Universitat de Lleida

Exposure assessment of Catalonian population to mycotoxins

Avaluació de l'exposició de la població catalana a micotoxines

German Cano Sancho

The present work is included in the following research projects

- Evaluación de la exposición de la población española a las toxinas de *Fusarium*. 2009. Ministerio de Ciencia e Innovación. AGL2008-05030-C02-01
- Aproximación integrada a la exposición humana simultánea a ocratoxina A y deoxinivalenol (MYCOEXPO). 2012. AGL2011-24862.
- Exposure assessment of Catalonian population to mycotoxins. Agreement between Catalonian Food Safety Agency and University of Lleida between 2008 and 2010.

Pre-doctoral fellowship

- 2009 University of Lleida doctoral grant UdL-Competitive projects
- 2010-2012 Catalonian Government fellowship. AGAUR FI-DGR 2010.

Pre-doctoral training and collaborating residencies

- 2010 Mathematics and Applied Informatics Unit- Centre de Jouy en Josas -Institut de National de Recherche Agronomique (INRA). Programming and execution of simulation models to assess the exposure of Catalonian population to the mycotoxin zearalenone and to analyse the sensitivity of the high percentiles
- 2010 TOXALIM UMR 1331 INRA/INP/UPS. In vitro methods to assess the toxicity of *Fusarium* spp. Mycotoxins.
- 2012 Departamento de Medio Ambiente, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). Cytotoxic effects of the simultaneous exposure of Caco-2 cells to mycotoxins and antioxidants.

University of Lleida, 2008-2013

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Lleida 2013

Acknowledgements / Agraïments/Agradecimientos

- *Nora por tu infinita paciencia, tu fuerza y sensatos consejos.*
- *Als meus pares per ser com sou, per fer-me com sóc.*
- *Vicent, Sònia i Antònio per fer-me créixer científicament a la par que com a persona.*
- *Companys de laboratori aka "Peluqueria Tecal", per fer d'aquests anys una gran acumulació de bons moments en un entorn a vegades difícil, sou els millors!*
- *Merci à J.P. Gauchi pour m'ouvrir les portes de votre laboratoire et le monde fascinant de la modélisation.*
- *Gràcies a tothom que ha col·laborat en els diferents treballs i que ha fet posible la realització d'aquesta tesis.*
- *Agraïr també a tothom amb qui he compartit els bons moments fora del laboratori durant aquest quatre anys, sense això res és possible.*

a Nora i als meus pares

"No es más quién más alto llega, sino aquel que influido por la belleza que le envuelve, más intensamente siente."

Maurice Herzog, alpinista

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Abbreviation List

Chemistry

AFB_1 Aflatoxin B_1

AFB_2 Aflatoxin B_2

AFG_1 Aflatoxin G_1

AFG_2 Aflatoxin G_2

AFM_1 Aflatoxin M_1

AFs Total aflatoxins (B_1+B_2+G_1+G_2)

DON Deoxinivalenol

D3G Deoxinivalenol 3-glycoside

ELISA Enzyme Linked Immunosorbent Assay

FB_1 Fumonisin B_1

FB_2 Fumonisin B_2

FBs Total fumonisins (B_1+B_2)

HT2 HT-2 toxin

HPLC High performance liquid chromatography

IAC Immunoaffinity chromatography (column)

NIV Nivalenol

OPA *o*-phthaldialdehyde solution

OTα Ocratoxin alpha

PAT Patulin

PBS Phosphate buffered saline

Sa Sphinganine

So Sphingosine

T2 T-2 toxin

TRCs Trichothecenes

ZEA Zearalenone

Governmental Organisations

ACSA Agència Catalana de Seguretat Alimentària (Catalonian Safety Food Agency)

EC European Commission

EFSA European Food Safety Agency

FAO Food and Agriculture Organization

IARC International Agency for Research on Cancer

JECFA Joint FAO/WHO Expert Committee on Food Additives

SCF Scientific Committee on Food

WHO World Health Organisation

Other abbreviations

ALARA As Low As Reasonably Achievable

BMD Benmarch Dose

$BMDL_{10}$ BMD increasing cancer rates 10 % over the control

CI_b "Bootstrapping" Confidence Interval

FFQ Food frequency questionnaire

HBV Hepatitis B viruse

KM Kaplan-Meier

LOAEL Lowest observed adverse effect level

LOD Limit of detection

LOQ Limit of quantification

NOAEL No observed adverse effect level

MoE Margin of exposure

MLE Maximum likelihood estimation

ND Non detect

Pdf Probability density function

PMTDI Provisional maximum tolerable daily intake

R3 3 days dietary record

R24 24 hours dietary recall

TD50 Median toxic dose

TDI Tolerable daily intake

TWI Tolerable weekly intake

Chapter 1. Summary

1.1 Summary (English)

Mycotoxins are toxic secondary metabolites produced by fungi that contaminate various agricultural commodities either before harvest or under post-harvest conditions. Their purpose is thought to be for fungal defence or competition. Although there are many species of toxigenic moulds, only a few mycotoxins, particularly those affecting cereals (maize, wheat, barley, oats and rice) and groundnuts are considered to be significant for humans. Several mycotoxins have been identified up to the present, but those of special interest in food and feed safety are: aflatoxins (B_1, B_2, G_1, G_2, M_1), fumonisins (B_1, B_2), ochratoxin A, patulin, trichothecenes (deoxynivalenol, T-2 toxin, HT-2 toxin), and zearalenone.

The general objective of the present thesis was to assess the exposure of Catalonian population to the major mycotoxins, aflatoxins group B and G, aflatoxin M_1, patulin and the *Fusarium* toxins deoxynivalenol, fumonisins, T-2 and HT-2 toxins, and zearalenone. To reach this general objective, several sub-objectives were developed: 1) To assess the dietary intake of Catalonian population in regard of those mycotoxin related foods, including the most vulnerable collectives like elderly, infants, immigrants or celiac sufferers. 2) To determine the mycotoxin levels in most relevant food categories, from a representative sample purchased around of the geography of Catalonia. 3) To combine accurately raw consumption and contamination datasets in order to obtain a thorough estimation of exposure levels of the population collectives, and a reliable characterization of the related health risk. 4) To assess the effect of any probable variable, as food processing or cooking, over the exposure assessment outputs. 5) To evaluate the novel biomarker ratio sphinganine (Sa) : sphingosine (So) as an indicator of exposure to fumonisins.

The global design consisted in one hand, of a nutritional study and in the other, of an analytical study. Food samples were selected to be the most susceptible commodities to mycotoxin contamination and to be commonly consumed in Catalonia. Between 2008 and 2009 the samples were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú,

l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. Almost 3000 individual samples were pooled to obtain more than 1300 analytical samples following the Total Diet Study designs. Samples were analysed to determine the levels of 1 to 6 mycotoxins depending of the matrices. Regarding brands, finally a wide range of trade brands (n=770) were involved in the study, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of Spanish market. Chemical analysis was performed by means of validated methods consisting on a preliminary extraction and clean-up of the mycotoxins, followed by a determination by HPLC coupled to fluorescence, absorbance or mass detection.

Nutritional study was carried out using a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins, excluding those foods not consumed in the region. Five response options, ranging from never to annually, were considered to report frequencies. Quantities were assessed by portion size with the aid of a series of colour photograph models. Five different population groups of each sex were considered: infants (0-3 years), children (4-9), adolescents (10-19 years), adults (20-65 years) and elders (>65 years). Finally, 76 elders, 720 adults, 235 adolescents, 69 children and 164 infant parents were interviewed during 2008-2009 by trained interviewers. Moreover, 70 celiac sufferers and 56 individuals with an ethnic dietary pattern were included in the nutritional study. Individuals were from 89 cities and towns from around of Catalonia. Combination of contamination with consumption datasets was computed with deterministic and probabilistic models. Each exposure estimated was compared with the respective safety level in order to characterise the expected health risk.

Considering results from our studies, after performing more than 4500 chemical analysis, global occurrence of all mycotoxins was found to be almost of 32 % of total samples. The highest levels of positive samples (above of LOQ) were reached for DON and FBs with percentage levels of 47 and 40 %, respectively. Lowest levels of positive

samples were found for A-type trichothecenes, T-2 and H-T2 (1 and 8 %, respectively), followed by aflatoxins (12 %). Corn snacks were one of the food matrices with highest percentages of *Fusarium* mycotoxins. Aflatoxins were most widely found in dried red pepper, followed by nuts. Very few samples were found to exceed the EU limits.

Results from exposure assessment computation, through direct and probabilistic methods identified infants, children and immigrants to be the main risk groups for most of the mycotoxins studied. Considering our results, each exposure profile from all population groups studied were far from the safety levels set by EFSA or WHO, including aflatoxins, fumonisins, patulin, zearalenone. The main health concern derived from mycotoxins was focused in A and B-type trichothecenes. T-2 and HT-2 toxins were found in few samples but their high toxicity derived in a very narrow margin of exposure. DON was found widely in most of cereal-based food analysed, also high consumed commodities, therefore considering our exposure models, high consumers of risk groups could exceed its safety level.

Derived from these results, the sub-objective 4 was focused mainly in the food processing and cooking effect on DON and ZEA. The goals of this work were, in one hand, to assess the effect of baking on stability of ZEA and DON, as well as the transference of DON from pasta to the boiling water, and in the other hand, to quantify the impact of DON depletion, during cooking of pasta, on the global exposure estimations. Results showed DON and ZEA to be stable during the bread making process, including fermentation with *Saccharomyces cerevisiae* and baking at 200 ºC. Our results showed a high transference of DON from pasta to boiling water reaching depletion levels of almost 75 %, correlated with those ones in water. Accordingly, these cooking depletion rates were computed through a stochastic exposure model to weight its impact on the final exposure estimates. Finally, statistically significant differences were found in most of parameters and population assessed, but to be moderately protective in the highest risk groups.

In order to reach the sub-objective 5, firstly, plasma and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and later urinary Sa

and So levels from corn food consumers and a control group were monitored for 2 weeks under controlled intake of corn foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection. Significant differences were not found in urine samples when Sa/So ratios were compared from corn food consumers and non-consumers, while significant differences were found in urine and plasma samples, but evidence of the mechanism of action of fumonisins was not apparent. Through a time-course study we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans.

1.2 Resum (Catalan)

Les micotoxines són metabòlits secundaris produïts per fongs que contaminen diferents productes agrícoles, tant abans de la collita com en condicions de post-collita. La seva funcionalitat és bàsicament per la defensa del propi fong o millorar les condicions de competència. Tot i que hi ha moltes espècies de fongs toxigènics, només unes poques micotoxines, particularment aquelles que afecten els cereals (panís, blat, sègol, civada o arròs) i fruits secs, són els més importants per als humans. Bastants micotoxines han estat identificades fins ara, però les més especials a nivell alimentari, tan a nivell humà com animal, són les següents: les aflatoxines (B_1, B_2, G_1, G_2, M_1), les fumonisines (B_1, B_2), l'ocratoxina A (OTA), la patulina, els tricotecens (deoxinivalenol, toxina T_2 i HT_2) i la zearalenona (ZEA).

L'objectiu general d'aquesta tesis doctoral era avaluar l'exposició de la població catalana a les micotoxines més importants: aflatoxines del grup B i G, aflatoxina M_1, patulina i les toxines de *Fusarium*, deoxynivalenol, fumonisines, toxines T2 i HT2 i zearalenona. Per assolir aquest objectiu general, es van desenvolupar alguns sub-objectius: 1) Avaluar la ingesta dietètica dels principals aliments relacionats amb la contaminació de les micotoxines, per part de la població catalana, incloent-hi grups més vulnerables com la gent gran, nadons, immigrants o celíacs. 2) Determinar els nivells de

micotoxines en una mostra representativa de les principals categories d'aliments. 3) Combinar acuradament les dades de contaminació amb les d'ingesta per tal d'obtenir una estimació precisa dels nivells d'exposició de cada grup de població, així com del risc que en deriva. 4) Avaluar l'efecte del processat o el cuinat dels aliments sobre els resultats finals de l'exposició. 5) Avaluar l'aplicació del biomarcador esfinganina:esfingosina com a indicador d'exposició a fumonisines.

