spices and liquorice up to 80 μg/kg (Table 7) (European Commission, 2010b). The European Union has not set any limit for contamination of animal by-products. However, some countries have appropriate regulation about this subject. In Romania, the maximum allowed

level for meat is 5 μg/kg (Boutrif and Canet, 1998). The Danish Veterinary and Food Administration set the guidelines for the control of OTA in pigs. Organs condemnation is enforced for OTA levels in kidney between 10 and 25 μg/kg, and entire carcase condemnation for levels above 25 μg/kg (Jørgensen et al., 2001). In Italy, the maximum admissible value for OTA in pork meat and derived products is 1 μg/kg (Ministero dell' Economia e delle Finanze, 1999). The FAO published in 2004 the "Worldwide regulations for mycotoxins in food and feed in 2003" (FAO, 2004), in which the regulations of at least 99 countries are listed. Some mycotoxins, including OTA, are also regulated in regions like MERCOSUR, Australia and New Zealand (Van Egmond et al., 2007). As OTA can be found in several foodstuffs, there is special concern to evaluate its occurrence in the dietary sources so as to recommend adequate intakes of the toxin, and as a consequence, to set the limits of the regulations according to consumption.

Regarding animal feed, the European Union has published in 2006 a Recommendation on the presence of some mycotoxins in products intended for animal feeding (European Commission, 2006c): 0.25 mg/kg in cereal and cereal products, 0.05 mg/kg in complementary and complete feedingstuffs for pigs, and 0.1 mg/kg in complementary and complete feedingstuffs for poultry. Maximum levels have not been established yet, and some countries have appropriate regulation about this subject.

Table 7. Maximum levels for OTA in foodstuffs (modified from European Commission, 2006a and 2010b).

Foodstuffs	Maximum levels (µg/kg)
Unprocessed cereals	5
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of baby foods and dietary foods for special medical purposes for infants	3
Dried vine fruit (currants, raisins and sultanas)	10
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5
Soluble coffee (instant coffee)	10
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15% vol) and fruit wine	2
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2
Processed cereal-based foods and baby foods for infants and young children	0.5
Dietary foods for special medical purposes intended specifically for infants	0.5
Spices	30 (as from 1.7.2010 until 30.6.2012)
Capsicum spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika)	
Piper spp. (fruits thereof, including white and black pepper)	
Myristica fragans (nutmeg)	15 (as from 1.7.2012)
Zingiber officinale (ginger)	
Curcuma longa (turmeric)	
Mixtures of species containing one or more of the abovementioned spices	
Liquorice (*Glycyrrizha glabra, Glycyrrhiza inflate* and other species)	
Liquorice root, ingredient for herbal infusion	20
Liquorice extract, for use in food in particular beverages and confectionery	80

5. RISK COMMUNICATION

The fundamental goal of risk communication is to provide meaningful, relevant and accurate information, in clear and understandable terms targeted to a specific audience. As a part of the risk analysis, it facilitates the decision alternatives by the risk managers, when communication among all interested parties takes place.

Diferent kinds of organizations are involved in this process: international organizations, governments, industries, consumer organizations, research institutions, and media. The Codex Alimentarius Commission, as well as the FAO, the WHO and the WTO (World Trade Organization) are the leading organizations involved in the risk communication. The WHO in cooperation with the FAO developed the International Food Safety Authorities Network (INFOSAN), to promote the exchange of food safety information and to improve collaboration among food safety authorities at national and international levels.

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II. OBJECTIVES and WORK PLAN

GENERAL OBJECTIVE

This Thesis work takes part of the main research line "Assessment of the exposure to mycotoxins" of the Applied Mycology Unit of the Food Technology Department of the University of Lleida, and was supported by national and international projects:

- The project "Presence of mycotoxins in foods in Catalonia and assessment of the exposure", founded by the Catalan Agency of Food Safety (ACSA).
- The Spanish project AGL 2008-05030-C02-01: "Exposure assessment of the Spanish population to *Fusarium* toxins".
- The BASELINE European project 222738: "Selection and improving of fit-for-purpose sampling procedures for specific foods and risks".

Due to the ubiquity of the mycotoxin ochratoxin A (OTA) and its toxicological characteristics, the general objective of the present work was to develop the exposure assessment step within the frame of the risk analysis (Figure 1), by the evaluation of the exposure to OTA of certain groups of people who live in the Spanish region of Catalonia and the characterization of the risk that this exposure may involve for this population.

Specific objectives were also proposed:

- Measurement of the levels of OTA in biological fluids (blood plasma and urine) in the Catalan province of Lleida, and study of the factors that may influence them.
- Measurement of the levels of OTA in certain foodstuffs sampled in Catalonia.
- Collection of food consumption data.
- Estimation of the daily intake of OTA based on the consumption and contamination data of foodstuffs, as well as on the levels of OTA in blood plasma.
- Comparison of the obtained daily intake estimations with reference intake values such as the latest proposed tolerable daily intakes, in order to obtain a preliminary characterization of the risk due to the observed intake of OTA.

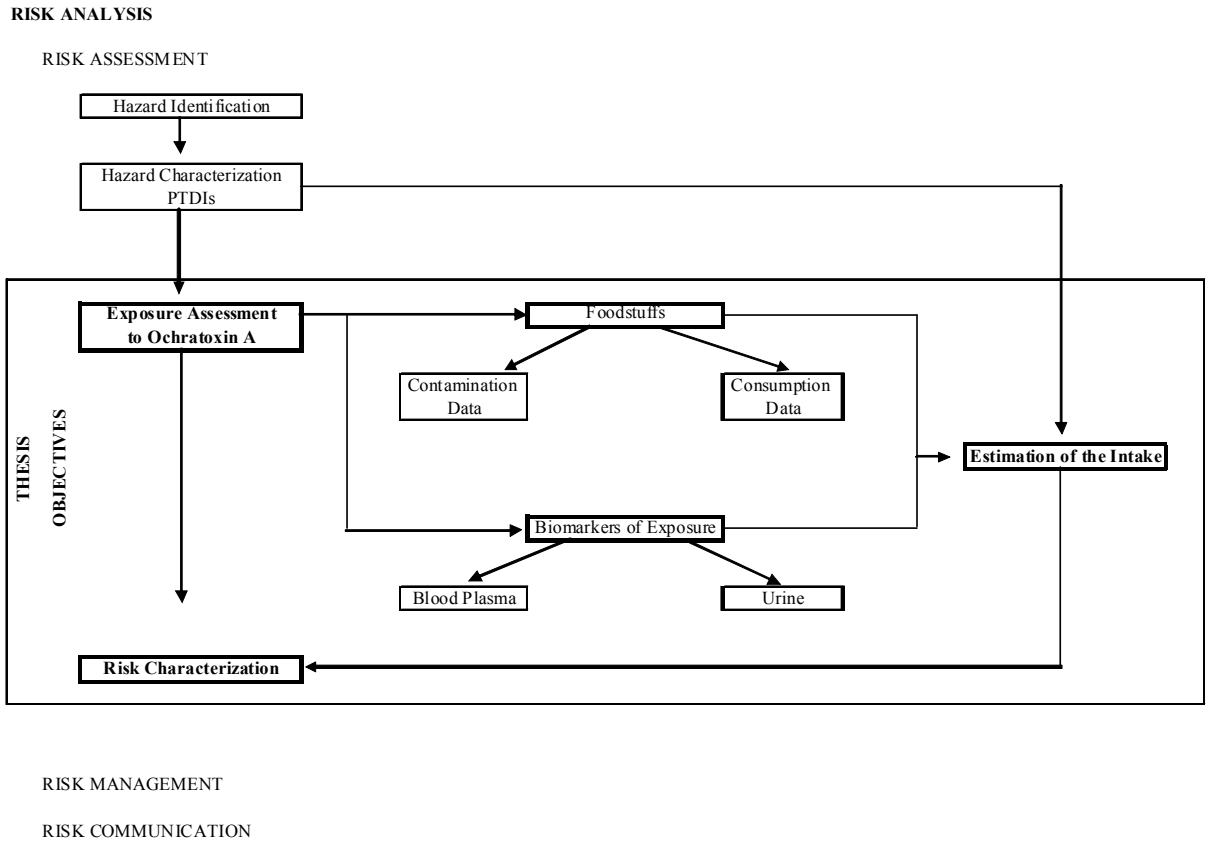


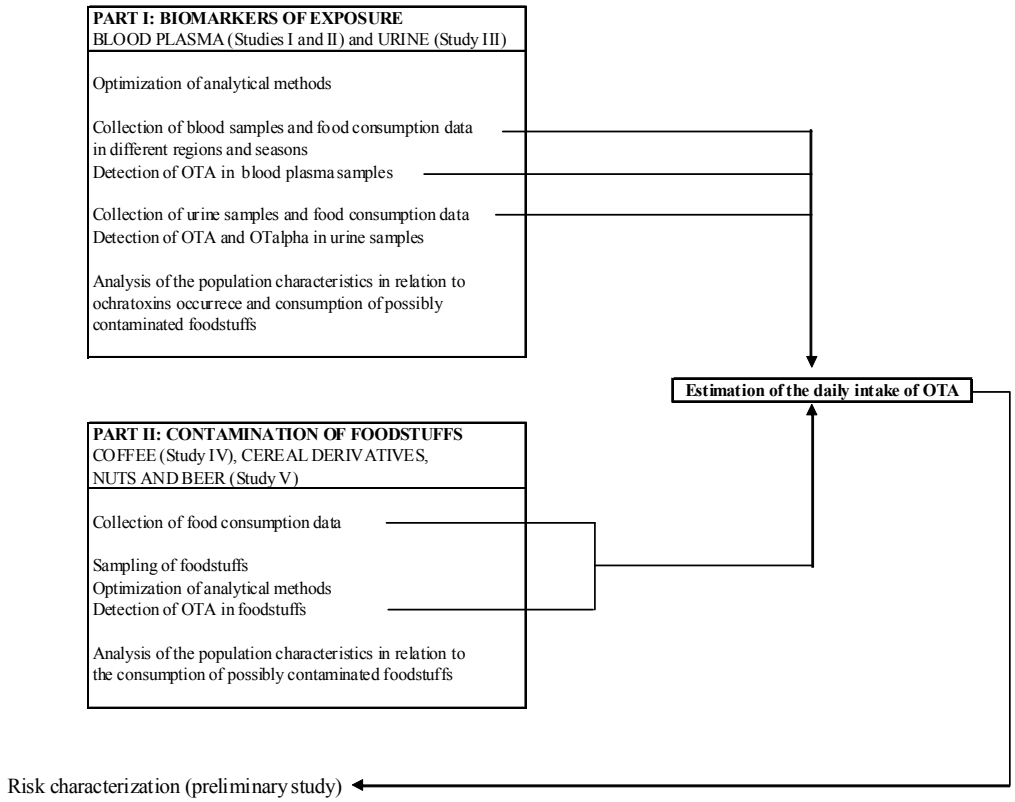
Figure 1. General and specific objectives in the frame of the risk analysis.

WORK PLAN

In order to achieve the mentioned objectives, the following work plan was proposed (Figure 2):

Bibliographic revision

Research work: Exposure assessment



Preparation of the Thesis document

Figure 2. Work plan.

In the studies on biomarkers, two independent samplings for blood collection were carried out with different donors in 2008 and 2009. Urine samples were obtained from persons different from those that had donated blood. The number of samples collected for the studies on biomarkers is shown in Table 1. Moreover, food consumption data used in the biomarkers studies (Part I) were obtained from the participants of the respective studies.

Table 1. Number of samples in the biomarkers studies.

	Part I: Biomarkers of exposure		
	Blood plasma		Urine
Studied population	Study I	Study II	Study III
Adults: only donors	279	325	72

Food consumption data used in the studies on food contamination (Part II) were obtained from the blood donors and also from other inhabitants of the province of Lleida, even though they were not blood donors, and therefore during a more extended period of time than the period of sampling of blood. In Study V, food consumption data of infants, children, and adolescents were also included (Table 2).

Table 2. Number of persons interviewed for consumption of OTA possibly contaminated foodstuffs.

Studied population	Part II: Contamination of foodstuffs	
	Study IV	Study V
Adults: blood donors and non-donors	885	905
Infants	-	164
Children	-	68
Adolescents	-	211

III. RESEARCH WORK

III. RESEARCH WORK

PART I:
Biomarkers of exposure

STUDY I

Assessment of the exposure to ochratoxin A in the province of Lleida, Spain

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ABSTRACT

Exposure to ochratoxin A (OTA) of 279 blood donors of nine localities of the province of Lleida (Spain) was assessed. OTA levels were detected in the blood plasma of the participants by HPLC-fluorescence detection with previous clean-up of the samples by immunoaffinity columns. Limit of detection was 0.075 ng/mL. Participants answered a questionnaire on consumption frequency of foods possibly contaminated with OTA. Foodstuffs were grouped: cereals and derived products, dried fruits and derived products, cacao and derived products, grape juice, wine, beer and coffee. The range of positive samples was 0.11 to 8.68 ng/mL and the median was 0.54 ng/mL. No differences were found between OTA plasma levels in men and women, neither in the different localities, but there were significant differences among three age groups. Highest consumed foods were cereals and derived products, followed by beer and wine. No correlation was found between food consumption and OTA plasma levels. OTA daily intake was estimated based on OTA plasma concentrations and on the food consumption data combined with food contamination data taken from the literature. Mean values were 1.69 and 1.96 ng/kg body weight/day, respectively. These values are below the latest proposed tolerable daily intake of 14 ng/kg body weight/day.

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1. INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that has nephrotoxic, immunotoxic, teratogenic and carcinogenic effects in animals. The main target organs for OTA toxicity are kidney and liver. In humans, it has been related to the etiology of the Balkan endemic nephropathy (BEN) and the outbreak of urinary tract tumours. IARC (International Agency on Cancer Research) has classified OTA as "possible human carcinogen (Group 2B)" (IARC, 1993). OTA is a toxic secondary metabolite of the fungi *Aspergillus* section *Circumdati, Aspergillus* section *Nigri*, *Aspergillus* section *Flavi, Penicillium verrucosum* and *Penicillium nordicum.* In tropical and subtropical climates the main OTA producers are species of *Aspergillus,* whereas in temperate climate the main producer is *Penicillium verrucosum* (Breitholtz et al., 1991). OTA can be found in a large number of foods, such as cereals and derived products, pulses, coffee, cacao, dried fruits, spices, vine fruit, wine, grape juice and beer. Animal feed can be also contaminated by OTA. As the toxin can be accumulated in animal tissues or excreted in milk, it is possible to find contaminated foodstuffs of animal origin, as pork, chicken, eggs and dairy products. This means that human exposure to this mycotoxin can be chronic.

Exposure of a population to OTA can be assessed in different ways, such as the determination of the levels of the toxin in human blood plasma and the estimation of the daily1intake of the toxin. Daily intake (DI, ng/kg body weight/day) of a study population can be estimated taking into account these plasma levels by means of the Klaassen equation (Klaassen, 1986). Estimation can also be done taking into account food consumption and food contamination data. Tolerable daily intakes (TDIs, ng/kg body weight/day) of the toxin have been proposed: 5 (The Nordic Group on Food Toxicology and Risk Evaluation, 1991; Scientific Committee on Food, 1998), 17 (EFSA, 2006) and 14 (JECFA, 2007).

In this paper we studied the relationship between OTA plasma levels and individual characteristics (sex, age and geographical location) of healthy inhabitants of some locations of the province of Lleida (Spain). Food consumption of OTA possibly contaminated food was assessed, and OTA DI was estimated. DI estimations were based on the inhabitants OTA plasma levels and on their consumption habits and food contamination data taken from the literature, and the results were compared it with the proposed TDIs.

2. MATERIALS AND METHODS

2.1. Collection of blood samples

Blood samples were collected during blood donation campaigns carried out in nine locations of the province of Lleida by the "Banc de Sang i Teixits" (blood bank) of the hospital Arnau de Vilanova of the city of Lleida. The collection period was from March to May 2008 in the cities of Alcarràs (n = 21), Almacelles (n = 30), Arbeca (n = 31), Artesa de Segre (n = 31), Balaguer (n = 25), Les Borges Blanques (n = 33), Lleida (n = 77), Tàrrega (n = 22) and Torà (n = 9). The distance between the furthest cities was 102 km. The total number of samples was 279, of which 132 were from men (44 aged 18 to 29, 45 aged 30 to 44, 43 aged 45 and more) and 147 from women (36 aged 18 to 29, 49 aged 30 to 44, 62 aged 45 and more). Availability of samples varied in each city, as samples were taken in accordance with the blood bank schedule for each location. Identity of the participants remained anonymous. This study was submitted and approved by the Ethic's Committee of the Hospital Vall d'Hebron, Spain.

Blood donors were informed about this study and were invited to participate by means of the donation of an extra amount of blood. Participants gave their written consent. 20 mL of blood were collected in Vacutainers containing EDTA as anticoagulant. Plasma was separated by centrifugation at 1000*g* for ten minutes at 4ºC and stored at -25ºC until analysis.

2.2. Analysis of OTA

Extraction: OTA was extracted from plasma according to the method proposed by Zimmerli and Dick (1995) with some modifications. 2 mL of plasma were mixed in a glass centrifuge tube with 10 mL of a solution containing 33.7 mL of 85% ortophosphoric acid and 118 g of sodium chloride per litre. Mixture was vortexed for one minute. After addition of 5 mL chloroform and two minutes vortexing, mixture was centrifuged for fifteen minutes. Organic phase was withdrawn and transferred to a 50 mL ambar flask. Extraction with 5 mL chloroform was repeated and the combined organic extracts were evaporated to dryness in a rotary evaporator.

Clean-up by immunoaffinity column (IAC): Dry extract in ambar flask was resuspended with 5 mL of a mixture of PBS (phosphate buffered saline, solution containing 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4, 8 g NaCl and 1 L H2O, pH = 7.4): acetonitrile, 95:5, by thoroughly rinsing the walls of the flask. Resuspended extract was loaded onto the IAC (Ochraprep, R-Biopharm, Rhône LTD) and allowed to pass through the column by gravity. This operation was repeated 3 times with 5 mL PBS:acetonitrile until a total volume of 20 mL. Afterwards, IAC was washed with 20 mL PBS and dried by passing air through it. OTA was eluted in an ambar vial with 1.5 mL desorption solution (acetic acid:

methanol, 2:98). Backflushing was done 3 times during the passage of the eluant through the IAC. 1.5 mL Milli-Q water was passed to obtain a volume of 3 mL in the collecting vial. Finally air was passed to collect the last drops of eluate.

Chromatographic analysis: High performance liquid chromatography (HPLC) was performed using a Waters 2695 Separations Volume (Alliance) coupled to the Waters 2475 Multi λ fluorescence detector. Waters Spherisorb ODS2 C18 column (5 μm, 4.6 x 150 mm) equipped with a Waters Spherisorb ODS2 guard column (5 μm, 4.6 x 10 mm) (Waters, Ireland) was used. The integration software used to manage the chromatographic data was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00). Mobile phase consisted of acetonitrile:Milli-Q water:acetic acid (51:47:2). Flow-rate was 1 mL/min. Injection volume was 100 μL. Excitation and emission wavelengths were 333 nm and 443 nm, respectively. Temperature of column and guard column was maintained at 40ºC. Retention time for OTA was 5.4 min.

Limit of detection and quantification were calculated using the signal-to-noise ratio of 3 and 10, respectively. Limit of detection was 0.075 ng/mL and limit of quantification was 0.23 ng/mL. Samples were considered as positive when their concentration was above the limit of detection. For calculations, concentrations of samples under the detection limit were considered as half the value of the limit of detection. Recovery rates for spiked samples (n = 5) at the levels of 0.2, 0.5, 1 and 5 ng OTA per mL plasma were 83%, 68.8%, 85.1% and 72%, respectively, with a coefficient of variation of 19.9%. Results have not been corrected for recovery. Repeatability was tested in the same day on five replicates at the spiking level of 1 ng/mL, and presented a relative standard deviation of 10%. Reproducibility was assessed in three different days also at 1 ng/mL, and the relative standard deviation was 9%.

Confirmation of OTA identity: positive samples were confirmed by derivatization of the toxin to its methyl ester according to the method described by Zimmerli and Dick (1995) with some modifications. Briefly, 1 mL of the sample obtained after IAC clean-up was evaporated. 2.5 mL methanol and 0.1 mL hydrochloric acid were added, the vial was closed and kept overnight at room temperature. The mixture was evaporated to dryness and resuspended in 1 mL desorption solution: Milli-Q water, 50:50. Derivatized samples were analyzed by HPLC in the same conditions for OTA analysis. The retention time for the OTA methyl ester was 10.5 minutes.

2.3. Collection of food consumption data

Blood donors were asked to answer a questionnaire about the consumption frequency of foods in which OTA is known or suspected to occur. The questionnaire included 32 food items, classified in the seven following groups: cereals and derived products (twelve items: breakfast cereals, bread, sandwich bread, pasta, stuffed pasta, rice, cookies, bakery products, cakes, pizza, “coca de recapte”, i.e. a local product similar

to pizza, snacks), dried fruits and derived products (eight items: almonds, hazelnuts, sunflower seeds, walnuts, pistachios, peanuts, nougat, marzipan), cacao and derived products (five items: chocolate powder, thick drinkable chocolate, bar chocolate, chocolates, chocolate nougat), grape juice (two items: pure and mixed with other fruits), wine (two items: red and sweet), beer, and coffee (three items: black coffee, espresso with milk, white coffee).

In this survey, participants indicated frequency and quantity of consumption of the listed foods for the period of one year. Standard portions of each food were defined and showed to the participants by means of a photographic album. Food consumption was calculated as grams of food per person and day from data of the food consumption questionnaires. Calculation of OTA consumption took into account food contamination data from the references listed in Table 1.

Table 1. OTA levels (ng/g) in certain food groups.

Food group	Mean OTA levels	Reference
Cereals and derived products	0.29	European Commission (2002)
Dried fruits and derived products	0.87	Food Standards Agency (2002)
Cacao and derived products	0.24	European Commission (2002)
Grape juice	0.55	European Commission (2002)
Wine	0.36	European Commission (2002)
Beer	0.03	European Commission (2002)
Coffee	0.72	European Commission (2002)

2.4. Calculation of OTA daily intake based on OTA plasma levels

Daily intake of OTA (k_0, ng OTA/kg body weight per day) can be estimated based on plasma OTA levels by means of the Klaassen equation (Klaassen 1986): $k_0 = Cl_p \times C_p/A$, where Cl_p is plasma clearance (mL/kg body weight per day); C_p is the plasma concentration of OTA (ng/mL) and A is the bioavailability of OTA. Cl_p value of 0.99 mL/kg bw/day was derived by Miraglia et al. (1996) from the proposed by Studer-Rohr et al. (1995). A was estimated as a 50% (European Commission, 2002). Thus, we estimated DI by means of Klaassen equation taking into account the proposed coefficients:

$$k_0 = 0.99 \times C_p/0.5 = 1.97 \times C_p$$

2.5. Calculation of OTA daily intake based on food consumption and food contamination data

OTA daily intake (ng OTA/kg body weight/day) was calculated as the consumption of the toxin (ng OTA/day/person) divided by the body weight (kg bw). In this case, an average body weight of 60 kg was assumed.

2.6. Statistical analysis

Normality of OTA plasma levels, food consumption data and intake estimations was tested by the Kolmogornov-Smirnov test. Non-parametric methods were used to test differences among groups of non-normally distributed data: Wilcoxon-Mann-Whitney rank sum test to assess differences between two groups, and Kruskal-Wallis test in the case of more than two groups. Distribution data were expressed as percentiles in order to facilitate the comparison of high and low values. Median values were presented as well, because due to non-normal distribution mean values are affected by outliers.

Box plots were used to describe distribution of OTA by age groups and locations. In these graphics, upper and lower limits of a box indicate 25^{th} and 75^{th} percentiles (first quartile or Q1, and third quartile or Q3, respectively). Thus, the box contains the 50% of the central data of the distribution. The line inside the box shows the median. T-shaped lines displayed outside the box are called "whiskers", and represent the distance among the lowest and highest observations within 1.5 times the interquartile range (IQR) and the Q1 and Q3, respectively. Outlier values are more than 1.5 times the IQR away from Q1 and Q3. In the boxplot, these values are represented by a circle. If an outlier is more than three times the IQR away from Q1 or Q3, it is classified as an extreme outlier and is represented in the graph by an asterisk.

Correlation between food consumption and OTA plasma levels was assessed by the Spearman correlation coefficient.

Statistical programs SAS 9.1.3 (Cary, NC, USA) and SPSS 17.0.0 (SPSS Inc.) were used for the analysis.

3. RESULTS

3.1. OTA distribution in plasma of blood donors in the province of Lleida

OTA was detected in the 98.6% of the samples, i.e., only four samples presented OTA levels below the limit of detection. Table 2 shows the distribution of OTA levels found in the study population by gender.

Table 2. OTA plasma levels (ng/mL) in inhabitats of the province of Lleida.

	n	Positive samples (%)	Mean ± SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Men	132	130 (98.5)	0.89 ± 1.03	0.57	0.12	8.03	0.13	2.92	4.05	4.17
Women	147	145 (98.6)	0.83 ± 1.11	0.49	0.11	8.68	0.17	2.42	2.94	7.60
Total	279	275 (98.6)	0.86 ± 1.07	0.54	0.11	8.68	0.16	2.51	4.05	7.60

[1] Minimum
[2] Maximum

Minimum values listed in Table 2 were the minimum detected by the present method of analysis. Only for the calculation of the incidence, values below the limit of detection were considered as negatives. For further data analysis, levels below the limit of detection were expressed as half the limit of detection.

48% of the samples had OTA levels up to 0.5 ng/mL, whereas the 75% of the study population presented concentrations up to 1 ng/mL. For only a 2.5% OTA plasma concentrations were above 6 ng/mL. Distribution of the data was not normal when testing the whole dataset neither by groups of gender or age. No significant differences were found among OTA plasma levels of men and women.

3.1.1. OTA distribution by age groups

Influence of age on OTA plasma concentrations was analysed (Table 3).

Table 3. OTA plasma levels by age groups.

Age group (years)	*n*	Mean ± SD	Median	Min[1]	Max[2]	Percentiles			
						5th	95th	97.5th	99th
A (18-29)	80	0.87 ± 1.19	0.57	0.12	8.03	0.15	2.72	5.04	8.03
B (30-44)	94	0.64 ± 0.55	0.47	0.11	3.04	0.18	1.65	2.47	3.04
C (≥45)	105	1.05 ± 1.29	0.66	0.14	8.68	0.16	2.92	4.17	7.60

[1] Minimum
[2] Maximum

Highest mean values were found in the group of people aged over 45, followed by the first age group. Significant differences were found among the three age groups (Pr > Chi square = 0.0416). No significant differences were found when comparing the groups A and B, neither A and C, but differences were significant between the groups B and C (Pr > Chi square = 0.0139). The higher the age of the participants, the higher the OTA levels in plasma. No significant differences were found between men and women within each age group. Figure 1 shows similar distributions of the groups A and C regarding outliers and extreme values.

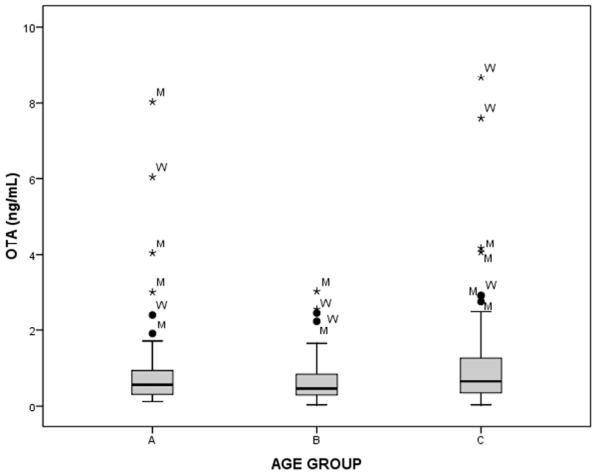

Figure 1. Boxplot of OTA plasma levels by age groups.
Outliers were labelled by sex (M = men, W = women).

3.1.2. OTA distribution by localities

According to the non-parametric test, similar levels of OTA in plasma were found in all the locations. A wider distribution was found in Almacelles, Lleida and Tárrega (Figure 2), with maximum values of 7.60, 8.03 and 8.68 ng OTA/mL plasma, respectively.

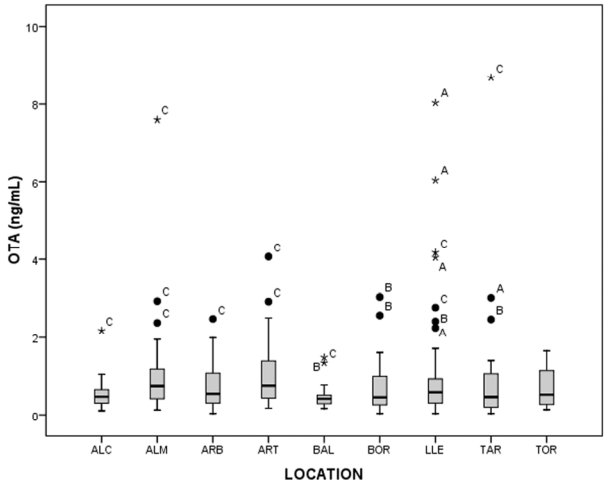

Figure 2. Boxplot of OTA plasma levels in different locations (ALC = Alcarràs, ALM = Almacelles, ARB = Arbeca, ART = Artesa de Segre, BAL = Balaguer, BOR = Les Borges Blanques, LLE = Lleida, TAR = Tárrega, TOR = Torà). Outliers were labelled by age group (A: 18 to 29 years, B: 30 to 44 years, C: ≥ 45 years).

3.2. Estimation of OTA intake based on OTA levels in plasma

The distribution of estimations of the DI by Klaassen equation is shown in Table 4. Mean value was 1.69 ng/kg bw/day.

3.3. Consumption of possibly contaminated food

Significant differences were found among food consumption of the different groups. Cereals and derived products were the highest consumed, followed by beer and wine. From all the groups, only cereals did not present non-consumption cases. Distribution of the consumption data was different for the studied groups and non-normally distributed (data not shown). Significant differences were found between consumption of men and women (Pr > Chi square = <0.0001). Differences were not significant among age groups and locations.

No significant correlation ($P < 0.05$) was found between food consumption (total and by food groups) and OTA plasma levels, neither when analysis was done by sex, age or location.

3.4. Estimation of OTA intake based on food consumption and on food contamination data from the literature

OTA intake by food groups is shown in Table 4.

Table 4. OTA daily intake estimations (ng/kg bw/day) by gender and for the total study population.

Population	Food groups	Mean ± SD	Median	Min	Max	Percentiles			
						5th	95th	97.5th	99th
Based on plasma levels of the toxin by Klaassen equation									
Men		1.75 ± 2.03	1.13	0.24	15.82	0.26	5.75	7.98	8.21
Women		1.64 ± 2.19	0.97	0.22	17.10	0.33	4.77	5.79	14.97
Total		1.69 ± 2.11	1.05	0.22	17.10	0.32	4.94	7.98	14.97
Based on food consumption and food contamination data									
Men	Cereals and derived products	1.12 ± 0.65	0.97	0.37	6.23	0.50	2.17	2.65	2.88
	Dried fruits and derived products	0.35 ± 0.60	0.18	0.00	5.88	0.01	1.19	1.51	1.73
	Cocoa derived products	0.07 ± 0.08	0.04	0.00	0.37	0.00	0.23	0.25	0.33
	Grape juice	0.09 ± 0.30	0.00	0.00	2.23	0.00	0.65	0.92	1.83
	Wine	0.62 ± 0.91	0.32	0.00	4.70	0.00	2.43	3.51	4.68
	Beer	0.08 ± 0.11	0.05	0.00	0.66	0.00	0.25	0.42	0.66
	Coffee	0.11 ± 0.10	0.09	0.00	0.67	0.00	0.30	0.37	0.45
	Total	2.43 ± 1.34	2.19	0.68	9.14	1.00	4.85	6.22	7.07

Women	Cereals and derived products	0.68 ± 0.34	0.65	0.03	2.12	0.24	1.45	1.61	1.79
	Dried fruits and derived products	0.33 ± 0.51	0.17	0.00	3.96	0.00	1.15	1.88	2.91
	Cocoa derived products	0.07 ± 0.19	0.03	0.00	1.10	0.00	0.24	0.32	0.55
	Grape juice	0.08 ± 0.34	0.00	0.00	2.75	0.00	0.39	0.92	2.75
	Wine	0.22 ± 0.38	0.02	0.00	2.34	0.00	1.17	1.17	1.19
	Beer	0.02 ± 0.05	0.00	0.00	0.38	0.00	0.11	0.21	0.25
	Coffee	0.13 ± 0.12	0.08	0.00	0.75	0.00	0.37	0.37	0.60
	Total	1.54 ± 0.79	1.34	0.42	4.68	0.64	2.96	3.79	4.15
Total	Cereals and derived products	0.89± 0.56	0.77	0.03	6.23	0.31	1.73	2.17	2.69
	Dried fruits and derived products	0.34 ± 0.55	0.18	0.00	5.88	0.01	1.19	1.64	2.91
	Cocoa derived products	0.07 ± 0.10	0.03	0.00	1.10	0.00	0.24	0.30	0.38
	Grape juice	0.08 ± 0.32	0.00	0.00	2.75	0.00	0.52	0.92	2.23
	Wine	0.41 ± 0.71	0.10	0.00	4.70	0.00	2.34	2.43	3.51
	Beer	0.05 ± 0.09	0.01	0.00	0.66	0.00	0.19	0.28	0.42
	Coffee	0.12 ± 0.11	0.08	0.00	0.75	0.00	0.31	0.37	0.60
	Total	1.96 ± 1.17	1.61	0.42	9.14	0.69	3.97	4.85	6.68

Intake of OTA was significantly different among food groups (Pr > Chi square = <0.0001), as well as between men and women (Pr > Chi square = <0.0001). Differences were not significant among age groups and locations. As reported before, main OTA intake by the whole studied population was due to cereals, followed by wine (European Commission, 2002), dried fruits and coffee, with minor contributions of the remaining food groups. Cereals were the main source for OTA intake for both men and women, being higher for men more than 1.5-fold. OTA intake due to wine by men was almost three times higher than its intake by women, whereas the toxin intake due to beer consumption was four times higher in men than in women. Mean daily intakes were similar in the rest of the food groups.

3.5. Comparison of the estimated DIs

Estimation of DI based on food consumption and contamination data was significantly different (Pr > Chi square = <0.0001) from DI estimation based on OTA plasma concentrations calculated by means of Klaassen equation (Table 4). Mean DI estimated from food data was higher than DI estimated from plasma; however, 97.5th and 99th percentile estimations were higher based on the latter method. Mean values and high percentiles were lower than the latest proposed TDIs of 17 ng/kg body weight/day (EFSA, 2006) and 14 ng/kg body weight/day (JECFA, 2007). However, DI estimation based on OTA plasma levels in the 99th percentile was above the TDI proposed by JECFA (2007), and the results for 97.5th and 99th percentiles were above the PTDI of 5 ng/kg body weight/day (The Nordic Group on Food Toxicology and Risk Evaluation, 1991; Scientific Committee on Food, 1998).