Per tant, el disseny global consistia, per una banda, d'un estudi nutricional i per una altra, un estudi analític. Les mostres d'aliments es van seleccionar per ser aquelles categories més susceptibles a la contaminació de micotoxines i al temps, consumides a Catalunya. Entre l'any 2008 i 2009 les mostres es van recollir en 6 supermercats o hipermercats de 12 de les principals ciutats de Catalunya (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa i Lleida), representatives del 72 % de la població. Quasi 3000 mostres individuals es van mesclar per tal d'obtenir més de 1300 mostres analítiques seguint el disseny dels estudis de Dieta Total. Les mostres es van analitzar per determinar el nivell de 1 a 6 micotoxines, en funció de la matriu alimentària. En quan a les marques, finalment un gran ventall de marques (n=770) es van considerar en l'estudi, les quals representen la majoria de la quota de mercat de Catalunya, així com de la resta del mercat espanyol. Les anàlisis químiques es van dur a terme amb mètodes degudament validats, que consistien en una extracció i purificació de les micotoxines, seguits per la determinació realitzada bàsicament per HPLC acoblat a un detector de fluorescència, absorbància o masses, depenent del cas.

L'estudi nutricional es va dur a terme mitjançant un qüestionari de freqüència de consum d'aliments (QFCA) desenvolupat per a la població catalana, incloent-hi aquells aliments consumits a la regió i que poden estar contaminats per micotoxines. Es van detallar 38 ítems, i les freqüències de consum anaven detallades de mai a anualment. Les quantitats es van determinar mitjançant racions prèviament pesades i amb l'ajuda de models fotogràfics. Cinc grups de població, d'ambdós sexes es van tenir en compte inicialment: nadons (0-3 anys), nens/es (4-9), adolescents (10-19 anys), adults (20-65 anys) i gent gran (> 75 anys). A més es van incorporar dos grups poblacionals per tenir

patrons alimentaris marcadament diferents a la població general, ja sigui per necessitats fisiològiques o culturals: immigrants i celíacs. Finalment entrevistadors prèviament entrenats van administrar els qüestionaris a 76 ancians, 720 adults, 235 adolescents, 69 pares de nens, 164 pares de nadons, 70 celíacs i 56 immigrants. Els individus entrevistats eren residents de 89 pobles i ciutats de Catalunya. La combinació de les dades de contaminació i de consum es va realitzar mitjançant models deterministes i probabilístics. Cada exposició estimada es va comparar amb el respectiu nivell de seguretat, per tal de caracteritzar el risc per la salut esperat.

Considerant els resultats dels nostres estudis, després de processar més de 4500 determinacions analítiques, es va trobar que el percentatge global de mostres contaminades per micotoxines era de gairebé el 32 % del total de mostres. El nivell més elevat de mostres positives (per sobre del LOQ) correspon a DON i FBs, amb percentatges del 47 i 40 % de mostres contaminades, respectivament. Els nivells més baixos de mostres positives es van trobar per als tricotecens tipus A, les toxines T-2 i HT-2 (1 i 8 %, respectivament), seguit per les aflatoxines (12 %). Els aperitius de panís va ser una de les matrius amb els percentatges més elevats de contaminació de les micotoxines de *Fusarium*. Les aflatoxines van ser trobades distribuïdes més àmpliament en pebre vermell, seguit en menor nombre en fruits secs (cacauets i festucs). Un nombre molt reduït de mostres van excedir els nivells de seguretat de la Comissió Europea.

Els resultats dels càlculs d'exposició, tant dels models deterministes com probabilístics, van mostrar que els principals grups de risc per a la majoria de micotoxines estudiades eren els nadons, els nens i els immigrants. Considerant aquests resultats, el perfil d'exposició de tots els grups de població es trobaven lluny dels nivells de seguretat establerts per la EFSA o la OMS per a les aflatoxines, fumonisines, patulina i zearalenona. Els nivells d'exposició més elevats es van trobar principalment per als tricotecens tipus A i B. Les toxines T-2 i HT-2 van ser determinades en un baix nombre de les mostres analitzades, tanmateix, la seva elevada toxicitat comporta un marge d'exposició molt baix per no excedir els nivells de seguretat. En canvi, el DON va ser determinat en un ampli ventall de mostres d'aliments a base de cereals, incloent categories d'alt consum com el pa, per tant considerant els resultats dels models

d'exposició, els majors consumidors dels grups de risc podrien excedir els nivells de seguretat.

Derivat d'aquests resultats, el sub-objectiu 4 es centra bàsicament en l'efecte del processat i el cuinat sobre el DON i la ZEA. Per tant, els objectius d'aquest treball van ser, per una banda, avaluar l'efecte del processat del pa sobre l'estabilitat de la ZEA i del DON, així com, la transferència del DON de la pasta a l'aigua de cocció; i per una altra banda, avaluar el impacte de la disminució de DON durant la cocció sobre les estimacions globals d'exposició a aquesta toxina. Els resultats dels experiments van mostrar que el DON i la ZEA són estables durant el processat del pa, incloent la fermentació amb *Saccharomyces cerevisiae* i la cocció a 200ºC. Per altra banda, els resultats mostren una elevada transferència de DON de la pasta a l'aigua de cocció, d'entorn al 75 %. En conseqüència, aquesta disminució pronunciada durant la cocció de la pasta es va introduir en el model d'exposició per tal d'avaluar el biaix derivat en les estimacions globals. Finalment, tot i que es van trobar diferències estadístiques significatives al aplicar la reducció derivada de la cocció de la pasta, aquestes variacions no eren suficients per a ser protectores en els grups de població de risc.

Per tal de respondre el sub-objectiu 5, en primer lloc, es van comparar els nivells plasmàtics i urinaris d'esfinganina, esfingosina i del seu ràtio, entre dos grups de població (exposats i no exposats a fumonisines). En segon lloc, els nivells urinaris d'aquests esfingolípids i el ràtio es va monitoritzar durant dues setmanes en dos grups de voluntaris (l'un amb consum de panís restringit i l'altre amb consum lliure). La determinació analítica es va realitzar mitjançant mètodes validats per HPLC amb detecció de fluorescència. No es van trobar diferències significatives entre les ràtios de les mostres urinàries de consumidors i no consumidors, mentre que es van trobar diferències en les mostres plasmàtiques, tot i que el mecanisme d'acció no era evident. En el darrer estudi, en el qual es va avaluar l'evolució al llarg del temps dels nivells i les ràtios d'esfinganina i esfingosina, es va observar el dia en el qual s'espera la màxima alteració.

1.3 Resumen (Spanish)

Las micotoxinas son metabolitos secundarios producidos por hongos y contaminan diferentes productos agrícolas, tanto antes de la cosecha como en condiciones de post-cosecha. Su funcionalidad es básicamente para la defensa del propio hongo o la mejora de las condiciones de competencia. A pesar de que hay muchas especies de hongos toxigénicos, sólo unas pocas micotoxinas, particularmente aquellas que afectan los cereales (maíz, trigo, centeno, avena o arroz) y frutos secos, son las más importantes para los humanos. Bastantes micotoxinas han sido identificadas hasta ahora, pero las más especiales a nivel alimentario, tanto a nivel humano como animal, son las siguientes: las aflatoxinas (B_1, B_2, G_1, G_2, M_1), las fumonisinas (B_1, B_2), la ocratoxina A (OTA), la patulina, los tricotecenos (deoxinivalenol, toxina T-2 y toxina HT-2) y la zearalenona (ZEA).

El objetivo general de esta tesis doctoral era evaluar la exposición de la población catalana a las micotoxinas más importantes: aflatoxinas del grupo B y G, aflatoxina M_1, patulina y las toxinas de *Fusarium*, deoxynivalenol, fumonisinas B_1 y B_2, toxina T-2, toxina HT-2 y zearalenona. Para lograr este objetivo general, se desarrollaron algunos sub-objetivos: 1) Evaluar la ingesta dietética de los principales alimentos relacionados con la contaminación de las micotoxinas por parte de la población catalana, incluyendo grupos más vulnerables como la gente mayor, bebés, inmigrantes o celíacos. 2) Determinar los niveles de micotoxinas en una muestra representativa de las principales categorías de alimentos. 3) Combinar cuidadosamente los datos de contaminación con las de ingesta para obtener una estimación precisa de los niveles de exposición de cada grupo de población, así como del riesgo que deriva. 4) Evaluar el efecto del procesado o el cocinado de los alimentos sobre los resultados finales de la exposición. 5) Evaluar la aplicación del biomarcador esfinganina (Sa) : esfingosina (So) como indicador de exposición a fumonisinas.

Por lo tanto, el diseño global consistió, por un lado, de un estudio nutricional y por otro, en un estudio analítico. Las muestras de alimentos se seleccionaron por ser aquellas categorías más susceptibles a la contaminación por micotoxinas y al tiempo,

consumidas en Cataluña. Entre el año 2008 y 2009 las muestras se recogieron en 6 supermercados o hipermercados de 12 de las principales ciudades de Cataluña (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa y Lleida), representativas del 72 % de la población. Se mezclaron casi 3000 muestras individuales para obtener más de 1300 muestras analíticas o composites, siguiendo el diseño de los estudios de Dieta Total. Las muestras se analizaron para determinar el nivel de 1 a 6 micotoxinas, en función de la matriz alimentaria. En cuanto a las marcas, finalmente un gran abanico de marcas (n=770) se consideraron en el estudio, las cuales representan la mayoría de la cuota de mercado de Cataluña, así como del resto del mercado español. Los análisis químicos se llevaron a cabo con métodos debidamente validados, que consistían en una extracción y purificación de las micotoxinas, seguidos por la determinación realizada básicamente por HPLC acoplado a un detector de fluorescencia, absorbancia o masas, dependiendo del caso.

El estudio nutricional se llevó a cabo mediante un cuestionario de frecuencia de consumo de alimentos (CFCA) desarrollado para la población catalana, incluyendo aquellos alimentos consumidos en la región y que pueden estar contaminados por micotoxinas. Se detallaron 38 ítems, y las frecuencias de consumo iban de nunca a anualmente, pasando por diario, semanal o mensual. Las cantidades se determinaron mediante raciones previamente pesadas y con la ayuda de modelos fotográficos. Cinco grupos de población de ambos sexos se tuvieron en cuenta inicialmente: bebés (0-3 años), niños (4-9 años), adolescentes (10-19 años), adultos (20-65 años) y gente mayor (> 75 años). Además se incorporaron dos grupos poblacionales por tener patrones alimentarios marcadamente diferentes a la población general, ya sea por necesidades fisiológicas o culturales: inmigrantes y celiacos. Finalmente entrevistadores previamente entrenados administraron los cuestionarios a 76 ancianos, 720 adultos, 235 adolescentes, 69 padres de niños, 164 padres de bebés, 70 celíacos y 56 inmigrantes. Los individuos entrevistados eran residentes de 89 pueblos y/o ciudades de Cataluña. La combinación de los datos de contaminación y de consumo se realizó mediante modelos determinísticos y probabilísticos. Cada exposición estimada se comparó con el respectivo

nivel de seguridad, con el fin de caracterizar el riesgo esperado para la salud.

Considerando los resultados de nuestros estudios, después de procesar más de 4500 determinaciones analíticas, se encontró que el porcentaje global de muestras contaminadas por micotoxinas era casi del 32 % del total de muestras. El nivel más elevado de muestras positivas (por encima del LOQ) corresponde a DON y FBs, con porcentajes del 47 y 40 % de muestras contaminadas, respectivamente. Los niveles más bajos de muestras positivas se encontraron en los tricotecenos tipo A, las toxinas T-2 y*HT-2 (1 y 8 %, respectivamente), seguido por los aflatoxinas (12 %). Los aperitivos de maíz fueron una de las matrices con los porcentajes de contaminación más elevados de las micotoxinas de *Fusarium*. Las aflatoxinas se detectaron principalmente en pimentón rojo, seguido en menor proporción en frutos secos (cacahuetes y pistachos). Un número muy reducido de muestras excedieron los niveles de seguridad legislados por la Comisión Europea.

Los resultados de los cálculos de exposición, tanto de los modelos deterministas como probabilísticos, mostraron que los principales grupos de riesgo para la mayoría de micotoxinas estudiadas eran los bebés, los niños y los inmigrantes. Considerando estos resultados, el perfil de exposición de todos los grupos de población se encontraban lejos de los niveles de seguridad establecidos por la EFSA o la OMS para las aflatoxinas, fumonisinas, patulina y zearalenona. Los niveles de exposición más elevados se encontraron principalmente para los tricotecenos tipos A y B. Las toxinas T-2 y HT-2 fueron encontradas en un bajo número de las muestras analizadas, aun así, su elevada toxicidad comporta un margen de exposición muy bajo para no exceder los niveles de seguridad. En cambio, el DON sí fue determinado en un amplio abanico de muestras de alimentos a base de cereales, incluyendo categorías de alto consumo como el pan. A la vista de estos resultados y considerando los resultados de los modelos de exposición, los mayores consumidores de los grupos de riesgo podrían exceder los niveles de seguridad.