3.6. Outliers evaluation

Data corresponding to the outliers derived from the age groups analysis (Figure 2) have been evaluated. Thus, the number of analysed points was 16 (ten men and six women). Six people belonged to the age group A, four to the age group B and six to the age group C. OTA plasma levels for these outliers ranged from 1.91 to 8.68 ng/mL. When comparing the OTA total DI estimated by the food consumption and food contamination data, it was observed that these estimations did not approach the estimated DI values by plasma concentrations except in only one case. This fact matches with the significant difference found among the two estimations of the DI obtained for the whole studied population.

4. DISCUSSION

OTA was present in the 98.6% of the plasma population, which shows how wide the exposure to the toxin is, and also that it is possible to find people who present levels below the limit of detection of our method.

Comparison of the incidence and mean levels of the present results with other studies is difficult, as the limits of detection of the analytical methods are different in each case. In Spain, the mean level found in Lleida (0.86 ng/mL) was lower than the mean value found in Madrid (1.19 ng/mL) (Burdaspal and Legarda, 1998) and higher than the mean values in Navarra (0.71 ng/mL) (Jimenez et al., 1998) and Granada (0.63 ng/mL) (Pérez de Obanos et al., 2001). Regarding other countries, mean values were higher than those found in Argentina (Pacin et al, 2008), Chile (Muñoz et al., 2006), Croatia (Peraica et al., 2001), Czech Republic (Ruprich and Ostry, 1993a and 1993b, Malir et al., 2001), Italy (Breitholtz-Emmanuelson et al., 1994, Palli et al, 1999), Japan (Ueno et al., 1998), Lebanon (Assaf et al., 2004), Morocco (Filali et al, 2002), Norway (Thuvander et al., 2001, Skaug, 2003), Sweden (Breitholtz et al., 1991, Thuvander et al., 2001) and Switzerland (Zimmerli and Dick, 1995); and lower than the levels found in Canada (Scott et al., 1998) and the UK (Gilbert, 2001). An alternative way is to compare the ranges of concentrations found. Thus, in Spain, values of OTA concentration in plasma (ng/mL) ranged from 0.22 to 6.96 in Granada (Pérez de Obanos et al., 2001), 0.12 to 5.58 in Madrid (Burdaspal and Legarda, 1998) and 0.52 to 4.00 (Jimenez et al., 1998). Maximum levels found in our study were higher than the ones found in the named regions of this country. Moreover, more than considering mean levels of the whole population, attention should be paid to the results of the high percentiles of population (risk population). In the present study, the estimated DI calculated from plasma levels for the 97.5^{th} and 99^{th} percentiles were higher than the lowest PTDIs of 5 ng/kg body weight/day (The Nordic Group on Food Toxicology and Risk Evaluation, 1991; Scientific Committee on Food, 1998). It should be noted that the study population consisting of only blood donors is not representative of the whole population, as children and elder people did not participate in the study.

Lack of differences between OTA plasma levels in men and women agrees with other studies (Breitholtz et al., 1991; Thuvander et al., 2001; Ueno et al., 1998). Differences among age groups were found, as well as a tendency to find higher OTA levels as the age rose. This result matches with the observed by Pacin et al. (2008), who found the highest levels in the people aged 51 to 60 in one of the two Argentinean cities studied. In Portugal, people over 40 years old presented the highest concentrations of the study done by Lino et al. (2008). Other studies (Jimenez et al., 1998, Ueno et al., 1998, Palli et al., 1999, Gareis et al., 2000, Grosso et al., 2003, Skaug, 2003, Assaf et al., 2004) showed no differences among age groups, although for each case the age groups were defined differently. The absence of differences among the studied localities might be expected as the

longest distance between them is 102 km, thus, small differences in dietary habits may also be expected.

It was not possible to relate the consumption of any food group with the presence of OTA in plasma. The absence of correlation between OTA potentially contaminated food consumption (total and by food groups) and OTA plasma concentrations (total and by population groups) can be due to the heterogeneous distribution of the toxin in foodstuffs, which can be present in very low concentrations or occasionally in high levels. Thus, there is an interest in distribution data regarding OTA contamination in foods at the local level by analysing foods from the local markets, or else the foods actually consumed by the study population. Although food groups were those considered to be potentially contaminated by OTA, nor animal derived products neither spices were included in the questionnaires. Over- or under-reporting of food consumption could have taken place, as the recall period was a year. Considering that it was observed that OTA half-life in plasma was 35.5 days (Studer-Rohr et al., 1995) recall period for the further studies should match with this. An advantage from this is that it will be easier for the participants to remember the consumed quantities. Also the influence of the changes of alimentary habits in the different seasons should therefore be suppressed.

The estimated mean DI of OTA based on plasma concentrations and on food consumption data was under the latest PTDIs. This matches with the fact that in any case in the literature the mean OTA calculated DI was above the PTDIs (Pacin et al., 2008, Scott et al., 1998, Muñoz et al., 2006, Peraica et al., 1999 and 2001, Ruprich and Ostry, 1993a, Palli et al., 1999, Ueno et al., 1998, Assaf et al., 2004, Filali et al., 2002, Lino et al., 2008, Pérez de Obanos et al., 2001, Burdaspal et al., 1998, Breitholtz et al., 1991, Thuvander et al., 2001, Gilbert et al., 2001), not even above the lowest published PTDI of 5 ng/kg body weight/day (The Nordic Group on Food Toxicology and Risk Evaluation, 1991).

Differences between DI estimations agreed with the result of Thuvander et al. (2001), in which estimated intake from food consumption and food contamination data (1.3 to 1.4 ng/kg bw/day) resulted higher than estimated DI based on plasma OTA concentrations (0.24 to 0.28 ng/kg bw/day). Breitholtz et al. (1991) found a good agreement between the results obtained with the two methods of calculation (0.36 and 0.35 ng/kg bw/day, respectively). Duplicate diet studies were performed by Gilbert et al. (2001). In this case, mean DI estimated from food consumed was lower than estimation from plasma concentrations. The European Commission (2002) also estimated the mean DI based on plasma concentration of OTA from six countries (Germany, Italy, Norway, Spain and the UK) and the resulting value was 0.67 ng/kg bw/day. However, when comparing the results of the two approaches, in Germany, Sweden and Norway the estimated DIs from plasma were lower than those deriving from food consumption, whereas the opposite result was obtained in Spain and in the UK. Thus, there is no clear tendency in the correlation of the

estimations. Normalization of the methods of data acquirement could be a good step for achieving comparable results. Besides, the proposed way of estimating OTA DI from plasma concentrations using the Klaassen equation involved approximations on plasma clearance (i.e., OTA elimination) and bioavailability of OTA. Bioavailability was considered as 50% as obtained for several animals by Hagelberg et al. (1989). The proposed value for OTA clearance was obtained by a single human experiment (Studer-Rohr et al., 1995). Even though coefficients used in Klaassen equation are approximated, the estimation of the OTA daily intake cannot be influenced by human bias as the estimation based in the participants' consumption data can be.

No differences were found for gender in DI estimation based on plasma, though estimated DI based on food consumption was different for men and women, as the amount of food consumed was higher in the first group. A reason for this can be a different metabolism of OTA for each gender that affects the presence of OTA in human plasma.

5. CONCLUSIONS

Our results indicate that the mean DI in the studied locations of the province of Lleida was lower than the latest PTDIs. However, as high percentiles showed higher values of DI, food and feed contamination should be controlled as a step for minimizing exposure to OTA. Exposure assessment methods should be improved. Regarding DI estimation based on plasma, further studies can be done on the kinetics of OTA in humans. This would be a good advance on exposure assessment, as it requires a single determination per person, and saves all the problems associated with the food sampling methods and consumption data collection.

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STUDY II

Ochratoxin A in adult population of Lleida, Spain: Presence in blood plasma and consumption in different regions and seasons

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ABSTRACT

Ochratoxin A (OTA) levels in blood plasma, as well as the consumption of possibly contaminated foodstuffs by adult inhabitants, were determined in three seasons in the plain and the mountain regions of the province of Lleida (Spain). Daily intake of the toxin was estimated in order to evaluate the exposure of the studied population. OTA was extracted from plasma through liquid-liquid extraction followed by immunoaffinity chromatography columns clean-up. Detection was done through HPLC-fluorescence, and limit of detection was 0.018 ng/mL. Consumption data of the participants were obtained by means of a food frequency questionnaire. Occurrence of OTA in plasma was 100%. Range was 0.06-10.92 ng/mL, and median was 0.50 ng/mL. Differences between genders, regions or seasons were not significant, whereas significant differences were found among age groups. Regarding food consumption, significant differences were found between genders, but not between age groups, regions, or seasons. OTA plasma levels were not correlated with food consumption. Distributions of the intake estimations based on plasma levels differed from those based on food consumption and contamination. Mean and median values of the daily intake estimations were below the latest provisional tolerable daily intake of 14 ng/kg bw/day, but some high percentiles were above it.

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1. INTRODUCTION

Ochratoxin A (OTA) is a toxic secondary metabolite produced by certain species of the fungal genera *Aspergillus* and *Penicillium.* This mycotoxin can be found in many foodstuffs from vegetal origin, as it can be produced during the growth period of the plants, as well as during the storage of the harvested commodities. The possibly contaminated foodstuffs are cereals and derived products, coffee, wine, grape juice, beer, cocoa, spices, and dried fruits. OTA can also be present in animal feed, and therefore in some products of animal origin. Due to its ubiquity, chronic ingestion of this toxin by the population is possible.

Toxicity studies in animals revealed OTA to be nephrotoxic, hepatotoxic, genotoxic, immunosuppressive, and carcinogenic, being the kidney its main target. In humans, its effect has been related to the aetiology of the Balkan Endemic Nephropathy and the appearance of urinary tract tumours. The IARC (1993) classified OTA as a possible human carcinogen (group 2B).

Assessment of the exposure to OTA by the population has been done previously by different methods, which are mainly the determination of the presence of the toxin in foodstuffs and its consumption, as well as the analysis of biomarkers (blood, urine). In addition, it is possible to estimate the daily intake of OTA through food consumption and contamination data, and also through the levels of OTA in blood plasma by means of the Klaassen equation (Klaassen, 1986). In order to complete the exposure assessment, the calculated value should then be compared with the Provisional Tolerable Daily Intake (PTDI), a value that is proposed by certain health authorities. The latest PTDIs were proposed by EFSA (2006) and JECFA (2007), and the values were 17 and 14 ng/kg bw/day, respectively.

Presence of OTA in plasma of inhabitants of different countries, as well as the estimation of the OTA daily intake (when possible), are listed in Table 1. Most studies determined OTA plasma levels in a certain period of time, but only some of these investigations analysed seasonal and regional variations in the investigated populations. In the province of Lleida, two geographic regions can be distinguished: the plain region and the mountain region. The mentioned study in Lleida determined OTA levels in different localities of the plain region in a period of three months. In the present study, the aim was to evaluate the possible variation in OTA plasma levels of adult inhabitants due to sampling in different regions and seasons, as well as in the consumption of possibly contaminated foodstuffs, taking into account population characteristics (sex and age). In addition to this, daily intake of OTA was estimated based on both OTA plasma levels and food consumption and contamination data.

Table 1. Presence of ochratoxin A in human blood plasma of apparently healthy inhabitants of different countries and estimation of its daily intake (when done).

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Algeria	n.a.	0.1	346	66.9	n.a.-9.0 (2.8)	-	Khalef et al., 1993
Argentina	2004-2005	0.012	435	63	<0.012-74.8	-	Pacin et al., 2008
General Rodriguez			236	63.8	<0.012-47.6 (0.1537)	0.32[a]-0.47[b]	Pacin et al., 2008
Mar del Plata			199	62.3	<0.012-74.8 (0.4319)	0.15[a]-0.21[b]	Pacin et al., 2008
Bulgaria	1984, 1986, 1989, 1990	1-2	360	11	1-27.2	-	Petkova-Bocharova and Castegnaro, 1991
	n.a.	0.1	16	100	0.2-10.4 (1.59)	-	Petkova-Bocharova et al., 2003
Canada (15 localities)	1994	0.12	144	100	0.29-2.37 (0.88)	1.2[a]-1.7[b]	Scott et al., 1998
Chile	2004	0.1	88	70	0.07-2.75	-	Muñoz et al., 2006
Colbún			44	50	0.07-2.75	0.84[b]	Muñoz et al., 2006
San Vicente de Tagua			44	91	0.22-2.12	1.4[b]	Muñoz et al., 2006
Costa Rica	n.a.	0.025	149	95	0.01-1.906 (0.622)	-	Quintana Guzman et al., 2007
Croatia	1997-1998	0.2	198	44	<0.2-1.3 (0.19)	0.26[a]	Domijan et al., 1999
	1997	0.2	983	47.6	(0.30)	0.40[a]	Peraica et al., 2001
	March	0.2	242	53.7	(0.36)	0.48[a]	Peraica et al., 2001
	June	0.2	249	59.4	(0.39)	0.52[a]	Peraica et al., 2001
	September	0.2	242	41.7	(0.25)	0.34[a]	Peraica et al., 2001
	December	0.2	250	35.6	(0.19)	0.25[a]	Peraica et al., 2001

Table 1 *(Continued)*

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Croatia	1985-1993	2	2566	0-2.4	0-10	-	Radić et al., 1997
Former Czechoslovakia	n.a.	0.1	143	24.5	<0.1-1.26	-	Fukal and Reisnerova, 1990
Czech Republic	1991-1992	0.1 - 0.5	594	40	0.05-37 (0.625)	0.74[c]	Ruprich and Ostry, 1993
	1994	LOQ = 0.1	809	90.7	0.1-13.7 (0.23)	-	Ostry et al., 2005
	1995	LOQ = 0.1	413	97.8	0.1-1.9 (0.24)	-	Ostry et al., 2005
	1997	LOQ = 0.1	398	94.5	0.1-2.1 (0.33)	-	Ostry et al., 2005
	2000	LOQ = 0.1	400	96.3	0.1-2.0 (0.35)	-	Ostry et al., 2005
	2002	LOQ = 0.1	186	95.7	0.1-1.3 (0.36)	-	Ostry et al., 2005
Denmark	1986-1988	0.1	144	54.2	n.d.- 13.2 (1.8)	-	Hald, 1991
	1986				n.d.-9.7 (1.5)	-	Hald, 1991
	1987				n.d.-9.4 (2.3)	-	Hald, 1991
	1988				n.d.-13.2 (1.6)	-	Hald, 1991
Germany							
Oberschleissheim	1977	0.1	165	50.9	0.1-14.4 (0.79)	-	Bauer and Gareis, 1987
Munich	1985	0.1	141	63.1	0.1-1.8 (0.42)	-	Bauer and Gareis, 1987
	1988	0.1	208	68.3	n.a.-8.4 (1.1)	-	Hadlock and Wagner, 1993
	1996-1998	0.06	927	98.1	<0.06-2.03 (0.27)	0.46[d]	Rosner et al., 2000

Germany	1990, 1991, 1995, 1997	0.05	102 (36 persons)	98	0.072-1.290 (0.368)	0.493[a]-0.725[b]	Märtlbauer et al., 2009
Hungary	n.a.	LOQ = 0.2	100	51	0.2-12.9	-	Kovács et al., 1995
	1995	0.2	355	81.8	<0.2-10.0	-	Solti et al., 1997
	1997	0.1	277	77	n.a.-1.4	-	Tápai et al., 1997
Italy	1994-1996	n.a.	138	97	0.12-2.84 (0.56)	0.77[a]	Palli et al., 1999
Ivory Coast	2001, 2004	n.a.	63	35	0.01-2.83 (0.83)	0.42[b]	Sangare-Tigori et al., 2006
	2001		42	47.6	0.01-2.83 (0.35)		Sangare-Tigori et al., 2006
	2004		21	9.5	1.23-2.32 (1.55)		Sangare-Tigori et al., 2006
Japan	1992, 1994, 1995, 1996	-	184	85	0.004-0.278 (0.068)	0.08[e]	Ueno et al., 1998
	1992	0.010	24	96	0.016-0.278 (0.093)		Ueno et al., 1998
	1994	0.004	34	38	0.004-0.263 (0.073)		Ueno et al., 1998
	1995	0.020	69	93	0.024-0.13 (0.062)		Ueno et al., 1998
	1996	0.010	57	98	0.019-0.151 (0.062)		Ueno et al., 1998
Lebanon	2001-2002	n.a.	250	33	0.1-0.87 (0.17)	0.23[a]	Assaf et al., 2004
Morocco	2000	n.a.	309	60	0.08-6.59 (0.29)	0.4[e]	Filali et al., 2002
Norway	1998	0.01	206	100	(0.18)	0.24[a] - 0.36[b]	Thuvander et al., 2001
Poland	1983-1984	1	1065	7.2	n.a.-40 (0.270)	0.4[f]	Golinski et al., 1991
	1983		397	6.3	(0.21)		Golinski et al., 1991
	1984		668	7.8	(0.31)		Golinski et al., 1991

Table 1 *(Continued)*

Country/City	Year/s of sampling	LOD (ng/mL)	*n*	% of positive samples	Range (mean) (ng/mL)	Intake estimation (ng OTA/kg bw/day)	Reference
Portugal	2001-2002	0.1	104	100	0.14-2.49	-	Lino et al., 2008
Coimbra	2002		29		0.19-0.96 (0.42)	0.56[a]	Lino et al., 2008
Verride	2001		31		0.25-2.49 (0.78)	0.59[a]	Lino et al., 2008
Ereira	2001		44		0.14-1.91 (0.44)	1.05[a]	Lino et al., 2008
Spain							
Madrid	1997	0.02	168	100	0.12-5.58 (1.192)	0.26[a]	Burdaspal and Legarda, 1998
Granada	1996-1997	0.22	83	86.8	<0.22-6.96 (0.63)	0.74[c]	Pérez de Obanos et al., 2001
Navarra	1996, 1997, 1998	0.52	75	53.3	0.52-4.00 (0.71)	-	Jimenez et al., 1998
Lleida	2008	0.075	279	98.6	0.11-8.68 (0.86)	1.69[b]	Coronel et al., 2009
Valencia	2008	0.01	168	100	0.15-5.71 (1.09)	1.47[a]-2.16[b]	Medina et al., 2010
Sweden	1989	0.3	297	12.8	0.3- >0.8 (0.1)	0.13[a]	Breitholtz et al., 1991
Östersund			99	29.3		0.04[a]	Breitholtz et al., 1991
Uppsala			99	3		0.03[a]	Breitholtz et al., 1991
Visby			99	6		0.35[a]	Breitholtz et al., 1991
Visby	1997	0.01	200	100	(i0.21)	0.28[a]- 0.42[b]	Thuvander et al., 2001
Switzerland	1992-1993	0.002	368	100	0.06- 6.02 (mean around 0.30)	-	Zimmerli and Dick, 1995
	n.a.	0.05	79 (8 persons)	100	0.20-0.88	-	Studer-Rohr et al., 2000

Tunisia	n.a.	0.1	140	52	0.1-8.8 (0.7-2.2)	-	Maaroufi et al., 1995a
	1991	LOQ = 0.1	25	82	(3.35)		Abid et al., 2003
	1994	LOQ = 0.1	140	77	(2.25)		Abid et al., 2003
	1997	LOQ = 0.1	20	71	(2.6)		Abid et al., 2003
	2000	LOQ = 0.1	20	62	(1.22)		Abid et al., 2003
	1996, 1998	LOQ = 0.1	62	100	0.12-8.06 (0.53)	-	Grosso et al., 2003
	n.a.	n.a.	20	71	0-7.5 (2.6)	-	Hassen et al., 2004
	n.a.	n.a.	20	62	0-3.2 (1.22)	-	Hassen et al., 2004
Tunisia	n.a.	0.125	105	28	0.12-3.4 (0.49)	-	Hmaissia-Khlifa et al., 2008
	n.a.	0.1	44	52.3	0.11-6.1 (0.77)	-	Hmaissia-Khlifa et al, 2010
Turkey	2007-2008	0.025	239	85.1	-	-	Erkekoğlu et al., 2010
	Winter				0.0306-0.887 (0.137)	0.182[a]	Erkekoğlu et al., 2010
	Summer				0.0279-1.496 (0.312)	0.408[a]	Erkekoğlu et al., 2010
United Kingdom	2001	0.1	50	100	0.4-3.11 (1.09)	1.46[a]-2.15[b]	Gilbert et al., 2001
Former Yugoslavia	1981-1989	5	3378	0 - 3.7	n.a.-50	-	Fuchs et al., 1991

n.a.: not available
n.d.: not detected
LOD: limit of detection
LOQ: limit of quantification
[a] According to Breitholtz et al. (1991): $k_0 = 1.34 \times Cp$
[b] According to Miraglia et al. (1996): $k_0 = 1.97 \times Cp$
[c] $k_0 = 1.18 \times Cp$
[d] $k_0 = 1.98 \times Cp$
[e] According to Kuiper Goodman et al. (1993)
[f] According to Mortensen et al. (1983)

2. MATERIALS AND METHODS

2.1. Sampling

A total of 325 blood samples were collected during the blood donation campaigns of the blood bank of the hospital Arnau de Vilanova of the city of Lleida, in different localities of the plain region (n = 243) and mountain region (n = 82) of the province of Lleida, during the months of October and November 2008 (autumn, n = 116), January and February 2009 (winter, n = 98), and July and August 2009 (summer, n = 111). The longest distance between the furthest cities in the plain region of the province (Agramunt, Albatarrec, Alcarràs, Alcoletge, Alfarràs, Alguaire, Artesa de Segre, Linyola, Lleida, Mollerussa, Penelles, Tàrrega, and Torres de Segre) was 73 km, whereas the longest distance between the furthest cities in the mountain region of the province (La Pobla de Segur, La Seu d'Urgell, Vielha) was 125 km. The longest distance between two cities was 176 km. The average elevation of the localities in the plain region was 249 m, and the elevation of the localities in the mountain region was above 500 m.

Blood donors were adults aged 18 to 68. From the total population, 160 participants were men and 165 were women. Participants were also classified in three age groups: A (from 18 to 29 years old), B (30 to 44 years old), and C (over 45 years old). 87 persons belonged to age group A, 116 to age group B, and 122 to age group C. Blood donors were informed about the study and invited to participate by donating 20 mL extra of blood. Volunteers signed a written consent for their participation. This study was approved by the Ethic's Committee of the Hospital Vall d'Hebron, Spain.

2.2. Plasma obtainment

Blood was withdrawn in Vacutainers® containing EDTA as anticoagulant. Plasma was separated by centrifugation at 1000 g for 10 min at 4º C and stored at -25º C until analysis.

2.3. Determination of OTA in plasma

2.3.1. Liquid-liquid extraction

Extraction of OTA from plasma was done as described by Coronel et al. (2009). Briefly, 2 mL of plasma were mixed in a glass centrifuge tube with 10 mL of a solution containing 33.7 mL of 85% orthophosphoric acid and 118 g of sodium chloride per litre, and vortexed for 1 min. 5 mL chloroform were added and the mixture was vortexed for 2 min and afterwards centrifuged for 15 min at 1000 g and 4º C. Separated organic phase was transferred to an amber flask. The extraction with

chloroform was repeated on the aqueous remaining phase, and combined organic extracts in the amber flask were evaporated to dryness in a rotary evaporator.

2.3.2. Clean-up by immunoaffinity chromatography (IAC) columns

5 mL of a mixture of PBS (phosphate buffered saline, solution containing 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4, 8 g NaCl and 1 L H_2O, pH = 7.4):acetonitrile, 95:5 were used to resuspend the dried extract by rinsing the walls of the flask. Liquid extract was passed through the IAC column (Ochraprep, R-Biopharm, Rhône LTD). Washing of IAC column was carried out by three repeated passages of 5 mL of PBS:acetonitrile (95:5) each. Afterwards, IAC column was washed with 20 mL PBS and air was passed through it. OTA was eluted in an amber vial with 1.5 mL methanol:acetic acid (98:2), and backflushing was done three times during elution. Finally, 1.5 mL Milli-Q water was passed through the column and collected together with the previously eluted OTA.

2.3.3. HPLC analysis

High performance liquid chromatography was carried out in a Waters 2695 Separations Module (Alliance) coupled to a Waters 2475 Multi λ fluorescence detector. Waters Spherisorb ODS2 C18 column (5 μm, 4.6 x 150 mm) and guard column (5 μm, 4.6 x 10 mm) were used (Waters, Ireland). Temperature of column and guard column was maintained at 40ºC. Injection volume was 100 μL. Mobile phase was acetonitrile:Milli-Q water:acetic acid (51:47:2), and flow rate was 1 mL/min. Excitation and emission wavelengths were 333 nm and 443 nm, respectively. Retention time for OTA was 5.4 min. The integration software used was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00).

2.3.4. Method validation

The validity of the analytical method was evaluated through its linearity, limit of detection, limit of quantification, intra-day repeatability, inter-day repeatability, and recovery rates.

The calibration curve was prepared by the serial dilution of a stock solution, and was linear in the range of 0.012 to 12.5 ng/mL (determination coefficient R^2 was 0.999). Limit of detection was 0.018 ng/mL and limit of quantification was 0.060 ng/mL. Both were determined using the signal-to-noise ratio of 3 and 10, respectively. Samples were considered as positive when their concentration was above the limit of detection. Recovery rates for the spiking levels of 0.06, 0.2, 0.5, 1, and 5 ng/mL were 69%, 78.4%, 81.2%, 85%, and 76.3%, respectively (n = 3). Results were not corrected for recovery. Intra-day repeatability was tested at the same day for each spiking level, and inter-day repeatability was assessed in three

different days at the spiking level of 1 ng/mL. Relative standard deviation intra- and inter-day was less than 10% in all cases.

2.4. Collection of food consumption data

The participants of the present study completed a food frequency questionnaire in which they indicated the frequency of consumption of certain possibly OTA contaminated foodstuffs in a one-year period. The questionnaire included 32 food items, classified in the seven following groups: cereals and derived products, dried fruits and derived products, cacao and derived products, grape juice, wine, beer, and coffee. Individual foodstuffs were listed in Coronel et al. (2009). Standard portions of each food were defined and showed to the participants by means of a photographic album.

2.5. Food consumption calculation

Individual consumption of possibly contaminated food (g foodstuff/person/day) was calculated from data of the food frequency questionnaires taking into account the frequency and amount of consumption of foodstuffs, and the weight of the food portions.

2.6. Estimation of the daily intake of OTA

2.6.1. Based on OTA plasma levels

Klaassen (1986) described the concentration of a chemical in plasma as well as in other tissues after repeated exposure. The "average" concentration (C^{av}_{∞}) could be described by the equation:

$$C^{av}_{\infty} = f \times D_{oral} / Cl_b \times \tau$$

Where f is the fraction absorbed, D_{oral} is the oral dose, Cl_b is the total body clearance, and τ is the constant time between administration or exposure. No units were stated in this equation.

Bretiholtz et al. (1991) adapted this equation to the estimation of the continuous mean daily intake of OTA (k_0, ng OTA/kg bw/day) as the following:

$$k_0 = Cl_p \times C_p/A,$$

Where Cl_p is the plasma clearance (mL/kg bw/day), C_p is the plasma concentration of OTA (ng/mL), and A is the bioavailability of OTA. These authors utilised the values of Cl_p and A as 0.67 mL/kg bw/day and 50%, respectively, and the equation to estimate the daily intake of OTA from the plasma levels remained

$k_0 = 1.34 \times C_p$. The values of the coefficients were derived taking into account the toxicokinetic data published by Hagelberg et al. (1989). Later on, Miraglia et al. (1996) took into account the value of 0.048 mL/min obtained by Studer-Rohr (1995) for the renal clearance of radioactivity during the elimination phase in an experiment with a human volunteer, and thus suggested the value of Cl_p as 0.99 mL/kg bw/day (if the body weight is 70 kg bw). Considering A = 50%, the proposed equation to estimate OTA daily intake is $k_0 = 1.97 \times C_p$. In another study, Ruprich and Ostry (1993) and Pérez de Obanos et al. (2001) considered the biological availability of OTA in monkey (*Macaca mulata*) (57%, Hagelberg et al., 1989) and the value of 0.67 mL/kg bw/day for Cl_p. Then, $k_0 = 1.18 \times C_p$. To our present knowledge, the above mentioned are the ways to estimate OTA daily intake when OTA plasma levels are available, and this estimation has been done mostly taking into account the coefficients proposed by Breitholtz et al. (1991) and Miraglia et al. (1996).

Additionally, Studer-Rohr et al. (2000) calculated the whole body clearance for radioactive OTA and metabolites/conjugates in an experiment with a human volunteer, and its value was 0.0935 mL/min. Taking this value into account, and the bioavailability of OTA in monkey (57%) as the best approximation to human, Klaassen equation adapted to the estimation of the OTA daily intake would be: $k_0 = 236.21 \times C_p/W$ (ng/kg bw/d), where W is the body weight (kg bw). Considering that the body weight of the volunteer in the experiment by Studer-Rohr (2000) was 62 kg, then $k_0 = 3.81 \times C_p$ (ng/kg bw/d). In the present analysis, daily intake of OTA will be estimated according to $k_0 = 236.21 \times C_p/W$, as both plasma OTA levels and individual body weights were available (estimation method A). OTA daily intake will also be calculated according to Miraglia et al. (1996) in order to compare estimations with previous studies (estimation method B).

2.6.2. Based on food consumption and contamination data

Consumption data provided by the participants together with previously published food contamination data (Table 2) were used to calculate the OTA daily intake (ng OTA/kg bw/day), as the food consumption (g foodstuff/person/day) divided by the individual body weight (kg bw) and multiplied by the food contamination (ng OTA/g foodstuff).

Table 2. OTA levels (ng/g) in certain food groups.

Food group	Mean OTA levels	Reference
Cereals and derived products	0.29	European Commission (2002)
Dried fruits and derived products	0.87	Food Standards Agency (2002)
Cacao and derived products	0.24	European Commission (2002)
Grape juice	0.55	European Commission (2002)
Wine	0.36	European Commission (2002)
Beer	0.03	European Commission (2002)
Coffee	0.72	European Commission (2002)

2.7. Statistical analysis

Normality of distributions (plasma levels of OTA, food consumption, and daily intake estimations) was tested by the Shapiro-Wilk test. Differences among medians of the population groups were evaluated by the Wilkoxon/Kruskal-Wallis non-parametric test. Correlations were analysed by the Spearman correlation coefficient. JMP® 8.0 (2008 SAS Institute) was used for the analysis.

3. RESULTS AND DISCUSSION

3.1. Occurrence of OTA in plasma in the province of Lleida

OTA was detected in all the samples (n = 325). Descriptive statistics of the total population classified by gender and age are listed in Table 3.

Table 3. Distribution of OTA plasma levels (ng/mL) according to gender and age.

	n	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Total	325	0.80	1.01	0.50	0.06	10.92	0.15	2.11	2.71	5.93
Men	160	0.79	0.90	0.50	0.06	7.13	0.13	1.99	2.71	4.29
Women	165	0.81	1.12	0.49	0.06	10.92	0.16	2.15	2.69	5.04
Age group										
A (18-29 years)	87	0.63	0.55	0.41	0.06	2.53	0.12	1.92	2.09	2.38
B (30-44 years)	116	0.82	1.02	0.48	0.06	6.47	0.15	2.18	3.60	6.13
C (> 45 years)	122	0.90	1.23	0.55	0.07	10.92	0.22	2.20	2.72	6.22

[1] Minimum
[2] Maximum

These results were not normally distributed. Half of the samples presented OTA levels up to 0.5 ng/mL, the 76.9% of the population presented levels up to 1 ng/mL, 21.2% ranged between >1 to 4 ng/mL, and only a 1.8% was contaminated in levels from >4 to 11 ng/mL. This type of distribution in which a high frequency of low values is found, as well as low frequencies of high values, can be observed in previous studies of exposure to OTA. A similar distribution and magnitude of contaminations could be observed in relation with the previously published study in Lleida (Coronel et al., 2009). In the present study, maximum value was higher than in the previous study (8.68 ng/mL); whereas mean and median were lower than in the former (0.86 ng/mL and 0.54 ng/mL, respectively). In addition, the distribution was similar when the population was sorted by gender and age.

Considering the non-normality of the distributions of OTA in most populations, and that the limits of detection of the analytical methods differ, an appropriate way to compare results would be to consider the median values, the high percentiles, or the ranges of concentrations found. However, this information is not always available in the literature. Table 1 lists results of studies on the presence of OTA in apparently healthy persons from different countries. If we consider, for example, the mean of the present study, it can be observed that it was higher than in most of the studies cited in Table 1.

In the following sections the results were analysed, in the first place, for the total population (i.e., all seasons and regions), and secondly, for the population by seasons or regions, and within each (when appropriate), the season, region, the gender and the group of age.

3.1.1. Distribution of OTA according to gender and age

No significant differences were observed between genders in the total population, which agrees with the previous study in Lleida, and, also with Assaf et al. (2004), Erkekoğlu et al. (2010), Medina et al. (2010), Studer-Rohr et al. (2000), Thuvander et al. (2001), Ueno et al. (1998), and Zimmerli and Dick (1995). Investigations in Argentina (Pacin et al., 2008) and Chile (Muñoz et al., 2006) determined the levels of OTA in two different populations each and found differences between genders in only one of the studied populations.

Regarding age, significant differences were found between age groups A and C ($p = 0.0071$). Mean, median and maximum levels of OTA in plasma were higher as age increased. No significant differences were found between genders within each age group. These results agree with the observed in the previous study in Lleida, except the differences among age groups (defined same as the present), which in that case were significant between groups B and C. Descriptive values listed in Table 3 were slightly lower than those found in the previous investigation. Differences in OTA plasma concentration with respect to age were found previously (Gilbert et al., 2001; Filali et al., 2002; Lino et al., 2008; Pacin et al., 2008), although in all cases the age groups were defined differently and results on differences among groups were diverse.

3.1.2. Distribution of OTA according to the regions

Descriptive values are listed in Table 4. OTA levels in the plain region of the province of Lleida were not significantly different from the levels found in the mountain region, as could be expected due to the dissimilar geographical situations. In contrast with our results, regional differences were indeed found in Europe in the countries of Croatia (Peraica et al., 1999), Germany (Bauer and Gareis, 1987), Portugal (Lino et al., 2008), Spain (Pérez de Obanos et al., 2001), Sweden (Thuvander et al., 2001), and Switzerland (Zimmerli and Dick, 1995); in the Mediterranean countries of Lebanon (Assaf et al., 2004) and Tunisia (Hmaissia-Khlifa et al., 2008 and 2010), as well as in the American continent: in Argentina (Pacin et al., 2008), Canada (Scott et al., 1998), and Chile (Muñoz et al., 2006). The compared regions were rural, urban, inland, and coastal regions and the authors of these investigations related the difference in OTA plasma levels with the particular dietary habits of each region. In this study, however, dietary habits were assessed (Section 3.5.), and differences between regions were not found either.