El sub-objetivo 4 se centró básicamente en el efecto del procesado y el cocinado sobre el DON y la ZEA. Se pretendió, por un lado, evaluar el efecto del procesado del pan sobre la estabilidad de la ZEA y del DON, así como, la transferencia del DON de la

pasta al agua de cocción; y por otro lado, evaluar el impacto de la disminución de DON durante la cocción sobre las estimaciones globales de exposición a esta toxina. Los resultados de los experimentos mostraron que el DON y la ZEA son estables durante el procesado del pan, incluyendo la fermentación con *Saccharomyces cerevisiae* y la cocción a 200ºC. Por otro lado, los resultados mostraron una elevada transferencia de DON de la pasta al agua de cocción, de entorno al 75 %. En consecuencia, esta disminución pronunciada durante la cocción de la pasta se incorporó en el modelo de exposición para evaluar el sesgo derivado en las estimaciones globales. Finalmente, a pesar de que se encontraron diferencias estadísticas significativas al aplicar la reducción derivada de la cocción de la pasta, estas variaciones no fueron suficientes para reducir el riesgo de forma considerable en los grupos de población de riesgo.

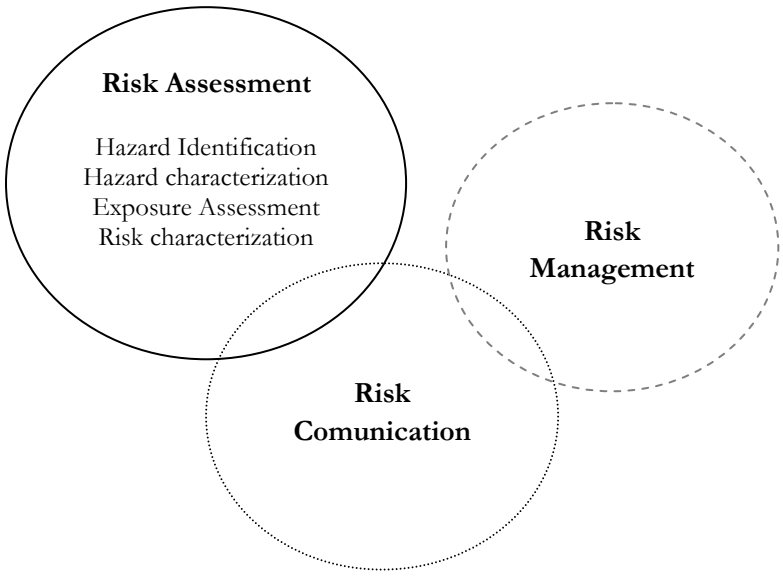
Para responder el sub-objetivo 5, en primer lugar, se compararon los niveles plasmáticos y urinarios de esfinganina, esfingosina y de su ratio, entre dos grupos de población (expuestos y no expuestos a fumonisinas). En segundo lugar, los niveles urinarios de estos esfingolípidos y el ratio se monitorizó durante dos semanas en dos grupos de voluntarios (el uno con consumo de maíz restringido y el otro con consumo libre). La determinación analítica se realizó mediante métodos validados por HPLC y detección de fluorescencia. No se encontraron diferencias significativas entre los ratios de las muestras urinarias de consumidores y no consumidores, mientras que se encontraron diferencias en las muestras plasmáticas, a pesar de que el mecanismo de acción no era evidente. En el último estudio, en el cual se evaluó la evolución a lo largo del tiempo de los niveles y los ratios de esfinganina y esfingosina, se halló el día en el cual se espera la máxima alteración.

Chapter 2. Introduction

2.1. Exposure assessment in the framework of risk analysis of chemicals in foods

The agro-food sector is of major importance for the European economy as a whole. A priority stated in the "White Paper of Food Safety" by EU is to offer a wide range of safe and high quality products coming from all Member States to the consumers. Food safety policy in the European Union (EU) is based on a comprehensive, integrated approach of risk analysis throughout the food chain, "*from farm to table*". Risk analysis has three main components: risk assessment (scientific advice and information analysis), risk management (regulation and control) and risk communication (see Figure 2.1.). Risk assessment at the international level provides the scientific basis for the establishment of Codex standards, guidelines, and other recommendations and includes dietary exposure assessments as an essential component. This ensures that safety requirements for food are protective of public health, consistent among countries, and appropriate for use in international trade. Risk assessments are performed in a four-step process: hazard identification, hazard characterisation, exposure assessment, and risk characterisation (European Commission, 2000; WHO, 2001).

Figure 2.1. Graphical scheme of risk analysis components



In the first stage, **hazard identification**, the effects that are considered as adverse are identified, irrespective of the dose needed or the specific mechanism involved to elicit this effect. The scientific tools available for hazard identification of chemicals in food range from well-tried and tested animal-based toxicity studies to the newest of molecular biology techniques. The majority of hazard identification studies are designed to demonstrate the nature of any adverse effect and both effect- and no-effect dose levels, the overall no observed-adverse-effect level (NOAEL) being central for derivation of acceptable or tolerable daily intakes. As an alternative, the calculation of benchmark doses (BMD) rather than NOAELs has been advocated.

Next step, **hazard characterisation**, is centred on the quantification of these effects, so that the dose–response relationships identified at this stage of the risk assessment can be compared with the potential for exposure: establishment of the dose–response relationship for critical effects, assessment of external vs internal dose, identification of the most sensitive species and strain, identification of potential species differences (qualitatively and quantitatively), characterisation of the mode of action/the mechanism for the critical effects and/or extrapolation from high to low dose and from experimental animals to humans. This process generally involves the identification of the NOAEL as a starting point; that is the exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects. Alternatively, a regression function fitted on the response data may be used to estimate the dose (the benchmark dose) at which adverse effects start to arise. For hazards that are considered not to have thresholds for their mode of action, low-dose extrapolation and other modelling approaches may be applied. A central theme to **hazard characterisation** is unravelling of the modes of action/the mechanism for the critical effects observed so that their relevance for the human situation can be addressed. As animal experiments are used as models for potential toxic responses in humans, and since the dosages used in the animal experiments are usually much higher than those experienced in human exposures to low molecular weight chemicals in food and diet, hazard characterisation involves both extrapolation from high to low dose and from experimental animals to humans.

Exposure assessment, as part of the risk assessment process, is defined as the qualitative and/or quantitative evaluation of the likely intake of biological, chemical or physical agents via food as well as exposure from other sources if relevant. Dietary exposure assessments combine food consumption data with data on the concentration of chemicals in food. Several methods can be used to estimate the intake of a food chemical, and the choice will depend on what information is available and how accurate and detailed the estimate needs to be. Because this step is the central subject of the present work, the highlights of this procedure will be most deeply developed in the next section.

The last step, **risk characterisation**, integrates the information collected in the preceding three steps; that is, integration of evidence, reasoning and conclusions collected in hazard identification, dose-response assessment and exposure assessment and the estimation of the probability, including attendant uncertainties, of occurrence of an adverse effect if an agent is administered, taken or absorbed by a particular organism or population. For genotoxic substances which interact with DNA, directly or after metabolic transformation (direct-acting genotoxic chemicals), the absence of a threshold in their mechanism of action is generally assumed, i.e. there is no dose without a potential effect. For non genotoxic substances a 100-fold uncertainty factor is routinely applied to the No-Observed-Adverse-Effect-Level (NOAEL) from an animal study to derive a health based limit value, e.g. Tolerable Daily intake (TDI). The 100-fold uncertainty factor is based on scientific judgement and allows for species differences and human variability (Renwick et al., 2003). For substances which are both genotoxic and carcinogenic, a NOAEL for tumour formation should not be regarded as a surrogate for a threshold; the NOAEL only defines the reference point on the dose-response curve where the study is unable to detect a significant increase in incidence. Consequently, the NOAEL approach is not appropriate for substances that are genotoxic and carcinogenic. Alternatively, the margin of exposure (MoE), defined as the ratio between a point on the dose-response curve for the adverse effect and human intake, has been proposed to characterise the risk of these contaminants. Mathematical analysis of the dose-response data from animal bioassays can be used to define the intakes necessary to produce a

given level of response, such as 10% or 25% cancer incidence. The most commonly used methods are the T25 approach (chronic daily dose which give tumours in 25 % of the animals above background at a specific tissue site) and BMD_{10} approach (the 95 % lower confidence interval on a BMD for a 10 % increase in tumour incidence determined by fitting dose-response data to various mathematical models) (EFSA, 2005, 2007, 2010).

2.2. Exposure assessment highlights

The current exposure assessment frameworks combine contamination and consumption datasets by means of different mathematical methods. The general equation to combine consumption with contamination data, is apparently simple (1), however, it is widely recognised the large drawbacks to obtain accurate estimations.

(1)

$$Dietary\ exposure = \frac{\Sigma(\text{Food chemical concentration x Food consumption})}{Body\ weight}$$

Inaccuracies are derived from each component of the equation: a) from contamination raw datasets (involving the food sampling design, chemical analysis methods or the left-censored management), b) from food dietary intake assessment methods (food consumption data sources, population groups…) and finally, 3) from the methods applied to combine and integrate the raw data. Each step in dietary exposure assessment is affected by scientific uncertainties, which contribute to the overall uncertainty of risk estimates. Although there are not definitive guidelines to identify and analyse the uncertainties, Scientific Committee asked by EFSA published a guidance listing the major sources of uncertainties and the most important method to analyse them (EFSA, 2006). The study design should decrease these uncertainties in order to provide the most realistic estimations as possible. Uncertainties from chemical analysis

will be derived from sampling, left-censored data, analytical bias and variation. Concerning food consumption estimation, will depend on the data available, the method administered, representativeness of the population sample, other sources of exposure or missing data.

Novel methods like biomarkers has been proposed as successful methods to assess the exposure of individuals to chemicals through estimation of the food contaminant or their metabolites in biological fluids or molecules induced specifically in response to the toxic effect.

2.2.1. Contamination data

2.2.1.1. Sampling

There exist at least two different sampling strategies in exposure assessment studies. The first strategy aims to obtain a representative picture of chemical levels present in food. This type of sampling is without a priori knowledge on what levels can be found. In the representative sampling strategy, the sample numbers of different varieties or brands can be stratified according to production or consumption figures (varieties) or market share (brands). The second sampling strategy, directed or targeted sampling, is aimed at sampling those products expected to contain higher levels in a cost effective way (Codex Alimentarius, 1993, 1999). Mycotoxins are examples of substances with an extremely high heterogeneity of the distribution even within a lot; therefore, sampling methods should be designed in order to reduce this source of variability. Total diet study designs are characterized to pool a variable number of samples in composites to be analysed. This strategy permits to increase the representativeness in a cost-effective way, and in turn reducing the variability. The main problem of this approach is derived from dilution effect when the number of pooled samples is too high, thus acute exposure assessment can be significantly biased.

2.2.1.2. Chemical analysis methods

Foods are complex mixtures of lipids, carbohydrates, proteins, vitamins, organic compounds, and other naturally occurring substances. Developments in analytical chemistry have improved both specificity and sensitivity of methods for determining chemicals in foods.

Advances in chemical analysis have come in the areas of sample purification techniques and in separation science with the development of high-performance liquid chromatography (HPLC) and associated detectors. For mycotoxins with suitable chromophores (patulin) or fluorophores (aflatoxins), the increased sensitivity of modern detectors has allowed analysts to achieve lower limits of detection. Other mycotoxins, which lack suitable chemical groups (trichothecenes and fumonisins), can be initially derivatised and then separated either by gas chromatography (GC) or by HPLC.

Parameters of importance in mycotoxin extraction are solvent type and composition of a mixture, solvent to sample ratio, type of matrix (processed or unprocessed), extraction method and physical aggregation of the sample. The type of matrix that is analysed can strongly influence the analytical recoveries from the extraction process. Original methods for mycotoxin analyses frequently relied on extract clean-up on open columns packed with materials such as silica or diatomaceous earth, which were eluted with various organic solvent mixtures. The development of antibodies raised against individual mycotoxins led to the introduction of immunoaffinity columns (IACs) in which a specific antibody is immobilized on a gel contained in a small column. The antibodies on the column will recognise and bind the specific mycotoxin and allow impurities to pass through the column, which is subsequently washed with phosphate-buffered saline (PBS). GC has been applied to the analysis of a range of mycotoxins, although for many of these compounds, which possess strong fluorescence or UV properties, HPLC methods have been more successful. Nevertheless, for the trichothecenes, of which the B group possesses weak UV absorption and the A group does not have a suitable absorption band, capillary GC has been extensively used. The polar nature of mycotoxins and their solubility in water and organic solvents such as

methanol and acetonitrile implies that they are readily amenable to separation on reversed-phase HPLC columns and this has resulted in a diverse array of methods. Chromatographic detection has mostly been achieved with UV and fluorescence detectors, although the relatively recent successful application of atmospheric pressure ionization techniques has resulted in the development of a range of LC-MS or LC-MS/MS methods capable of very low detection limits. For mycotoxins with useful UV absorption bands, UV detection has been universally applied. Thus mycotoxins such as patulin (wavelength maximum 276 nm), deoxynivalenol (wavelength maximum 219 nm) and moniliformin (wavelength maximum 229 nm) are routinely quantified by UV detection. However, a number of other mycotoxins, such as the aflatoxins, ochratoxin A and zearalenone, possess fluorescence bands. Fluorescence detection has a number of advantages. In measuring light emitted rather than absorbed, it can frequently achieve lower detection limits than UV detection and as analytical interferences may not absorb and fluoresce at the same wavelengths as the analyte of interest, the fluorescence chromatograms are frequently less prone to interference from co-eluting compounds.