No differences had been obtained before among individuals from localities in the plain area of the province. When analysing the population of each region by gender and age, the only significant differences were found in the plain region between the age groups A and C ($p = 0.0019$), and B and C ($p = 0.0059$). Group C presented the highest values. If we compare the assessments carried out in Spain with the present results, these mean values were higher than those found in Granada (0.63 ng/mL, Pérez de Obanos et al., 2001) and Navarra (0.71 ng/mL, Jimenez et al., 1998); and lower than the mean levels in Madrid (1.19 ng/mL, Burdaspal and Legarda, 1998) and Valencia (1.09 ng/mL, Medina et al., 2010).

Table 4. Distribution of OTA plasma levels (ng/mL) according to seasons and regions.

	n	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Region										
Plain	243	0.79	1.04	0.48	0.06	10.92	0.13	2.11	2.59	4.92
Mountain	82	0.83	0.93	0.53	0.21	6.47	0.26	1.99	3.48	4.67
Season										
Autumn	116	0.73	0.65	0.52	0.06	4.24	0.22	1.85	2.16	3.36
Winter	98	0.87	1.48	0.42	0.07	10.92	0.15	2.13	4.87	7.24
Summer	111	0.80	0.81	0.55	0.06	6.47	0.14	2.13	2.62	2.78

[1] Minimum
[2] Maximum

3.1.3. Distribution of OTA according to the seasons

When the total population was analysed, median contamination of samples in the three seasons presented no significant differences amongst ($p = 0.0746$). However, the maximum values of the samples and the highest frequency of high values were detected in the samples collected in winter (Table 4). Also in winter, differences were significant between age groups A and C, and B and C. The maximum values in the three seasons were detected in women. Pérez de Obanos et al. (2001) found no significant differences among seasons either, and the highest values were also found in winter.

When the variation of the seasons in each region was evaluated separately, significant differences were found between summer and autumn in the mountain region ($p = 0.0194$), and the highest median was found in summer. To our present knowledge, few evaluations on the seasonal variability of OTA plasma concentrations have been carried out, and the highest OTA levels were found in the months of spring (Ruprich and Ostry, 1993), or summer (Burdaspal and Legarda, 1998; Peraica et al., 2001, Erkekoğlu et al., 2010). The present results show no defined trend with respect to the seasonal influence, as different results were obtained according to the analysed population.

3.2. Estimation of the daily intake of OTA considering OTA plasma levels

Results of the daily intake estimations based on OTA plasma levels are listed in Tables 5 and 6. These were obtained following the estimation methods A and B stated in section 2.6.1.

Estimations of the OTA daily intake according to the estimation method A were around 1.5-fold higher than the values obtained according to the estimation method B. Mean and median values for both estimations were far below the latest PTDI of 14 ng/kg bw/day (JECFA, 2007), but different results can be observed when the high percentiles are considered. For example, the 99th percentile of the total population, for the estimation A, was above the PTDI, whereas for estimation B it was below it. Maximum values for the total population were in both cases above the PTDI. Most of the maximum values classified as in Tables 5 and 6 were also above the PTDI, and in a higher frequency in the estimation A.

Table 1 includes the estimations of the daily intake from OTA plasma levels, when done, in other studies on OTA presence in plasma. As mentioned in section 2.6.1., different ways of estimation can be observed, and these are specified in the footnotes of the table. The estimated daily intake values derived from estimation method A (Table 5) were higher than all the listed cases, whereas the estimations derived from estimation method B were higher than most of the values found in other countries.

Differences of daily intake results between population groups, regions, and seasons were analysed in the same way as OTA levels in plasma (section 3.1.), and identical results to those were obtained through the estimation method B. This is logical, as results are a multiple of the concentration of OTA in plasma. In contrast, different results to the mentioned were observed in some cases in the analysis of the daily intake data calculated by means of the estimation method A, in which, apart from different clearance values, the body weight of the individuals was included in the calculation. The differences were significant between men and women in the following cases: in the total population (p = 0.0258, higher medians in women), in the age group A (p = 0.0374, women's median higher than men's), in the plain region (p = 0.0245, higher medians in women), and in autumn (p = 0.0023, higher medians in men). Apart from gender, differences were not significant among age groups in the total population. These results show the influence of the inclusion of the individual body weight in the estimation of the daily intake. In the estimation method B, body weight was assumed as 70 kg. Mean body weight of this population was 74 kg; in women it was 67 kg, and in men, 81 kg. From these results we can observe that if the body weight of the individuals is normalised, information derived from genders can be lost.

Table 5. OTA daily intake estimation (ng/kg bw/day) based on OTA plasma levels and coefficient derived from Studer-Rohr et al. (2000).

	n	**Mean**	**SD**	**Median**	**Min[1]**	**Max[2]**	**Percentiles**			
							5th	**95th**	**97.5th**	**99th**
Total	325	2.66	3.58	1.61	0.16	43.71	0.46	7.73	9.13	17.76
Men	160	2.34	2.74	1.40	0.16	22.75	0.44	6.28	7.75	12.77
Women	165	2.96	4.23	1.88	0.22	43.71	0.57	8.61	9.97	16.46
Age group										
A	87	2.16	1.94	1.46	0.16	8.69	0.42	6.96	7.74	8.56
B	116	2.68	3.24	1.50	0.22	20.37	0.43	7.16	13.32	18.17
C	122	2.99	4.62	1.74	0.28	43.71	0.71	8.27	9.79	20.07
Region										
Plain	243	2.63	3.74	1.61	0.16	43.71	0.40	7.73	8.94	15.13
Mountain	82	2.72	3.09	1.64	0.61	20.37	0.78	7.26	13.06	15.42
Season										
Autumn	116	2.42	2.32	1.51	0.22	14.26	0.63	6.46	8.55	12.51
Winter	98	2.90	5.33	1.45	0.28	43.71	0.44	8.04	15.09	23.38
Summer	111	2.69	2.64	1.84	0.16	20.37	0.42	7.73	8.24	9.13

[1] Minimum
[2] Maximum

Table 6. OTA daily intake estimation (ng/kg bw/day) based on OTA plasma levels and coefficient derived from Miraglia et al. (1996).

	n	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Total	325	1.57	1.99	0.98	0.11	21.51	0.30	4.15	5.33	11.69
Men	160	1.55	1.77	0.99	0.12	14.04	0.26	3.92	5.33	8.46
Women	165	1.59	2.20	0.97	0.11	21.51	0.31	4.24	5.29	9.94
Age group										
A	87	1.25	1.08	0.80	0.12	4.98	0.24	3.78	4.12	4.70
B	116	1.61	2.01	0.95	0.11	12.74	0.30	4.29	7.08	12.08
C	122	1.77	2.43	1.08	0.14	21.51	0.43	4.33	5.35	12.24
Region										
Plain	243	1.55	2.05	0.95	0.11	21.51	0.26	4.15	5.11	9.69
Mountain	82	1.64	1.84	1.05	0.40	12.74	0.51	3.92	6.86	9.19
Season										
Autumn	116	1.45	1.28	1.02	0.11	8.36	0.43	3.65	4.26	6.61
Winter	98	1.71	2.91	0.82	0.14	21.51	0.29	4.98	9.60	14.27
Summer	111	1.59	1.59	1.08	0.12	12.74	0.27	4.20	5.17	5.47

[1] Minimum
[2] Maximum

3.3. Consumption of foodstuffs possibly contaminated by OTA

Individual consumption (g foodstuff/person/day) was not normally distributed by food groups neither in the total consumption calculated as the sum of the food groups. Cereals were the most consumed food group (median value: 158.32 g/person/day), followed by beer (median of 43.40 g/person/day), wine (median of 27.78 g/person/day), and dried fruits, cacao, coffee, and grape juice (medians: 8.41, 6.68, 6.20, 0.00 g/person/day, respectively).

When the total population was analysed, consumption by men was significantly higher than women's consumption ($p < 0.0001$), and therefore significant differences between genders were found when the analysis was done by regions (plain region: $p < 0.0001$; mountain region: $p = 0.0011$) and by seasons (autumn: $p = 0.0082$; winter: $p = 0.0455$; summer: $p < 0.0001$). No differences were found among age groups, regions, or seasons. These results on consumption agreed with the lack of seasonal differences observed in the plasma levels of OTA. However, no significant correlation was found between OTA levels in plasma and individual consumption, which agrees with the previous study in Lleida (Coronel et al., 2009).

3.4. Estimation of the daily intake of OTA considering food consumption and food contamination data from the literature

Results of the estimation of the OTA daily intake based on food consumption and contamination data are listed in Table 7. It can be observed that in any case the values exceeded the established PTDIs; moreover, the maximum daily intake of the total population (5.59 ng/kg bw/day) was less than half the latest PTDI of 14 ng/kg bw/day. In the total population, differences were significant between genders (p = 0.0012). Differently from the estimation of the daily intake based on OTA plasma concentrations, in this case the highest values were observed in men, and also in men in the analysis by regions and seasons. No significant differences were found among age groups, nor regions or seasons.

Table 7. OTA daily intake estimation (ng/kg bw/day) based on food consumption and contamination data.

	n	**Mean**	**SD**	**Median**	**Min[1]**	**Max[2]**	**Percentiles**			
							5th	**95th**	**97.5th**	**99th**
Total	325	1.60	0.86	1.39	0.34	5.59	0.56	3.21	3.70	4.22
Men	160	1.76	0.95	1.49	0.34	5.59	0.63	3.37	3.86	5.25
Women	165	1.44	0.73	1.26	0.37	4.18	0.49	2.88	3.24	3.62
Age group										
A	87	1.56	0.80	1.36	0.34	3.85	0.59	3.26	3.58	3.73
B	116	1.60	0.92	1.37	0.37	5.59	0.56	3.25	3.71	4.89
C	122	1.62	0.85	1.45	0.46	5.48	0.61	3.13	3.26	4.22
Region										
Plain	243	1.63	0.89	1.44	0.34	5.59	0.57	3.26	3.75	4.73
Mountain	82	1.50	0.74	1.27	0.45	3.57	0.56	2.89	3.17	3.38
Season										
Autumn	116	1.62	0.91	1.46	0.39	5.59	0.55	3.25	4.18	4.97
Winter	98	1.63	0.81	1.41	0.34	3.75	0.63	3.22	3.51	3.71
Summer	111	1.55	0.85	1.31	0.35	5.48	0.57	3.00	3.40	3.82

[1] Minimum
[2] Maximum

3.5. Relation of the estimations of the daily intake based on OTA levels in plasma and based on food consumption

The correlation between the OTA daily intake estimated from plasma concentrations of the toxin (obtained by estimation methods A and B) and the daily intake estimated by means of consumption data was not significant (p = 0.5052 in the first case, and p = 0.3823 in the second). This is in agreement with the lack of correlation observed between the consumption of possibly contaminated foodstuffs and the OTA levels in plasma of the population.

Significant differences were found among the three OTA daily intake estimations. Distributions of the three estimations differed, as can be seen in the descriptive statistics and percentiles (Tables 5, 6, and 7). Taking into account that the distributions were not normal, it could be observed that median OTA daily intake obtained by estimation A were similar to the median daily intake estimations derived from food consumption data; whereas daily intake median levels derived from estimation B was lower than DI estimated from food consumption. The distribution of the OTA daily intake estimations based on food consumption did not show any group of risk, as consumption-derived estimation reached a maximum of 5.59 ng/mL, whereas the maximum values of other distributions were 43.71 ng/mL (estimation A) and 21.51 ng/mL (estimation B). Thus, although similar median results were obtained from food consumption and contamination data, information on the high percentiles (groups of risk) could not be retrieved when the estimation was based on food consumption and contamination data. Therefore, it would be useful that the evaluations of the exposure through food consumption and contamination data were complemented with the analysis of biomarkers. More accurate correlations might be obtained if other sources of OTA intake are considered (such as animal derived products) in the food frequency questionnaires and if data on foodstuffs contamination are more recent and derived from local food sources. Another way is to analyse the foodstuffs actually consumed by the population, as in the duplicate-diet study carried out by Gilbert et al. (2001). Moreover, exposure to mycotoxins is possible through other ways different from diet, as contaminated environments in certain workplaces.

4. CONCLUSIONS

The results of this study showed that OTA was present in the plasma of the total studied population, and even though the levels were low in a high frequency, some outliers were detected. No differences were observed in OTA plasma levels from samples collected in different regions and seasons, and they were not correlated with the consumption of possibly contaminated foodstuffs by the blood donors. As similar results were obtained in the previous study in the plain region of Lleida, it would be interesting to evaluate regional variations in more distant localities, and seasonal variations in different years.

Regarding the assessment of the exposure of the population to OTA, median estimations of the OTA daily intake were below the PTDIs, but estimations of the daily intake through Klaassen equation showed that high percentiles and maximum values were near, and in some cases, above the latest PTDIs. The estimations calculated through food consumption and contamination data were below these values. A more precise estimation of the OTA daily intake through consumption and contamination data could be obtained if the methods for the collection of food consumption are improved, and if the concentrations of OTA are determined in foodstuffs sampled in the local stores. Unfortunately, no correlation could be observed between the OTA plasma levels and the food consumption, and thus no conclusion could be achieved with respect to any special food group. However, as population risk groups were observed, it is important to control and minimize the contamination of foodstuffs.

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STUDY III

Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain

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ABSTRACT

Ochratoxin A (OTA) as well as its metabolite ochratoxin α (OTα) were detected in human urine in order to assess the exposure to OTA of a group of 72 adult inhabitants of the city of Lleida (Spain). Urine samples were enzymatically treated; OTA and OTα were separated by liquid-liquid extraction, and detected by HPLC-fluorescence. Exposure to OTA was also evaluated by the estimation of its daily intake from food contamination data from the literature and from food consumption data provided by the participants, who filled in a food frequency questionnaire (FFQ) and a three-day food consumption record (3DR). OTA occurrence (12.5%, limit of detection = 0.034 ng/mL) was lower than OTα occurrence (61.1%, limit of detection = 0.023 ng/mL). The range of concentrations was 0.057 to 0.562 ng/mL and 0.056 to 2.894 ng/mL for OTA and for OTα, respectively. It could be observed for positive samples that the FFQ data were related to the OTA concentration in urine, whereas the 3DR data were related to the OTα levels in urine. The OTA estimated daily intake of the participants was lower than 30% of the latest proposed tolerable daily intake of 14 ng/kg body weight/day in the worst cases of exposure.

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1. INTRODUCTION

Ochratoxin A (OTA), a dihydroisocoumarin derivative linked over a 7-carboxygroup to L-phenyl-alanine by an amide bond, is a fungal secondary metabolite produced by certain *Aspergillus* and *Penicillium* species. It has been classified as possibly carcinogenic to humans (group 2B) by the IARC (1993). The main effects in humans related to the exposure to the toxin were nephropathies and development of tumours in the urinary tract. The latest provisional tolerable daily intakes (PTDIs) of the toxin by EFSA (2006) and JECFA (2007) were 17 ng/kg bw/day and 14 ng/kg bw/day, respectively.

This mycotoxin can be found in several foodstuffs from vegetal origin such as cereals and cereal products, coffee, beer, grape juice, dry vine fruits and wine, cacao products, nuts, as well as spices. Animal derived products such as meat, milk, or eggs may present OTA contamination due to the ingestion of contaminated feed, although these sources are considered negligible for human exposure (EFSA, 2006), except certain pork products, in which higher concentrations of OTA may occur. The ubiquity of OTA makes possible its chronic ingestion by humans.

OTA toxicokinetics, biotransformation pathways, and toxicodynamics were updated in Ringot et al. (2006); and an experiment on a single person who ingested radio-labelled OTA was performed by Studer-Rohr et al. (2000). So far, biomonitoring studies have been based on the determination of OTA levels in blood plasma or serum (Coronel et al., 2009, 2010; Erkekoğlu et al., 2010; Märtlbauer et al., 2009), urine (Table 1), and in few occasions, in human milk (Biasucci et al., 2010; Hassan et al., 2006; Muñoz et al., 2010a), levels that were related in some investigations with food consumption. From the named fluids, an advantage of considering OTA in urine as a human OTA biomarker is that, unlike blood, the sample collection method is non-invasive, even though OTA levels found in urine are smaller in comparison to those found in plasma (Gilbert et al., 2001; Muñoz et al., 2010b). Table 1 shows the occurrence of OTA in urine samples obtained in previous studies, according to the type of sampling that was performed in each investigation.

Table 1. Occurrence of OTA in urine samples in different countries according to the type of sampling[a] (modified from Duarte et al., 2009).

Country	City	*n*	% Positive	Mean ± SD (ng/mL)	Range (ng/mL)	Reference
			Point sampling			
Croatia		35	94	2.39 ± 1.29	0.99 - 5.22	Domijan et al., 2003
Germany	Dortmund	13	100	0.07 ± 0.05	0.02 - 0.14	Muñoz et al., 2010a
India		152[b]	40	n.a.	0.005 - 0.03	Castegnaro et al., 1990
Portugal	Coimbra	60	70	0.027 ± 0.004	0.021 - 0.105	Pena et al., 2006
	Lisbon	43	72.1	0.026 ± 0.017	ND - 0.071	Duarte et al., 2009
	Bragança	30	93.3	0.024 ± 0.014	ND - 0.069	Duarte et al., 2010
	Porto	30	73.3	0.021 ± 0.014	ND - 0.062	
	Coimbra	30	53.3	0.014 ± 0.007	ND - 0.034	
	Alentejo	40	90	0.023 ± 0.012	ND - 0.064	
	Algarve	25	80	0.024 ± 0.015	ND - 0.068	
Sierra Leone		190 rainy season	25	6.4 ± 3	0.6 - 72.2	Jonsyn-Ellis, 2000
		244 dry season	26	5.3 ± 5.6	0.05 - 148	
			24-hour sampling			
Bulgaria	Gorno-Peshtene	5	100	0.0508 ± 0.044	0.010 - 0.330	Petkova-Bocharova et al., 2003
	Beli Izvor	11	100	0.168 ± 0.111	0.010 - 1.910	
Hungary	Besenyotelek		73	0.022	0.006 - 0.065	Fazekas et al., 2005
	Füzesabony		57	0.018	0.007 - 0.029	
	Debrecen		56	0.008	0.006 - 0.011	
	Komadi		56	0.010	0.006 - 0.019	
	Kaposvar		59	0.008	0.006 - 0.012	
Italy		38 healthy 3 patients[c]	58	n.a.	0.012 - 0.046	Pascale and Visconti, 2000
UK		50	92	0.013	<0.01 - 0.058	Gilbert et al., 2001

Table 1 *(Continued)*

Country	City	*n*	% Positive	Mean ± SD (ng/mL)	Range (ng/mL)	Reference
		Morning and afternoon sampling				
Portugal	Coimbra	30 morning	43.3	0.019 ± 0.041	0.011 - 0.208	Manique et al., 2008
		30 afternoon	46.7	0.018 ± 0.027	0.008 - 0.110	
Spain	Valencia	31 morning	80.6	0.032 ± 0.031	0.007 - 0.124	Manique et al., 2008
		31 afternoon	83.9	0.028 ± 0.019	0.008 - 0.089	

n.a.: not available
ND: not detected
[a] Type of sampling that was performed in the studies: "point sampling" refers to the collection of a single sample at a certain moment of the day; "24-hour sampling" implies the collection of the urine during a period of 24 hours, followed by the pooling of all the samples; and "morning and afternoon sampling" means the collection of the samples at those two moments of the day.
[b] BEN (Balkan endemic nephropathy) and UTT (tumours of the urinary tract) patients and control families.
[c] Kariomegalic interstitial nephritis patients.

In addition to the occurrence of OTA in urine, it is also possible to find derivatives of the toxin that result from its biotransformation. Although the mechanisms are not yet completely understood, the known pathways are common to the metabolism of xenobiotics, whose goal is to detoxify ingested drugs or poisonous compounds. The biotransformation pathways are divided in phase I reactions (detoxification or bioactivation reactions, by the modification of the OTA molecule) and phase II reactions (conjugation reactions to facilitate the excretion of the compounds), both enzyme-mediated. The major pathway of detoxification of OTA is the hydrolysis of its amide bound, which results in phenylalanine and OTα, a less toxic compound. Phase II reactions are mainly the glucuronidation and sulphate conjugation, and in a minor frequency the formation of *O*-labile ester conjugates as well as the glutathione conjugation. Unchanged OTA and phase I OTA-derivatives can also suffer these phase II transformations. The presence of OTA glucuronide or sulphate conjugates in rat urine and bile was suggested by Kühn et al. (1995) and Li et al. (2000) and in human urine by Castegnaro et al. (1991), Kane et al. (1986), and Pena et al. (2006). These compounds are excreted by renal (urine) or biliary (faeces) routes. Recently Muñoz et al. (2010b) optimized a method for the simultaneous detection of OTA and OTα in urine that included an enzymatic treatment of the samples in order to produce the cleavage of the glucuronide or sulphate conjugates of both toxin and metabolite.

Thus, assessment of exposure to OTA of a certain population can be performed by determining the presence of the toxin in human fluids, as well as the intake of the

toxin, or the contamination of foodstuffs. Therefore, the aim of this study was to assess the exposure of a group of adult inhabitants of the city of Lleida (Spain) to ochratoxin A, by means of its detection in urine as well as by the detection of its metabolite OTα, and by the estimation of the daily intake of OTA from the analysis of the food consumption data provided by the participants.

2. MATERIALS AND METHODS

2.1. Sampling

Seventy-two inhabitants of the city of Lleida, Spain (28 men and 43 women) were informed about this study and gave their written consent for their participation. Collection of samples was done during the months of October, November, and December 2009. Participants provided 100 mL of urine collected at first hour in the morning in a sterile disposable container. Samples were stored at -17 ºC. Volunteers were asked to fill in two types of food consumption questionnaires (see section 2.8.) and also provided information about gender, age, weight, height, and occupation. Three age groups were defined: A (18 to 29 years old), B (30 to 44 years old), and C (over 45 years old). According to this classification, the number of individuals in each age group was 24.

2.2. Standards and reagents

OTA standard was purchased from Sigma-Aldrich Chemie (Steinheim, Germany), whereas OTα standard was purchased from Biopure (Tulln, Austria). $NaHCO_3$ was provided by Panreac Química S.A. (Spain). Chloroform, isopropanol, methanol, and acetic acid (100%) were purchased from VWR International (Fontenay sous Boise, France). Orthophosphoric acid was provided by Scharlau Chemie (Barcelona, Spain). Solvents used as mobile phase were HPLC grade. Boron trifluoride-methanol solution (14%) was purchased from Sigma-Aldrich (Steinheim, Germany). Enzyme β-glucuronidase from *Helix pomatia* (116400 units/mL b-glucuronidase, 1015 units/mL sulfatase) was obtained from Sigma-Aldrich (Steinheim, Germany). The enzyme was used diluted to an activity of 60 units/mL in a sodium acetate 0.02 M buffer solution at pH 5.

2.3. Samples enzymatic treatment

Enzymatic treatment stated in Muñoz et al. (2010b) was applied, with some modifications: 100 μL of the enzyme dilution were added to 10 mL of the urine sample and then incubated at 37 ºC for 17 hours before the extraction of the toxins.

2.4. Extraction

The method of extraction was a modification of the liquid-liquid extraction method developed by Muñoz et al. (2010b). The enzyme-treated sample (10 mL) was mixed with 10 mL of 1% $NaHCO_3$, and adjusted to pH 3 with 1 M orthophosphoric acid. 6 mL of a mixture chloroform:isopropanol (97:3) were added, and stirred for 20 minutes by a magnetic stirrer. The mixture was centrifuged at 1000 *g* for 20 minutes at 4 ºC. 4 mL of the organic layer were transferred to a vial and evaporated to dryness under stream of nitrogen at 40 ºC. The extract was prepared for chromatographic analysis by resuspension in 2 mL methanol:water (50:50). The vial was vortexed, and the liquid was then filtered through a 0.45 μm pore size syringe filter (Advanced Microdevices PVT. LTD., Ambala Cantt., India).

After extraction, samples were not cleaned-up. Muñoz et al. (2010b) tested the sample purification procedure by immunoaffinity columns and obtained acceptable method performance parameters for OTA. However, immunoaffinity columns are specific only for OTA and therefore OTα is not retained.

2.5. HPLC analysis

The high performance liquid chromatography analysis was performed on a Waters 2695 Separations Volume (Alliance) equipped with a Waters Spherisorb ODS2 C18 analytical column (5 μm, 4.6 x 250 mm) and a Waters Spherisorb ODS2 guard column (5 μm, 4.6 x 10 mm) (Waters, Ireland). Temperature of column and guard column was maintained at 40 ºC. The detector was a Waters 2475 Multi λ fluorescence detector. Excitation and emission wavelengths were 333 nm and 450 nm, respectively. Two mobile phases were used to achieve a gradient. Phase A consisted on acetic acid 2%:methanol (63:34), and phase B was methanol:isopropanol (90:10). The gradient was as follows: 0-15 min 95% A, 15-16 min 95 to 60% A, 16-30 min 60% A, 30-31 min 60 to 5% A, 31-33 min 5% A, 33-34 min 5 to 95% A, 34-45 min 95% A. Flow-rate was 1 mL/min and injection volume was 100 μL. Retention time for OTA was 25 min, whereas for OTα it was 8.4 min. The integration software used to manage the chromatographic data was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00).

2.6. Method validation

The evaluated quality parameters of the analytical procedure were linearity, limit of detection (LOD), limit of quantification (LOQ), intra-day repeatability, inter-day repeatability, and recovery rates.

2.7. Confirmation of positive samples

Positive samples were confirmed by the methyl-esterification of the detected toxins. The procedure followed the method published by Patel et al. (1997) with some modifications: 1 mL of the sample prepared for the chromatographic analysis, which was resuspended in methanol:water (50:50), was evaporated under stream of nitrogen at 40 ºC. 0.5 mL of a 14% boron trifluoride-methanol solution were added to the dry extract, and then heated in the capped vial for 20 minutes at 60 ºC. Afterwards the sample was evaporated to dryness at 40 ºC under stream of nitrogen, and finally resuspended in 1 mL methanol:water (50:50). These samples were analysed in the same HPLC conditions as for the ochratoxins analysis. Retention time for OTA methyl ester was 31.4 min, and 22.6 min for OTα methyl ester.

2.8. Food consumption data

Participants were asked to provide written information about their food consumption habits. For this, they filled in two types of questionnaires: a food consumption frequency questionnaire (FFQ) and a 3-day food consumption record (3DR). In the first case, the volunteers indicated the amount and frequency of consumption of eighteen possibly OTA contaminated foodstuffs within the period of a year. Foodstuffs were classified in five groups according to European Commission (2002) and Food Standards Agency (2002) (Table 2). In the second case, participants registered daily the quantity of all the foodstuffs and beverages ingested during the three days previous to the urine sample collection.

Table 2. OTA levels (ng/g) in certain food groups.

Food group	Mean OTA levels	Reference
Cereals and derived products	0.29	European Commission (2002)
Dried fruits and derived products	0.87	Food Standards Agency (2002)
Cacao and derived products	0.24	European Commission (2002)
Wine	0.36	European Commission (2002)
Beer	0.03	European Commission (2002)
Coffee	0.72	European Commission (2002)

2.9. Calculation of OTA daily intake based on food consumption and food contamination data

Individual food consumption data (g foodstuff/person/day) were obtained from the FFQ and 3DR by converting the quantities stated by the participants to previously defined portions. As a previous step and in order to obtain any useful information, food consumption was normalized by dividing the data by the individual body weight. OTA daily intake (ng OTA/kg bw/day) was calculated as the normalized food consumption (g foodstuff/kg bw/day) multiplied by the OTA food contamination (ng OTA/g foodstuff) data stated in Table 2.

2.10. Statistical analysis

Wilkoxon and Kruskal-Wallis non-parametric tests were used to evaluate differences between groups. Spearman correlation coefficient was used to test correlations. JMP® 8.0 (2008 SAS Institute) was used for the analysis.

3. RESULTS

3.1. Validation parameters for OTA and OTα analysis

The calibration curve was linear in the range of 0.024 ng/mL to 12.5 ng/mL for both mycotoxins, and the coefficients of determination (R^2) were 0.9994 and 0.9990 for OTA and OTα, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the signal-to-noise ratio of 3 and 10, respectively. In the case of OTA, LOD was 0.034 ng/mL and LOQ, 0.112 ng/mL. Regarding OTα, LOD was 0.023 ng/mL and LOQ, 0.076 ng/mL. Recovery assays were performed on spiked ochratoxin-free urine samples. Recovery rates in the tested concentration levels for spiked samples (n = 3) with OTA and OTα, as well as intra-day repeatability, are listed in Table 3. Intra-day repeatability was assessed at the same day for each spiking level, and expressed as relative standard deviation (RSDr). Inter-day repeatability (expressed as relative standard deviation) was assessed in three different days at the level of 2 ng/mL for each toxin. In this case, relative standard deviation was 4.16% for OTA, and 8.59% for OTα.

Table 3. Recovery rates and intra-day repeatability for OTA and OTα.

Spiking level ng/mL	OTA		OTalpha	
	Recovery (%)	RSDr (%)	Recovery (%)	RSDr (%)
0.5	69.17	3.48	70.37	5.35
1	71.63	2.95	72.29	6.09
2	73.03	4.27	69.62	2.41

3.2. Urine contamination: Presence of OTA and OTα by population groups

Positive samples were considered as those in which mycotoxin contamination was above the limit of detection (LOD) of the method of analysis. OTA was detected in only nine samples (12.5%), whereas OTα was detected in 44 samples (61.1%) (Tables 4-5). Figure 1 shows the distribution of OTA and OTα in the analysed population. An outlier of 21.26 ng/mL occurred in the OTα distribution, in a male individual of age group C. In order to describe the proportion of non detected samples (ND), the graphic was built assigning the value of zero to these ND and the outlier was excluded.

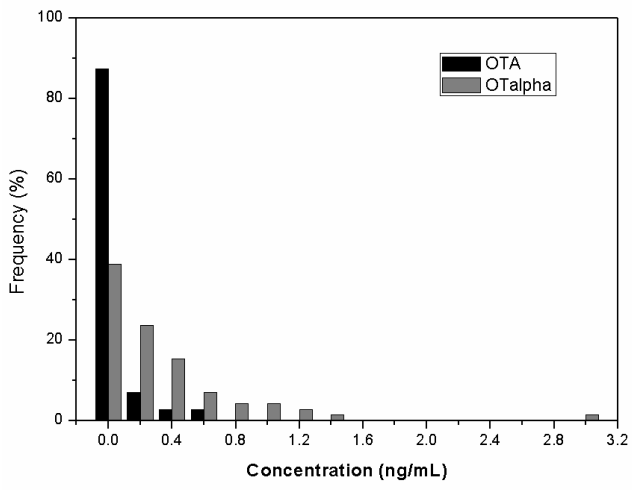

Figure 1. Distribution of OTA and OTα in urine samples. Outlier (21. 26 ng/mL) was not included in this plot.

Descriptive statistics on OTA and OTα concentrations of the study population are listed in Tables 4 and 5, respectively, where population groups were defined by sex and age. No significant differences of ochratoxin levels were found between sex groups (p = 0.7116) neither among age groups (p = 0.2108). It was observed that no significant correlations were found between OTA and OTα levels, neither in the total samples (ρ = 0.0029, p = 0.9806) nor in the positive ones (ρ = 0.0990, p = 0.5276).

Table 4. Descriptive statistics of OTA positive samples in urine (ng/mL).

	n	**Positive samples (%)**	**Mean**	**SD**	**Median**	**Min[1]**	**Max[2]**
Total	72	9 (12.5)	0.237	0.181	0.185	0.057	0.562
Men	29	3 (10.3)	0.304	0.233	0.238	0.111	0.562
Women	43	6 (14.0)	0.204	0.163	0.140	0.057	0.445
A	24	5 (20.8)	0.280	0.219	0.238	0.057	0.562
B	24	1 (4.2)	0.111	-	0.111	0.111	0.111
C	24	3 (12.5)	0.208	0.144	0.185	0.077	0.363

[1] Minimum
[2] Maximum
A: group of age from 18 to 29 years old; B: group of age from 30 to 44 years old; C: group of age over 45 years old.

Table 5. Descriptive statistics of OTα positive samples in urine (ng/mL).

	n	**Positive samples (%)***	**Mean**	**SD**	**Median**	**Min[1]**	**Max[2]***
Total	71	43 (60.6)	0.441	0.508	0.248	0.056	2.894
Men	28	18 (64.3)	0.387	0.370	0.254	0.056	1.387
Women	43	25 (58.1)	0.480	0.593	0.223	0.092	2.894
A	24	11 (45.8)	0.775	0.800	0.441	0.092	2.894
B	24	15 (62.5)	0.410	0.354	0.278	0.056	1.387
C	23	17 (73.9)	0.251	0.230	0.196	0.099	1.073

[1] Minimum
[2] Maximum
*Occurrence and maximum values excluding the outlier of 21.62 ng/mL OTα, which belongs to a male individual from age group C.
A: group of age from 18 to 29 years old; B: group of age from 30 to 44 years old; C: group of age over 45 years old.

3.3. Food consumption and estimation of the daily intake of OTA

Results of food consumption and estimation of the daily intake are shown separately in the Tables 6 and 7, respectively for each method of data collection (FFQ and 3DR).

Table 6. Consumption of possibly OTA contaminated foodstuffs and estimated OTA daily intake, calculated from FFQ data.

	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
						5th	95th	97.5th	99th
Individual food consumption (g foodstuff/person/day)									
Total	306.46	201.61	263.61	93.37	1206.18	102.89	619.42	879.18	1128.68
Men	329.97	205.93	292.17	93.37	1095.47	99.49	614.27	789.31	973.01
Women	291.15	199.68	231.02	99.93	1206.18	122.47	594.60	796.58	1038.56
A	347.74	226.06	303.46	135.62	1206.18	154.69	635.16	881.72	1076.40
B	313.44	233.59	241.32	99.93	1095.47	133.38	772.06	929.65	1029.14
C	256.11	119.55	263.61	93.37	562.94	98.59	438.17	497.31	536.69
Normalized food consumption (g foodstuff/kg body weight/day)									
Total	4.41	2.64	3.92	1.07	16.08	1.67	9.49	10.60	14.09
Men	4.36	2.68	4.22	0.91	13.04	1.22	9.14	10.80	12.15
Women	4.70	3.51	3.61	1.43	20.10	1.90	11.59	13.70	17.45
A	5.20	3.09	4.29	2.13	16.08	2.44	9.68	12.43	14.62
B	4.44	2.81	3.45	1.26	13.23	1.84	9.42	11.10	12.38
C	3.56	1.57	3.58	1.07	7.31	1.47	5.90	6.54	7.00
OTA daily intake (ng OTA/ kg body weight/day)									
Total	1.19	0.59	1.08	0.40	3.77	0.49	2.29	2.64	3.10
Men	1.30	0.62	1.18	0.40	2.81	0.50	2.44	2.66	2.75
Women	1.11	0.57	1.03	0.45	3.77	0.49	1.65	2.37	3.20
A	1.30	0.73	1.16	0.49	3.77	0.67	2.52	3.09	3.50
B	1.22	0.58	1.05	0.45	2.81	0.55	2.35	2.58	2.72
C	1.03	0.39	1.04	0.40	1.71	0.47	1.63	1.67	1.69

[1] Minimum
[2] Maximum
A: group of age from 18 to 29 years old; B: group of age from 30 to 44 years old; C: group of age over 45 years old.