A number of mycotoxins do not absorb in the UV range and for these, suitable derivatization methods have been developed to allow UV or fluorescence detection. Examples of these are T-2 toxin and the fumonisins. In the case of fumonisins, a number of fluorescent derivatives such as fluorescamine, *o*-phthaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde have been used. Although the aflatoxins are inherently fluorescent, quenching can occur in certain eluents. For optimum detection limits of the aflatoxin B_1 and G_1 analogues in reversed-phase chromatography, they are frequently derivatised either in a pre-column method with trifluoroacetic acid or post-column by a number of techniques. These include reaction with an elemental solution of iodine in a post-column reaction coil at 60 ºC, reaction with bromine generated from potassium bromide in a post-column electrochemical cell (Kobra cell), reaction with pyridinium hydrobromide perbromide added post-column or hydrolysis using a post-column UV lamp and reactor coil (Shephard, 2008).

2.2.1.3. Treatment of left-censored data

Analytical methods are defined by a limit of detection (LOD) and a limit of quantification (LOQ). Results below these limits cannot be expressed quantitatively with a sufficient level of credibility. Several techniques can be used to handle the so-called left-censored values. In statistical terminology, left-censoring occurs when a data point is below a certain value, but it is not known by how much. In risk assessment the purpose of measuring the presence of contaminants is to characterise the distribution of one or more substances in the food with the aim of estimating exposure to the substances in the population of a country or in the entire European Union (EU) when consuming average or extremely high amounts of food. This kind of estimation requires knowledge of the entire range of contaminant concentrations, including low levels. Statistical methods to handle left-censored observations of chemical contaminants fall into four general categories: substitution methods (Hornung and Reed, 1990; Glass and Gray, 2001), log-probit regression methods (Hawkins et al., 1991; Mulhausen and Damiano, 1998), maximum likelihood estimation methods (Finkelstein and Verma, 2001) and non-parametric methods (Schmoyer et al., 1996; She, 1997).

In the field of food safety, the most commonly used recommendations to handle left-censored data are the ones made by WHO under the activities of the Global Environment Monitoring System (GEMS/Food-EURO, 1995). Details of these recommendations may be found in the report of the workshop, "Reliable Evaluation of Low-level Contamination of Food" (EUR/ICP/EHAZ.94.12/WS04). More recently EFSA has produced a Scientific Report resulted from estimation of the accuracy of methods currently used and recommendations for more advanced alternative statistical approaches (EFSA, 2010).

2.2.1.3.1. Substitution

The substitution of non-detects with other values is widely used in food risk assessment. As mentioned in the previous section, WHO produced recommendations for replacing the non-detect samples by LOD/2, or 0 and LOD according to the

percentage of non-detects in the sample. Similar guidelines were provided in the case of non-quantified values. It has been widely recognised that the substitution method is biased, the bias being a function of true variability in the data, the percentage of censored observations and the sample size (El-Sharaawi and Esterby, 1992). Another disadvantage of substitution is that it does not consider the overall distribution of detect samples. Despite its drawbacks, the substitution method is still widely used, mainly with the justification that it is easy to implement, it is widely understood and that the upper bound practice leads to conservative estimates for exposure assessment calculations, i.e. over-estimation of the mean and under-estimation of the variability.

2.2.1.3.2. Parametric methods

Maximum likelihood estimation

The consensus view is that the maximum likelihood estimation (MLE) method is the best approach from a methodological perspective. The sample parameters are those estimates that maximise the likelihood function after the definition of a parametric distribution to best fit the data. The method may be traced back to Fisher (1922) and has underpinned the modern science of applied statistics throughout most of the 20th century. This method is very versatile as it can be applied to data that do not necessarily follow the well-known (log-) normal distribution.

MLE uses three pieces of information (Helsel, 2005):

1. Values above detection limits.

2. Proportion of data below the detection limit.

3. Assumption on the shape of the distribution of positive values.

Data below and above the detection limit are assumed to follow a given statistical distribution. The parameters of the chosen distribution are estimated to best fit the distribution of the observed values above the detection limit, compatibly with the percentage of data below the limit. The estimated parameters are the ones that maximise

the likelihood function.

The log-probit regression method

In the log-probit regression (LPR) method (also called regression on order statistics) the data are sorted and a linear relationship is assumed between the logarithm of occurrence values and the inverse cumulative normal distribution of the observations plotted position, often determined by the Blom's formula (Helsel, 2005). This is a linear equation which is solved for each non-detect observation. LPR was recommended for analysing left-censored data (Hawkins et al., 1991). A number of variations were suggested for the LPR approach, mainly to address departures from the log-normal distribution (Helsel, 2005; Hewett and Ganser, 2007), although it was suggested that the LPR or its substitutes should not be applied to complex datasets (Hewett and Ganser, 2007), i.e. datasets with multiple LOD values.

2.2.1.3.3. Non-parametric methods

Non-parametric methods are so named because they do not involve computing "parameters" such as the mean or standard deviation, of a given distribution. Instead they use the relative magnitude (ranks) of data. The standard non-parametric technique for censored data is the Kaplan-Meier (KM) method (Kaplan and Meier, 1958). This method is based on the empirical cumulative distribution function. The advantage of such approach is the possibility of estimating the mean, together with the median and other quantiles, in the presence of non-detect values, without relying upon distributional assumptions (Tressou et al., 2004). With the KM method, the weight of the censored data is distributed over the different observed values below the censoring values, i.e. LODs and LOQs, and zero. It is therefore redundant to apply KM when there is only one LOD value, as it would be equivalent to substituting the censored values with zero or the largest observed value below the LOD. Because it is non-parametric, the KM method tends to be insensitive to outliers, which occur frequently in environmental data (Antweiler and Taylor, 2008). This method is available in many statistical packages, but because it was originally intended for right-censored datasets, the concentration data

must be "flipped" before analysis (i.e. the left-censored dataset must be converted to a right-censored dataset) (EFSA, 2010).

2.2.2. Consumption data

Food consumption data reflect what either individuals or groups consume in terms of solid foods, beverages, including drinking-water, and supplements. In principle, to assess food consumption, four different types of data can be used: food supply data, data from household consumption surveys and data from dietary surveys among individuals; moreover total diet studies are becoming the standard gold method in high scale exposure assessment studies (Kroes et al., 2002).

- *Food supply data*. Food supply data are calculated in food balance sheets (FBSs), which are accounts, on a national level, of annual production of food, changes in stocks, imports and exports, and agricultural use and industrial use. The result is an estimate of the average value per head of the population, irrespective of, for instance, age or gender. Food supply data refer to food availability, which gives only a crude (over-estimated) impression of potential average consumption. Food and nutrient losses prior to consumption, due to processing, spoilage, trimming and waste may not be adequately accounted for. EUROSTAT publishes FBSs for the member countries of the European Union.

- *Household consumption surveys*. Food available at the household level may be estimated by budget surveys and by consumption surveys. In household consumption surveys, the amounts of foods and drinks brought into the household are also recorded. For the most part, only the expenditures of meals taken at home are noted. Some household surveys may even measure changes in food stocks, in addition to acquisition. In general, household surveys do not provide information on how food is handled within the household, or on actual consumption by its members.

- *Dietary surveys*. In contrast to FBSs and household surveys, data from individual

surveys provide information on average food and nutrient intake and their distribution over various well-defined groups of individuals. These data more closely reflect actual consumption.

- *Food records*. Food records, dietary records or food diaries are kept for a specified time period, usually 1–7 days. If total daily intake of energy and/or nutrients is required, the food records should include all foods and beverages consumed at meals and in between, in quantified amounts. In a precise weighed record the respondent notes the weights of all ingredients used in the preparation of the meals, as well as the inedible waste, the total cooked weight of meal items, the cooked weight of the individual portion and plate waste.

- *24-hour recall method*. In the 24-hour recall the subject is asked by a trained interviewer to recall and describe the kinds and amounts of all foods and beverages ingested during the immediate past, mostly a 24- or 48-hour period. Dietary recalls maybe administered in person or by telephone interview. Food quantities are usually assessed by using household measures, food models, or photographs.

- *Food frequency method*. A food frequency questionnaire (FFQ) consists of a structured list of individual foods or food groups. The aim of the FFQ is to assess the frequency with which these items are consumed during a specified time period (e.g. daily, weekly, monthly, yearly). Brief FFQs may focus on one or several specific chemicals. FFQs maybe qualitative, semi-quantitative or completely quantitative. The FFQ is often used to rank individuals by food or nutrient intakes and also by food group intakes into categories so that high and low intakes maybe studied.

- *Dietary history method*. With the aid of the dietary history method, a trained interviewer assesses an individual's total usual food intake and meal pattern. 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 actual foods eaten during the preceding 24 hours. In addition, the

interviewer completes a check list of foods usually consumed. Finally as a cross-check, the respondent is often asked to complete a 3-day estimated record. The reference time frame is often the past month or several months, or may reflect seasonal differences if the time frame is the past year.

- *Total diet studies (TDS).* For estimating dietary exposure of the population, FAO/WHO recommends the use of TDS. TDS have become a reference approach in exposure assessment studies. In TDS, representative samples of widely consumed foods are collected and analysed for the constituents of interest. The accuracy of population intakes estimated using total diet study results depends on the extent to which the foods analysed represent important dietary sources of the chemical. We can distinguish three main approaches irrespectively of the combination:
    - *Market Basket.* It 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. Each food group is analysed for a number of additives, contaminants and nutrients.
    - *Individual food items.* A list of foods representing the products most commonly consumed is composed based on national food consumption surveys for several age–sex groups. All selected food items are prepared according to methods most commonly used and analysed.
    - *Duplicate portion.* A replicate of the portion consumed by each individual is collected and analysed as eaten.

Dietary patterns in Catalonia have been assessed subsequently from 1992 to 2003 in two individual studies, ENCAT-92 (n=2641) and ENCAT-02 (n=2060). These studies were carried out using two 24-h recall and a food frequency questionnaire administered by trained interviewers. Previously, in 1986 it was carried out the first nutritional questionnaire in Catalonia and no more research has been done to date (Jiménez et al., 1988). In rest of Spain at national level, there are few studies devoted to assess the nutritional status. EnKid Study 1998-2000 was carried out to young population and

children between 1 and 14 years old by means of 24-hours recall (Serra-Majem et al., 2001). AESAN (Agencia Española de Seguridad Alimentaria y Nutrición) also conducted a couple of studies, one of them using a 24-hours recall and the other a food record with similar purposes (Requejo et al., 2002; Ortega et al., 2010). In 2005, EFSA's Scientific Committee published an opinion on exposure assessment recommending the urgent collection of available food consumption data at an aggregated level followed by an expanded collection of data at a detailed level. As a first response, EU Member States collaborated on the establishment of the EFSA Concise European Food Consumption Database, which is operational since the end of February 2008. At the end of 2008, EFSA started projects to establish the EFSA Comprehensive European Food Consumption Database built on existing information for adults at a detailed level. It is anticipated that when the Comprehensive Database is operational it will greatly improve the accuracy of EFSA exposure assessment calculations (EFSA, 2011b).

2.2.3. Methods to combine consumption and contamination raw datasets

A number of different methods exist to combine and integrate consumption estimates with chemical concentration data, ranging from quick worst-case estimations to refined methods aimed at assessing actual exposure. The selection of the method usually depends on a number of factors, including the purpose of the assessment (target chemical substance, population group, degree of required accuracy, etc.) or availability of information. When separate datasets are available for food consumption, as measured in food consumption surveys, and chemical concentration, one of both approaches is usually applied to combine or integrate the data to provide an estimate of exposure: (i) point estimate, direct method or deterministic approach and (ii) probabilistic or stochastic analyses. The usefulness of both probabilistic models and deterministic models is dependent on the availability and quality of the input data.

2.2.3.1. Direct method, point estimate or deterministic approach

A "point estimate" of dietary exposure is simply a single value that describes some parameters of consumer's exposure. For example, an average consumer's exposure is calculated as the product of the average consumption of the foods of interest with the average residues of the substance of interest in those foods. A point estimate is not inherently "conservative" or "realistic". The conservatism incorporated into the analysis is determined by the data and assumptions that are used in calculating the estimate. Point estimates can range from initial screening methods that use very few data and generally include very conservative assumptions to refined exposure assessment that includes extensive underlying data in order to realistically calculate the actual exposure estimates

2.2.3.2. Probabilistic methods

For substances requiring further refinement beyond screening methods or point estimates of exposure, as described above, a probabilistic analysis of exposure variability can be conducted. Conceptually, population exposure must be thought of as a range of values, rather than a single value, because individual members of the population experience different levels of exposure. Factors that contribute to this "variability" include age (owing to differences in body weight and the type and amount of food consumed), sex, ethnicity, nationality, region, and personal preferences, among others. Variability in dietary exposure is often described using a "frequency distribution". Sometimes, the frequency distribution is approximated as a continuous probability distribution. In both cases, the horizontal axis corresponds to the level of exposure, and the vertical axis corresponds to the relative proportion of the population.

The structure of a probabilistic model is similar to that of the deterministic models described in section above, in that it is based on the same basic equations. The fundamental difference is that at least one variable is represented by a distribution function instead of a single value. A single value is selected from the probability distribution of each variable. These values are then used in the model algorithms to produce a single estimate of exposure. This process is then repeated many times, with

new values for each stochastic input parameter selected each time, to produce a probability distribution of possible exposures. Monte Carlo simulation methods and Latin Hypercube sampling are commonly employed in probabilistic exposure modelling. Representing input parameters as distributions of possible values rather than single values means that the inherent variability in model parameters can be incorporated into the exposure assessment along with any uncertainty associated with parameter estimates (Fryer et al., 2006).

2.2.4. Biomarkers

Biological markers or biomarkers are indicators of changes or events in biological systems. Biomarker-based methodologies may be employed to determine human exposure to food chemicals. The use of biochemical indicators has been proposed as a suitable method to assess individual exposure to contaminants. The WHO defined in 1993, a biomarker as "any parameter that can be used to measure an interaction between a biological system and an environmental agent, which may be chemical, physical or biological". This method allows effective exposure assessment considering variability among food contamination levels, cooking effect, individual consumption, variations in toxicokinetics or toxicodynamics. Among the potential valuable application of biomarkers in epidemiologic studies and in clinical trials, there is the possibility of measuring them earlier than the observed true endpoint of interest, given their property of relating the effect of exposures or treatments on cellular and molecular changes to the true endpoint/outcome. They involve usually two main stages. In the first of these human volunteer studies (or - for contaminants - total diet studies) are undertaken to establish whether a quantitative relationship can be established between the dietary intake of the chemical in question and the amount of the corresponding biomarker detected in an appropriate body fluid or tissue. In most cases the biomarker is either the food chemical itself or a metabolite. The chosen body fluid is frequently urine or blood, although other options exist including especially breast milk, but also hair, adipose tissue, buccal swaps, exhaled air and faeces. Interpretation of biomarkers of effect is hampered

by lack of knowledge on the metabolism of most non-nutrients and their mechanisms of action in humans *in vivo*. Before a biochemical indicator can be used as a measure of dietary intake, it must be evaluated with respect to its sensitivity to the intake of those contaminants. If these indicators are to be used as measures of dietary exposure, however, the epidemiologist is obviously responsible for ensuring that the exposure measure is a valid representation of long-term intake. Several strategies are available to define the relationships between long-term dietary intake and biological levels: (i) animal studies; (ii) geographic correlation of intake and biological marker; (iii) correlation with individual intake; (iv) dietary manipulation in humans and (v) repeated measures (Willet, 1998).

One of the first applications of biomarkers to human exposure to food chemicals is illustrated in the studies on the mycotoxin aflatoxin B_1 in the late 1980s. The use of serum aflatoxin B_1-albumin adducts as biomarkers of aflatoxin exposure has been validated in experimental and human sample analyses (Wild et al., 1990a, b, 1992). The use of urinary aflatoxin B1-N7-guanine adduct validated in the laboratory with human samples, provides a measure of acute exposure to aflatoxin B1 (AFB1) and reflects a relatively short-term (24-48 h) exposure (Groopman et al., 1992a, b, 1993). Biomarkers have also been investigated as a means of assessing exposure to the fumonisin class of mycotoxins. Based on the biological perturbation induced by fumonisins, the elevations of sphinganine (Sa) to sphingosine (So) in tissues, urine and blood have been proposed as potential biomarkers of fumonisin exposure in various animal species (Wang et al., 1992, 1999; Riley et al., 1993; Morgan et al., 1997; van der Westhuizen et al., 2001; Kim et al., 2006; Tran et al., 2006; Cai et al., 2007). Several studies have been conducted to assess the effectiveness of this biomarker in humans, but the results did not allow an accurate validation (van der Westhuizen et al., 1999, 2008, 2010; Abnet et al., 2001; Qiu and Liu, 2001; Solfrizzo et al., 2004; Nikiema et al., 2008; Silva et al., 2009). The individual Sa and So basal levels, as well as the basal Sa/So ratio, vary depending on unknown parameters, being related to nutrition factors (Abnet et al., 2001; Shephard et al., 2007). The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (51 $\mu g\ kg^{-1}$ bw day-

[1]). Considering that the PMTDI is 2 μg kg^{-1} bw day^{-1}, low sensitivity should be expected when we apply this biomarker in a human population (Kim et al., 2006; Cai et al., 2007; Voss et al. 2007). Turner et al. (2008) emphasized urinary DON as a good tool to assess exposure to this contaminant at the individual level. In contrast, they reported several uncertainties to resolve the full validating of this biomarker to apply in epidemiological studies.

2.3. Mycotoxins in food

Mycotoxins are toxic secondary metabolites produced by fungi and contaminate various agricultural commodities either before harvest or under post-harvest conditions. Their purpose is thought to be for fungal defence or competition. Although there are many species of toxigenic moulds, only a few mycotoxins, particularly those affecting cereals (maize, wheat, barley, oats and rice) and groundnuts are considered to be significant for humans. Several mycotoxins have been identified up to the present, but those of special interest in food and feed safety are: aflatoxins (B_1, B_2, G_1, G_2, M_1), fumonisins (B_1, B_2), ochratoxin A, patulin, trichothecenes (deoxynivalenol, T-2 toxin, HT-2 toxin), and zearalenone.

2.3.1. Aflatoxins B and G group

Aflatoxins B_1, B_2, G_1 and G_2 (Fig. 2.2) are mycotoxins that can be mainly produced by moulds of *Aspergillus* species, like *A. flavus*, *A. parasiticus* and *A. nomius*, and can occur in a wide range of important raw food commodities including cereals, nuts, spices, figs and dried fruits (Malone et al., 2000; Otta et al., 2000).

Among the naturally occurring aflatoxins, aflatoxin B_1 is the most important compound with respect to both, prevalence and toxicity for man and animals.Aflatoxin B_1 (AFB_1) is the most carcinogenic mycotoxin known and there is evidence from human studies that aflatoxins are major risk factors for hepatocellular carcinoma, therefore classified in group 1 by International Agency for Research on Cancer (JECFA, 1998;

IARC, 2002; Wang and Tang, 2004).

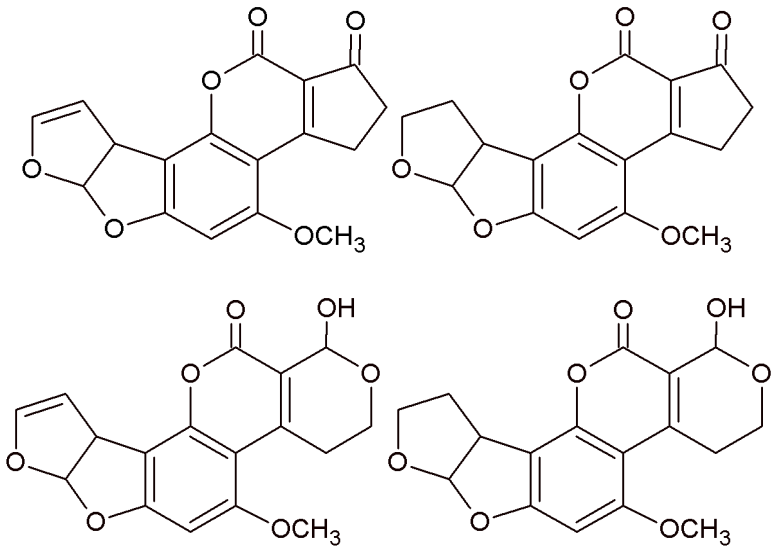


Figure 2.2. Molecular structure of Aflatoxins B_1,B_2, G_1 and G_2

AFB_1 is metabolized, mainly in the liver, to AFB_1-8,9-exo-epoxide and 8,9-endo-epoxide but it is the exo-epoxide that binds to DNA to form the predominant 8,9-dihydro-8-(N_7-guanyl)-9-hydroxy AFB_1 (AFB_1-N_7-Gua) adduct. AFB_1-N_7-Gua can result in two secondary lesions, an apurinic site and a more stable ring opened AFB_1–formamidopyrimidine (AFB_1–FAPY) adduct; the latter is far more persistent *in vivo* than AFB_1-N_7-Gua. The major human cytochrome P450 (CYP) enzymes involved in aflatoxin metabolism are CYP3A4, 3A5, 3A7 and 1A2 and the predominant site of metabolism is the liver.

Many studies in poultry, pigs and rodents showed that exposure to aflatoxin results in suppression of various aspects of the cell-mediated immune response. Some of these effects may be mediated through altered cytokine expression. Studies in different animal species indicate that aflatoxin exposure can severely affect growth (Wild and Gong, 2009).

Occurrence of AFs in foods from Spanish market has been previously reported in several studies where corn-based products and other cereals, pulses, dried fruits and nuts, snacks, breakfast cereals, bread, herbs or spices were analysed. Studies performed

in peanuts from the Spanish market by thin layer chromatography showed incidence values ranging from 1.5 to 10.8 % of positive samples and contamination levels close to 5 μg kg^{-1}, with the exception of several cases with highest contamination level of AFB_1, up to 120 μg kg^{-1} in peanuts in-shell (Sanchis et al., 1986). Subsequent surveys carried out in Spain with pistachios showed percentages of positive samples from 19 to 59 % and variable AFB_1 contamination levels: from 0.12-0.29 μg kg^{-1} as reported by Ariño et al. (2009), to 0.57-98.5 μg kg^{-1} (Burdaspal and Legarda, 1998; Burdaspal et al., 2005; Fortuny et al., 2007). On the other hand, the AFs have not commonly been detected in cereal-based foods, like corn flakes or corn snacks (Sanchis et al., 1986, 1995; Santamarina et al., 1986; Blesa et al., 2004), with the exception of a recent study, where 12 out of 46 breakfast cereal samples were found above the LoQ (0.2 μg kg^{-1}), the maximum level being 0.13 μg kg^{-1} (Ibañez-Vea et al., 2011). One study has recently reported the presence of AFs in paprika, with 59 % of positive samples (n=64) and a maximum value of 7.3 μg kg^{-1} (Santos et al., 2010).

2.3.2. Aflatoxin M_1

Aflatoxin M_1 (AFM_1) is the main monohydroxylated derivative of AFB_1 formed in liver by means of cytochrome p450-associated enzymes (Fig. 2.3.). Mammals that ingest AFB_1 contaminated diets excrete amounts of the principal 4-hydroxylated metabolite AFM_1 into milk. AFM_1 can be found in the milk of the AFB_1 feeding animal after 12-24 hours after intake, being the highest levels after two or three days. The results of studies on heat processing and storage at low temperature of dairy products indicate that such processes do not cause an appreciable change in the amount of AFM_1 in these products. Manufacture of fermented dairy products, concentration and drying of milk do not affect AFM_1.