Table 7. Consumption of possibly OTA contaminated foodstuffs and estimated OTA daily intake, calculated from 3DR data.

	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
						5th	95th	97.5th	99th
Individual food consumption (g foodstuff/person/day)									
Total	254.21	140.82	226.67	26.10	773.50	96.12	542.13	629.45	758.47
Men	251.47	112.55	237.17	95.37	566.28	131.91	470.73	533.67	553.24
Women	256.00	157.78	218.50	26.10	773.50	87.27	574.06	743.86	764.48
A	291.90	163.14	260.13	128.23	773.50	145.06	696.68	761.16	768.56
B	253.03	138.51	239.80	26.10	588.58	88.89	506.78	547.98	572.34
C	216.12	110.20	200.75	26.40	566.28	95.52	377.91	464.92	525.74
Normalized food consumption (g foodstuff/kg body weight/day)									
Total	3.78	2.36	3.15	0.37	15.47	1.42	7.71	9.08	12.16
Men	3.27	1.49	2.80	1.22	7.35	1.73	6.35	6.87	7.16
Women	4.11	2.76	3.41	0.37	15.47	0.98	8.48	10.63	13.48
A	4.54	2.99	3.96	1.71	15.47	1.98	10.01	12.75	14.38
B	3.77	2.15	3.12	0.37	8.53	1.03	7.85	8.26	8.42
C	3.00	1.51	2.83	0.54	7.35	1.26	5.81	6.59	7.05
OTA daily intake (ng OTA/ kg body weight/day)									
Total	1.10	0.52	1.00	0.22	3.01	0.44	2.04	2.31	2.60
Men	1.05	0.62	0.90	0.22	3.01	0.27	2.21	2.51	2.60
Women	1.12	0.45	1.03	0.39	2.43	0.51	1.82	1.99	2.24
A	1.15	0.39	1.14	0.50	2.09	0.60	1.60	1.81	1.98
B	1.16	0.69	1.05	0.22	3.01	0.31	2.40	2.67	2.88
C	0.97	0.42	0.92	0.27	2.00	0.40	1.68	1.83	1.93

[1] Minimum
[2] Maximum
A: group of age from 18 to 29 years old; B: group of age from 30 to 44 years old; C: group of age over 45 years old.

Consumption (individual, g foodstuff/person/day; and normalized, g foodstuff/kg bw/day) and OTA daily intake were significantly different among food groups ($p < 0.0001$), cereals being the most consumed and therefore the most important contributors to the daily intake, as calculated from FFQ as well as from 3DR data (data not shown). OTA mean and median estimated daily intake were below the latest PTDIs, as well as the maximum values of the distributions, representing a

range of 1.3% – 22.2% of the PTDI proposed by EFSA (2006), and 1.6% – 26.9% of the PTDI proposed by JECFA (2007).

It can be seen in Tables 6 and 7 that the individual consumption and the normalized consumption stated by the participants in the FFQ were higher than the values stated in the 3DR, for the population in general as well as for the sex and age groups. The correlation between data from FFQ and 3DR was significant in the case of the normalized consumption ($\rho = 0.2417$, $p = 0.0423$). The remaining correlations were not significant (individual consumption: $\rho = 0.2095$, $p = 0.0796$; OTA daily intake: $\rho = 0.1132$, $p = 0.3471$).

Differences within population groups (sex and age) were analysed taking into account individual consumption, normalized consumption, and OTA daily intake calculated from data derived from the FFQ and 3DR questionnaires. Thus, statistically significant differences were found between men and women ($p = 0.0031$) when considering individual consumption from the FFQ data, with men having the highest values. Also between the age groups A and C, taking into account the normalized food consumption (from the FFQ data: $p = 0.0390$; from the 3DR data: $p = 0.0192$). In the case of 3DR data, differences between these two age groups were not significant but a low p-value was obtained for individual consumption ($p = 0.0582$) and for daily intake ($p = 0.0969$).

3.4. OTA and OTα levels in urine in relation with food consumption and estimated OTA daily intake

The relation between OTA and OTα concentration in urine and food consumption or OTA daily intake was analysed, considering in first place the whole contamination dataset and, in second place, only the toxin-positive samples.

Considering the FFQ data, and in the first analysis, no relation was found between the OTA or OTα urine levels and the food consumption (neither individual nor normalized) or the OTA daily intake. In the second analysis, a strong positive correlation was found between the normalized food consumption and OTA levels ($\rho = 0.7500$, $p = 0.0199$). A slight correlation ($\rho = 0.5833$, $p = 0.0992$) was found between OTA estimated daily intake and OTA positive samples. However, it has to be remarked that the number of OTA positives was too small ($n = 9$) to have an accurate analysis.

When the analysis was done taking into account the total data obtained by the 3DR, no correlation was found between ochratoxins levels in urine and food consumption or OTA daily intake, neither when the OTA positives were tested separately. Only in the case of the positive OTα samples ($n = 43$), positive correlations were found between the OTα levels in urine and the individual consumption of possibly contaminated foodstuffs ($\rho = 0.3821$, $p = 0.0115$), the normalized consumption ($\rho = 0.4097$, $p = 0.0064$), and the OTA daily intake

(ρ = 0.3620, p = 0.0171). This same analysis was performed considering the consumption data of the day previous to the collection of the urine sample, but no correlation was found in any case.

Therefore, considering positive samples, it seems that the FFQ data were related to the OTA concentration in urine, whereas the 3DR data were related to the OTα levels in urine.

3.4.1. Outlier's evaluation

The OTα concentration outlier (21.26 ng/mL) had no relation with the values of food consumption, neither with the OTA daily intake reported in the FFQ or in the 3DR.

4. DISCUSSION

Regarding OTA, if we compare our results with those of the point samplings listed in Table 1, it can be observed that mean of positives in the present study (0.237 ng/mL) was lower than mean levels found in Croatia and Sierra Leone, and higher than the rest of studies cited, including those that carried out the other two ways of sampling. Considering the maximum value (0.562 ng/mL), it was higher than most of the cited in Table 1, except Beli Izvor (Bulgaria), Croatia, and Sierra Leone. Thus, the here obtained OTA levels in urine were in general higher than the levels presented in the literature, but by contrast, the incidence of OTA positive samples of the present study (12.5%) was far below the stated in other previous studies. A different way of presenting results was employed by Akdemir et al. (2010), who made a point sample collection and normalised 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. In that case, average OTA concentration was 14.34 ng/g creatinine. In our study first morning urine was analysed; the aim was not to represent daily excretion, but to have a point observation.

Not all the investigations in Table 1 analysed differences among population groups. The lack of significant differences between genders in this study agrees with the results found in Hungary (Fazekas et al., 2005) and Portugal (Duarte et al., 2009, 2010; Manique et al., 2008). Only in the case of Pena et al. (2006), significant differences were found between men and women but only in the age group of 20-39 years old. When testing differences among age groups, Duarte et al. (2009) did not find significant differences either.

Recently, Muñoz et al. (2010b) determined both OTA and OTα in a pilot study in Dortmund, Germany. In that study, human plasma and urine were analysed, and both ochratoxins were found in all the samples (n = 13). Mean OTA levels in urine (0.07 ± 0.05 ng/mL) were lower than OTA levels in plasma (0.25 ± 0.03 ng/mL),

whereas OTα mean levels in urine (2.88 ± 2.24 ng/mL) were higher than OTα levels in plasma (0.95 ± 0.46 ng/mL). The present study agrees with the previous one in the fact that mean OTα concentrations were higher than OTA mean levels, but not in the occurrence (with similar LOD in both cases), neither in the mean concentration amounts, which in this case were higher for OTA (0.237 ± 0.181 ng/mL) and lower for OTα (0.441 ± 0.508 ng/mL).

The presence of OTA in urine in a smaller amount than its metabolite OTα is in agreement with the findings by Støren et al. (1982) in rats, who found that, independent of the route of administration of OTA, 6% of a given dose was excreted as the toxin, 1 to 1.5% as 4-(R)-hydroxy-OTA, and 25 to 27% as OTα in the urine. A higher quantity of OTα than OTA in urine is expected, as ingested OTA is metabolised in order to facilitate its excretion. Moreover, due to enterohepatic circulation, remnant OTA can be converted in the large intestine to OTα and still recirculated until its excretion. Concerning humans, Studer-Rohr et al. (2000) studied the toxicokinetic profile of OTA in one person after the ingestion of ^{3}H-labeled OTA. They measured radioactivity at elution times of OTA and possible metabolites/conjugates in blood plasma and urine. In urine between 42% and 54% of the radioactivity was recognized as unchanged OTA. An additional activity peak in a different retention time was detected, and represented between 14% and 20% of the total activity in urine, possibly indicating the presence of OTA metabolites and/or conjugates. However, these metabolites were not identified. No further studies have been carried out with humans to elucidate the metabolic pathways of OTA. Thus, the here presented results do not agree with the quantity of OTA in urine of the study conducted in a human volunteer. It is not possible to compare the results of OTA derivatives because those were not accurately identified in the previous study.

Differences between values estimated from FFQ and 3DR data obtained from a certain population were also found in previous studies (some examples are Haftenberger et al., 2010; Trinidad Rodríguez et al., 2008; Willet et al., 1985). This may be explained in part by the sources of error inherent to each method for data collection. For example, the 3DR does not depend on the memory of the participants, whereas the FFQ does. The season when data were collected (in this case, autumn) can also influence results, as consumption of certain foodstuffs may be higher or lower depending on the time of the year. Another source of error is that some participants may tend to over- or underestimate the consumption of certain foodstuffs (Willet et al., 1985). This may also explain the difference in the magnitude and significance of the correlations between the estimated consumption and daily intake from data of the two questionnaires, as well as the finding of significant differences between population groups only when the normalized consumption was analysed.

Gilbert et al. (2001) found a positive correlation between the urine concentration of OTA and the consumption of OTA, in a 92% incidence (n = 50). In the present study, OTA incidence was far lower, but positive correlations were also found between positive samples and consumption of possibly OTA contaminated food, as

well as OTA daily intake. They also suggested comparing the level of OTA excreted in urine with the previous day intake. Such analysis was evaluated here (section 3.4.), but no correlation was found. Regarding the relation between the data obtained by the two types of questionnaires and the levels of OTA and OTα, it could be seen that the significant correlations were found only in the case of positive samples, and that OTA levels were correlated to FFQ data, whereas OTα levels had significant correlations with 3DR data. The first case matches with the fact that the frame of time considered for the FFQ is a period of interest for a chronic exposure, and that an extended period of time is needed to excrete OTA as the native form due to its high affinity to plasma proteins and its enterohepatic recirculation. In contrast, metabolism derivative OTα is excreted more easily and faster than OTA, and therefore the second correlation case may be explained by the brief recall period of the consumption record. A special case is that of the outlier, which showed no relation between the ingested and the presence of OTA or OTα. This may express a different ability of the individual's metabolism, and another possibility is that the participant may not have accurately filled the questionnaires.

5. CONCLUSIONS

OTA and OTα incidence in urine of the studied population confirm its exposure to OTA and evidence the usefulness of OTA and particularly OTα in urine as a biomarker of the exposure to ochratoxin A, especially by the fact that sample collection is non-invasive as in the case of blood. Due to the low incidence of OTA and the high incidence of OTα in this study, we agree with Muñoz et al. (2010b) and also recommend the detection of this metabolite in biomonitoring studies. However, further research is needed to elucidate the relation of ingested OTA and excreted OTA and metabolites in humans. Interindividual differences in metabolism abilities should be also considered.

The estimated OTA DI in the present study does not imply a high risk, as in the worst case it was lower than 30% of the PTDI.

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III. RESEARCH WORK

PART II:

Contamination of foodstuffs

STUDY IV

Ochratoxin A in Spanish retail ground roasted coffee: Occurrence and assessment of the exposure in Catalonia

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ABSTRACT

Occurrence of ochratoxin A (OTA) in ground roasted coffee from different brands and types available in Spain was assessed. Based on these data, exposure of the Catalan population to OTA through coffee consumption was estimated. Coffee samples were purchased in hypermarkets and supermarkets of twelve cities of Catalonia, Spain, and composite samples were prepared for analysis. OTA was extracted, cleaned-up by immunoaffinity columns, and detected by HPLC-fluorescence detection. Mean OTA content (n = 72) was 2.17 ± 0.79 ng/g (range 1.21-4.21 ng/g, 49% occurrence). An additional sampling was done by brands (n = 45), mean OTA contamination being 2.07 ± 0.61 ng/g (range 1.30-5.24 ng/g, 95% occurrence). Coffee consumption data were obtained by means of a food frequency questionnaire. Mean coffee consumption per capita was 11.58 ± 8.73 g/person/day. OTA daily intake (DI) was estimated by means of deterministic and probabilistic methods. In both cases, estimated DI (around 0.22 ng/kg bw/day) was below the latest PTDI value of 17 ng/kg bw/day suggested by EFSA.

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1. INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that can be found in several foodstuffs, such as cereals and derived products, maize, beer, wine, grape juice, dried vine fruit, spices, cocoa and coffee. It can also be present in products derived from non-ruminant animals fed with OTA contaminated feed. This toxin is produced in food by certain species of the *Penicillium* and *Aspergillus* genera; with the latter being the most frequent producers in coffee. The International Agency for Research in Cancer (IARC, 1993) has classified OTA as possible human carcinogen (group 2B). Kidney is considered to be the main target organ for OTA, and animal studies have shown nephrotoxic, teratogenic, immunotoxic and carcinogenic effects. The World Health Organization/Food and Agricultural Organization Joint Expert Committee on Food Additives (JECFA) has set in 2007 a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg bw/week, retaining this value from previous OTA evaluations (JECFA 1996 and 2001), whereas the European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain established a PTWI of 120 ng/kg bw/week (EFSA, 2006). JECFA and EFSA PTWI values correspond to the Provisional Tolerable Daily Intake (PTDI) values of 14 and 17 ng/kg bw/day, respectively.

Concerning coffee contamination, the European Union has set the OTA maximum level for roasted coffee beans and ground roasted coffee at 5 ng/g, whereas for instant coffee the limit was established at 10 ng/g (European Commission, 2006). In the Spanish market, several kinds of coffee are available: mainly ground roasted and instant, and the decaffeinated variant within each kind. Regarding ground roasted coffee, two types of varieties can be found: the locally called “naturally roasted” coffee (roasted coffee) and the “torrefacto roasted” coffee (sugar roasted coffee), i.e., coffee that has been roasted in the presence of up to a 15% of sugar. Mixtures of both in different proportions are commercialized as final product.

The Scientific Cooperation (SCOOP) (European Commission, Directorate-General Health and Consumer Protection, 2002) calculated the point estimation of the OTA daily intake (DI) through several foodstuffs in different European countries, taking into account contamination and consumption data from previous studies. In the case of Spain, contamination data were taken by the study done by Burdaspal and Legarda (1998). Analysed samples were ground roasted coffee and roasted coffee beans, and the number of samples was 29. Consumption data were obtained from the Spanish population in general (Dirección General de Política Alimentaria, 1998). The estimated OTA DI through coffee was 0.15 ng/kg bw/day.

The aim of this study was to assess the occurrence of OTA in coffee from different brands and types available in Spain, and to assess the exposure to OTA through coffee consumption particularly in the adult population of Catalonia. For this purpose, a higher number of samples than the previous Spanish study was analysed, and different sampling methods were utilised. The assessment of the exposure to OTA by the Catalan population was achieved by determining the

contamination of ground roasted coffee samples available in the local markets, and by determining the consumption of coffee from the local population by means of food frequency questionnaires. From these data, estimation of daily intake of OTA was performed by the analysis of simple distributions as a part of a complete probabilistic method which would include more foodstuffs possibly contaminated by OTA. The analysis by simple distributions is a method that employs distributions of food consumption but uses a fixed value for the contamination variable (Kroes et al., 2002). Deterministic and probabilistic methods were used to model the consumption data.

2. MATERIALS AND METHODS

2.1. Sampling

In order to assess differences among brands, 45 samples of four nationally available ground roasted coffee brands with four types per brand (100% naturally roasted, mixture 50% naturally roasted and 50% sugar roasted, decaffeinated 100% naturally roasted, and decaffeinated mixture 50% naturally roasted and 50% sugar roasted) were analysed. Three samples per brand and per type were analysed, except the decaffeinated mixture 50% naturally roasted and 50% sugar roasted of one brand, which was not available in markets.

In order to assess the exposure of Catalan population to OTA through coffee, in the month of November 2008 a total of 204 samples of different kinds of ground roasted coffee of 27 different brands were purchased in hypermarkets and supermarkets of twelve cities of the region of Catalonia, Spain (Barcelona, Girona, L´Hospitalet de Llobregat, Lleida, Manresa, Mataró, Reus, Sabadell, Tarragona, Terrasa, Tortosa and Vilanova i la Geltrú); these cities account for 72% of the total population in Catalonia. Three samples (packages of 250 g each), when available, were randomly taken in six markets per city. Samples taken in each market were pooled and a total of 72 coffee composites was obtained for analysis. Sampled coffee sorts were: roasted (100% naturally roasted, mixture 50% naturally roasted and 50% sugar roasted, mixture 70% naturally roasted and 30% sugar roasted), and decaffeinated (100% naturally roasted, mixture 50% naturally roasted and 50% sugar roasted).

2.2. OTA chemical analysis

Extraction: 5 g of ground coffee were weighed in an amber flask and 100 mL of a 1% $NaHCO_3$ solution were added. Mixture was blended for ten minutes by means of a magnetic stirrer. The homogenised sample was filtered by gravity (Whatman Nº 1 filter).

Clean-up by immunoaffinity columns (IAC): 5 mL of the filtrate were mixed with 5 mL of PBS (phosphate buffered saline, solution containing 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4, 8 g NaCl and 1 L H2O, pH = 7.4). Diluted extract was loaded onto the IAC (Ochraprep, R-Biopharm, Rhône LTD) and allowed to pass through it by gravity. After washing the column with 20 mL PBS and drying it with air, OTA was eluted with 1.5 mL desorption solution (methanol:acetic acid, 98:2) in an amber vial. Backflushing was done three times. Finally, 1.5 mL Milli-Q water was passed to obtain a total volume of 3 mL.

Chromatographic analysis: High performance liquid chromatography (HPLC) was performed on the clean-up-step eluate on a Waters 2695 Separations Volume (Alliance) coupled to a Waters 2475 Multi λ fluorescence detector. Waters Spherisorb ODS2 C18 column (5 µm, 4.6 x 150 mm) equipped with a Waters Spherisorb ODS2 guard column (5 µm, 4.6 x 10 mm) (Waters, Ireland) was used. Mobile phase consisted of acetonitrile:Milli-Q water:acetic acid (51:47:2). Flow-rate was 1 mL/min. Injection volume was 100 µL. Excitation and emission wavelengths were 333 nm and 443 nm, respectively. Temperature of column and guard column was maintained at 40ºC. Retention time for OTA was 5.4 min. The integration software was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00).

Validation: Calibration curve was prepared by serial dilution of a stock solution, with eight points of different concentrations from 0.098 to 12.5 ng/mL; each concentration was injected three times. Determination coefficient R^2 was 0.999. Limit of detection (LOD) was 1.16 ng/mL (signal:noise ratio 3:1). Samples were considered positive when their concentration was above the LOD. Recovery rates for spiked samples (n = 5) at the levels of 2, 3, 5 and 8 ng/g were 79%, 78%, 82% and 102%, respectively. Coefficient of variation was 17%. Results were not corrected for recovery. Repeatability was assessed at the same day at the level of 2 ng/g (n = 5), and reproducibility was tested on three different days also at 2 ng/g. Relative standard deviations were 11.63% and 1.92%, respectively.

Confirmation of OTA identity: Positive samples were confirmed by derivatization of the toxin to its methyl ester according to Patel, Hazel, Winterton, & Gleadle (1997), with some modifications. Briefly, 0.5 mL of eluate were transferred to a 3 mL vial and evaporated to dryness at 40ºC under a stream of nitrogen. Dry extract was resuspended in 0.5 mL 14% boron trifluoride-methanol. Vial was capped and heated at 60ºC for 20 minutes. After heating, derivatised extract was evaporated to dryness at 40ºC under a stream of nitrogen and resuspended in 1 mL mobile phase. Derivatised samples were analyzed by HPLC in the same conditions as for OTA analysis. The retention time for the OTA methyl ester was 11.6 minutes.

2.3. Consumption data

Coffee consumption data were obtained by means of a food frequency questionnaire that included 32 food items with potential OTA contamination. The survey was administered by trained interviewers from January 2008 to February 2009 to 885 inhabitants of several localities in the province of Lleida (389 men and 496 women) aged 18 to 66, out of which 753 were coffee consumers (332 men and 421 women). Participants indicated frequency and quantity of consumption of the listed foods. Participants were asked to answer frequency of consumption as their average consumption in the period of days, weeks, months or in a year, as well as no consumption. We assumed that the consumption pattern of the surveyed population was the same for all the inhabitants of the region of Catalonia.

2.4. Assessment of the exposure to OTA by coffee by simple distributions

Exposure to OTA was assessed by the estimation of the toxin's daily intake. DI was calculated as the individual coffee consumption multiplied by a unique mean value of contamination so as to obtain simple distributions of the intake. The contamination value was the mean value obtained from the detected and non-detected concentrations in samples. Contamination of samples in which OTA was not detected was considered to have a value of half the limit of detection of the method, as suggested by GEMS/Food-EURO (1995) according to the proportion of results under the LOD in the total analysed samples. Consumption data were modelled by means of deterministic and probabilistic methods. In the deterministic method, consumption data were those obtained by the questionnaires done to the interviewed population. In the probabilistic estimation, the mentioned consumption data were modelled in a semi-parametric way and also in a non-parametric way (Gauchi & Leblanc, 2002).

2.4.1. Deterministic estimation

OTA daily intake (ng OTA/kg bw/day) was calculated by multiplying coffee OTA mean contamination (ng/g) by coffee individual consumption data (g/kg bw/day).

2.4.2. Probabilistic modeling of consumption data

Consumption data (g/kg bw/day) were randomized by two different methodologies: semi-parametric and non-parametric. Randomization was performed in Microsoft ® Office Excel 2003 and its add-in, Simtools.

2.4.2.1. Semi-parametric procedure

This procedure consisted on fitting the consumption data to a parametric distribution and thereafter randomizing the consumption original distribution according to the fitted distribution. Goodness-of-fit tests (Chi-square, Kolmogorov-Smirnov and Anderson-Darling) were performed for the Normal, Lognormal, Gamma, Weibull, and Exponential distributions. Fitting was qualitatively assessed by the construction of Q-Q plots, which displayed the observed values of the consumption dataset against theoretical or expected values of the named distributions. In these plots, a 45-degree reference line is drawn. If the distribution of both datasets is the same, the points should fall approximately along this reference line. The randomization algorithm took into account scale and shape factors of the fitted Gamma distribution and the percentage of non-consumers from the total population. Number of iterations was 5000. Fitting distributions and goodness-of-fit tests were performed with the software Statgraphics Plus 5.1.

2.4.2.2. Non-parametric procedure

Randomization of consumption was performed by randomly sampling values from the original consumption distribution. Number of iterations was 5000.

2.5. Statistical analysis

Concerning the estimated DI, nonparametric testing was performed by means of the Kruskal Wallis test to evaluate differences among groups of population (SAS 9.1.3, Cary, NC, USA).

3. RESULTS AND DISCUSSION

3.1. OTA occurrence in different ground coffee brands

43 out of 45 samples of four different brands presented OTA concentrations above the LOD of the method, and positive samples ranged from 1.30 to 5.24 ng/g. Mean OTA concentration of the positive samples was 2.07 ± 0.61 ng/g. No significant differences were found among brands neither among coffee types. The maximum was the only value that surpassed the limit set by the European Union (5 ng/g). Excluding this value, the range of contamination was 1.30 to 2.75 ng/g, which is below the established limit.

3.2. OTA occurrence in ground coffee in Catalonia

Out of 72 composite samples, 35 were positive, i.e., 48.6% of the samples presented contamination values above the LOD of the method. The positive levels

ranged from 1.21 to 4.21 ng/g and the mean concentration of positive samples was 2.17 ± 0.79 ng/g. Mean coffee contamination was higher than the previously found in Spain (0.88 ng/g, n = 29, Burdaspal et al., 1998). Contamination distribution is showed in Figure 1.

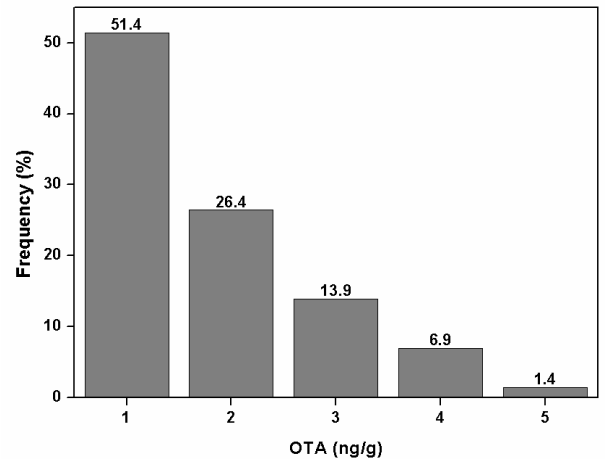

Figure 1. Contamination frequency distribution (percentage) of coffee composites. Non detected samples were assigned the value of half the limit of detection (LOD = 1.16 ng/g).

Similar OTA levels were found when analysing samples by brands and by composites. However, incidence was different in both samplings. This can be due to the method of sampling itself (restricted to certain brands in the first case), or else to the heterogeneity of the distribution of the mycotoxin.

Regarding OTA occurrence in ground coffee in the region of Catalonia (48.6%), Van der Stegen et al. (1997) and the European Commission (2002) assessed the occurrence of OTA in groups of countries, and showed a similar occurrence to the here presented, with 47% (n = 633) and 46% (n = 1184) occurrence, respectively. Van der Stegen et al. (1997) screened OTA levels in different kinds of coffee in eight European countries (Belgium, Finland, France, Germany, Italy, the Netherlands, Switzerland and the United Kingdom), and OTA levels in roasted coffee ranged from 0.5 to 8.2 ng/g. The European Commission (2002) studied the dietary intake of OTA through several foodstuffs by the population of Denmark, Finland, France, Germany, Norway, Sweden, the Netherlands, The United Kingdom, Italy, Greece, Spain, and Portugal. Each country provided food contamination and consumption data. OTA levels in roasted coffee reached 13.1 ng/g. However, in the other studies done by individual countries (Brazil, Canada, Denmark, Germany, Hungary,

Japan, Spain, United Kingdom, United States), OTA occurrence in commercial roasted coffee was different for each case and varied from 7 to 100%, whereas OTA levels varied from 0.1 to 17 ng/g, as showed in the compilation of Codex Alimentarius Commission (2008). Thus, our results concerning coffee contamination remain within the ranges of contamination detected in Spain and in other countries.

3.3. Coffee consumption

3.3.1. Coffee consumption per capita (g/person/day)

Study population was stratified in order to assess group differences by sex and age (Table 1). Descriptive statistics of coffee consumption (g/person/day) by the total interviewed population and by only consumers are listed in Table 1. Figure 2 shows the histogram of the consumption data by only consumers. A similar shape was observed when the histogram was built by sex and age groups.

The amount of ground roasted coffee needed to prepare a cup of coffee for the defined portion in this study was 6.2 g. Thus, median of consumption by only consumers indicates two cups of coffee per day. A similar result was obtained for data acquired in 2006 for consumption of coffee and infusions in the whole country (Dirección General de Industrias Agroalimentaria y Alimentación, 2007): 3.76 kg of coffee were bought per capita and year, which is equivalent to 10.3 g per person and per day. No significant differences were observed between men and women. Median consumption by men was higher. However, the rest of descriptive statistics were similar in both sexes. Significant differences were observed between age groups A (18-29 years old) and B (30-44 years old) (Pr > Chi square = <0.0001), and A and C ($\geq$ 45 years old) (Pr > Chi square = 0.0004), whereas between B and C differences were not significant. When comparing medians, consumption by the youngest group of population was half the consumption of the rest. Group B presented the highest values of consumption regarding mean, maximum and 99th percentile. Regarding the total population, results of the analysis of group differences were similar to those obtained for only consumers.

Table 1. Coffee consumption (g/person/day) by all participants and by only consumers classified by sex and age groups.

	n	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Total population	885	9.85	9.05	6.81	0.0000	62.88	0.00	24.80	31.00	38.08
Men	389	9.87	8.89	7.95	0.0000	62.00	0.00	24.80	31.00	37.20
Women	496	9.84	9.18	6.20	0.0000	62.88	0.00	24.80	31.00	40.30
Age group (years)										
A (18-29)	290	7.37	7.77	6.20	0.0000	43.40	0.00	21.25	25.68	31.00
B (30-44)	308	11.52	10.09	12.40	0.0000	62.88	0.00	24.80	37.20	46.50
C (≥ 45)	287	10.58	8.55	7.97	0.0000	55.80	0.00	25.21	31.00	38.08
Only consumers	753	11.58	8.73	12.40	0.0170	62.88	0.61	25.21	31.00	40.30
Men	332	11.56	8.54	12.40	0.0170	62.00	0.41	24.80	31.00	37.20
Women	421	11.59	8.89	9.30	0.0170	62.88	0.61	25.68	31.00	40.30
Age group (years)										
A (18-29)	236	9.05	7.67	6.20	0.0170	43.40	0.20	24.80	31.00	31.00
B (30-44)	262	13.54	9.61	12.40	0.0340	62.88	1.77	27.90	37.20	49.60
C (≥ 45)	255	11.90	8.15	12.40	0.0849	55.80	1.77	25.68	31.00	38.08

[1] Minimum
[2] Maximum

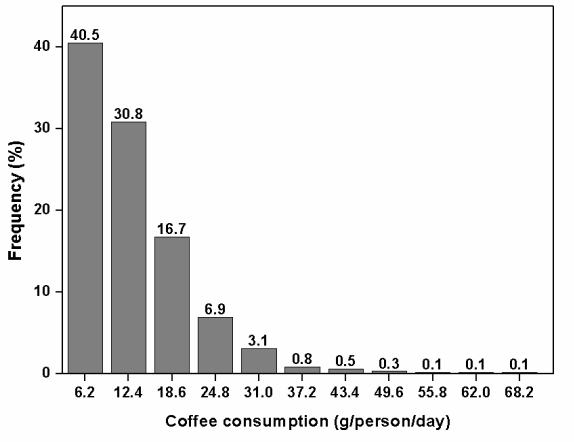

Figure 2. Frequency distribution (percentage) of coffee consumption (g/person/day) by only consumers.

3.3.2. Coffee consumption per body weight (g/kg bw/day)

As a previous step to the calculation of the daily intake of OTA, individual coffee consumption was divided by the individual body weight (kg bw). For consumers, levels ranged from 0.0002 to 1.86 g/kg bw/day and the mean consumption was 0.16 ± 0.14 g/kg bw/day. When considering the consumer population, significant differences were observed between consumption of men and women (Pr > Chi square = 0.0009); this shows the influence of the body weight value in the magnitude of the intake when included in its calculation. Regarding differences among age groups, results were equal to the obtained in the analysis for consumption per capita.

3.4. Assessment of the exposure to OTA by coffee

Histogram of coffee consumption (Figure 2) shows a distribution whose shape approaches to the Gamma distribution. For the semi-parametric procedure, goodness-of-fit tests showed no fitting to any of the proposed distributions in Materials and Methods section, except in the case of the Anderson-Darling test that showed a value of ≥ 0.10 for the Gamma distribution. Q-Q plots were built to qualitative check the fitting results (Figure 3). Therefore, randomization of consumption was performed according to the Gamma distribution.

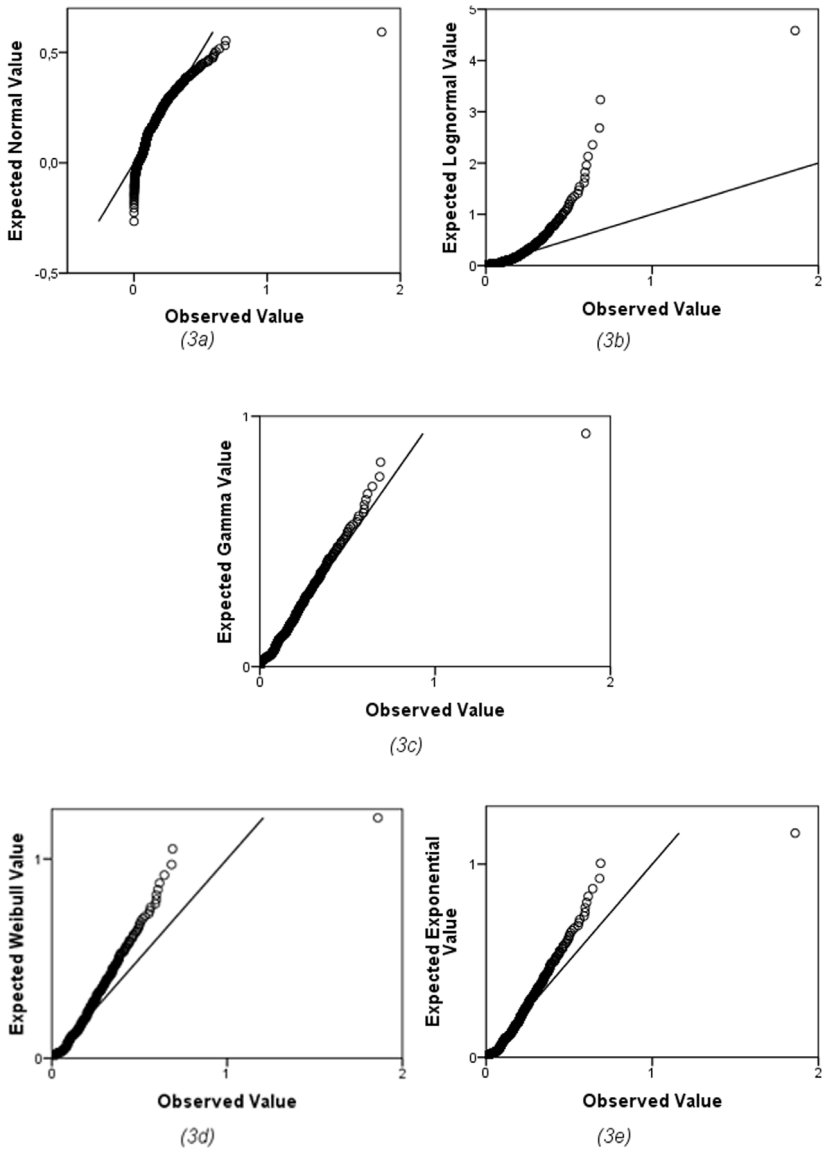

Figure 3. Q-Q plots of Normal (3a), Lognormal (3b), Gamma (3c), Weibull (3d), and Exponential (3e) distributions for consumption of coffee by the consumer population.

Descriptive statistics of the deterministic and probabilistic estimations (semi-parametric and non-parametric) of the intake of OTA by coffee are shown in Table 2. Deterministic analysis was performed separately for the whole population and for only consumers.

Table 2. OTA daily intake estimations (ng/kg bw/day).

Estimation	*n*	Mean	SD	Median	Min[1]	Max[2]	Percentiles			
							5th	95th	97.5th	99th
Deterministic										
Total population	885	0.19	0.19	0.14	0.0000	2.51	0.0000	0.52	0.63	0.80
Only consumers	753	0.22	0.18	0.18	0.0003	2.51	0.0119	0.53	0.66	0.80
Probabilistic										
Semi-parametric	5000	0.22	0.19	0.17	0.0001	1.60	0.0212	0.62	0.73	0.87
Non-parametric	5000	0.19	0.19	0.14	0.0000	2.51	0.0000	0.52	0.62	0.75

[1] Minimum
[2] Maximum

Similarities could be observed when comparing deterministic estimation distribution for only consumers and semi-parametric distribution, as well as in the comparison of the deterministic estimation distribution for all the population and the non-parametric distribution. High percentiles obtained by all the tested methods were also similar. Mean of semi-parametric distribution was equal to the mean of the deterministic distribution calculated for only consumers, and median values for both were almost equal. However, when considering the high percentiles, values of intake were higher in the case of semi-parametric estimation. Maximum was higher in the deterministic estimation. The distribution of the semi-parametric estimation resulted to be similar to the deterministic estimation distribution; although it can be observed that the modeling excluded the maximum value of the deterministic estimation. Regarding non-parametric treatment, mean, median, and extreme values were equal to those obtained from the deterministic distribution of all the surveyed population, whereas high percentiles values were very similar. It has to be taken into account that no specific criteria were used to randomize the data, as done in the semi-parametric case.

3.4.1. OTA DI differences within population groups

Significant differences were observed for all population groups (Table 3) except between the age groups B (30-44 years old) and C ($\geq$ 45 years old) in the deterministic estimation. Similarities were detected only when the analysed dataset was the original and not the randomized datasets that were used in the probabilistic analysis.

Table 3. *P* values for difference tests between population groups (gender and age) for the estimated daily intake of OTA. Differences are significant at $P < 0.05$.

Estimation	Pr > Chi-square				
	Sex	Age group*			
		All	AB	AC	BC
Deterministic					
Total population	0.0131	<.0001	<.0001	<.0001	0.5761
Only consumers	0.0009	<.0001	<.0001	0.0004	0.0941
Probabilistic					
Semi-parametric	<.0001	<.0001	<.0001	<.0001	<.0001
Non-parametric	<.0001	<.0001	<.0001	<.0001	0.0042

*Age group (years): A (18-29), B (30-44), C (≥ 45).

3.5. Exposure assessment

OTA mean DI by coffee consumption estimated by different methods (0.19 to 0.22 ng/kg bw/day) resulted to be far below the latest PTDI value of 17 ng/kg bw/day (EFSA, 2006), even in the high percentiles of exposure (Table 2). These estimations represent less than the 2% of the PTDI, whereas the 99th percentile estimated DIs were around the 5% of those values. Only the maximum value of 2.51 ng/kg bw/day, which was an outlier, represented a 15% of the PTDI.

As mentioned in section 2, the European Commission (2002) assessed OTA DI in several European countries. In the case of the intake of OTA by coffee, the estimated DI in Spain was 0.15 ng/kg bw/day, which is slightly lower than the here estimated. In the rest of the countries, DI varied from 0.06 (Italy) to 0.42 ng/kg bw/day (Finland). In all these cases, as well as in the present study, intake estimations were calculated assuming that OTA present in the roasted coffee was fully extracted in the coffee brew. However, studies evidence that the percentage of extraction of the toxin varies according to the brewing process, such as infusion, espresso, moka, or drip brew (Pérez de Obanos, González-Peñas, & López de Cerain, 2005; La Pera, Avellone, Lo Turco, Di Bella, Agozzino, & Dugo, 2008). Therefore, OTA intake can be even lower than the previously estimated values.

4. CONCLUSIONS

From the results obtained in this study, we can observe that coffee contamination by OTA in the Spanish region of Catalonia was below the limit set by the European Union, except one sample, and that the incidence was low. Consumption per capita was similar to previously obtained data for the whole country. Control of the raw

material, throughout processing, until the finished product is obtained, can contribute to minimize the exposure to mycotoxins. Suggestions on this subject were presented on the draft of a proposed "Code of practice for the prevention and reduction of ochratoxin A contamination in coffee" (Codex Alimentarius Commission, 2008).

Differences were observed in the consumption among population groups. Moreover, when the consumption per kg body weight was analysed, results of the analysis among groups were different to those obtained by analysing consumption per capita. Estimated OTA daily intake for the studied population (adults from 18 to 66 years old) was below the latest PTDI. However, attention should be paid to the worst cases of high contamination and high consumption, in combination to other sources of the toxin. Deterministic procedure and probabilistic treatment of consumption data utilized to obtain simple distributions of the intake showed similar results of descriptive statistics, but not in the case of group differences. The probabilistic method was presented as step for the exposure assessment method to be used when more foodstuffs are included in the analysis.

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STUDY V

Assessment of the exposure to ochratoxin A by its detection in certain foodstuffs sampled in Catalonia, Spain

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ABSTRACT

Ochratoxin A (OTA) was analysed in composite samples of cereal-based baby foods, beer, breakfast cereals (corn- and wheat-based), loaf bread, peanuts, and pistachios. Foodstuffs were collected in hypermarkets and supermarkets of twelve cities of the Spanish region of Catalonia, and composite samples were prepared for analysis: liquid-liquid extraction, followed by immunoaffinity column clean-up and HPLC-fluorescence detection. Consumption data of the selected foodstuffs were collected by means of a food frequency questionnaire. The studied population was grouped by age in infants, children, adolescents, and adults, and exposure to OTA through the mentioned foodstuffs, and through wine and coffee, was assessed. Exposure assessment was done through deterministic and probabilistic modelling of the contamination and consumption data. OTA occurrence and mean of positive samples (ng/g or ng/mL, for beer) were the following: 8.7% and 0.233 in baby foods; 88.7% and 0.022 in beer; 2.8% and 0.728 in corn-based breakfast cereals; 25% and 0.293 in wheat-based breakfast cereals; 12.9% and 0.283 in loaf bread; 41.7% and 0.241 in peanuts; 2.9% and 0.228 in pistachios. The median estimated daily intake of OTA through the considered foodstuffs by each age group was below the latest provisional tolerable daily intakes (PTDIs) of 17 and 14 ng/kg bw/day recommended by EFSA (2006) and JECFA (2007), respectively, ranging from 1 and 2% of those values in adolescents and children, to 3 and 11% in adults and infants.

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1. INTRODUCTION

Ochratoxin A (OTA) is a fungal secondary metabolite produced by some species of the genera *Aspergillus* and *Penicillium*. Studies on animals have characterised this mycotoxin as nephrotoxic, hepatotoxic, neurotoxic, immunotoxic, teratogenic and carcinogenic. Chronic human exposure to OTA has been related to the development of urinary tract tumours and the Balkan Endemic Nephropathy, and the International Agency for Research on Cancer classified OTA as possibly carcinogenic to humans (group 2B) (IARC, 1993).

Sources of human exposure to OTA are mainly foodstuffs of vegetal origin, and it is possible to find OTA in cereals and derivatives, wines and grape juices, coffee, beer, nuts and dried fruits, spices, and in a minor extent, in animal by-products. Considering such ubiquity and the mentioned toxic effects, international authorities have proposed values of tolerable daily or weekly intakes of the toxin, which indicate the dose that can be safely consumed daily/weekly over a lifetime without incurring in appreciable adverse health effects (WHO, 1999). Thus, provisional tolerable daily intake (PTDIs) of 17 and 14 ng/kg bw/day were recommended by EFSA (2006) and JECFA (2007), respectively.

Control of the presence of mycotoxins in the European Union has been favoured by the establishment of maximum levels in certain foodstuffs. Thus, in the case of OTA, maximum levels were set for unprocessed cereals and derivatives, cereal-based baby foods, coffee, wines, dried vine fruit and grape juices, ranging from 0.5 μg/kg in foodstuffs intended for babies and infants, to 10 μg/kg in dried vine fruit and soluble coffee (European Commission, 2006). More recently, maximum levels for liquorice and some spices were also set (European Commission, 2010).

Exposure to OTA by humans can therefore be assessed by the detection of the toxin in possibly contaminated foodstuffs, as well as by the evaluation of the dietary habits of a population, especially of the consumption of those foodstuffs. Thus, the evaluation of the exposure of the inhabitants of the Spanish region of Catalonia to OTA was the aim of this study. For this, certain possibly contaminated foodstuffs were collected in several localities of the region and OTA levels were therein determined. The following step was to estimate the intake of OTA due to the consumption of the analysed foodstuffs. Therefore consumption data of Catalan individuals were used together with the obtained contamination data to quantitatively estimate the daily intake of OTA. Two methodologies were used to perform this estimation: an analysis by simple distributions and a probabilistic analysis. The first one is a method that employs distributions of food consumption but uses a fixed value for the concentration variables, whereas probabilistic analysis utilises distributions of both the food consumption and contamination data, and simulates dietary exposure by drawing random values from each input distribution (Kroes et al., 2002).

2. MATERIALS AND METHODS

2. 1. Sampling

Samples of breakfast cereals made of corn and of wheat and rice, cereal-based baby foods (multicereals), beer, loaf bread (white and wholemeal), peanuts, and pistachios, were purchased in the months of June to November 2008 in hypermarkets and supermarkets of twelve cities of the region of Catalonia, Spain: Barcelona, Girona, L´Hospitalet de Llobregat, Lleida, Manresa, Mataró, Reus, Sabadell, Tarragona, Terrasa, Tortosa, and Vilanova i la Geltrú. These cities account for 72% of the total population of Catalonia.

Samples were bought in six stores per city, and three samples of each foodstuff, when available, were randomly picked in each store. Samples corresponding to each store were pooled to obtain a composite sample per store. The number of composites per foodstuff is listed in Table 2.

2.2. OTA chemical analysis

2.2.1. Conditioning of food samples

Breakfast cereals and loaf bread: Breakfast cereals were crushed (Moulinex crusher DPA139). Loaf bread was let dry and afterwards crushed.

Beer: Samples were degassed by ultrasound treatment for 40 minutes, and pH was adjusted to 7.2 by adding 2M NaOH.

Peanuts: Most of the samples were purchased unshelled. If peanuts were shelled, shells were taken off and afterwards nuts were milled (Moulinex crusher DPA139).

Pistachios: Whole pistachios (shells and nuts) were milled (FOSS 1093 Cyclotec^{TM} Sample Mill).

2.2.2. Extraction of OTA

5g of sample were mixed with 20 mL (peanuts and pistachios) or 25 mL (breakfast cereals, loaf, and cereal-based baby food) of 60% acetonitrile in an amber flask. Mixture was blended for ten minutes in the capped flask by means of a magnetic stirrer and afterwards filtered (Whatman Nº 1 filter).

2.2.3. Clean-up of samples by immunoaffinity chromatography columns (IACs)

This step consisted on mixing certain volumes of the filtered liquid extract of a foodstuff (except beer) with a certain volume of phosphate buffered saline (PBS) solution, and then on loading this mixture onto the IAC (Ochraprep, R-Biopharm, Rhône LTD). The mixture was let pass by gravity. PBS was prepared by dissolving in 1 L of water the following: 0.2 g potassium chloride, 0.2 g potassium di-hydrogen phosphate, 1.2 g di-sodium hydrogen phosphate anhydrous and 8 g sodium chloride (Panreac, Barcelona, Spain). pH was adjusted to 7.4 with sodium hydroxide. For each foodstuff, volumes of extract and PBS were the following:

Breakfast cereals, loaf bread, and cereal-based baby food: Two mL of filtrate were diluted with 22 mL PBS.

Peanuts and pistachios: Four mL of filtrate were diluted with 44 mL PBS.

Beer samples were not mixed with PBS; 150 mL of the sample (adjusted to pH 7.2) were let pass through the IAC.

In all cases, after the diluted extracts passed through the IACs, columns were washed with 20 mL PBS, then air was passed through them and wash liquid was discarded. The final step of the clean-up procedure was the elution of OTA in an amber vial. For that, 1.5 mL desorption solution (methanol: acetic acid, 98:2) was loaded onto the IAC. During elution, backflushing (reversing the flow in the IAC) was performed 3 times. Finally, 1.5 mL Milli-Q water was passed and a final volume of 3 mL was obtained. Air was passed to collect the last drops of eluate.

2.2.4. HPLC analysis

Analysis of the clean-up final extracts was done by high performance liquid chromatography (HPLC) using a Waters 2695 Separations Module (Alliance) coupled to the Waters 2475 Multi λ fluorescence detector. Waters Spherisorb ODS2 C18 column (5 µm, 4.6 x 150 mm) equipped with a Waters Spherisorb ODS2 guard column (5 µm, 4.6 x 10 mm) (Waters, Ireland) was used. The integration software used to manage the chromatographic data was Empower 2 (2006 Waters Corporation, Database Version 6.10.00.00). Mobile phase consisted of acetonitrile:Milli-Q water:acetic acid (51:47:2). Flow-rate was 1 mL/min, and injection volume was 100 µL. Excitation and emission wavelengths were 333 nm and 443 nm, respectively. Temperature of column and guard column was maintained at 40ºC. OTA retention time was 5.4 min.

2.2.5. Validation of the analytical methods

Validation of the methods of analysis of the different foodstuffs was performed by the evaluation of their linearity, recovery, repeatability, and limit of detection (LOD). LOD was calculated using the signal-to-noise ratio of 3. A calibration curve was built for the

analysis of each foodstuff by serial dilution of a stock solution in the range 0.012 – 12.5 ng/mL and it was linear in that range. Coefficient of determination (R^2) was higher than 0.998 for all cases. Recovery rates were evaluated by spiking the samples with certain amounts of OTA standard solutions. Inter-day repeatability was evaluated in three different days for a certain concentration in each foodstuff. Results of the validation assays are shown in Table 1.

Table 1. Results for recovery in the different spiking levels, repeatability (intra- and inter-day), and LOD for each foodstuff.

Foodstuff	Spiking level (ng/g)*	Recovery rate (%)	RSD intra-day (%)	RSD inter-day (%)	LOD (ng/g)*
Baby foods	0.3	101.67	7.90		0.180
	0.5	102.00	1.47		
	0.8	99.06	2.38		
	1	93.25	0.46	4.27	
Beer	0.05	89.40	4.06		0.003
	0.2	85.73	2.95		
	0.5	89.91	12.59	6.30	
Breakfast	0.8	90.94	2.82		0.098
cereals	1.5	106.83	2.93	2.59	
	3	100.42	3.96		
	5	100.45	2.65		
Loaf bread	0.5	103.90	4.32		0.139
	0.8	104.00	0.72		
	1.5	110.13	2.27		
	3	99.65	1.02	2.24	
Peanuts	0.5	71.48	7.79		0.072
	0.8	99.30	4.98		
	1	93.94	1.37	4.19	
Pistachios	0.5	100.96	0.73		0.129
	1	88.76	1.39		
	1.5	96.08	1.85	7.45	
	2	97.84	6.23		

* ng/mL in the case of beer.

2.3. Consumption data

Data of consumption of the listed foods were obtained by means of a food frequency questionnaire (FFQ), which included 33 foodstuffs possibly contaminated by OTA (Coronel et al., 2009). The survey was administered by trained interviewers from January 2008 to February 2009 to inhabitants of several localities in the Catalan province of Lleida. Gender, age and weight of the participants were also recorded. Population was classified according to their age in infants (0-3 years old, n = 164), children (4-9 years old, n = 68), adolescents (10-17 years old, n = 211), and adults (18-65 years old, n = 905). Parents were interviewed for infants' responses. The number of participants classified by gender was, in the adolescent group, 89 males and 122 females; and in the adult group, 396 males and 509 females. Food consumption of infants and children was assumed to be equal for both genders.

For calculation purposes, individual consumption data (g foodstuff/person/day) obtained from the FFQs was normalised by dividing them by the corresponding individual body weight (g foodstuff/kg body weight/day).

2.4. Estimation of the daily intake of OTA

Estimation of the OTA daily intake (ng OTA/kg bw/day) was performed by deterministic and probabilistic procedures, taking into account the contamination data of OTA in the analysed foodstuffs (ng OTA/g foodstuff) and the normalised consumption data (g foodstuff/kg bw/day) of the surveyed population. Results of the intake estimations were listed as descriptive statistics such as means, median and high quantiles of the obtained distributions. Measures of asymmetry of the distributions (skewness and kurtosis) were also shown.

Data of the contamination distribution in coffee were taken from a previous publication (Coronel et al. 2010), in which the sampling procedure was the same as in this work. Additional contamination data of red and dessert wine were also included: distribution data of OTA presence in samples previously collected in Spain (Bellí et al., 2004; Valero et al., 2008) were incorporated in this work in order to complete the analysis of the exposure to OTA, as wines consumption is considered to be an important source of OTA in the diet.

2.4.1. Treatment of contamination censored data: Alternatives for the values below the limit of detection of the method of analysis

Contamination censored data (values below the LOD or non detectable results, from now on: ND) were treated as advised by GEMS/FOOD-Euro (1995) to obtain a simple estimate of the mean. The alternatives depend on the proportion of results below the LOD:

A) If the proportion is ≤ 60%, the value of LOD/2 should be used for the results less than LOD.

B) If the proportion is >60% but ≤ 80%, and with at least 25 results quantified, two estimates should be produced: using zero and LOD for the results less than LOD.

C) If the proportion is >80%, two estimates should be produced: using zero and LOD for the results less than LOD and the estimation of other descriptive statistics will not be practicable.

Thus, values for mean contamination could be one value if ND data were replaced by LOD/2, or two if ND were replaced by zero and LOD.

2.4.2. Deterministic approach

The deterministic estimation of the intake was achieved by the analysis of simple distributions, which consider the average value of contamination of a foodstuff, and the individual values of consumption of the study population (Kroes et al., 2002). Contamination mean values were calculated according to the above mentioned criteria (Section 4.1). The estimation of the daily intake was performed by multiplying the individual consumption data of each foodstuff by its mean contamination obtained after the treatment of the ND. Total OTA daily intake was the sum of the individual OTA intakes through the different foodstuffs, and therefore two exposure scenarios were obtained (ND replaced by zero and ND replaced by the LOD). The values replaced by the LOD/2 were included in both estimations.

2.4.3. Probabilistic approach

The probabilistic or stochastic procedure used was based on the Mixed Parametric-Parametric (*MP-P*) method reported extensively in Gauchi and Leblanc (2002) and Cano-Sancho et al. (2011), as advantages against non parametric methods were hence elucidated, being the most important the fact that the latter can possibly lead to less reliable estimations, especially of the high quantiles.

In this methodology, a mixed probability density function (*pdf*) was fitted to each food consumption, and a parametric *pdf* was fitted to each food contamination (Gauchi and Leblanc, 2002):

1) The appearance of the consumption histograms was irregular (Figure 3), especially for those foods whose consumption is seasonal or sporadic, such as dessert wine, pistachios or peanuts. Data in the histograms could be divided in two: non consumers and consumers. Thus, a mixed distribution was fitted as follows:

$$U_{\pi_0,j}^{[D]} = \left\{ \left[U\left(0, c_{i_{\min}(\pi_0),j}\right) \right] j, h; \left[\Gamma\left(r, \lambda, \theta\right) \right]_{\pi_{0,j}}, \left(1-h\right) \right\}$$

Where $\left[U\left(0, c_{i_{\min}(\pi_0),j}\right) \right]$ is the continuous uniform distribution defined on the interval $\left(0, c_{i_{\min}(\pi_0),j}\right)$ with $c_{i_{\min}(\pi_0),j}$ as the minimal consumption of the foodstuff *j*, in the sample π_0 (this part corresponds to the non consumer class). $\left[\Gamma\left(r, \lambda, \theta\right) \right]_{\pi_{0,j}}$ is the Gamma or Lognormal fitted consumption distribution for the foodstuff *j* (this part corresponds to the consumers class). $U_{\pi_0,j}^{[D]}$ means a sampling from a discrete uniform distribution: a random number *u* is drawn from a continuous uniform distribution defined on [0; 1]. If *u* is less than or equal to *h* (proportion of non consumers), then a new random number *u'* is drawn from $\left[U\left(0, c_{i_{\min}(\pi_0),j}\right) \right]_j$, otherwise a new random number is drawn from $\left[\Gamma\left(r, \lambda, \theta\right) \right]_{\pi_{0,j}}$.

2) Food contamination treated censored data (Section 4.1) were fitted in most cases to the gamma distribution and in some cases to the lognormal distribution for each population group. As in the deterministic estimation, two exposure scenarios were obtained (ND replaced by zero and ND replaced by the LOD).

3) The *MP-P* method used to estimate the normalised exposure *k* of the *S* simulation set was built as follows:

$$\hat{E}_k^{[MP-P]} = \sum\nolimits_{j=1}^{p} \tilde{c}_{k(\hat{F}_{U,j}),j} \tilde{t}_{k(\hat{F}_{Tj}),j}$$

Where $\tilde{c}_{k(\hat{F}_{U,j}),j}$ is a random normalised consumption for the foodstuff *j*; the random deviate *k* is drawn from $\hat{F}_{U,j}$, the corresponding cumulative distribution function (*cdf*) adjusted of the distribution consumption defined above. $\tilde{t}_{k(\hat{F}_{Tj}),j}$ is a random contamination for the foodstuff *j*; the random deviate *k* is drawn from $\hat{F}_{T_j}$, the fitted gamma *cdf* of contamination. In case of wheat flakes, the mean contamination was used in the simulations because not enough data were available for computations.

The mean of normalised exposures over the simulation set *S* was then estimated using the following equation, where *n* was the number of random deviates drawn (10000 in the present study):

$$\hat{E}_S^{[MP-P]} = \frac{1}{n}\sum_{k=1}^{n} \hat{E}_k^{[MP-P]}$$

Other descriptive statistics were directly computed on the histogram built with the simulations of the *S* set.

Pseudo-parametric bootstrap confidence intervals were built as reported by Gauchi and Leblanc (2002), by randomly drawing *B* samples of size n_{π_0} in the exposure simulation set *S* (being *B* equal to 10000). The boundaries of the 95% confidence interval were calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution.

Statistical program SAS 9.0 (Cary, NC, USA) was used for the probabilistic analysis.

2.5. Other statistical analysis

Differences between population groups (sorted by gender and age) were evaluated by means of the non-parametric tests Wilkoxon and Kruskal-Wallis.

3. RESULTS AND DISCUSSION

3.1. Presence of OTA in foodstuffs sampled in Catalonia

Table 2 shows the occurrence of OTA in the foodstuffs considered in this work, and Figures 1 and 2 show the distributions of the contamination by OTA. Data of contamination in loaf bread were corrected for water content, as the measured dry weight was 77% of the fresh samples. The correction factor was then 0.77. Data of contamination in pistachios were corrected for the shells proportion. It was observed that the edible part constituted a 56% of the total weight. Thus, assuming the worst case in which all the detected toxin was in the edible portion, the correction factor was 0.56^{-1}, which equals to 1.79.

Table 2. OTA levels (ng/g, and ng/mL for beer and wines) in food composites and samples of wines.

Foodstuffs	n	Positive samples	% of positive samples	Mean of positives	SD	Median	Min[d]	Max[e]
Baby foods	69	6	8.7	0.233	0.036	0.225	0.195	0.293
Beer	71	63	88.7	0.022	0.023	0.015	0.004	0.126
Breakfast cereals								
Corn-based	71	2	2.8	0.728	0.764	0.728	0.188	1.268
Wheat/rice-based	28	7	25.0	0.293	0.141	0.270	0.180	0.570
Coffee[a]	72	35	48.6	2.171	0.790	1.960	1.210	4.210
Loaf bread	70	9	12.9	0.283	0.181	0.196	0.162	0.658
Peanuts	72	30	41.7	0.214	0.138	0.173	0.084	0.774
Pistachios	70	2	2.9	0.228	0.133	0.228	0.134	0.321
Red wine[b]	120	18	15.0	0.513	0.807	0.165	0.070	3.190
Dessert wine[c]	141	70	49.6	3.288	6.890	0.797	0.057	48.680

[a] Data taken from Coronel et al., 2010.
[b] Data taken from Bellí et al., 2004.
[c] Data taken from Bellí et al., 2004 and Valero et al., 2008.
[d] Minimum
[e] Maximum

With the exception of beer, the frequency of contamination of the analysed foodstuffs was lower than 50%. Mean of positives of baby foods, breakfast cereals, and loaf bread, were below the maximum levels established by the European Commission (2006): 0.50 and 3 ng/g, respectively, considering breakfast cereals and loaf bread as products derived from cereals. Still no legislation has been set regarding beer and nuts, but the mean of positives of these foodstuffs were lower than the levels found in the rest of foodstuffs.

As concerns to baby foods, the mean contamination of the positive samples was higher than that found in a previous study in Spain, in which the mean level was 0.187 ng/g and two samples of 0.706 and 0.740 ng/g exceeded the European limit of 0.5 ng/g (Araguás et al., 2005), and similar to the mean contamination levels found in Turkey (0.221 ng/g) (Kabak, 2009). The observed mean contamination was lower than the found in a study in Canada, where means of positive samples ranged from 0.28 ng/g to 2.40 ng/g, according to the type of cereal used in the formula (Lombaert et al., 2003). Considering ranges, levels detected in an Italian study were in the range of <0.06-0.74 (Beretta et al., 2002), with four samples above the European limit of 0.5 ng/g.

In Portugal, levels of OTA in baby foods ranged between 0.034-0.212 ng/g (Alvito et al., 2010). The percentage of positive samples in the present study (8.7%) was lower than in the rest of the named studies: Canada (26.1%), Italy (16.8%), Portugal (37%), Spain (70%), and Turkey (17%).

Many studies on the occurrence of OTA in beer have been done. A summary of the sample collections carried out from 1992 to 2007 in several countries of beers from diverse origins was presented by Kabak (2009). Apart from those, there are studies done in Belgium (Anselme et al., 2006), Brazil (Kawashima et al., 2007), and Japan (Kumagai et al., 2008; Aoyama et al., 2010). In most cases, mean OTA levels were below 0.070 ng/mL, with the exception of those found in Korea (0.25 ng/mL, Park et al., 2005), a sample of Scottish origin (0.201 ng/mL, Medina et al., 2005), Belgian beers (0.103 ng/mL, Anselme et al., 2006) and non alcoholic beers purchased in Iranian supermarkets (0.108 ng/mL, Mahdavi et al., 2007). Incidence ranged from zero to 100%, but was mostly above 50%. Bertuzzi et al. (2011) analysed 106 beer samples collected in 25 European countries. The incidence was 67.9% and levels ranged between <0.002-0.189 ng/mL, with a mean of the total samples of 0.019 ng/mL. Particularly, we could observe that the mean value of this study (0.022 ng/mL) was lower than other mean values found in Spain: 0.044 (Araguás et al., 2005) and 0.0358 ng/mL (Medina et al., 2005). Thus, the mean level of this study was in the range of those found in the literature, and incidence was also similar to the observed in previous studies.

Contamination mean level of positive samples in corn-based breakfast cereals were around two-fold higher than in the case of wheat and rice-based breakfast cereals, and the same was observed in the median and maximum values of both distributions, although occurrence was higher in the second case. Roscoe et al. (2008) and Ibáñez-Vea et al. (2011) determined the contamination of OTA and other mycotoxins in breakfast cereals of different compositions sampled in Canada and Spain, respectively. In both cases a higher incidence was also observed in the wheat and rice-based ones, but mean contamination was lower in the corn-based (0.12 and 0.10 ng/g, respectively) than in the wheat and rice-based (0.30 and 0.16 ng/g, respectively). Both studies showed lower mean contaminations than in this study, which also occurred in samples collected in Greece (0.18 ng/g, Villa and Markaki, 2009). Mean contamination of positive samples in the two types of cereals of this study matched with the observed by Araguás et al. (2005) (0.265 ng/g) and Kabak (2009) (0.752 ng/g). In this study, incidence in both types of breakfast cereals was low, especially in the case of the corn-based. Such incidences were lower than the found in other studies: up to 100%, (Araguás et al., 2005); 60% (Villa and Markaki, 2009); 18% (corn-based) to 38% (wheat-based) (Roscoe et al., 2008); 5% (corn-based) to 88% (wheat and rice-based) (Ibáñez-Vea et al., 2011).

Regarding bread contamination, Duarte et al. (2010) compiled the occurrence of OTA in different types of bread worldwide. Mean values of wheat bread positive samples ranged from 0.07 ng/g in Switzerland (Legarda and Burdaspal, 2001) to 13 ng/g in Morocco (Zinedine et al., 2007a), although most of them were below 0.50 ng/g. Incidence was between 65 and 100% for most of the listed studies, and in some exceptions it was below 20%. Therefore, the present results were similar to most of the data of previous studies. In addition, when comparing our results with other samples collected in Spain, we could observe that mean values were lower than those found by Legarda and Burdaspal (2001) and Osnaya et al. (2006), with 0.45 ng/g and 2.19 ng/g, respectively.

Few data are available on OTA contamination of nuts. From these, we could observe that OTA levels in this study were below those found in Tunisia (0.1-3, Ghali et al., 2009, and 11-203 ng/g, Zaied et al., 2010). The low incidence of OTA in pistachios observed here (2.9%) was even lower than the observed in the mentioned studies (16% and 25%, respectively), whereas in Morocco, Zinedine et al. (2007b) found no contamination above the limit of quantification of their detection method (0.027 ng/g). A higher incidence than in the pistachio samples was observed for peanuts (41.7%), and it was similar to the observed in Tunisia (44%, Ghali et al., 2009) and higher than in the samples analysed in Morocco and in other study in Tunisia (25 and 24%, Zinedine et al., 2007b and Zaied et al., 2010, respectively). Mean of positives were lower than the observed in Côte d'Ivoire (0.373 ng/g, Sangare-Tigori et al., 2006), Morocco (0.68 ng/g, Zinedine et al., 2007b), and Tunisia (2.4 ng/g and 60 ng/g, Ghali et al., 2009 and Zaied et al., 2010, respectively).

Contamination and occurrence data of red and dessert wines and coffee were previously discussed in the articles from which data were taken (Bellí et al., 2004, Valero et al., 2008, Coronel et al., 2010).

The comparison of the present results with other works indicates that most of the values found were similar or lower than previously published data.

Figures 1 and 2 show the histograms for contamination levels of the foodstuffs listed in Table 2. In all cases it can be observed that the shape of the distributions does not show any pattern in common, and that for most of the samples the levels of contamination were non detectable.

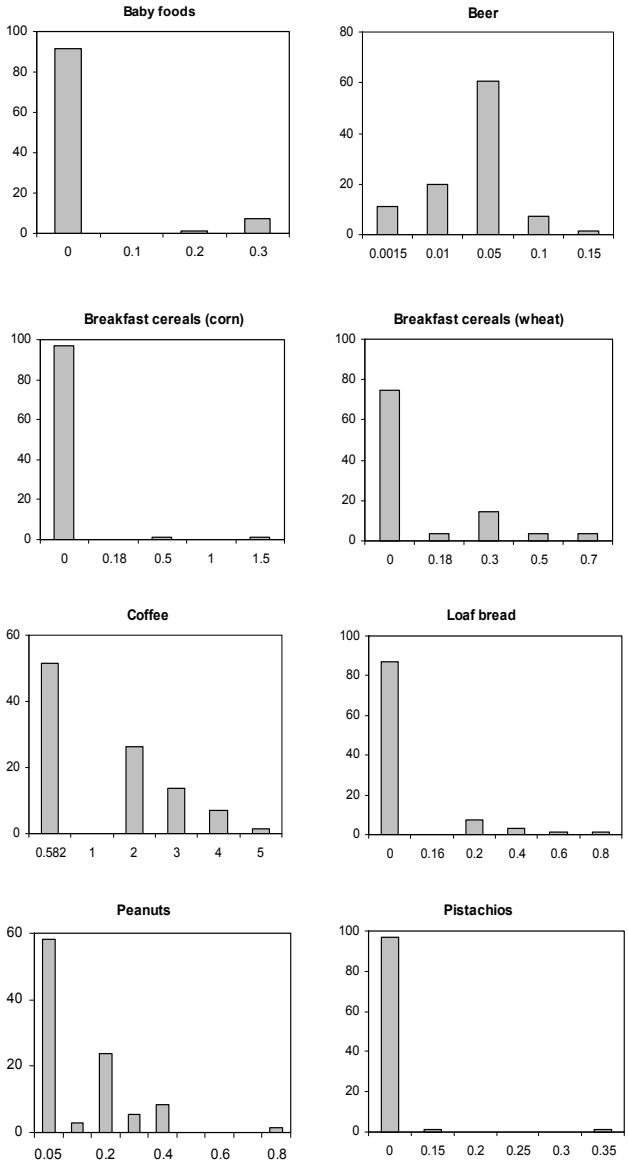

Figure 1. Contamination histograms in food composites. (relative frequencies vs. OTA contamination, ng/g, and ng/mL in the case of beer) In order to build these plots, non detected values were replaced by zero (baby foods, breakfast cereals, loaf bread, pistachios) or by the LOD/2 (beer, c offee, peanuts).

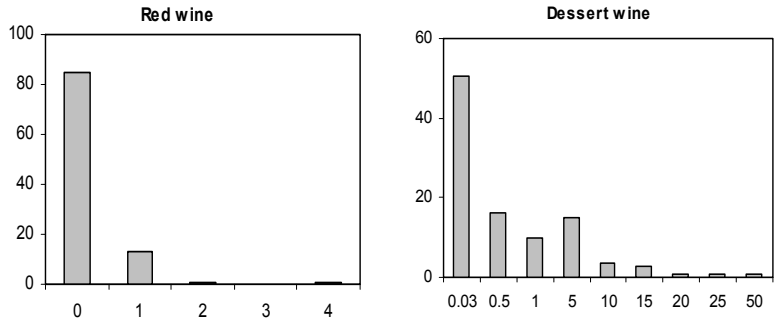

Figure 2. Contamination histograms in wine samples (relative frequencies vs. OTA contamination, ng/mL). In order to build these plots, non detected values were replaced by zero (red wine) or by the LOD/2 (dessert wine).