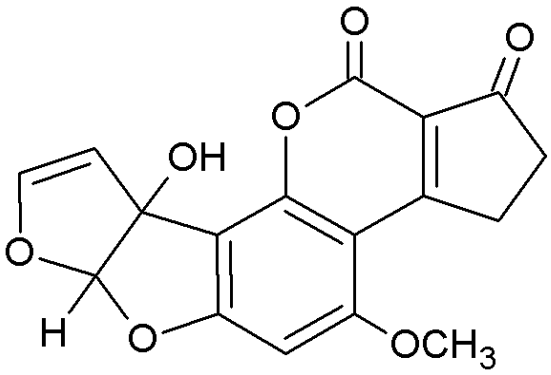


Figure 2.3. Molecular structure of aflatoxin M_1 (AFM_1)

AFM_1 is cytotoxic, as demonstrated in human hepatocytes *in vitro* and its acute toxicity in several species is similar to that of AFB_1. In ducklings and rats, the acute and short-term toxicity of AFM_1 was similar to or slightly less than that of AFB_1. AFM_1 can also cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalians cells *in vitro*, in insects, lower eukaryotes and bacteria. However, AFM_1 is less mutagenic, and genotoxic than AFB_1. Although AFM_1 is less toxic than AFB_1, it has been classified as a possible human carcinogen, Group 2B agent by International Agency for Research on Cancer (IARC) (IARC, 1993). Shuaib et al. (2010) reviewed those works designed to assess the reproductive health effects of aflatoxins. Despite the authors found few studies published with these objectives, they highlighted the high rate of aflatoxins in human breast milk from developing countries, and the possible relation with health effects of young population.

Occurrence of AFM_1 in milk and cheese samples from Spanish market has been previously reported in several studies; however, exposure assessment of Catalonian population to this contaminant has not been performed until now. An early study carried out in Spain showed low incidence of contamination of commercial milk by AFM_1 with 7.3% of positive samples (LOD = 20 ng kg^{-1}) and a range of 20-40 ng kg^{-1} (Burdaspal et al., 1983), however, a subsequent study reported higher contamination levels with a contamination range of 20-100 ng kg^{-1} (Blanco et al., 1988). Later studies found 45.9-86 % of samples were below 10 ng kg^{-1}, and 4-6.6% among 10-20 ng kg^{-1}. AFM_1 mean levels reported were 10.5 and 17.3 ng kg^{-1} in UHT milk samples (Díaz et al., 1995; Jalón

et al., 1994; Rodríguez et al., 2002).

2.3.3. Fumonisin B_1 and B_2

Fumonisin B_1 (FB_1) and B_2 (FB_2) (Fig. 2.4.) are mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum* that commonly contaminate corn (Nelson et al. 1992). Fumonisins (FBs) occur mainly in maize and maize-based foods, therefore populations with high maize consumption could be exposed to significant amounts of these mycotoxins through the ingestion of fumonisin contaminated maize (Marasas, 1996; Shephard et al., 1996; Visconti et al., 1996; WHO, 2001).

FB_1 is stable during most types of processing. Dry milling of maize results in the distribution of FB_1 into the bran, germ and flour. FB_1 is stable in polenta (maize porridge). However, the concentration of FB_1 is reduced during the manufacture of corn starch by wet milling, since FB_1 is water-soluble. A number of factors make it difficult to extract FB_1 from processed food. Nixtamalisation (calcium hydroxide processing) and ammoniation lead to hydrolysed FB_1 (AP_1 or HFB_1) and aminopentol, respectively. These treatments reduce the fumonisin content while increasing the concentration of hydrolysed fumonisins without eliminating the toxic product (SCF, 2003).

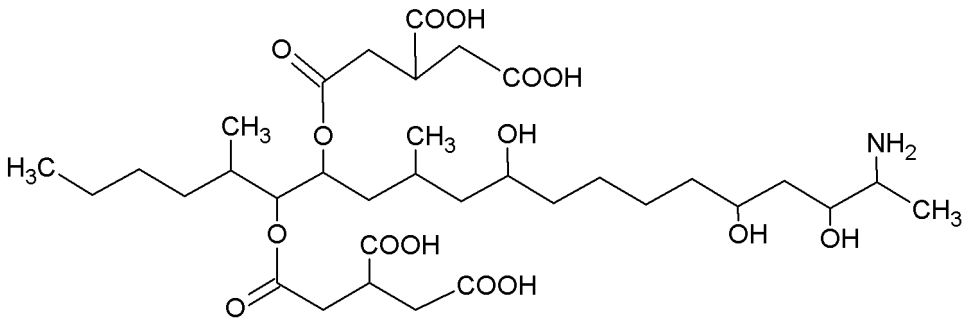


Figure 2.4. Molecular structure of Fumonisin B_1 (FB_1).

FB_1 is poorly absorbed when given orally (less than 6 %) and rapidly eliminated by biliary excretion in several animal species including laying hen, swine, cow, rat, mouse and non-human primates. Enterohepatic recycling is clearly important in some animal

species. Small amounts (less than 1 %) are excreted in urine.

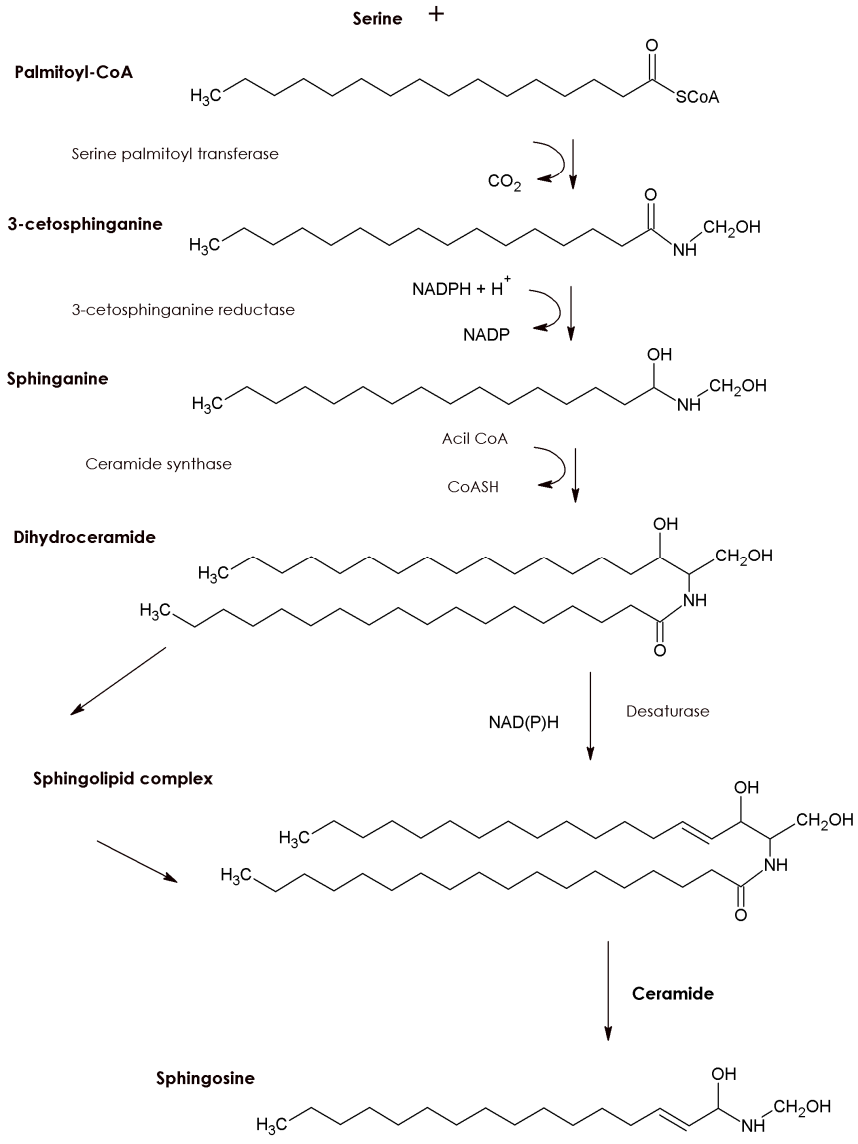


Figure 2.5. Cycle of ceramide (CER)

FBs have a remarkable structural similarity to sphingolipids (Merrill and Sweeley, 1996; Riley et al., 2001). This group of mycotoxins, especially FB_1, potently inhibit the enzyme ceramide (CER) synthase which catalyzes the acylation of sphinganine (Sa) and reacylation of sphingosine (So) (Fig. 2.5.). The inhibition of CER synthase by FBs increases the intracellular Sa concentration, main contributor to the toxicity and carcinogenicity of FB_1 (Wang et al., 1991; Merrill et al., 1993; Yoo et al., 1996; Riley et

al., 2001). Based on this biological perturbation, elevation of Sa to So, or Sa 1-phosphate to So 1-phosphate, ratios in tissues, urine and blood, have been proposed as potential biomarkers of fumonisin exposure in various animal species (Shephard, 2007).

Human exposure to fumonisin contaminated commodities has been linked to oesophageal and liver cancer in South Africa and China (Sydenham et al., 1990; Yoshizawa et al., 1994). Acute and chronic toxicity of FBs has been largely demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al., 1988, 1991). FB_1 is a cancer promoter, but a poor cancer initiator. It is not genotoxic because FB_1 does not induce unscheduled DNA synthesis in primary rat hepatocytes (Norred et al., 1992).

Based on toxicological evidence, the International Agency for Research on Cancer (IARC) has classified FB_1 as possibly carcinogenic (group 2B) to humans (IARC, 2006). In the Mediterranean countries, maize is more widely used for animal nutrition than for human consumption, but some countries traditionally use the maize in regional cooking as broa (maize-based bread from Portugal) and polenta (from Italy). As a consequence of the use of maize grits as adjunct in brewing process, aflatoxins and FBs can be also found in beer (Torres et al., 1998; Pietri et al., 2009).

Natural occurrence of FBs in corn or corn-based foods has been studied largely (SCOOP, 2003), and surveys have been conducted in several cases in corn-based products marketed in Spain. A previous study conducted in Spain showed low contamination levels of FBs in corn snacks and corn flakes but the authors found contaminated samples of sweet corn with respective mean values of 46.27, 17.03 and 55.96 $\mu g\ kg^{-1}$, all of them determined by HPLC (Velluti et al., 2001). D'Arco et al. (2008) compared organic and conventional maize-based food and they found higher percentage of contaminated samples in organic commodities (78 %) than in conventional food (16 %), moreover, the maximum and highest mean was found in the organic category (235 vs 51 $\mu g\ kg^{-1}$). Another previous study performed by ELISA kit with beer samples showed a contamination range of $85.53 \pm 0.34 – 4.76 \pm 1.21\ \mu g\ kg^{-1}$ (Torres et al., 1998).

2.3.4. Patulin

Patulin (4-hidroxy-4H-furo[3,2-c]pyran-2(6H)-one) (Fig. 2.6.) is a mycotoxin produced by a wide range of fungal species of the *Penicillium*, *Aspergillus*, *Byssochlamys*, *Eupenicillium* and *Paecilomcyes* genera of which *Penicillium expansum*, a common contaminant of damaged fruits is the most important (Fuchs et al., 2008). *P. expansum*, known as the causal agent of the blue mold rot in pome and stone fruits, attacks apples, pears, plums, peaches, apricots, cherries, blackcurrants, grapes, melons and strawberries (Snowdon, 1990; Larsen et al., 1998). However, the most common fruits where patulin has been detected are apples and pears, usually cold stored under refrigeration (Barkai-Golan et al., 2008).

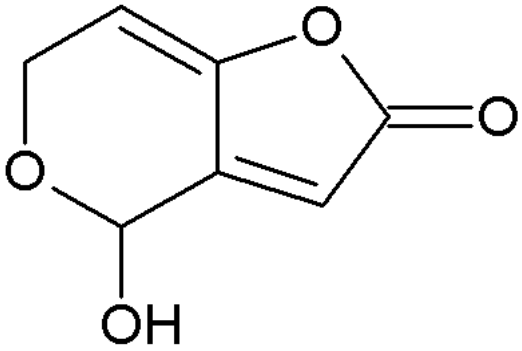


Figure 2.6. Molecular structure of patulin

Patulin has a strong affinity for sulfhydryl groups. Patulin adducts formed with cysteine are less toxic than the unmodified compound in acute toxicity, teratogenicity, and mutagenicity studies. Its affinity for SH-groups explains its inhibition of many enzymes. Acute toxic signs consistently reported in all studies were agitation, in some cases convulsions, dyspnoea, pulmonary congestion, oedema, and ulceration, hyperaemia and distension of the gastro intestinal tract. As described for other mycotoxins, patulin can alter the immune response of the host. Numerous *in vitro* studies have demonstrated that patulin inhibits several macrophage functions. *In vivo* studies using mice indicate variable effects of patulin on the immune system. These effects include an increased number of splenic T lymphocytes and depressed serum immunoglobulin concentrations, depressed delayed hypersensitivity responses and increased neutrophil numbers and

resistance to *Candida albicans* infection. The few studies of long-term toxicity on patulin showed an absence of tumors in rats orally exposed. Patulin did not increase revertant frequency in the Ames test using several strains of *Salmonella* Typhimurium, but some studies have shown mutagenic activity in *Saccharomyces cerevisiae* strains and in *Bacillus subtilis*. A study performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concludes that this mycotoxin has neither teratogenic nor reproductive effects, but shows embryotoxicity combined with maternal toxicity (JECFA, 2002). However, there is insufficient evidence that patulin has a carcinogenic effect in experimental animals and it has not yet been tested in humans; therefore the International Agency for Research on Cancer (IARC) classified patulin in group 3 or as "not carcinogenic to humans" (IARC, 1993).