3.2. Consumption of foodstuffs

Table 3 shows the proportion of the total population, classified by age groups, of the people who consumed the studied foods, sorted by each kind of foodstuff. In the case of infants, even though there was only one surveyed food product, a high percentage of consumers could be observed. FFQ for children included the same foodstuffs as for adolescents and adults (except alcoholic beverages), however, this age group showed no consumption of nuts nor coffee. Clear differences were observed in the percentages of consumers of each foodstuff when comparing the age groups. For example, in the case of breakfast cereals and loaf bread, the consumer percentage decreased as the age increased. These foodstuffs presented the highest percentages of consumers in the groups of children and adolescents. Regarding adults, the highest consumer percentages were observed for beer, coffee, and red wine.

Further information about the normalised quantities of food consumption is listed on Tables 4 and 5, for the total population and the consumer population.

Table 3. Percentage of the consumer population for each foodstuff.

	INFANTS (*n*=164)		CHILDREN (*n*=68)		ADOLESCENTS (*n*=211)		ADULTS (*n*=905)	
	***n* consumers**	**Consumer population (%)**	***n* consumers**	**Consumer population (%)**	***n* consumers**	**Consumer population (%)**	***n* consumers**	**Consumer population (%)**
Baby foods	133	81.1						
Beer					44	20.9	586	64.8
Breakfast cereals (corn)			49	72.1	143	67.8	382	42.2
Breakfast cereals (wheat and rice)			49	72.1	143	67.8	383	42.3
Coffee			0	0	93	44.1	767	84.8
Loaf bread			38	55.9	140	66.4	440	48.6
Peanuts			0	0	84	39.8	407	45.0
Pistachios			0	0	71	33.6	460	50.8
Red wine					26	12.3	597	66.0
Dessert wine					25	11.8	184	20.3
Total	133	81.1	55	80.9	201	95.3	900	99.4

Table 4. Descriptives of the normalised consumption (g/kg bw/day) of foodstuffs by population groups, considering all the surveyed population.

ALL POPULATION	Mean	SD	Median	99th quantile	Mean	SD	Median	99th quantile
	INFANTS (*n* = 164)				CHILDREN (*n* = 68)			
Baby foods	12.65	12.20	10.00	49.13				
Breakfast cereals (corn)					0.71	0.81	0.26	3.26
Breakfast cereals (wheat)					0.71	0.81	0.26	3.26
Loaf bread					0.53	0.83	0.08	3.00
Total consumption	12.65	12.20	10.00	49.13	1.94	1.80	1.86	7.21
	ADOLESCENTS (*n* = 211)				ADULTS (*n* = 905)			
Beer	0.19	0.71	0.00	3.34	1.30	2.20	0.38	9.63
Breakfast cereals (corn)	0.26	0.40	0.08	2.05	0.10	0.19	0.00	0.60
Breakfast cereals (wheat)	0.26	0.40	0.08	2.05	0.10	0.19	0.00	0.60
Coffee	0.03	0.07	0.00	0.30	0.14	0.14	0.11	0.56
Loaf bread	0.36	0.62	0.07	2.78	0.22	2.18	0.00	1.74
Peanuts	0.05	0.19	0.00	0.82	0.03	0.15	0.00	0.57
Pistachios	0.03	0.12	0.00	0.78	0.03	0.19	0.00	0.48
Red wine	0.05	0.34	0.00	0.98	0.93	1.94	0.22	7.66
Dessert wine	0.02	0.17	0.00	0.46	0.03	0.20	0.00	0.41
Total consumption	1.25	1.54	1.07	6.02	2.89	4.07	1.83	13.88

Table 5. Descriptives of the normalised consumption (g/kg bw/day) of foodstuffs by population groups, considering the consumer population.

ONLY CONSUMERS	Mean	SD	Median	99th quantile	Mean	SD	Median	99th quantile
	INFANTS (*n* = 133)				CHILDREN (*n* = 55)			
Baby foods	133.00	15.60	15.00	50.08				
Breakfast cereals (corn)					0.98	0.81	1.00	3.37
Breakfast cereals (wheat)					0.98	0.81	1.00	3.37
Loaf bread					0.94	0.93	0.54	3.00
Total consumption	133.00	15.60	15.00	50.08	2.40	1.70	2.22	7.32
	ADOLESCENTS (*n* = 201)				ADULTS (*n* = 900)			
Beer	0.91	1.34	0.48	5.89	2.01	2.47	1.14	11.39
Breakfast cereals (corn)	0.38	0.44	0.24	2.43	0.24	0.22	0.18	0.85
Breakfast cereals (wheat)	0.38	0.44	0.24	2.43	0.24	0.22	0.18	0.85
Coffee	0.07	0.09	0.02	0.35	0.16	0.14	0.13	0.59
Loaf bread	0.54	0.70	0.20	2.85	0.46	3.12	0.12	2.43
Peanuts	0.12	0.29	0.03	1.16	0.08	0.22	0.02	1.16
Pistachios	0.10	0.19	0.05	0.94	0.06	0.26	0.02	0.57
Red wine	0.43	0.90	0.12	3.65	1.42	2.25	0.75	7.88
Dessert wine	0.21	0.48	0.03	1.88	0.14	0.43	0.04	2.44
Total consumption	1.32	1.56	1.12	6.13	2.91	4.07	1.86	13.88

As an example, histograms of consumption for each foodstuff by the adults are shown in Figure 3. The shapes of these histograms were irregular, and showed the proportion of consumers and non consumers.

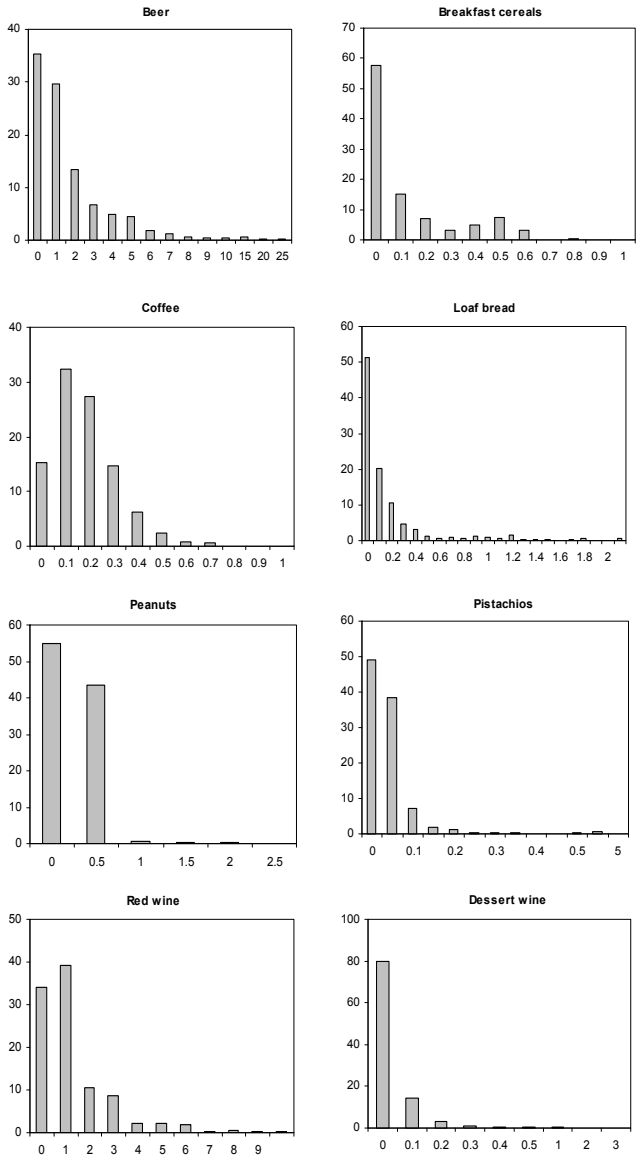

Figure 3. Consumption histograms (relative frequencies) of the adult population for each foodstuff (g/kg bw/day).

3.3. Estimation of the daily intake of OTA

3.3.1. Deterministic estimation

The results of the mean contamination values derived from the treatment of the ND data are listed in Table 6. These alternatives were used in the calculation of the OTA daily intake of each foodstuff, and results of the estimations for the total population are shown in Table 7.

Table 6. Mean values of OTA contamination in different foodstuffs derived from treated ND data used to perform the deterministic estimation.

	n	% ND	Censored data treatment*	Mean contamination, ND replaced by:		
				LOD/2	ZERO	LOD
Baby foods	69	91.3	C	-	0.020	0.185
Beer	71	11.3	A	0.020	-	-
Breakfast cereals						
Corn-based	71	97.2	C	-	0.020	0.195
Wheat/rice-based	28	75.0	C	-	0.073	0.208
Coffee	72	51.4	A	1.354	-	-
Loaf bread	70	87.1	C	-	0.036	0.158
Peanuts	72	58.3	A	0.110	-	-
Pistachios	70	97.1	C	-	0.007	0.132
Red wine	120	85.0	C	-	0.077	0.119
Dessert wine	141	50.4	A	1.639	-	-

*According to criteria described in Section 2.4.1 of Materials and Methods.

The total population (consumers and non consumers) of each age group was considered in this analysis, as the percentage of consumers in all cases was high (above 80%), and in the case of adolescents and adults, above 95%. Another reason was to obtain results to make possible the comparison with the probabilistic estimation, in which the percentages of consumers and non consumers were taken into account. An example of the shapes of the estimated daily intake distribution obtained by the deterministic method is shown in Figure 4, where adult exposure in the two exposure scenarios (ND = 0 and ND = LOD) is described.

Table 7. Deterministic estimation of the daily intake of OTA (ng/kg bw/day) for the total surveyed population.

	ND = 0	ND = LOD	ND = 0	ND = LOD
	Infants		**Children**	
N	164	164	68	68
Mean	0.25	2.34	0.08	0.37
SD	0.24	2.26	0.08	0.35
Median	0.20	1.85	0.07	0.32
0.90th quantile	0.51	4.69	0.19	0.81
0.95th quantile	0.73	6.79	0.19	0.84
0.99th quantile	0.98	9.09	0.33	1.42
Skewness	1.07	1.07	1.05	1.05
Kurtosis	1.17	1.17	1.26	1.25
	Adolescents		**Adults**	
N	211	211	905	905
Mean	0.16	0.29	0.47	0.57
SD	0.40	0.45	0.53	0.68
Median	0.06	0.21	0.39	0.47
0.90th quantile	0.33	0.63	0.87	1.01
0.95th quantile	0.58	0.81	1.03	1.22
0.99th quantile	1.24	1.42	1.76	1.91
Skewness	8.08	6.73	7.30	9.36
Kurtosis	80.82	63.08	84.49	125.55

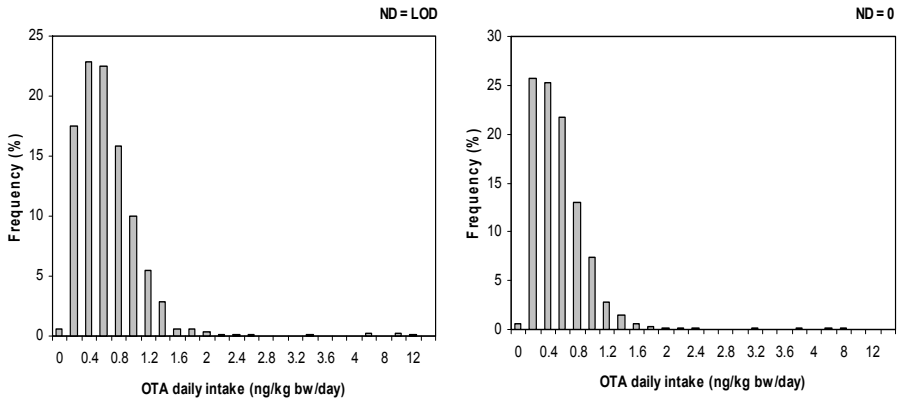

Figure 4. Histograms of the OTA daily intake by the adult population estimated by the deterministic method, in the ND = 0 and ND = LOD scenarios.

In the best case exposure scenario (ND = 0), adults presented the highest mean daily intake, followed by infants, adolescents, and children. In the worst case scenario (ND = LOD), the highest mean was observed for infants, followed by adults, children, and adolescents. The observed increase in the descriptive values was not proportional for each age group, instead, depended on the values of the LOD of the different methods of analysis of the considered foodstuffs (consumption of each age group was the same for both estimations). However, results agreed in the fact that the most exposed groups were infants and adults.

In all groups, mean and median estimated OTA daily intakes due to the consumption of the studied foodstuffs were below the latest PTDIs for both exposure scenarios, and the same was observed for the high quantiles: values reached a 14% of the PTDI of 14 ng/kg bw/day and a 11% of the PTDI of 17 ng/kg bw/day, respectively, excluding the infants. The highest values were observed for the high quantiles in the infants group, especially in the ND = LOD scenario and these were closer to the PTDI (65% and 53% of the mentioned PTDIs). Even though the contamination of the baby foods was similar to the foods consumed by the rest of the population (Table 6), it is worth to note that infants have a less varied diet than the rest of the population, and thus the contribution of a specific foodstuff to the total diet (in this case baby foods) may be higher when comparing it with the dietary habits of the other age groups.

Differences among age groups were tested in total and by pairs taking into account data of the total population (consumers and non consumers). These resulted significant in all cases ($p < 0.0001$, both for ND = 0 and ND = LOD) but not when comparing children and adolescents ($p = 0.4882$ for ND = 0 and $p = 0.1083$ for ND = LOD). In a further analysis, adult population group was divided in three sub-groups of age: group A (18-29 years old, $n = 314$), group B (30-44 years old, $n = 308$), and group C (≤ 45 years old, $n = 283$). Significant differences were also found among these groups ($p < 0.0001$), with medians of 0.30, 0.42 and 0.45 ng/kg bw/day, respectively (for ND = 0), and 0.42, 0.47 and 0.52 ng/kg bw/day, respectively (for ND = LOD). When analysing differences between age groups by pairs, differences were significant between groups A and B ($p = 0.0006$ for ND = 0 and $p = 0.0362$ for ND = LOD) and A and C ($p < 0.0001$ for ND = 0 and $p = 0.0001$ for ND = LOD).

Gender was also considered to evaluate differences in the exposure of the adolescent and adult population. Thus, total OTA daily intake by adolescents showed no significant differences ($p = 0.4229$ for ND = 0 and $p = 0.3720$ for ND = LOD), whereas in adults, differences were indeed significant ($p = 0.0175$ for ND = 0 and $p = 0.0027$ for ND = LOD), with men presenting the highest median intake (0.41 and 0.50 ng/kg bw/day for each scenario) than women (0.36 and 0.44 ng/kg bw/day).

Taking into account the significant differences between genders in the adult population, it could be expected that such differences were found within the age groups. Therefore, differences were analysed in the same gender, for age groups

and by pairs. For men, differences were significant between groups A and B (p = 0.0182 for ND = 0 and p = 0.1871 for ND = LOD) and A and C (p = 0.0021 for ND = 0 and p = 0.0077 for ND = LOD). Similarly, in the case of women, differences were significant between groups A and B (p = 0.0206 for ND = 0 and p = 0.1734 for ND = LOD) and A and C (p < 0.0001 for ND = 0 and p = 0.0057 for ND = LOD). It could be observed that when testing differences between groups A and B in men and women, differences were significant when ND = 0, but not when ND = LOD.

3.3.2. Probabilistic estimation and confidence intervals

Table 8 shows the results of the probabilistic estimation of the daily intake of OTA in the different population age groups, for the two alternatives of replacement of the ND values.

Mean values were similar to the obtained by the deterministic estimation, slightly higher for infants and children, and slightly lower for adolescents and adults. As regards to medians, all of them were slightly lower, and an especial case could be observed for the infant population, in which the median was almost equal to zero. Taking into account the high quantiles, in most of the cases these were higher than the deterministic estimations, but in all cases OTA daily intake was lower than the latest PTDIs for both exposure scenarios (ND = 0 and ND = LOD). Infant population was the most exposed group, too.

Regarding the analysis of differences between population groups, it was performed for the same groups and pairs as in the deterministic estimation. Thus, in all cases differences were significant and in all cases p was <0.0001, with only one exception: differences between age groups B and C in men were not significant (p = 0.1365) in the ND = 0 scenario.

Table 8. Probabilistic estimation of the daily intake of OTA and confidence intervals of the descriptive statistics.

	OTA daily intake (ng/kg bw/day)				Confidence intervals			
	ND =0	ND = LOD	ND =0	ND = LOD	ND =0	ND = LOD	ND =0	ND = LOD
	Infants		Children		Infants		Children	
N	10000	10000	10000	10000				
Mean	0.28	2.42	0.09	0.39	[0.14; 0.47]	[2.05; 2.81]	[0.06; 0.14]	[0.32; 0.46]
SD	1.11	2.48	0.16	0.30	[0.44; 2.07]	[2.03; 2.97]	[0.07; 0.39]	[0.24; 0.40]
Median	0.0012	1.77	0.05	0.32	[0.0003; 0.0042]	[1.34; 2.19]	[0.03; 0.07]	[0.24; 0.40]
0.90th quantile	0.58	5.74	0.21	0.80	[0.24; 1.06]	[4.72; 6.74]	[0.14; 0.33]	[0.63; 1.03]
0.95th quantile	1.46	7.23	0.30	0.98	[0.65; 2.77]	[5.85; 8.87]	[0.19; 0.55]	[0.75; 1.27]
0.99th quantile	4.94	11.00	0.68	1.39	[2.23; 12.33]	[8.18; 15.34]	[0.29; 3.20]	[0.97; 2.36]
Skewness	9.17	1.69	8.40	1.40				
Kurtosis	129.70	4.15	128.13	3.08				
	Adolescents		Adults		Adolescents		Adults	
N	10000	10000	10000	10000				
Mean	0.14	0.28	0.37	0.53	[0.10; 0.24]	[0.23; 0.36]	[0.30; 0.45]	[0.46; 0.60]
SD	0.46	0.45	0.53	0.48	[0.12; 1.12]	[0.18; 0.23]	[0.31; 0.93]	[0.34; 0.81]
Median	0.07	0.20	0.23	0.41	[0.05; 0.08]	[0.17; 0.23]	[0.19; 0.27]	[0.35; 0.47]
0.90th quantile	0.28	0.52	0.79	1.05	[0.21; 0.38]	[0.44; 0.62]	[0.62; 1.00]	[0.87; 1.21]
0.95th quantile	0.44	0.68	1.14	1.31	[0.30; 0.61]	[0.54; 0.85]	[0.83; 1.54]	[1.07; 1.58]
0.99th quantile	1.04	1.37	2.39	2.14	[0.58; 5.39]	[0.81; 5.36]	[1.46; 5.71]	[1.51; 3.87]
Skewness	25.56	15.22	6.86	4.42				
Kurtosis	1015.77	364.75	86.05	50.95				

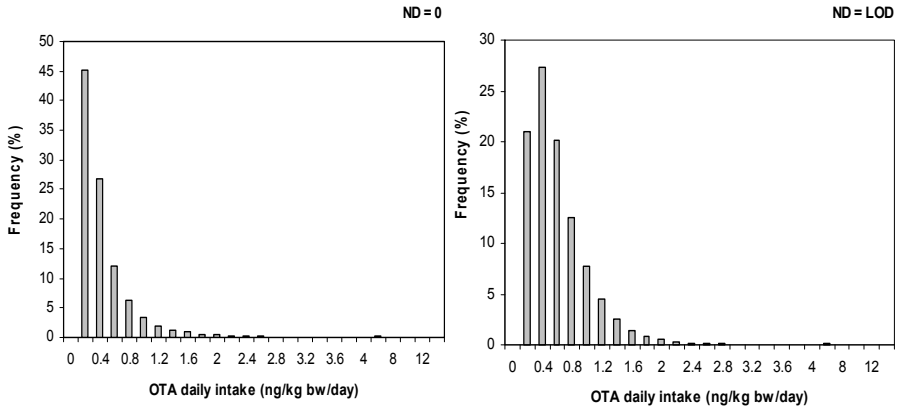

Figure 5. Histograms of the OTA daily intake by the adult population estimated by the probabilistic method, in the ND = 0 and ND = LOD scenarios.

Figure 5 shows the distribution of the exposure obtained through the probabilistic method, for the adult population. The shape of the distribution is much more defined than the obtained by the deterministic approach. This can be explained by the number of samples included in the analysis (n = 10000) and the model used to perform the simulations. In this case, the simulation process achieved exposure values that were not obtained by the deterministic estimation, as the probability to obtain a higher number of different exposures cases was higher, which is confirmed by the high values of skewness and kurtosis. The mentioned characteristics make possible the calculation of confidence intervals of the estimated distribution descriptives. These confidence intervals are listed in Table 6, and complete the description of the probabilistic estimation. In all cases but one the estimated daily intake values of Table 6 remained within the range defined by the confidence intervals. The range of the intervals increased in the highest quantiles, which was expected as the precision in the estimation of the confidence intervals of these quantiles is lower than in the case of mean or median values (Beirlant and Devroye, 1999; Breiman et al., 1990).

When checking the deterministic estimations against the probabilistic confidence intervals, it could be observed that all but seven values were within these ranges. Taking into account that in addition similar descriptive statistics were obtained through the deterministic method, we can state that the probabilistic method is a useful tool for the estimation of the exposure descriptives and the obtainment of precision indicators related to those values.

4. CONCLUSIONS

The present work assessed the exposure of the Catalan population to OTA by determining the contamination levels of certain foodstuffs sampled in Catalonia, and by considering data of consumption of this population. The levels of contamination by OTA of the sampled foodstuffs were below the limits established by the European Commission, and the consumer population was almost the total surveyed population, which indicates that although the contamination levels were low, a major part of the population was exposed to OTA. However, a more complete exposure assessment can be reached by the inclusion of other foodstuffs considered to be contaminated by OTA, raisins and spices, or animal by-products, which were not included in this study due to the lack of consumption data. The analysis of pasta would have added important information to this study, but unfortunately this food product was not selected for sampling.

Exposure was quantified by the estimation of the daily intake of OTA, which was achieved by deterministic and probabilistic methods. Both estimations showed that the exposure levels were lower than the PTDIs, but differences among population groups were confirmed and still differences among adult population subgroups were also found. However, non significant differences between population groups could be observed only when the deterministic data was analysed for differences. We could observe that the probabilistic estimation gave similar results to the obtained by the deterministic methodology, but had the additional feature of the calculation of confidence intervals for the estimated descriptive values of the exposure distributions.

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IV. GENERAL DISCUSSION

GENERAL DISCUSSION

As mentioned in the Introduction, the assessment of the exposure can be done by means of two main methodologies: through the collection of food consumption data and the determination of the levels of contamination in foodstuffs, and through the use of biomarkers of the exposure. The results obtained in this research work are discussed as follows according to these two main subjects. In particular, some points are addressed, which were not fully considered in the discussion sections of the isolated chapters.

1. ASSESSMENT OF THE EXPOSURE THROUGH FOOD CONTAMINATION AND CONSUMPTION DATA

The assessment of the exposure to OTA through food contamination and consumption data was done in all the studies of this work. The characteristics of those data and the methodologies used to perform the exposure assessment are discussed below.

1.1. Collection of food consumption data and surveyed population

The first stage of this work, as mentioned in the work plan, was the study of biomarkers of exposure to OTA. The collection of samples of biological fluids was complemented by the collection of the participants' consumption data, and a food frequency questionnaire (FFQ) was designed with this purpose (ANNEX 1).

The foodstuffs included in the FFQ were classified in food groups, according to the criteria of the SCOOP Report (European Commission, Directorate-General Health and Consumer Protection, 2002; from now on the "SOOP Report") on the assessment of dietary intake of ochratoxin A by the population of EU member states. Those food groups were: cereals and derived products, dried fruits, coffee, wine, beer, cocoa derived products, meat products (pork and poultry), fruit juices (including grape juice), spices, and pulses. In this work, the selected categories were: cereals and derived products, cocoa derived products, dried fruits and derivatives, wine, beer, coffee, and grape juice. Spices were excluded due to their low rate of consumption of 0.5 g/day (WHO-GEMS/Food, 2003), whereas poultry meat, juices of fruits different from grape, and pulses were excluded due to the low OTA contamination observed in the samples analysed in that report: no positive samples were observed in any of the poultry meat samples (n = 41), whereas contamination for fruit juices and pulses were 0.1 and 0.01 μg/kg, respectively.

Thus, the FFQ included 32 food items, classified in seven food groups:

- **Cereals and derived products:** twelve items: breakfast cereals, bread, loaf bread, pasta, stuffed pasta, rice, cookies, bakery products, cakes, pizza, "coca de recapte", which is a local product similar to pizza, and snacks.
- **Dried fruits and derived products:** eight items: almonds, hazelnuts, sunflower seeds, walnuts, pistachios, peanuts, nougat, and marzipan.
- **Cocoa derived products:** five items: chocolate powder, thick drinkable chocolate, bar chocolate, chocolates, and chocolate nougat.
- **Coffee:** three items: black coffee, espresso with milk, white coffee.
- **Wine:** two items: red and sweet
- **Beer**
- **Grape juices:** two items: pure and mixed with other fruits.

Two food items that were not included in the questionnaire were pork offal and raisins. As these foods are susceptible to OTA contamination, they should have been included, although a low consumption rate is expected.

Participants indicated the frequency and quantity of consumption of the listed foods for the period of one year. Standard portions of each food product were defined and showed to the participants by means of a photographic album. FFQs were designed to obtain data of a recall period of a year, even though there was a possibility that foodstuffs listed were not being consumed at the moment the interview was carried out, for example, some of the listed foods were consumed only in certain seasons, such as thick drinkable chocolate or marzipan.

As mentioned, this questionnaire was used, in the first place, during the collection of blood samples, to interview the blood donors that participated in the two studies on OTA in plasma (Studies I and II). Later on, during the development of this work, inhabitants of the province of Lleida were also interviewed using this questionnaire in order to get additional data on consumption, even though they were not donors. Individuals aged differently from the blood donors could be interviewed, although they were mostly adults. Thus, the last study on food contamination (Study V) included consumption data of infants, children, and adolescents, in addition to those of adults. However, in the following paragraphs of this discussion, only the results of the adult population will be considered, as their exposure data was evaluated in all the studies of this work and can therefore be compared.

1.2. Contamination of foodstuffs

The data of contamination of foodstuffs used in this work were obtained from two main sources:

1) Contamination data of foodstuffs classified according to the SCOOP Report (2002) as summarised by EFSA (2006): cereals and derived products, cocoa derived products, wine, beer, coffee, and grape juice. Contamination of dried fruits and derivatives was estimated from data of a survey carried out by the UK Food Standards Agency (2002). Mean values of contamination were calculated from data provided by the countries that participated in the assessment study, considering detected and non detected levels. Non detected levels were replaced by the value corresponding to half the limit of detection (LOD/2) of each analytical method.

These data were used in the studies on biomarkers of exposure (Part I: Studies I and II on OTA in plasma, and III on OTA and OTα in urine) for the estimation of the daily intake of OTA. A summary of these contamination data, in decreasing order of levels, is listed in Table 1. This table was also included in the mentioned studies.

Table 1. OTA mean contamination levels (ng/g) in certain food groups (modified from EFSA, 2006).

Food group	Mean contamination	Reference
Dried fruits and derived products	0.87	Food Standards Agency (2002)
Coffee	0.72	SCOOP (2002)
Grape juice	0.55	SCOOP (2002)
Wine	0.36	SCOOP (2002)
Cereals and derived products	0.29	SCOOP (2002)
Cacao and derived products	0.24	SCOOP (2002)
Beer	0.03	SCOOP (2002)

2) Data of contamination of food products sampled in stores of Catalan cities that, due to their number of inhabitants, represented the 72% of the Catalan population. The sampling was carried out during a period of six months, from June to November 2008. Sampling and selection of these foodstuffs was done within the frame of a project for the evaluation of the exposure of the Catalan population to mycotoxins, supported by the Catalan Food Safety Agency.

The foodstuffs collected for OTA analyses belonged to the food groups included in the FFQs, and were: breakfast cereals, loaf bread, coffee, beer, peanuts, pistachios, and cereal-based baby foods. The contamination data obtained from the sampled

foodstuffs were used in the studies on food contamination (Part II, Studies IV and V) for the estimation of the daily intake of OTA. The data on contamination of wine were taken from previous studies, in which samples were purchased by the research group (Bellí et al., 2004 and Valero et al., 2008). The sampling in those cases was for red and dessert wines collected in Catalonia and other Spanish regions. Cocoa derivatives were not sampled, as at the moment of the selection of foodstuffs scarce information on OTA contamination for such food products was available. Moreover, the analysis of pasta would have added important information to this study, but unfortunately this food product was not selected for sampling.

Table 2 shows the mean contamination values of the analysed foodstuffs, calculated in two ways: as the mean of positive samples, and as the mean of the total samples. The latter values were calculated as in the SCOOP Report (2002), in order to obtain results that could be compared to those values. The highest values were observed in coffee, followed by cereal derivatives, dried fruits, and beer. Low occurrences were observed in these samples (below 50%), with the exception of beer (89%), and mean contamination of positive samples was in all cases below the limits set by the European Commission (2006).

Table 2. OTA mean contamination levels (ng/g) in the foodstuffs analysed in this work, sorted by food groups according to the EFSA (2006).

Analysed foodstuffs sorted by food groups	Mean contamination of positive samples	Mean contamination of all samples*
Dried fruits: peanuts and pistachios	0.22	0.11
Coffee	2.17	1.35
Grape juice: not analysed	-	-
Wine: red and dessert	1.90	0.88
Cereals: breakfast cereals and loaf bread	0.43	0.19
Cacao and derived products: not analysed	-	-
Beer	0.02	0.02

* Non detected levels were replaced by LOD/2, as in SCOOP Report (2002).

When comparing these values with those listed by the SCOOP Report and the UK Food Standards Agency (Tables 1 and 2), and considering the mean contamination of all samples (as calculated in the SCOOP Report), it can be observed that contamination levels found in dried fruits (peanuts and pistachios) and cereal derivatives (breakfast cereals and loaf bread) were lower in this work, whereas OTA levels in coffee were higher. Mean values of beer were slightly lower in this study. The first comparison is not completely accurate, as the number of food items included in the groups of dried

fruits and derivatives and in the group of cereals and derivatives were higher in the SCOOP Report.

1.3. Assessment of the exposure to OTA by the estimation of its intake through food consumption and contamination data

The estimation of the daily intake of OTA, when contamination and consumption data were considered, was achieved by means of two modelling methodologies: deterministic and probabilistic (Introduction, Section 3.3.1.4). Briefly, deterministic methods use a fixed value of contamination and the distribution values of consumption, whereas probabilistic methods use the values of the distributions of both contamination and consumption. Thus, according to the availability of data, only the deterministic method was used in the biomarkers studies (I, II and III), as only consumption distributions were available; whereas both deterministic and probabilistic methods were used in the contamination studies (IV and V).

Regarding the consumption data of this work, and as mentioned previously, inhabitants of the province of Lleida that did not participate in the studies on OTA in plasma were also interviewed for food consumption. Then, the datasets of food consumption used in the studies on food contamination (Studies IV and V) were built up from data provided by blood donors and non-donors. Table 3 shows the data of food consumption and contamination used for the estimation of the OTA daily intake in the two types of studies.

Table 3. Sources of contamination and consumption data for the exposure studies carried out in this work.

	Estimation of the intake	
	Biomarkers studies	**Contamination studies**
Contamination data		
SCOOP (2002): Broad food categories (Table 1)	X	
Contamination surveys: Selected foodstuffs (Table 2)		X
Consumption data (FFQs)		
Blood donors	X	X
Interviewed persons that did not donate, belonging to the same age groups of donors, and also aged differently		X

Below the results of consumption from the mentioned sources are discussed:

1.3.1. Consumption of possibly contaminated foodstuffs by participants of biomarkers studies

Table 4 shows the median values of the individual consumption (g/person/day) of the food groups as listed in the studies on biomarkers (Studies I, II and III), in decreasing order of amounts. Even though values were different in each study, it can be observed that the most consumed foodstuffs were cereals and derivatives, followed by beer, wine, dried fruits and derivatives, cacao and derivatives, coffee, and grape juice. The order of amounts was the same for Studies I and II (OTA in plasma), and similar for Study III (OTA and OTα in urine), in which the difference was observed for the consumption of cacao. As regards to quantities, a lower value of consumption was observed in Study III (OTA and OTα in urine), which can be explained by the fact that the FFQ included less food items than the FFQs used in Studies I and II (OTA in plasma). Even though all food groups (except grape juices) were included in Study III, specific items were not detailed, which might have led participants not to include such foodstuffs. Thus, the more detailed the questionnaire, the more accurate the estimation, although a long interview may tire out the participants.

Table 4. Median individual consumption (g/person/day) by the participants of the biomarkers studies.

Food groups	Studies		
	OTA in plasma (I)	OTA in plasma (II)	OTA and OTα in urine (III)
Cereals and derived products	158.43	158.32	174.99
Beer	24.66	43.40	17.42
Wine	16.38	27.78	6.41
Dried fruits and derived products	12.11	8.41	3.95
Cocoa and derived products	8.63	6.68	0.11
Coffee	7.08	6.20	6.20
Grape juice	0.00	0.00	-
Total	335.99	371.21	263.61

1.3.2. Consumption of analysed foodstuffs by the total surveyed population

The consumption of foodstuffs sorted by groups, but taking into account only the foodstuffs analysed in the last contamination study (Study V, which includes data of coffee contamination of Study IV), is listed in Table 5. Consumption data for these studies were those collected from blood donors and also individuals that did not participate in the studies on OTA in plasma, as shown in Table 3. In this section, only the data of consumption by adults will be considered, as the results observed for only this population group can be compared with the consumption of the participants of the studies on biomarkers. Thus, in this case, the order of amounts was the following: beer, wine, cereal derivatives (breakfast cereals and loaf bread), coffee, and dried fruits (peanuts and pistachios), and differs from the observed in the biomarkers studies. This is reasonable as the food groups did not include all the food items listed in FFQs used in the studies on biomarkers. However, it can be observed that, when the food groups were made up of only one item (wine, beer, coffee), values were in the range of those found in the biomarkers studies.

Table 5. Median individual consumption (g/person/day) of the foodstuffs analysed in Study V.

Analysed foodstuffs sorted by food groups	Individual consumption
Cereals: breakfast cereals and loaf bread	6.90
Beer	28.49
Wine	19.23
Dried fruits: peanuts and pistachios	0.45
Cocoa and derived products: not analysed	-
Coffee	6.20
Grape juice: not analysed	-
Total	127.01

1.3.3. Consumption of possibly contaminated foodstuffs by the total surveyed population

Finally, the consumption data of all the foodstuffs included in the FFQs by all the surveyed population is listed in Table 6. These results indicate the total amounts of consumption of each food group, and therefore evidence the differences in the quantities when comparing them with the amounts listed in Table 4, except beer, wine, and coffee. In this way, an evidence of the contribution of the analysed foodstuffs to the total consumption is given. This information should be considered when planning future exposure assessments

as concerns to the selection of food products to be analysed for OTA occurrence.

Table 6. Median individual consumption (g/person/day) by the total population surveyed (blood donors and non donors)

Food groups	Individual consumption
Cereals and derived products	164.90
Beer	28.49
Wine	19.23
Dried fruits and derived products	10.21
Cocoa and derived products	8.60
Coffee	6.20
Grape juice	0.00
Total	355.59

1.3.4. Estimation of the intake of OTA

In this section the results of the daily intake of OTA obtained through the two estimation methodologies (deterministic and probabilistic) will be discussed.

1.3.4.1. Deterministic procedure

Tables 7 and 8 list the median daily intake of OTA estimated by the deterministic procedure, in the biomarkers studies and in the contamination studies. Values were in all cases below the PTDIs, even the high percentiles of the distributions (data of high percentiles are shown in each study).

Table 7. Median OTA daily intake (ng/kg bw/day) by the participants of the biomarkers studies.

Food groups	Studies		
	OTA in plasma (I)	**OTA in plasma (II)**	**OTA and OTα in urine (III)**
Cereals and derived products	0.77	0.64	0.74
Dried fruits and derived products	0.18	0.10	0.06
Cocoa and derived products	0.03	0.02	0.00
Wine	0.10	0.12	0.04
Beer	0.01	0.02	0.01
Coffee	0.08	0.07	0.06
Grape juice	0.00	0.00	-
Total	1.61	1.39	1.08
% PTDI EFSA (2006), 17 ng/kg bw/day	9.47	8.18	6.35
% PTDI JECFA (2007), 14 ng/kg bw/day	11.50	9.93	7.71

Table 8. Median OTA daily intake (ng/kg bw/day) by the population considered in the contamination studies.

Analysed foodstuffs sorted by food groups	OTA daily intake	
	ND = ZERO	**ND = LOD**
Cereals: breakfast cereals and loaf bread	0.00	0.02
Dried fruits: peanuts and pistachios	0.00	0.00
Cacao and derived products: not analysed	-	-
Wine: red and dessert	0.03	0.04
Beer	0.01	0.01
Coffee	0.23	0.23
Grape juice: not analysed	-	-
Total	0.39	0.47
% PTDI EFSA (2006), 17 ng/kg bw/day	2.29	2.76
% PTDI JECFA (2007), 14 ng/kg bw/day	2.79	3.36

Similar values of daily intake could be observed within the biomarkers studies (I, II, III), but not when comparing them with the results of the contamination studies. In such studies, lower values of intake were found for cereal derivatives and dried fruits. Regarding individual foodstuffs, a lower intake of OTA due to wine was also observed, and in the case of beer, the values of the estimated daily intake were similar in both biomarkers and contamination studies. The only foodstuff through which the OTA estimated intake was higher in the contamination study than in the biomarkers studies was coffee.

Different results in both types of estimations are reasonable, and can be explained by the reasons mentioned in the following paragraphs:

In the case of grouped foodstuffs (cereal derivatives and dried fruits), the main reason is the fact that the study on contamination (Study V) did not include a wide variety of foodstuffs in these two food groups.

The use of different methodologies used to estimate the daily intake also leads to different results, even if a similar consumption of the individual foodstuffs (wine, beer, and coffee) is assumed for both types of studies (contamination and biomarkers). In the studies on biomarkers a general value of contamination was used (SCOOP Report, 2002), and this mean value of contamination was obtained from several sources, each one having a different limit of detection (LOD). The value of the LOD/2 was used to replace the non detected data in each sampling set. In contrast, in the contamination study (Study V) a different methodology was applied to treat the non detected data, by replacing them by a certain value (zero, LOD, or LOD/2) according to the proportion of non detected values in the sample sets of each food product (criteria proposed by GEMS/FOOD-Euro, 1995).

Also in relation to the previous paragraph, the occurrence in the sample sets of each foodstuff and the value of the limit of detection of the methods of analysis also influence the value of contamination used for the estimation of the intake through that foodstuff. If the occurrence is low, which implies a high number of non detected levels, most of the values of the distribution will be replaced by the values of the mentioned alternatives (zero, LOD, or LOD/2), and if the LOD for a certain food is also low, a low mean contamination value will be obtained, and such value will be used in the deterministic estimation. This may explain the low values of intake found for wine and beer, and the high value for coffee. In this latter case, a similar consumption was observed in biomarkers and contamination studies, but the contamination found in the samples collected in this work was higher than the contamination value used in the biomarkers studies, and the LOD for this foodstuff was also higher.

1.3.4.2. Probabilistic procedure

This methodology was used only in the contamination studies (Part II, Studies IV and V), as contamination and consumption distributions were available. Those studies showed different methodologies to model contamination and consumption data. In the first one, the daily intake of OTA due to a single food product (coffee) was modelled, whereas in the second, an improved methodology was employed for modelling the intake of OTA through several sources. In this case, the exposure analysis was completed by the construction of confidence intervals.

Probabilistic modelling takes into account all the possible values of consumption and contamination to estimate the intake, and therefore situations that may not have occurred in the surveyed population (data used in the deterministic estimation), can be obtained in the simulations: for example the high consumption of highly contaminated foodstuffs. This means that, having a certain number of observations on consumption and contamination, a more complete description of the exposure than in the deterministic estimation can be obtained. This could be observed in the high percentiles of the simulated distributions in Study V. However, the mean and median results obtained through both methodologies in that study were similar. In contrast to the deterministic procedure, this kind of analysis is more complex, but has the advantage of the information on the accuracy of results given by the confidence intervals.

1.4. Additional remarks

Considering the mentioned above, it can be stated that an accurate estimation of the intake of a contaminant (in this case, OTA) by a certain population should be done by collecting consumption data of the studied population and sampling foodstuffs in the region where the population lives, at the same period of time. However, a study on food consumption by the selected population should be done previous to the collection of food samples. If consumption and contamination data are obtained, and if tools are available, it will be possible to perform a complete probabilistic estimation of the intake, in addition to the deterministic modelling of the intake. This is the most commonly used methodology to estimate the exposure of a population due to the simplicity of its calculation, and to the availability of data, as mean values of contamination and consumption are accessible, for example from national surveys carried out to assess the dietary habits of a population.

Finally, estimations of the intake through food contamination and food consumption data gave results that were below the established PTDIs (Tables 7 and 8), even though data sources and methodologies of analysis were different.

2. BIOMARKERS OF EXPOSURE

The studies on biomarkers of exposure evaluated the presence of OTA in blood plasma, and OTA and OTα in urine, in adults living in the Catalan province of Lleida. Consumption data of possibly contaminated foodstuffs by the participants of these studies were also collected. The estimation of the daily intake of OTA of this population was achieved by two ways: in the first place, in the studies on blood plasma, by means of the Klaassen equation, considering the plasma levels of the toxin. In the second place, in both plasma and urine studies, by considering the consumption data given by the participants, and data of contamination of foodstuffs taken from the SCOOP Report (2002) and the UK Food Standards Agency (2002). This second methodology was discussed in the previous section.

2.1. OTA in blood plasma as a biomarker of exposure to OTA

The high incidence of OTA in plasma (Studies I and II) confirmed the exposure of the studied population to this mycotoxin. The particular feature of the detection of OTA in blood plasma is that it is possible to estimate the daily intake of each individual by only knowing the levels of the toxin in this fluid, by means of the Klaassen equation: $k_0 = Cl_p \times C_p/A$, where C_p is the plasma concentration of OTA (ng/mL), Cl_p is the plasma clearance (mL/kg bw/day), and A is the bioavailability of OTA. Different values of Cl_p and A have been used in the literature, and the more frequently utilised coefficients derived from their combination are cited in Table 1 of Study II.

This equation was used in both studies on OTA in plasma (I and II), and the coefficient utilised was the suggested by Miraglia et al. (1996). In addition to this, in the second study on OTA in plasma (Study II), an alternative version of the equation was proposed, in which the body weight of the individual could be considered. Thus, in the first case, the daily intake was a multiple of the concentration of OTA in plasma; whereas in the second, an individual characteristic (the body weight) was considered. The influence of this modification in the equation could be observed, as different results were obtained when comparing them with the first method:

- Analyses of differences regarding gender and age: significant differences in the OTA intake regarding gender could be observed, even if the plasma levels were not significantly different. In contrast, no significant differences were observed among age groups.
- When comparing the median daily intake of OTA with the estimated from food consumption and contamination data, results were similar in the second case, whereas in the first, values were lower.

Moreover, this equation can be improved by further studies, as values of bioavailability and clearance used in Klaassen equation are approximations:

bioavailability was not tested in humans, and the data on OTA clearance was tested in a single experiment with a human volunteer.

Finally, it could be observed that in some cases the estimation of the daily intake through plasma levels was above the PTDIs, which is different from the observed in the intake estimations from food consumption and contamination data, in which no case was found to present values above the PTDIs. This may be due to the fact that the questionnaires utilised to collect consumption data from the participants were referred to a recall period of consumption of a year, whereas the levels of OTA in plasma may be related to a shorter exposure period. Such period probably matches the half life of OTA, which was observed to be 35.5 days in a human volunteer (Studer-Rohr, 1995). Still the frame of time of exposure to OTA related to the levels of the toxin in plasma has to be defined.

Despite the assumptions made to use this equation, the estimation of the OTA daily intake through it requires a single determination per person, and saves all the problems associated with the food sampling and the collection of consumption data. In addition, if exposure took place by airborne sources, it would also be reflected in this estimation.

2.2. OTA and OTα in urine as biomarkers of exposure to OTA

OTA and its metabolite OTα were also found in urine (Study III), and it was observed that the incidence of OTα was higher than the incidence of OTA. The presence of OTA in urine has not been studied in humans as thoroughly as in plasma, but the observed results contribute to the study of urine as a possible alternative biomarker of exposure to OTA. In contrast to the observed in the studies on OTA in plasma, in which no relation was observed between those levels and food consumption, the results observed in Study III indicated a relation between the consumption of possibly contaminated foodstuffs and the levels of ochratoxins, as positive correlations were observed between food consumption (data from FFQ) and OTA levels of positive samples, and between consumption and OTA daily intake (data from 3DR) and OTα levels of positive samples. However, it is not yet possible to estimate the daily intake of OTA from its concentrations in urine.

In addition, it is important to determine which species to detect in order to get an adequate exposure assessment. Thus, considering the metabolic pathways mentioned in Study III, the low incidence of OTA, the higher incidence of OTα in urine, and the relations observed between contamination levels and consumption data, the presence of the metabolite OTα in urine might be a better indicator of the exposure to OTA than OTA itself. The non-invasive sample collection should also be reminded. However, more studies need to be carried out to confirm this hypothesis, and these should consider the metabolic pathways of OTA and the interindividual differences in metabolism abilities.

2.3. Occurrence of OTA in plasma and urine in relation with gender and age

No significant differences were found between genders in the levels of OTA neither in plasma nor in urine (in this case, the same was observed for OTα). Despite the absence of significant differences, in all studies (OTA in plasma, and OTA and OTα in urine) the highest median levels were found in men. This agrees with the results cited in the Introduction (Section 3.3.2.1). Differences between genders were expected, as sex is one of the multiple factors considered to influence the toxicological effects of a xenobiotic compound.

As regards to differences among age groups, these were significant among the OTA levels in plasma (higher medians for people aged above 45), but not in the OTA and OTα levels of urine samples (highest medians for people aged 18-29). It is interesting to observe that higher amounts of both OTA and OTα in urine were found in the youngest group of age, whereas the highest levels in blood were found in the elder group. A possible explanation for this can be that the younger group of population may have a higher ability to eliminate the toxin, or, which is the same, that the efficiency of the removal of OTA from the body decreases with age, leading to higher plasma levels (Gilbert et al., 2001). In addition to this, due to the long half-life of OTA in human blood, a continuously exposed person could have higher blood concentrations of OTA with time (Sangare-Tigori et al., 2006).

2.4. Plasma *versus* urine

The following points highlight certain aspects of the two biological fluids studied in this work:

- Collection of biological fluids

The obtainment of blood samples was only possible through the collaboration with the Blood Bank of the hospital Arnau de Vilanova, of the city of Lleida, with the approval of the Ethic Committee of the Hospital Vall d'Hebron (Barcelona). One of its tasks is to collect blood in different localities of the province of Lleida. Thus, the collection on samples for these studies was done following the Blood Bank schedule for each location, and a selection of the localities was done according to this schedule and the objectives of this work. Blood donors were informed about the study and asked to participate by giving an amount of blood (20 mL), extra from the 500 mL donated to the blood bank, and to answer to a food frequency questionnaire related to their consumption of possibly OTA contaminated foodstuffs. Blood samples were taken by nurses. Thus, the number of samples collected per locality depended on the number of donors per city, on the decision of the donor to participate in the study, and on the time needed by the interviewer to collect the food

consumption data. The examined population consisted only of apparently healthy adults, who were allowed to donate blood.

As regards to urine, collection of samples was easier than blood sampling, as the methodology was not invasive and no specialised personal was needed. However, the decision of the volunteers was also determinant, as their involvement in the study also implied the recording of the food consumed during the three days previous to the collection of the sample, and participants were asked to complete a food frequency questionnaire.

- Methods of analysis and alternatives of detection

The analytical method used to detect OTA in plasma was laborious and expensive, due to the need of a liquid-liquid extraction with solvents followed by immunoaffinity chromatography clean-up. In contrast, analysis of OTA and OTα in urine was easier, as only a liquid-liquid extraction was needed. As observed in the tables that summarize the previous studies on the OTA levels in plasma and urine (Tables 5 and 6 of the Introduction), these levels were lower in urine, and therefore analytical methods need to be sensitive enough to detect such levels.

Apart from the two ochratoxins detected in this study, other alternatives are available for the detection of OTA and its metabolites. Methods have been developed for the detection of other ochratoxins in blood plasma (Muñoz et al., 2010) and in mostly in urine (Muñoz et al., 2010; Schaut et al., 2008; Li et al., 2000). This is useful for toxicological studies, but in the case of exposure assessment, it has to be clearly stated which compound to detect.

- OTA levels in body fluids in relation with consumption of possibly contaminated food and the daily intake estimated from food consumption and contamination data

As observed in the studies on OTA in plasma (I and II), no significant correlation could be established between the consumption of possibly contaminated food (data retrieved from the FFQ) and the levels of OTA in plasma. As regards to the daily intake estimated considering those data, correlation was also non significant, which agrees with the first result, and even though values were in some cases similar, statistical tests indicated that the median values of the distributions were significantly different.

A different result was observed in the Study III (OTA and OTα in urine), in which positive significant correlations were found between food consumption and OTA levels in urine (when consumption data were obtained through the FFQ), and between food consumption and OTα levels in urine (when consumption data were obtained through the 3DR). These results were obtained

by analysing only the data of the individuals that presented detectable levels of ochratoxins (positive samples), which agrees with the analysis of plasma samples, for which the occurrence was 98 and 100% in Studies I and II, respectively. Concerning the estimation of the daily intake of OTA, which was done by considering only food contamination and consumption data, it was positively correlated to the presence of OTα in urine.

Therefore, the relation between OTA and/or OTα levels in body fluids could be observed at least in one of the two fluids studied. The reasons for the results of correlations were discussed in the respective studies, and can be summarised as follows:

Regarding the consumption questionnaires:

- The recall period of the FFQ was a year, which is a period of time of interest for the assessment of a chronic exposure. Therefore, over- or under-reporting of food consumption could have taken in the FFQs due to this long recall frame of time, as this method depends on the memory of the participants. In addition, it was observed that OTA half-life in human plasma was 35.5 days, which may also explain the lack of correlation observed in the studies in plasma. Concerning the correlation with OTA levels in urine, in the first place it has to be reminded that the number of samples in the analysis was low ($n = 9$) to have an accurate analysis. As mentioned, a possible explanation for the correlation might be that the long recall period matched the extended time needed to excrete the native form of OTA (not metabolised), due to its high affinity to plasma proteins and the enterohepatic recirculation.
- The brief recall period of the 3DR may explain the positive correlations observed with OTα, as this metabolite is excreted faster than OTA.
- Individual metabolism abilities are also a factor to be considered, as a certain amount of toxin can be metabolised differently from one person to another, leading to results in fluids that would not be related to the food consumption.

Regarding the contamination data of foodstuffs:

- The values of contamination used in the estimation of the intake (considering contamination data) did not correspond with food sampled in the region where the exposure was studied neither with the period of sampling of the fluids.
- Some possibly contaminated foodstuffs were not included in the questionnaires, such as spices or animal by-products. Other sources of exposure, such as contaminated dusts, were not considered.

Finally, it would be interesting to compare the levels of OTA in both plasma and urine in the individuals of a same population.

2.5. Additional remarks

Factors on which exposure to OTA (or other mycotoxins) depend, such as food contamination and dietary habits, vary with time. It is known that mycotoxin contamination of food products is heterogeneous, and in addition, the origin of food products is diverse due to the trade facilities, such as importation of goods. Therefore the exposure assessment of a certain population should be referred to a certain time-frame. In the second study on OTA in plasma (Study II) it was observed that seasonal and regional samplings did not lead to significantly different results of exposure, and therefore the period of time for the exposure assessment of the studied population (one year) should be extended in order to evaluate possible differences. It was also observed in all the studies that exposure varied according to the characteristics of the assessed population, such as gender and age, but a common tendency was not found among the different studies.

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V. CONCLUSIONS and FUTURE RESEARCH

CONCLUSIONS

The results obtained in this Thesis work led to the following conclusions:

- The different methodologies (analysis of body fluids and foodstuffs) utilised showed that the population studied in the present work was exposed to OTA, and that the mean and median levels of exposure were below the latest PTDIs of 17 and 14 ng/kg bw/day. These values do not imply a significant health risk. However, attention has to be paid to the risk groups of exposure, i.e. the population at the high percentiles of the exposure distributions, in which the exposure was above the PTDIs.
- OTA contamination levels of the studied foodstuffs were below the limits established by the European Community, with the exception of a coffee sample, and occurrence was also low in most cases.
- As regards to food consumption of OTA probably contaminated foodstuffs, the most consumed foodstuffs were cereals, followed by wine and beer.
- Deterministic and probabilistic modelling of food contamination and consumption data showed similar results, but the latter modelling methodology had the additional feature of the calculation of confidence intervals for the estimated descriptive values of the exposure distributions.
- The presence of OTA in blood plasma was useful to evidence the exposure of the population to this mycotoxin and allowed the estimation of the daily intake, showing a high occurrence of low levels. This feature makes the presence of OTA in blood plasma a useful tool for the exposure assessment as an alternative for food sampling and the collection of consumption data. OTA plasma levels were not significantly different in the seasons or regions studied.
- The presence of OTA and especially its metabolite OTα in urine was found to be an alternative biomarker of the exposure to OTA, especially by the fact that the obtainment of samples is non-invasive as in the case of blood, although further studies are needed to establish the relation between the levels of ochratoxins in urine and the intake of OTA.
- OTA levels in plasma were not correlated with the consumption of possibly contaminated foodstuffs, whereas OTA and OTα levels in urine were correlated with the consumption of possibly contaminated foodstuffs. The values of daily intake estimated through OTA plasma levels were higher than those estimated from consumption and contamination data.
- Presence of OTA in plasma and urine, as well as OTα in urine was not different between men and women, although consumption of possibly contaminated foodstuffs and the intake estimated from it was observed to vary with gender in

most cases. Ochratoxins levels in body fluids and food consumption by men were higher than by women.

- No clear tendency with respect to age was observed in common within the studies: significant differences in the presence of OTA in plasma were observed with respect to age, but not in the case of urine, whereas food consumption and intake estimations differences according to age varied in an opposite way.
- Many different methodologies are used to assess the exposure worldwide, and therefore the normalization of the methods of data obtainment could be a good step for achieving comparable results.

FUTURE RESEARCH

The discussion of the previous points leads to the following questions: Which is the period of time of exposure that the presence of OTA in plasma or OTA and OTα in urine refers to? Do the levels of ochratoxins in plasma or urine refer to long term or short term exposures? Which is the relation of these levels with food consumption? Thus, some points for future research are:

- Foodstuffs of animal origin or spices should be also included in the questionnaires. Although low occurrences are expected, the contamination levels might be high.
- The analysis of cereal-based foodstuffs as pasta or bakery products should be done, as well as animal derived products and spices.
- The recall period of the questionnaires used in the biomarkers studies should be adjusted taking into account the toxicokinetics properties of the analysed compounds (OTA or OTα).
- A better follow-up study might include the detection of OTA in blood plasma, and OTA and OTα in urine, in a same population, and the use of both food frequency questionnaires and three-day food consumption records. The analysis of foods “as consumed” may complete the study.
- Metabolism and toxicokinetics of OTA (absorption and excretion pathways) can be better clarified in order to establish a relation between the ingestion of OTA and its levels or its metabolites levels in body fluids. This seems a difficult task as the individual metabolism abilities vary among persons, and besides, tests are carried out in animals, although recently studies with cells are being developed. In addition, the presence of other xenobiotics or nutrients as well, may influence the metabolism or toxicity of these compounds.

VIII. ANNEXES

ANNEX 1

CUESTIONARIO DE FRECUENCIA DE CONSUMO DE ALIMENTOS (FOOD FREQUENCY QUESTIONNAIRE)

A continuación le preguntaré con qué frecuencia acostumbra a tomar los alimentos del siguiente listado: NUNCA (**N**), DIARIAMENTE (**D**), SEMANALMENTE (**S**), MENSUALMENTE (**M**) o ANUALMENTE (**A**) (márquelo con una cruz) y seguidamente, el número de veces que los acostumbra a tomar (indíquelo en la línea de puntos).

	CÓDIGO	ALIMENTO	CANTIDAD	N	D	S	M	A
CEREALES	101	Cereales de desayuno	….bol					
	131	Pasta: macarrones, espaguetis,..	….plato pequeño					
			….plato mediano					
			….plato grande					
	132	Pasta rellena	….plato pequeño					
			….plato mediano					
			….plato grande					
	141	Arroz	….plato pequeño					
			….plato mediano					
			….plato grande					
	111	Pan	….panecillo					
			….rodajas					
	121	Pan de molde	….rodajas					

	CÓDIGO	ALIMENTO	CANTIDAD	N	D	S	M	A
	151	Galletas	….unidades					
	161	Productos de bolleria: croissants, palmeras, magdalenas	....unidad pequeña					
			…unidad grande					
	171	Pasteles	….porciones					
	181	Pizza	….porciones					
	191	Coca de recapte	….porciones					
ZUMOS	212	Zumo de uva	….vaso					
			….brick pequeño					
	214	Zumo de uva con otra fruta	….vaso					
			….brick pequeño					
SNACKS	311	“Snacks”: ganchitos, doritos, cheetos ...	….platos					
FRUTOS SECOS	411	Almendras	….puñados					
	421	Avellanas	….puñados					
	461	Pistachos	….puñados					
	471	Cacahuetes	….puñados					
	431	Semillas de girasol (pipas)	….bolsa pequeña					
			….bolsa grande					
	441	Nueces	….unidades					

DULCES	611	Turrón	….porciones					
	621	Mazapán y/o panellets	….unidades					
	631	Cacao soluble	….cucharaditas					
	641	Chocolate a la taza	….tazas					
	642	Chocolate en tableta	…. porciones					
	651	Bombones	….unidades					
BEBIDAS	711	Vino tinto	….copa					
	721	Vinos de postre (Oporto, Pedro Ximenez, Mistela, …)	….copa					
	731	Cerveza	….quinto					
			….mediana/tercio					
			….lata					
			….caña					
	811	Café	….tazas					
	812	Cortado	….tazas					
	813	Café con leche	….tazas					

ANNEX 2

The following review was prepared at the beginning of this reseach work as a part of the bibliographic revision carried out throughout the development of this Thesis.

Review: Ochratoxin A: presence in human plasma and intake estimation

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ABSTRACT

Ochratoxin A (OTA) is a fungal toxic secondary metabolite that can be found in several foodstuffs and thereby ingested by humans. One way to assess exposure of humans to OTA is the determination of the levels of this mycotoxin in blood plasma from a certain population. Such studies have been done in many countries, both in healthy people and nephropathy patients. Relationships with individual characteristics were investigated in several cases. Thus, most studies found no correlation with age, either with gender. However, the few studies that found correlation between OTA plasma levels and gender showed that men presented the highest values. When sampling was done over more than one season, the highest OTA plasma levels were found mostly in summer. Differences within regions of a country were related to dietary habits of each area. OTA levels of group populations showed variations from year to year, whereas intraindividual repetitions showed no specific trend. Daily intake of the toxin can be estimated from OTA plasma concentrations by the Klaassen equation. OTA toxicokinetics are considered in this review. Calculated daily intake of OTA by different studies did not overpass the proposed tolerable daily intakes of OTA.

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INTRODUCTION

Ochratoxin A (OTA) is a toxic secondary metabolite of the fungi *Aspergillus* section *Circumdati, Aspergillus* section *Nigri*, *Aspergillus* section *Flavi, Penicillium verrucosum* and *Penicillium nordicum.* It is nephrotoxic, hepatotoxic, teratogenic, immunosuppressive and carcinogenic in animals. OTA has been related to the development of Balkan Endemic Nephropathy (BEN). It is also associated with urinary tract tumours (Smith et al., 1994). It has been classified as 2B (possibly carcinogenic) by the International Agency of Research in Cancer (IARC, 1993).

Toxicity of mycotoxins in animals is quantified by the determination of the highest dose of the toxin for a specific endpoint (i.e., the sign that constitutes one of the target outcomes of the toxicology study) at which no adverse effects are observed. This threshold dose is called NOAEL (no observed adverse effect level, mg/kg body weight per day). However, it is necessary to extrapolate to humans the results of the studies in animals. To characterize the hazard to humans of mycotoxins that have a threshold dose, a "safe dose" such as a provisional tolerable daily intake (PTDI) is estimated. It is calculated as the NOAEL divided by a safety factor. TDI is the dose that can be safely consumed daily over a lifetime without incurring appreciable adverse health effects, and which involves a biologically insignificant risk (WHO, 1987 and 1999; Edler et al., 2002). Table 1 shows the PTDIs of OTA suggested by different organizations.

Table 1. Provisional tolerable intakes of OTA.

PTDI (ng OTA/kg body weight/day)	PTWI (ng OTA/kg body weight/week)	Reference
5		The Nordic Working Group on Food Toxicology and Risk Evaluation, 1991
4		Health Canada (Kuiper-Goodman and Scott, 1989; Kuiper Goodman, 1996)
5		Scientific Committee on Food, European Commission, 1998
17*	120	EFSA, 2006
14*	100	JECFA, 1996, 2001, 2007

PTDI, Provisional Tolerable Daily Intake; PTWI, Provisional Tolerable Weekly Intake.
*Calculated values on the basis of the proposed weekly intake.

With regard to carcinogens, it is generally presumed that there is no threshold dose, and that there will always be some risk, then a TDI, which is based on a threshold as the NOAEL, is generally not determined. A mean for linear extrapolation can be the tumorigenic dose (TD), or its lower confidence interval, which is derived from all the experimental data in the observable range, and represents the dose at which 5, 10 or 25 % of the animals are responding (TD_{05}, TD_{10} or TD_{25}, respectively). Dividing the TD_{05}, which is not a threshold, by a factor of 5000, gives a value, the negligible cancer risk intake (NCRI) that is equivalent to a response level of 10^{-5} in animals. This value provides an appropriate estimate of safe intake for non-threshold carcinogens.

Dietary direct sources of OTA for humans are cereals and derived products, pulses, nuts, peanuts, dried fruits, coffee, cocoa, spices, wines and beer. The intake of OTA-contaminated feed may lead to residues in the blood, the kidney, the liver, the muscle of animals and also in eggs. Then, products of animal origin can also be a dietary source of OTA for humans (Bauer and Gareis, 1987). The Official Journal of the European Union published in 2006 (European Commission, 2006) the regulation to set the maximum levels for certain contaminants in foodstuffs. Regarding OTA, these levels vary from 0.5 μg/kg in baby foods to 10 μg/kg in soluble coffee and dried vine fruits. The European Union has not set any limit for contamination of animal products. However, some countries have appropriate regulation about this subject. In Romania, the maximum allowed level for meat is 5 μg/kg (Boutrif and Canet, 1998). The Danish Veterinary and Food Administration set the guidelines for the control of OTA in pigs. Organs condemnation is enforced for OTA levels in kidney between 10 and 25 μg/kg, and entire carcase condemnation for levels above 25 μg/kg (Jørgensen et al., 2001). In Italy, the maximum admissible value for OTA in pork meat and derived products is 1 μg/kg (Ministero dell' Economia e delle Finanze, 1999). The FAO published in 2004 the "Worldwide regulations for mycotoxins in food and feed in 2003" (FAO, 2004), in which the regulations of at least 99 countries are listed. Some mycotoxins, including OTA, are also regulated in regions like MERCOSUR, Australia and New Zealand (Van Egmond et al., 2007). As OTA can be found in several foodstuffs, there is special concern to evaluate its occurrence in the dietary sources so as to recommend adequate intakes of the toxin, and as a consequence, to set the limits of the regulations according to consumption.

Intake of foods contaminated with mycotoxins and the level of contamination in these foods are the factors on which exposure to mycotoxins depend. Assessment of exposure to mycotoxins can be done by the estimation of both factors. In the first case, intake of mycotoxins by the population can be assessed by means of consumption data of foods that are considered to contain mycotoxins. These data can be obtained from studies of dietary consumption such as Total Diet Studies brought about in many countries, as well as from individual surveys about dietary habits. With regard to food contamination, the

distribution of mycotoxins in foods is non-homogeneous, then appropriate sampling is necessary (Gilbert, 1996) to have a reasonable estimation of the contamination of foodstuffs. European Union established the methods of sampling and analysis for the official control of the levels of OTA and other mycotoxins in foodstuffs (Commission Regulation EC No 401/2006). In addition to this, the limit of detection of the detection methods has to be defined when analyzing food in an exposure study, as it is important to determine the level at which samples are positive.

Another way of estimating intake, particularly intake of OTA, is the determination of the levels of this mycotoxin in the blood plasma of the population. Daily intake can be calculated through the Klaassen equation (Klaassen, 1986), which considers OTA concentration in plasma and other biological factors.

This paper reviews the studies on assessment of exposure to OTA by the determination of the levels of this mycotoxin in the plasma of certain populations, and by the determination in many cases of the daily intake of OTA by means of the named plasma levels and the comparison with the PTDIs. Influence of characteristics of the study population (age, gender, geographic location and sampling season) on OTA plasma levels is also considered.

OTA chemical properties

OTA, (R)-N-[(5chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)-carbonyl]-L-phenylalanine, consists of a dihydrocoumarin moiety linked to a molecule of L-β-phenylalanine via an amide bond. OTA is a colourless, crystalline compound soluble in polar organic solvents, slightly soluble in water and soluble in diluted aqueous bicarbonate solutions (Valenta, 1998). A survey of the molar absorption coefficient ε of OTA in several solvents was presented by Valenta (1998). The UV absorption spectrum varies with pH and solvent polarity (Kuiper-Goodman and Scott, 1989).

Studies of the levels of OTA in plasma

Table 2 shows OTA concentrations found in plasma or serum of healthy volunteers in different countries of Europe and some countries all over the world. Surveys on the concentration of OTA in human plasma or serum have been carried out in several countries with two sorts of participants: healthy people (Table 2) and people suffering nephropathic diseases (Hult et al., 1982; Breitholtz-Emanuelsson et al., 1994; Maaroufi et al., 1995; Maaroufi et al., 1996; Radić et al., 1997; Jimenez et al., 1998; Malir et al., 2001; Grosso et al., 2003; Vrabcheva et al., 2004; Castegnaro et al., 2006; Sangare-Tigori et al., 2006; Dinis et al., 2007). Some of these studies found that OTA levels in

plasma were higher for the nephropathic patients. People who belonged to the healthy people group were in most of the cases only blood donors. Then, this group may not be representative of the whole population, as the results of exposure refer to a healthy population aged between 18 and 60. In some cases the study population belonged to both groups.

Table 2. Occurrence of OTA in blood plasma of inhabitants of different countries.

Country	Method	Year of sampling	Number of persons (men, women)	Occurrence % (number of positive samples)	Limit of detection ng/mL	Range of concentrations (mean) ng/mL	Reference
Argentina	HPLC		435 (335, 100)	63 (274)	0.012	<0.012 - 74.8	Pacin et al., 2008
Mar del Plata		2004	199 (142, 57)	63.8 (127)		n.a. - 47.6 (0.1537)	
General Rodriguez		2005	236 (193, 43)	62.3 (147)		n.a. - 74.8 (0.4319)	
Canada 15 collection centres	HPLC	1994	144 (72, 72)	100 (144)	0.12	0.29-2.37 (0.88)	Scott et al., 1998
Chile	HPLC	2004	88	70 (62)	0.1	0.07-2.75 (0.605)	Muñoz et al., 2006
Croatia	HPLC	1997	983	100 (983)	0.2	(0.3)	Peraica et al., 2001
Czechoslovakia	RIA	n.a.	143	24.5 (35)	0.1	0.1 - 1.26	Fukal and Reisnerova, 1990
Czech Republic	HPTLC	1990	644	22	0.5	0.5-12 (0.37)	Ruprich and Ostry, 1993b
	HPTLC	1991, 1992	594 (496, 98)	40 (240)	0.1 - 0.5	0.05 - 37 (0.625)	Ruprich and Ostry, 1993a
	n.a.	1994	809	90.7 (734)	0.1	0.1 - 13.7 (0.23)	Malir et al., 2001
	n.a.	1995	413	97.8 (404)	0.1	0.1 - 1.9 (0.24)	Malir et al., 2001
	n.a.	1997	398	94.5 (376)	0.1	0.1 - 2.1 (0.33)	Malir et al., 2001
Germany	HPLC						Bauer and Gareis, 1987
Oberschleissheim		1977	165	50.9 (84)	0.1	0.1 - 14.4	
Munich		1985	141	63.1 (89)	0.1	0.1 - 1.8	
Italy	HPLC	1992	65	100 (65)	0.05	0.12 - 2 (0.53)	Breitholtz-Emmanuelson et al., 1994

Italy		HPLC	1994, 1995, 1996	138	97 (134)	n.a.	0.12 - 2.84 (0.56)	Palli et al., 1999
Japan				184 (130, 154)	85 (156)		0.004-0.278 (0.068)	Ueno et al., 1998
		ELISA	1992	24 (18, 6)	96 (23)	0.01	0.016-0.278 (0.093)	
		ELISA	1994	34 (25, 9)	38 (14)	0.004	0.004-0.263 (0.073)	
		ELISA	1995	69 (46, 23)	93 (64)	0.02	0.024-0.13 (0.062)	
		HPLC	1996	57 (41,16)	98 (56)	0.01	0.019-0.151 (0.062)	
Lebanon		HPLC	2001-2002	250 (164, 86)	33 (82)	n.a.	0.1-0.87 (0.17)	Assaf et al., 2004
Morocco		HPLC	2000	309 (213, 96)	60 (185)	n.a.	0.08 - 6.59 (0.29)	Filali et al., 2002
Norway		HPLC	1998	206 (106, 100)	100 (206)	0.01	(0.18)	Thuvander et al., 2001
		HPLC	2000	210 (141, 69)	100 (210)	0.01	0.021 - 5.534 (0.397)	Skaug, 2003
Portugal		HPLC		104 (37, 67)	100 (104)	0.1	0.14-2.49	Lino et al., 2007
Spain		HPLC						
	Granada		1996, 1997	83 (0, 83)	86.75 (72)	0.22	<0.22 - 6.96 (0.63)	Perez de Obanos et al., 2001
	Madrid		1997	168 (88, 80)	100 (168)	0.02	0.12 - 5.58 (1.192)	Burdaspal and Legarda, 1998
	Navarra		1996, 1997, 1998	75 (44, 31)	53.3 (40)	0.52	<0.52 - 4.00 (0.71)	Jimenez et al., 1998
Sweden		HPLC	1989	297 (193, 104)	12.8 (38)	0.3	(0.1)	Breitholtz et al., 1991
		HPLC	1997	200 (134, 66)	100 (200)	0.01	(0.21)	Thuvander et al., 2001
Switzerland		HPLC	1992, 1993	368 (205, 163)	100 (368)	0.005	0.06 - 6.02 (0.30)	Zimmerli and Dick, 1995
UK		HPLC	2001	50	100 (50)	0.1	0.4 - 3.11 (1.09)	Gilbert, 2001

n.a.: not available.