The occurrence of patulin in apple-based products, mainly apple juice, has been reported by several researchers, but few studies on occurrence of patulin in baby apple-based food products have been reported. Previous studies conducted with apple juice marketed in Spain showed mean levels of patulin ranged from 13.8 to 19.4 $\mu g\ l^{-1}$, maximum levels of 118.7 and 170 $\mu g\ l^{-1}$, and values over the limit established by the European Union in a range of one to eleven samples per study (Rovira et al., 1993; Prieta et al., 1994; González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009). Infants and children are considered to be more susceptible to different toxins than adults, because of their lower body weight, higher metabolic rate, lower ability to detoxify and because of incomplete development of some organs and tissues, and they may be expected to be a risk group due to their highest dietary intake of apple-based food. Exposure assessment of Spanish population to patulin from apple-based products consumption has been estimated using hypothetical intake data in previous studies (González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009).

2.3.5. Trichothecenes

Trichothecenes have a common tetracyclic, sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system and are divided into four groups (A-D) according to

their different chemical functionalities. The stable epoxide group between C12 and C13 seems to account for many of the typical toxic effects of trichothecenes. Epidemiological surveys have demonstrated that the predominant type A and B trichothecenes are widely distributed in cereals and feeds as natural pollutants, whereas C (characterised by a second epoxide at C7,8 or C9,10) and D trichothecenes (containing an ester-linked macrocycle at C4,16) occur rarely in food and feed. Type A trichothecenes include T-2 toxin, HT-2 toxin and 4,15-diacetoxyscirpenol, and type B toxins include deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyldeoxynivalenol and nivalenol. Type A trichothecenes possess an ester function at the C8 position whereas for type B trichothecenes, a carbonylic functionality at C8 is characteristic.

2.3.5.1. A-type. T-2 and HT-2 toxins

T-2 toxin and HT-2 toxin are members of a large group of fungal sesquiterpenes, commonly denoted as trichothecenes. T-2 toxin and HT-2 toxin are non-volatile compounds, which are stable at neutral and acidic pH (Fig. 2.7). The fewer free hydroxyl groups and the lacking keto group at C8 of type A trichothecenes make them less polar compared with the related type B trichothecenes. They are produced by various *Fusarium* species, including *F. sporotrichoides, F. poae, F equiseti, F. acumninatum*, as well as species from the genera *Myrothecium, Cephalosporium, Verticimonosporum, Trichoderma, Trichothecium* and *Stachybotrys*. Generally, the *Fusarium* species grow and invade crops, and may produce T-2 toxin and HT-2 toxin under moist cool conditions already prior to harvest. T-2 toxin and HT-2 toxin and other trichothecenes are predominantly found in cereal grains (particularly in oats) and products thereof (SCF, 2001).

T-2 toxin is rapidly absorbed after ingestion in most animal species and it is distributed in the organism with little or no accumulation in any specific organs. The plasma half-life for T-2 toxin is less than 20 minutes. T-2 toxin is rapidly metabolised by deacetylation, hydroxylation, glucuronide conjugation and de-epoxidation. The main biotransformation pathway is deacetylation of the C-4 acetyl group of T-2 toxin, which leads to HT-2 toxin (the sole metabolite of T-2 toxin with isolated microsomes from

liver, kidney and spleen of various animals) (Li et al., 2011).

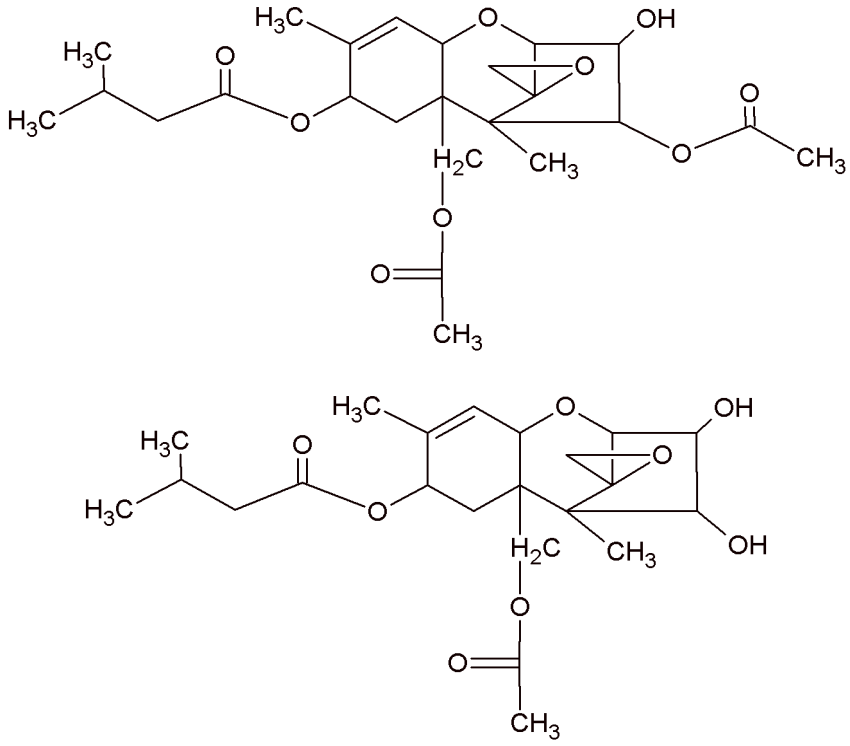


Figure 2.7. Molecular structure of T2 and HT2 toxins

T2 toxin is a potent inhibitor of protein synthesis and mitochondrial function both *in vivo* and *in vitro*, and shows immunosuppressive and cytotoxic effects. Moreover, it has been reported to have extremely toxic effects on skin and mucous membrane (Visconti et al., 1991; Visconti, 2001; Sudakin, 2003; Eriksen and Pettersson, 2004). The assessment by the SCF of the genotoxicity of T-2 toxin indicated a positive effect in several conventional tests for genotoxicity *in vitro* and in rodents *in vivo*, in particular for clastogenic effects, but these effects were observed primarily at concentrations also known to inhibit protein and DNA synthesis and produce cytotoxicity. The SCF reported limited evidence for tumourigenicity of T-2 toxin in experimental animals (induction of hepatocellular- and pulmonary adenomas in male mice). Despite T2 toxin toxic effects having been widely studied in animals, its toxicology has never been assessed in humans (EFSA, 2011c).

To date, few studies have been published reporting neither the occurrence of T2 toxin and HT2 toxin in Catalonia nor in the rest of Spain. In a previous study conducted

in Spain, T2 toxin was not found in any sample out of the 25 samples analyzed, above the LOD of 0.030 μg g^{-1} neither in corn flakes, sweet corn nor in corn snacks (Cerveró et al., 2007). Leblanc et al. (2005) quantified HT2 toxin in one sample out of 238 composite samples with a level of 0.270 μg g^{-1}.

2.3.5.2. B-type. Deoxynivalenol

Deoxynivalenol (DON) is a mycotoxin produced by fungi of the *Fusarium* genus, i.e. *Fusarium culmorum* and *Fusarium graminearum*, which are abundant in various cereal crops (wheat, maize, barley, oats, and rye) and processed grains (malt, beer and bread). Environmental conditions that favour DON production in the field are low temperature and high humidity (Fig. 2.8.). Cold and wet weather, which tends to delay harvest, permits continued growth of the mould on the crop, thereby increasing the chance of higher concentrations of DON being produced (SCF, 1999).

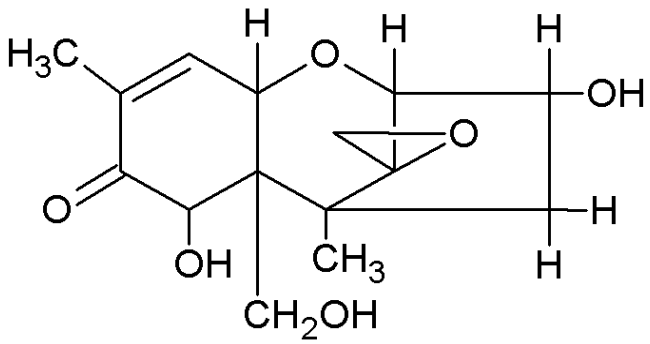


Figure 2.8. Molecular structure of deoxynivalenol (DON)

The compound is cytotoxic to a variety of cells including fibroblasts and lymphocytes. At the cellular level, the key toxic event is related to protein synthesis inhibition at the ribosomal level during the elongation-termination step as observed in rabbit reticulocytes. The effects in the whole animal, however, are more complex and involve secondary effects. On cells of the immune system, DON may be immunosuppressive or immunostimulatory, depending on the length and dosage. Monogastric animals, especially swine, show the greatest sensitivity to DON, while

chickens and turkeys, followed by ruminants, appear to have higher tolerance. Acute effects of food poisoning by DON in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in stool (Rotter et al., 1996).

Occurrence of DON has been widely reported in raw foods and foodstuffs in European countries (JECFA, 2001), confirming that food processing methods do not completely remove mycotoxins (Hazel and Patel, 2004). There are several previous studies carried out to assess the incidence of DON in foodstuffs from Spanish market. In the earliest study the range of 0.038-0.195 $\mu g\ g^{-1}$ was reported for corn flakes, 0.035-0.061 $\mu g\ g^{-1}$ for sweet corn and 0.028-0.109 $\mu g\ g^{-1}$ for fried corn snacks (Cerveró et al., 2007). Moreover, in other recent study, concentrations of 0.030-0.121 and 0.026-0.080 $\mu g\ g^{-1}$, were found in breakfast cereals and fried snacks, respectively (Castillo et al., 2008).

2.3.6. Zearalenone

Zearalenone (ZEA) is a non-steroidal oestrogen mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals, including corn and wheat, in temperate and warm regions around the world (Fig. 2.8.). ZEA is a resorcyclic acid lactone containing an unsaturated bond at C1'-C2' and a ketone function at position C6'. Either the double bond or the ketone, or both, can be reduced yielding a series of congeners and stereoisomers (Fig. 2.9). The reductions are important because they affect the biological activity, as well as the physical properties of the molecule. Reduction is also important because it represents a mechanism whereby organisms can biotransform ZEA. As might be expected, not all of the products of biotransformation have equivalent physical characteristics or bioactivity (Jelinek et al., 1989; Jiménez et al., 1996; Kuiper-Goodman et al., 1987).

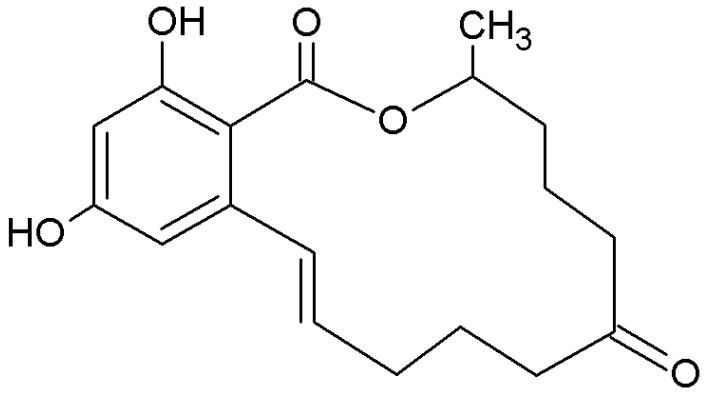


Figure 2.9. Molecular structure of zearalenone (ZEA)

Absorption of ZEA has been reported as extensive and quick in rats and rabbits (Kuiper-Goodman et al., 1987; Ramos et al., 1996), being estimated in the range of 80-85% in pigs (Biehl et al., 1993). ZEA and some of its metabolites have been shown to competitively bind to oestrogen receptors. Thus, the toxicity is associated with reproductive problems in specific animals and possibly in humans (Gromadzka et al., 2009). Fertility problems have been observed in animals such as swine and sheep (Krska et al., 2003). ZEA may be an important etiologic agent of intoxication in infants or foetuses exposed to this mycotoxin, with results in premature thelarche, pubarche, and breast enlargement (EFSA, 2011b).