The results in the literature were expressed as mean concentrations (ng OTA/mL plasma) and as occurrence (% of positive samples). However, samples were positive depending on the limit of detection of the method that was used in each assay. For this reason, some authors consider more appropriate to express occurrence in terms of concentration ranges (Jimenez et al., 1998; Pérez de Obanos et al., 2001). They express occurrence as a percentage of positive samples within certain concentration ranges. Thus, a better description of occurrence is obtained. For statistical purposes, when OTA was not detected by the analytical method, the assigned value of concentration used in most cases was half the limit of detection.

As shown in Table 2, the lowest occurrence of OTA in human plasma (12.8%) was found in Sweden; however, the limit of detection of the method was high compared to the rest of the studies considered. Taking into account the data of all the studies listed on Table 2, OTA plasma levels were analysed in 7664 persons, from which a 74% was positive. Even though the methods and limits of detection were different, the average values for the minimum, maximum and mean of all the studies listed in the table were calculated, and resulted to be 0.15, 9.15 and 0.45 ng OTA/mL plasma, respectively. The lowest mean value of this group was found in the Japanese population, whereas the maximum was found in Argentina.

Factors influencing OTA concentrations in plasma

Some studies tried to establish differences on OTA plasma levels according to the regions where the population lived and the season when the samples were obtained, as well as those due to gender and age. Regional dietary habits of the population were considered in their discussions, although these assumptions were not always supported by experimental data. However, there are studies that established an experimentally-based correlation between the detected plasma levels and food consumption by two possible ways: determination of OTA on the food consumed by the study population, using duplicate diet samples (Gilbert et al., 2001; i.e., food samples provided by the participants, in the consumption way) and establishment of food consumption patterns by means of food questionnaires that were answered by the study population (Thuvander et al., 2001; Muñoz et al., 2006).

OTA and age

In order to analyse the correlation between the age of the participants and the levels of OTA in plasma, age groups were defined. No relation was found between OTA concentrations in plasma and the age of the participants in several studies (Jimenez et al., 1998; Scott et al., 1998; Ueno et al., 1998; Palli et al., 1999; Gareis et al., 2000; Grosso et al., 2003; Skaug, 2003; Assaf et al., 2004; Jonsyn-Ellis, 2007).

Four age groups (21-30, 31-40, 41-50 and 51-60 years) were compared in the Argentinean cities of Mar del Plata and General Rodriguez and significant differences were found among age groups in both cities. In the first city, the highest

OTA levels were found in the 51 to 60 years group, and in the 31 to 40 years group in the second (Pacin et al., 2008). OTA plasma concentrations in the UK were higher in the age group from 30 to 44 (Gilbert et al., 2001). Lino et al. (2008) found the highest OTA concentrations in people aged over 40 in a rural area of Portugal. Filali et al. (2002) found no significant differences among age groups in Morocco, but analysed the frequency and the levels of exposure: the 40 to 50 years old group was the most exposed but the levels found were not high (0.23 ng OTA/mL plasma), whereas the 60.4 % of the 18 to 30 years old group was exposed to 0.42 ng OTA/mL plasma.

Only few studies have shown a relationship between age and OTA plasma levels. However, OTA-related age groups do not match from one study to another.

OTA and gender

Most of the studies on OTA levels in human plasma found no relation between OTA plasma levels and the sex of the participants (Breitholtz et al., 1991; Ueno et al., 1998; Thuvander et al., 2001).

In Switzerland, males living in the south of the Alps had higher OTA plasma concentrations than women from the same region and than the inhabitants of the north of the Alps (Zimmerli and Dick, 1995). In Tuscany (Italy) men had a significantly higher mean value of OTA in plasma than women (Palli et al., 1999). In Morocco, female population was less exposed than the male population (Filali et al., 2002). In Portugal, OTA was detected in serum of the inhabitants of three populations, Verride and Ereira (rural areas) and Coimbra. In the rural populations, OTA levels in men were higher than in women (Lino et al., 2008). Pacin et al. (2008) studied OTA concentrations in the Argentinean cities of Mar del Plata and General Rodriguez, and significant differences were found between men and women only in General Rodriguez, with men having the highest concentrations. Inhabitants of two agricultural zones in Chile were studied for OTA concentrations in plasma. No significant differences were found between men and women in the zone of Colbún, but in the zone of San Vicente de Tagua, the mean of OTA levels in women was significantly higher than that from men (Muñoz et al., 2006). Taking into account these data, it can be observed that when OTA levels in plasma were related to gender, in most of the cases male population presented the highest values.

OTA and season

There are studies in which samples were collected in different seasons of a year, and some other studies repeated measurements in the study population to determine differences in OTA levels from one year to another.

In Denmark, 144 blood samples were collected from January 1986 until December 1989. This study showed an inconsistent seasonal variability of OTA concentration: peak values were found in different months each year (September,

October and March) (Hald, 1991). In Granada, southern Spain, no significant differences were found between OTA plasma levels and season in two different years: 1996 and 1997 (Pérez de Obanos et al., 2001). In Madrid, central Spain, no relationship was found among the levels detected in the total study population (men and women) and the season (summer or winter). For men, OTA mean levels were lower in summer (1.119 ng/mL) than in winter (1.241 ng/mL), whereas levels of women were higher in summer (1.747 ng/mL), with a concentration of 0.974 ng/mL in winter (Burdaspal and Legarda, 1998). In Navarra, northern Spain, Jimenez et al. (1998) detected OTA in plasma of healthy people and nephropathy patients in the months of April, June, October and November 1996; January, February, March 1997 and January 1998. Levels found in the months of June and October 1996 were significantly lower than the obtained in the rest of the months.

Differences in plasma levels of OTA with season were found in a study made in five Croatian cities. Samples were taken four times in a year, in the months of June, September and December 1997, and March 1998. The highest frequency of the samples containing OTA in quantities above the limit of detection and the highest mean were found in June, whereas the month with the lowest frequency of positive samples was December (Peraica et al., 2001). The study done by Ruprich and Ostry (1993a) in the district of Brno, Czech Republic, lasted 13 months, and showed an increase of OTA levels in plasma in the spring months. In Italy, a group of 138 participants provided blood samples in the months of July and October 1994. The samples collected in the summer period of the study year presented the highest OTA levels in plasma (Palli et al., 1999). Fuchs et al. (1991) also found higher values in the summer period in a non-endemic village in Croatia. Hence, summer was the season during which the highest levels of OTA have been found.

Repeated measurements

Repeated measurements in groups

Ueno et al. (1998) studied OTA levels in plasma in Tokyo for the years: 1992, 1994, 1995 and 1996, in a total of 184 healthy volunteers. No significant differences were observed between the means in the four years. Palli et al. (1999) studied the levels of OTA serum of in healthy adults in Tuscany, Italy: 138 volunteers provided blood samples in the months of July and October 1994, and 69 of them agreed to provide an additional blood sample approximately one year later, in the months of October 1995 and January 1996. No correlation was found between the first and second measurements, moreover, in the repeated collection period, OTA levels showed a high variation and presented a different seasonal pattern from the observed in the previous year. In Germany, OTA plasma levels of a group of 20 persons varied over the period of three years (Märtlbauer et al., 1996). In Croatia, 6909 human sera samples were collected over a period of ten years, in endemic and control villages, and it was observed that OTA levels varied from year to year (Radić et al., 1997).

Intraindividual fluctuations

Ruprich and Ostry (1993a) repeated the quantification of OTA in serum of four persons, twice for three people, and three times for the forth one. Repetitions were done from 14 until 62 days after the first extraction. OTA concentrations decreased from the first determinations, with a variation from 37 ng/mL to < 0.33 ng/mL in the most extreme case.

Variations were also found in repeated measurements in three Japanese men after more than three months of the first extraction (Kawamura et al., 1993). In Canada, serum levels of one person were 0.25 ng/mL in 1990 and 1.29 ng/mL in 1991 (Kuiper-Goodman et al., 1993). In Germany, levels of OTA varied in a period of three years for a group of 20 persons. The highest differences were observed in two people, with ranges of 0.3 to 1.2 ng/mL and 0.3 to 1.3 ng/mL (Märtlbauer et al., 1996). In the study presented by Zimmerli and Dick (1995), serum levels of one Swiss person were almost equal in the months of May and November 1993, and March and June 1994. A special case is that of the nephropathic patients. In the study presented by Jimenez et al. (1998), repetitions of samples taken in April 1996 from four haemodialysis patients were done in January 1998; levels of OTA in the repeated measurements were lower.

All these different results show no specific trend in the behaviour of OTA in the individual cases. Therefore toxicokinetics of OTA have to be considered and related to the intake of the mycotoxin as well as the dietary habits of the persons. Palli et al. (1999) state that OTA serum levels are a short-term biomarker with high within-subject variability; therefore they have limited use at the individual level but can be used to characterize populations or subgroups of subjects.

OTA and geographic location

It has been observed that the distribution of OTA in plasma varies among regions within a country. Factors that have been considered to have influence on the regional variations of OTA are dietary habits and climate. In the first case, it is known that dietary habits are different on each zone in a country. For example, Zimmerli and Dick (1995) found that OTA plasma concentrations were higher in the south of the Alps than in the northern alpine region, which was related to the different food consumption habits of each region. Thereby, it was observed that in the south region consumption of rice, pasta, bread, biscuit, olive oil, cheese, grapes and tomatoes was higher than in the north, as well as a higher consumption of corn and alcoholic beverages, especially by men. Concerning the climate, Maaroufi et al. (1995) stated that the climate and the traditional way of food storage could influence the exposure of the population to OTA, as climatic conditions favour the proliferation of fungi during food storage. For example, when comparing OTA plasma levels of inhabitants of rural and urban areas in the same country, differences were observed. Peraica et al. (1999) found that the frequency of OTA positive samples was higher in rural than in urban populations, indicating that

locally produced food may influence the exposure of OTA. Other authors also stated that rural populations are more exposed to OTA than the urban ones: Lino et al. (2008) studied OTA levels in serum of the inhabitants of two farming villages in Portugal (Verride and Ereira) and of the city of Coimbra. Significantly higher concentrations were found in Verride population. In Chile, OTA plasma levels were studied in two different agricultural zones: Colbún and San Vicente de Tagua. The levels in the last were significantly higher than the levels found in Colbún (Muñoz et al., 2006).

Other studies compared levels of OTA in different cities. In Canada, Scott et al. (1998) compared the levels of the toxin in the population of inhabitants of fifteen cities and found significant differences among some of them. Breitholtz et al. (1991) determined OTA concentrations in plasma of inhabitants of three districts of Sweden: Uppsala, Östersund and Visby. The highest frequency of positive samples and highest levels of OTA were found in Visby. It should be noted that Visby is situated on an island, unlike Uppsala and Östersund. The study was later repeated in the city of Visby, together with the city of Oslo (Norway) and in this case levels found in Visby were slightly higher than the levels found in Oslo (Thuvander et al., 2001). On another study, OTA plasma levels were significantly different in two cities of Argentina, Mar del Plata and General Rodriguez, with medians of 0.11 ng/mL and 0.24 ng/mL, respectively. Differently from General Rodriguez, Mar del Plata is a coast city (Pacin et al., 2008). Peraica et al. (2001) studied the plasma concentration of blood donors in five different cities from Croatia: Varadzin, Zagreb and Osijek, located in the inland of the country, and in Split and Rijeka, located on the Adriatic coast. The results showed that the higher frequency of positive samples and the highest mean concentration (0.56 ng/mL) occurred in Osijek, meanwhile the lowest frequency of positive samples and lowest mean concentration (0.13 ng/mL) was found in Rijeka. The authors related these differences to different dietary habits in each region: the coast and the inland. Bauer and Gareis (1987) detected OTA in human serum of inhabitants of the German cities of Oberschleissheim (0.42 ng/mL) and Munich (0.79 ng/mL) in 1977 and 1985, respectively. In later studies, OTA levels found in Jena were 0.34 ng/mL and 0.17 ng/mL in Munich (Gareis et al., 2000; Rosner et al., 2000). In Lebanon, OTA mean levels in Beiruth/Month Lebanon, Bekaa and in the North and South Lebanon were similar (0.17; 0.16; 0.16 and 0.18 ng/mL respectively), though a higher frequency of positives samples was found in the regions of Bekaa and in the South of Lebanon. This difference was related to the fact that in the first two regions, consumption of cereals and burghul is important, whereas in Beirut/Mont Lebanon, cereals consumption has been replaced by fat-containing foodstuffs (Assaf et al., 2004). In Spain, mean values varied in three different regions: 1.19 ng/mL in Navarra (Jimenez et al., 1998), 0.63 ng/mL in Madrid (Burdaspal and Legarda, 1998) and 0.71 ng/mL in Granada (Pérez de Obanos et al., 2001).

From the above mentioned it can be seen that most of the authors relate dietary habits of each particular region (rural, urban, inland or coast areas) to the differences found in levels of the toxin in plasma of their inhabitants.

Estimation of OTA intake from OTA plasma levels

As an introduction to the estimation of the intake of OTA from the levels of the toxin in human plasma, toxicokinetics of OTA will be reviewed.

OTA toxicokinetics

Toxicokinetic studies of OTA have been done in different animal species: pigs, rabbits and chickens (Galtier et al., 1981); fish, quail, mouse, monkey (Hagelberg et al., 1989), rat (Hagelberg et al., 1989; Li et al., 1997; Mantle, 2008) and vervet monkeys (Stander et al., 2001). Concerning humans, the toxicokinetic profile of OTA was studied in one human volunteer, and intra-individual fluctuations of OTA plasma levels were studied in eight persons (Studer-Rohr et al., 2000). A method for the detection of OTA in plasma, kidney and liver of rats has been validated as a tool for toxicology studies (Vettorazzi et al., 2008).

The pathway of OTA in the organism consists basically of three steps: absorption, distribution and excretion. OTA is absorbed in the stomach and in the gastrointestinal tract, after which it is bound to serum proteins, transported via the portal venous system and distributed to the different tissues and organs. OTA is accumulated in liver and kidneys. Finally, OTA is eliminated through renal, faecal or milk excretion.

Absorption

After ingestion, OTA is rapidly absorbed from stomach and the small intestine in rats and mice (Pfohl-Leszkowicz and Manderville, 2007). The percentage of OTA absorbed differs among species: 66% in pigs, 56% in rats and rabbits and 40 % in chicken (Galtier et al., 1981). Moreover, bioavailability may vary in the presence of food components (Pfohl-Leszkowicz and Manderville, 2007). Once OTA reaches the bloodstream, it is bound to serum proteins (Galtier et al., 1981, Hult and Fuchs, 1986, Hagelberg et al., 1989), mainly albumin (Chu, 1971; Chu, 1974) and other macromolecules (Stojkovic et al., 1984; Hagelberg et al., 1989), in a proportion that varies depending on the species. In the study done by Hagelberg et al. (1989), the fraction of unbound toxin ranged from 0.02% in rat and man to 0.2 % in quail, and in fish it was a 22%. Erytrocites contain only traces of OTA (Ringot et al., 2006). Stojkovic et al. (1984) found that human and porcine plasma macromolecules had higher affinity than albumin, though the concentrations of those high-affinity molecules were lower than the concentrations of the low-affinity molecules (i.e., low molecular weight plasma proteins and albumin).

Circulating plasma levels

In animals, the concentration of the toxin and its metabolites in tissues and plasma depends on the animal species and sex (Kumagai, 1985; Heussner et al., 2002), the dose administered, the way of administration (crystalline or naturally occurring in feed, oral ingestion or intravenous administration), the duration of administration, the diet composition as well as the health status of the animal (Ringot et al., 2006). Species-specific factors such as half-life and the degree of serum binding also influence the levels of OTA in the organism (Hagelberg et al., 1989; Kuiper-Goodman and Scott, 1989). The species difference in serum half-life of OTA was reviewed by Petzinger and Ziegler (2000). Humans´ serum half-life of OTA was determined to be 35.5 days (Studer-Rohr et al.., 2000). Generally, the elimination half-life of OTA is longer in blood than in tissues, which may be related, in part, to the higher binding affinity of the toxin to blood proteins (Chang et al., 1979; Hagelberg et al., 1989; Fuchs and Hult, 1992; Marquardt and Frohlich, 1992; Li et al., 1997; Studer-Rohr et al., 2000).

Fuchs and Hult (1992) considered that OTA bound to serum albumin and other serum macromolecules is a mobile storage of toxin in the body, readily available to several tissues over a prolonged period of time. According to these authors, a process such as the enterohepatic circulation of OTA in the human body could maintain the saturation of the specific binding macromolecules for a long time. Enterohepatic circulation is the circulation of bile from the liver to the small intestine, and from there back to the liver via the hepatic portal system. This process involving OTA has been studied in rodents (Kumagai and Aibara, 1982, Fuchs et al., 1988b, Roth et al., 1988) and prerumiant calves (Sreemannarayana et al., 1988). In these cases the toxin was secreted toghether with the bile and afterwards reabsorbed in the intestine. OTA can also be reabsorbed in kidney, and it was suggested that this process facilitates the residual persistence of the toxin, and then the renal toxicity in rodents (Stein et al., 1985, Albassam et al., 1987). Stojkovic et al. (1984) stated that OTA binding to low molecular weight plasma constituents would facilitate the accumulation of the toxin into the kidney, as these small molecules can pass through the glomerular membrane. If food containing very low concentrations of OTA is consumed over a long period of time or even daily, exposure of the kidney to such very low doses may become toxicologically significant (Fuchs and Hult, 1992).

Ichiyama and Saito (1987), on an *in vitro* study, found that OTA had a high affinity to serum albumin but little affinity to soluble tissue proteins of liver and kidney of rats. The authors suggested that OTA *in vivo* may be present in kidney or liver mainly in the form of serum albumin-bound OTA, and that this argumentation can be applied to the explanation of the distribution of OTA in meat products.

Tissue distribution

As mentioned, OTA levels in tissues and blood of animals depend on several factors. Tissue distribution of OTA was studied in trout, quail and mice using whole-body autoradiography with [^{14}C] labelled OTA. The presence of the labelled toxin was observed in the liver, bile and intestinal content (Fuchs and Hult, 1992) and there was a large difference in the accumulation of the toxin for other organs. In birds, labelled OTA disappeared rapidly from the body and did not accumulate in the organs, but the deposition of the toxin was observed in the egg yolk (Fuchs and Hult, 1992). Other studies showed that OTA accumulated in organs such as kidneys, liver, muscle, lung, heart, intestine, testicles, spleen, brain, skin; and also in the fat, in different proportions, according to the dose and frequency of administration (Madsen et al., 1982; Harwig et al., 1983; Mortensen et al., 1983; Kane et al., 1986, Fuchs et al., 1988a; Hald, 1991; Breitholtz-Emanuelsson et al., 1992; Ferrufino-Guardia et al., 2000; Canadas et al., 2005a; Canadas et al., 2005b; Gaou et al., 2005; Canadas, 2006) with the kidneys and liver as the principal targets, followed by the muscle, lungs and heart. Auodia et al. (2008) observed a protective effect of micronized wheat fibres in terms of OTA concentrations in plasma, kidney and liver of rats when these fibres were included in their OTA naturally contaminated diet.

Canadas et al. (2005a, 2005b, 2005c, 2006) and Gaou et al. (2005) demonstrated that OTA tissue distribution was also sex-dependent in rats. They observed that except in brain, male rats accumulated higher amounts of OTA in liver, kidney and lung than female rats (Gaou et al., 2005; Canadas, 2006). Other studies showed transference of OTA in utero of mammals such as mice, rat and swine (Kuiper-Goodman and Scott, 1989: Hallen et al., 1998), and also transference via milk (Ferrufino-Guardia et al., 2000). In humans, OTA levels of foetal serum (Zimmerli and Dick, 1995) and placenta (Miraglia et al., 1998) doubled the maternal serum concentration.

Elimination

Elimination of OTA can take place through renal, biliary, faecal and milk routes, being the first two the principal ones.

In humans and monkeys, the major route of excretion is renal elimination (Studer-Rohr et al., 2000; Stander et al., 2001), whereas the biliary excretion is the main way for rats and mice (Storen et al., 1982; Moroi et al., 1985; Kuiper-Goodman and Scott, 1989; Li et al., 1997) and also for fish and quail (Fuchs and Hult, 1992). The relative contribution of each excretory route is influenced by the route of administration, the dose, the degree of binding with plasma proteins and the enterohepatic circulation of OTA (Pfohl-Leszkowicz and Manderville, 2007). Differences in effectiveness of enterohepatic circulation of OTA might partially be responsible for the differences in retention of the toxin in plasma among species (Fuchs and Hult, 1992).

Concerning filtration through the kidneys, renal excretion ability can be measured by the *clearance*. Clearance is done mainly by *glomerular filtration,* the process in which the fluids are filtered in the glomerular capillaries of the kidney. Fuchs and Hult (1992) considered that the reduced filtration of the toxin through the kidney agrees with the fact that glomerular impermeability for large proteins (such as albumin) is also restricted. Then, in the case of albumin deficiency, OTA is eliminated from plasma in a very short time (Fuchs and Hult, 1992; Hagelberg et al., 1989; Pfohl-Leszkowicz and Manderville, 2007). After its excretion, OTA is reabsorbed in the nephrons (Gekle et al., 2005). This process retards its excretion and may lead to the accumulation of the toxin in the kidneys (Ringot et al., 2006).

OTA metabolites can be cleared in the kidneys at a faster rate than OTA. Li et al. (1997) studied in rats the pharmacokinetics of OTA and some analogues as ochratoxin B (ochratoxin without chloride), ochratoxin C (OTA ethyl ester); as well as some metabolites as ochratoxin α (OTα), hydroxylated ochratoxin A (OTA-OH) and the open form of ochratoxin A (OP-OTA). The study included the determination of biliary, renal and estimated metabolic clearances of these compounds. Clearance data suggested significant differences among the various forms of OTA with regard to the preferred routes of elimination (renal, biliary, and/or metabolism). The results of the complete study demonstrated that OTA was very slowly cleared from the body and that its metabolites were cleared at a much faster rate, and via the bile and kidney.

OTA and its metabolite OTα are also excreted in faeces. Faecal excretion is mainly due to biliary excretion. Concerning milk excretion, several studies evaluated OTA presence in milk of many species: rabbit (Galtier et al., 1977; Ferrufino-Guardia et al., 2000), rats (Breitholtz-Emanuelsson et al., 1993b; Hallen et al., 1998), sows (Mortensen et al., 1983) and cows (Ribelin et al., 1978; Breitholtz-Emanuelsson et al., 1993a). Presence of OTA in human milk has been reported by Gareis et al. (1988), Breitholtz-Emanuelsson et al. (1993a), Micco et al. (1995) and Skaug et al. (2001).

Intake of OTA can be calculated by the Klaassen equation, which takes into account plasma clearance of the toxin.

Plasma clearance

Plasma clearance is determined by all the individual metabolizing/eliminating organ clearances and involves mainly liver and kidney clearances (Toutain and Bousquet-Mélou, 2004). From a pharmacological point of view, any clearance (total or for a given organ) should be defined as the ratio of two terms: the rate of drug elimination and the driving concentration of the drug in the system. For plasma clearance, the rate of drug elimination is the total rate of body elimination, and the driving concentration is the plasma concentration, as shown in Equation (1) (Toutain and Bousquet-Mélou, 2004).

Plasma clearance = Total (body) rate of elimination /plasma concentration (1)

The estimation of a plasma (total) clearance (Cl_{tot}) can be done if the drug is totally eliminated by a route which is experimentally measurable (e. g. urine, faeces). In most instances, total plasma clearance is the sum of organ clearances (Equation 2):

$$Cl_{tot} = Cl_{renal} + Cl_{liver} + Cl_{other} \quad (2)$$

Being Cl_{renal} the renal clearance, Cl_{liver} the hepatic clearance, and Cl_{other} all other clearance mechanisms. Generally, Cl_{other} is negligible and the plasma clearance is often the sum of renal and hepatic clearance. Moreover, if Cl_{liver} is negligible, Cl_{tot} reduces to Cl_{renal} (Toutain and Bousquet-Mélou, 2004).

Renal clearance

Seldin (2004) reviewed the development of the renal clearance concept. A preliminary definition of clearance in his work is the following: "The renal clearance of a substance is the volume of plasma required to supply the amount of this substance excreted in the urine during a given period of time" (Equation 3):

$$C_s = (U_s \, x \, V) / P_s \quad (3)$$

Where s is any substance, C_s is the clearance of the substance (mL/min), U_s is the concentration of the substance in urine (mg/mL), V is the urine flow rate (mL/min), and P_s is the concentration of the substance in plasma (mg/mL). However, the author makes clear that the kidney does not completely remove a substance from the total renal plasma flow, and that the kidneys free a fraction (virtual volume) of each mL of total plasma flow of any substance. Taking this into account, the concept of clearance would be: "The clearance of any substance is the virtual volume of plasma flow required to supply the amount of a substance excreted in one minute".

Measure of renal clearance

Renal function can be assessed by different means: measures of the blood urea concentration, urea excretion, glomerular filtration rate, and renal plasma flow or filtration fraction. The most common way is to determine the glomerular filtration rate, which can be done by the determination of the clearance of markers such as inulin or creatinin, being the first the most appropriate (Seldin, 2004). To summarize, and taking into account all the above mentioned considerations, plasma clearance can be assessed by the inulin or creatinine clearance.

Intake calculation from OTA plasma concentrations

Daily intake of OTA (k_0, ng OTA/kg body weight per day) can be calculated from the concentration of the toxin in plasma by the equation proposed by Klaassen, (1986) (Equation 4):

$$k_0 = Cl_p \times C_p/A \quad (4)$$

Where Cl_p is plasma clearance (mL/kg body weight per day); C_p is the plasma concentration of OTA (ng/mL) and A is the bioavailability of OTA (fraction of toxin taken up). In this equation it was assumed that plasma clearance involves only glomerular filtration.

Different values of A and Cl_p have been suggested. For most of the animals studied by Hagelberg et al. (1989), the bioavailability of OTA was around 50%. Few authors give to A the value of 0.57 (Ruprich and Ostry, 1993a; Pérez de Obanos et al., 2001).

Hagelberg et al. (1989) and Studer-Rohr et al. (1995) considered renal clearance as the estimation of plasma clearance and calculated its value. The first group obtained a value of 0.033 mL/min, corresponding to 0.67 mL/kg bw day and the second, 0.048 mL/min, corresponding to 0.99 mL/kg bw day. Taking these considerations into account, calculation of intake from Klaassen equation remains to be (Equations 5 and 6):

$$k_0 = 0.67 \times C_p/0.5 = 1.34 \times C_p \quad (5)$$

$$k_0 = 0.99 \times C_p/0.5 = 1.97 \times C_p \quad (6)$$

According to Gilbert et al. (2001), neither of these two equations is completely accurate, as estimation of plasma clearance in Equation (6) was based on a single human experiment (Studer-Rohr et al., 1995), whereas Equation (5) takes into account the clearance of inulin instead of OTA (Hagelberg et al., 1989). This author compared the OTA intake calculated from plasma using both equations with the intake calculated from the diet, and showed that the intake estimations from plasma were wide ranging and not close to the levels determined in the diet. Miraglia et al. (1996) considered that the calculation based on the plasma analysis gives an underestimation of the intake, as only renal clearance is considered for the estimation of plasma clearance.

Some of the studies on levels of OTA in human plasma used the obtained mean concentrations to calculate the daily intake of the toxin by the participants. Their results are shown in Table 3. When comparing the intakes obtained in the studies listed on Table 3 with the PTDIs of Table 1, it can be seen that none of them was superior even to the lowest PTDI.

Intake calculation from OTA consumption data and OTA plasma levels related to OTA intake

Occurrence of OTA in different countries varied in different regions. This can be related to the differences in alimentary habits of the populations. There are studies that assessed the food consumption habits of the population by means of

questionnaires in order to assess OTA intake and relate it with the levels of the toxin in blood. Thuvander et al. (2001) detected OTA in plasma of Norwegian and Swedish inhabitants. In this study, participants were also invited to fill in a questionnaire on food consumption during the two months previous to the study. The foods included in the questionnaire were those potentially contaminated by OTA. Daily intake was calculated taking into account OTA plasma levels and food consumption data. In this last case, data from other studies on OTA levels in foods were used to calculate the intake, as food analysis had not been included in the study. The estimation of DI based on food consumption data (1.3 – 1.4 ng/kg bw.d) was higher than the DI estimation based on plasma OTA levels (Oslo, 0.24 ± 0.15 ng/kg bw.d; Visby, 0.28 ± 0.22 ng/kg bw.d). The authors found no correlation between OTA plasma levels and the estimated DI from consumption data in the questionnaires and from food contamination data from the literature. In addition, plasma levels of OTA were not correlated with the total amount consumed. According to Berthiller et al. (2007), humans and animals consuming parts of mycotoxin contaminated plants are exposed not only to the native mycotoxins, but also to their metabolites formed by the plants. It is possible that these compounds, when metabolized by the human body, might derive in OTA and therefore the DI estimated from OTA plasma levels would be different from the DI estimated from food contamination data. Gilbert et al. (2001) assessed human exposure to OTA by the analysis of food and body fluids of 50 individuals during a month, taking control samples a month before. The analyzed food consisted on duplicate portions of all the food and drinks the people consumed, and the body fluids were urine and blood. Food samples were composited for analysis, whereas blood and urine samples were collected once a week and composited for analysis. The study population also kept a food diary for the two months the study lasted. Finally, OTA daily intake was calculated from food and plasma levels. The correlation between OTA consumption (levels of OTA in food) and plasma and urine levels was evaluated. The results showed that the correlation between the urine concentration of OTA and OTA consumption was more statistically significant than the correlation between plasma levels and OTA consumption. Muñoz et al. (2006) calculated the daily intake of OTA of a study population of two regions of Chile (Colbún and San Vicente de Tagua), based on its plasma levels, and correlated it with food consumption. The consumption data were obtained from the study population, which answered a food questionnaire about their alimentary habits. The questionnaire was based on the local alimentary habits, and the foods included were susceptible to contamination with OTA. The only foods correlated with OTA plasma levels were cereals, consumed by men in Colbún, and chicken consumed by men in San Vicente de Tagua.

Breitholtz et al. (1991) calculated OTA daily intake from plasma levels of the toxin and compared it with the DI calculated from food analyses and food consumption data by Olsen et al. (1991). A good agreement between OTA plasma levels and OTA level in food was found. Assaf et al. (2004), together with the determination of OTA levels in plasma, determined the contamination of some grains (wheat, burghul, pea, lentil and rice) and beer. These foodstuffs were obtained from markets from all over the country. DI was calculated from OTA plasma levels, but daily intake of OTA from these foodstuffs was not estimated, as the authors considered that the studied foods were only a part of the potential contaminated foods.

Table 3. OTA daily intakes of some populations.

Country	OTA daily intake (ng/kg bw/day)			Reference
	Calculated as 1.34 × Cp	Calculated as 1.97 × Cp	Others	
Argentina				Pacin et al., 2008
General Rodriguez	0.32	0.47	-	
Mar del Plata	0.15	0.21	-	
Canada	1.2	1.7	-	Scott et al., 1998
Chile				
Colbún	-	-	0.84	Muñoz et al., 2006, based on
San Vicente de Tagua	-	-	1.4	Miraglia et al., 1996
Croatia	0.53	-	-	Peraica et al., 1999
	0.4			Peraica et al., 2001
Czech Republic	-	-	0.74	Ruprich and Ostry, 1993a Calculated as 1.18 × Cp
Italy	0.77	-	-	Palli et al., 1999
Japan	-	-	0.08	Ueno et al., 1998, based on Kuiper Goodman et al., 1993
Lebanon	0.23	-	-	Assaf et al., 2004
Morocco	-	-	0.4	Filali et al., 2002, based on Kuiper Goodman et al., 1993
Norway	0.24	0.36	-	Thuvander et al., 2001

Table 3. Continued

Portugal					Lino et al., 2007
	Coimbra	0.56	-	-	
	Ereira	0.59	-	-	
	Verride	1.05	-	-	
Spain					Perez de Obanos et al., 2001
	Granada	-	-	0.74	Calculated as 1.18 × Cp
	Madrid	0.26		-	Burdaspal et al., 1998
Sweden					
	Östersund	0.04	-	-	Breitholtz et al., 1991
	Uppsala	0.03	-	-	Breitholtz et al., 1991
	Visby	0.35	-	-	Breitholtz et al., 1991
	Visby	0.28	0.42	-	Thuvander et al., 2001
UK		1.46	2.15	-	Gilbert et al., 2001

CONCLUSIONS

Factors such as age, gender, season and geographic location influence OTA presence in plasma of the population. In particular, season and geographic location have a remarkable effect. Estimation of OTA intake of a certain population through OTA plasma levels could, however, be refined. Further studies on the OTA bioavailability and plasma clearance should be done to have a better approach for the coefficients in the Klaassen equation. Few studies exist on the simultaneous calculation of OTA intake, both from plasma OTA concentrations and OTA consumption of potentially contaminated foodstuffs for the comparison of both results. Chronic exposure of certain foodstuffs, such as those consumed throughout the year without seasonal influence, should be assessed separately. Special groups of consumers, such as children and elder people, or people following a special diet, should also be considered in the assessment studies.

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