To our knowledge, only one study was carried out to assess the occurrence of ZEA in maize-based food for human consumption from the Spanish market (Cerveró et al., 2007). The authors reported percentages of positive samples ranging between 40% and 80% (LOD = 3 $\mu g\ kg^{-1}$), with mean values of 114.0 ± 10.6 $\mu g\ kg^{-1}$ in corn flakes, 11.1 ± 6.5 $\mu g\ kg^{-1}$ in sweet corn and 91.2 ± 45.2 $\mu g\ kg^{-1}$ in corn snacks..

2.4. Legislation and governmental recommendations

Until the late 1990s setting of mycotoxin regulations was mostly a national affair. Gradually, several economic communities (e.g. EU (European Union), MERCOSUR (Mercado Común del Sur), Australia and New Zealand) harmonized their mycotoxin regulations, thereby overruling existing national regulations. Current regulations are

increasingly based on scientific opinions of authoritative bodies, for example the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JECFA) and the European Food Safety Authority (EFSA). At the same time, requirements for adequate sampling and analytical methods put high demands on other professional organizations, for example AOAC International and the European Standardization Committee (CEN) (Van Egmond et al., 2007).

Until 2003 the European Union only regulated the maximum levels of aflatoxins in foods intended for human and animal consumption because they were the most studied and harmful mycotoxins (European Commission, 2001). Subsequently the rest of mycotoxins have been set in Commission Regulation (EC) Nº 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, and revised in the Commission Regulation (EC) Nº 1126/2007 of 28 September 2007 and Commission Regulation (EC) Nº 165/2010 of 26 February 2010 amending Regulation (EC) Nº 1881/2006 (See Annex 2). At least 99 countries in the world had mycotoxin regulations for food and/or feed in 2003, an increase of approximately 30% compared with 1995. Regulations have become more diverse and detailed with newer requirements with regard to official sampling procedures and analytical methods. Harmonisation of tolerance levels is occurring in several free-trade zones but remains as a big challenge in other regions of the global trade market.

Taking into account the high variability of mycotoxin levels on foods, especially for aflatoxins, several regulations have been developed in order to establish accurate sampling guidelines. In the Commission Regulation (EC) Nº 401/2006 of 23 February 2006 the sampling methods for the official control of the levels of mycotoxins in foodstuffs and sample preparation and methods of analysis used for the official control of the levels of mycotoxins were established. Sampling methods are focused mainly on official control and performance criteria for analysis of patulin, DON, ZEA, fumonisins and T2-HT2 toxins are detailed in terms of recovery and relative standard deviation.

Risk assessment regulations from European Commission are primarily based on known toxic effects. The Joint Expert Committee on Food Additives (JECFA—a

scientific advisory body of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO)) has evaluated their hazard in several sessions. The resulting report provided good and detailed insight into the process of risk assessment of mycotoxins. The reports addressed several concerns about the mycotoxins considered their properties and metabolism, toxicological studies, and final risk evaluation (FAO, 2001).

The European Food Safety Authority (EFSA) was set up in January 2002, following a series of food crises in the late 1990s, as an independent source of scientific advice and communication on risks associated with the food chain. As the risk assessor, EFSA produces scientific opinions and advice to provide a sound foundation for European policies and legislation and to support the European Commission, European Parliament and EU Member States in taking effective and timely risk management decisions. Regulation (EC) 178/2002 (European Commission, 2002), which establishes EFSA, stipulates that risk assessment should be objective, based on scientific evidence, and should be undertaken in an independent, objective and transparent manner.

The Scientific Committee on Food (SCF) issued between 1999 and 2002 a group of opinion series on *Fusarium* toxins, considering DON, NIV, ZEA, FBs, T2 and HT2 toxins evaluating the most relevant topics on toxicity in order to derive temporary TDI (t-TDI) (SCF, 1999, 2000, 2001, 2002).

Based on recent data in the most sensitive animal species, the pig, and taking into account comparisons between pigs and humans, the Panel on Contaminants in the Food Chain established a new tolerable daily intake (TDI) for zearalenone of 0.25 μg^{-1} kg b.w. (being 0.20 μg^{-1} kg b.w.). Estimates of chronic dietary exposure to zearalenone based on the available occurrence data are below or in the region of the TDI for all age groups and not a health concern (EFSA, 2011).

The European Commission (EC) asked the European Food Safety Authority (EFSA) for a scientific opinion on the risk to human and animal health related to the presence of T-2 and HT-2 toxin in food and feed. In particular the opinion should consider any new results of toxicological studies published since the assessment by the

SCF in 2001, in order to assess if the combined t-TDI of 0.06 μg^{-1} kg b.w. for T-2 and HT-2 toxin is still appropriate. Consequently, the Panel on Contaminants in the Food Chain established a group tolerable daily intake (TDI) of 100 ng^{-1} kg b.w. for the sum of T-2 and HT-2 toxins (EFSA, 2011).

The health effects of aflatoxins have been reviewed by a number of expert groups including the Scientific Panel on Contaminants in the Food chain (CONTAM Panel) of the European Food Safety Authority (EFSA). In January 2007 the CONTAM Panel adopted an opinion related to the potential increase in consumer health risk by a possible raising of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. The Scientific Committee for Food (SCF) endorsed the IARC conclusions in 1994 and concluded that even very low levels of exposure to aflatoxins could contribute to risk of liver cancer. Risk assessment for substances that are both genotoxic and carcinogenic were recommended by EFSA (2005) by means of the margin of exposure approach.

2.5. References

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Chapter 3. Objectives

This thesis work takes part of the research line "Assessment of the exposure of consumers 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", funded 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".

The general objective of the present thesis was to assess the exposure of Catalonian population to the major mycotoxins, aflatoxins group B and G, aflatoxin M_1, patulin and the *Fusarium* toxins deoxynivalenol, fumonisins, T-2 and HT-2, and zearalenone.

To reach this general objective, several sub-objectives were developed:

- To determine the mycotoxin levels in most relevant food categories, from a representative sample purchased around of the geography of Catalonia.
- To assess the dietary intake of Catalonian population in regard of those mycotoxin related foods, including the most vulnerable collectives like elderly, infants, immigrants or celiac sufferers.
- To combine accurately raw consumption and contamination datasets in order to obtain a thorough estimation of exposure levels of the population collectives and a reliable characterization of the related health risk.
- To evaluate the novel biomarker ratio sphinganine:sphingosine as an indicator of exposure to fumonisins.
- To assess the effect of any probable variable, as food processing or cooking, over the exposure assessment outputs.

Chapter 4. Global methodology

4.1 Global study design

The present study was developed in the framework of the "Project to Assess the Exposure of Catalonian Population to the Mycotoxins 2008-2009". The global design consisted in one hand, of a nutritional study and in the other hand, of an analytical study (See Fig. 4.1). The nutritional study was carried out to assess specifically the dietary habits of Catalonian population related with those foods that can be contaminated with mycotoxins. The analytical study was developed to provide an accurate estimation of the mycotoxins levels in the Catalonian market.

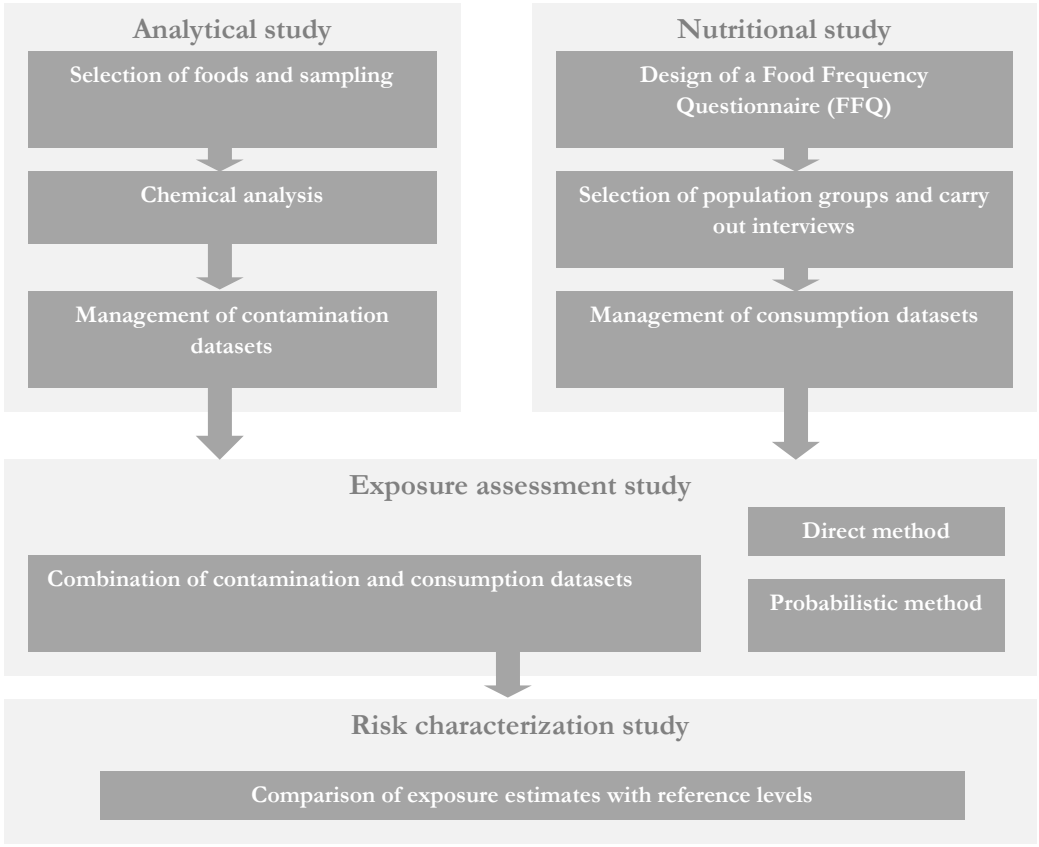

Figure 4.1 Global study design

The mycotoxins included in the project were aflatoxins B_1, B_2, G_1, G_2 and M_1, fumonisin B_1 and B_2, ochratoxin A, patulin, trichothecenes A-type (T-2 toxin and HT-2 toxin) and B-type (deoxynivalenol) and zearalenone. Ochratoxin A has not been included in the present work because was the main topic of another doctoral thesis, but it was performed in the framework of the present study.

4.2 Analytical study

4.2.1 Food selection

The food selection was based on the published bibliography reporting the most relevant commodities contaminated by mycotoxin and also consumed in the region (Table 4.1).

The most important foods that can be contaminated by patulin are those apple-based. In Catalonia, the most important apple-based foods consumed are apple juice, solid apple-based food (jam or apple pure) and apple-based baby foods.

Aflatoxins group B and G were determined in breakfast cereals, baby foods, corn snacks, canned sweet corn, dried figs, peanuts, pistachios and dried red pepper. Aflatoxin M_1 was determined in whole milk, and dairy products like cheeses and natural yoghurts.

Fumonisin B_1 and B_2 are mainly produced in corn, therefore, they were determined in corn-based food like canned sweet corn, corn snacks, breakfast cereals, cereal baby foods and beer because corn syrup is commonly used in its fabrication.

The trichothecenes were determined in bread, sliced bread, breakfast cereals, corn snacks, sweet corn, beer and pasta.

Out of these foods consumed by most of the population, a selection of special foods, consumed by collectives with specific nutritional or cultural requirements were included in the study: free-gluten foods intended for celiac sufferers (mainly bread, pasta and pastries) and ethnic foods from retail shops specialized in imported regional products (cuscus, corn semolina and flour).

Taula 4.1 Summary of the mycotoxins analysed in each food category

Food	AFs	AFM_1	PAT	FBs	TRCs	ZEA
Milk		X				
Cheese		X				
Yogurt		X				
Peanuts	X					
Pistachios	X					
Dried figs	X					
Sweet corn	X			X	X	X
Corn flakes	X			X	X	X
Wheat flakes	X				X	X
Corn snacks	X			X	X	X
Cereal baby foods	X			X	X	X
Bread					X	
Sliced bread					X	X
Pasta					X	X
Red pepper	X					
Beer				X	X	X
Apple juice			X			
Solid apple-based foods			X			
Baby apple-based foods			X			
Ethnic foods	X			X	X*	X
Free-gluten foods	X			X	X*	X

*In these categories only the TRC (trichothecenes) deoxynivalenol was determined

4.2.2 Food sampling and analysis

Food samples were selected to be the most susceptible commodities to mycotoxin contamination and to be commonly consumed in Catalonia. Between 2008 and 2009 the samples were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population (See Fig. 4.2).

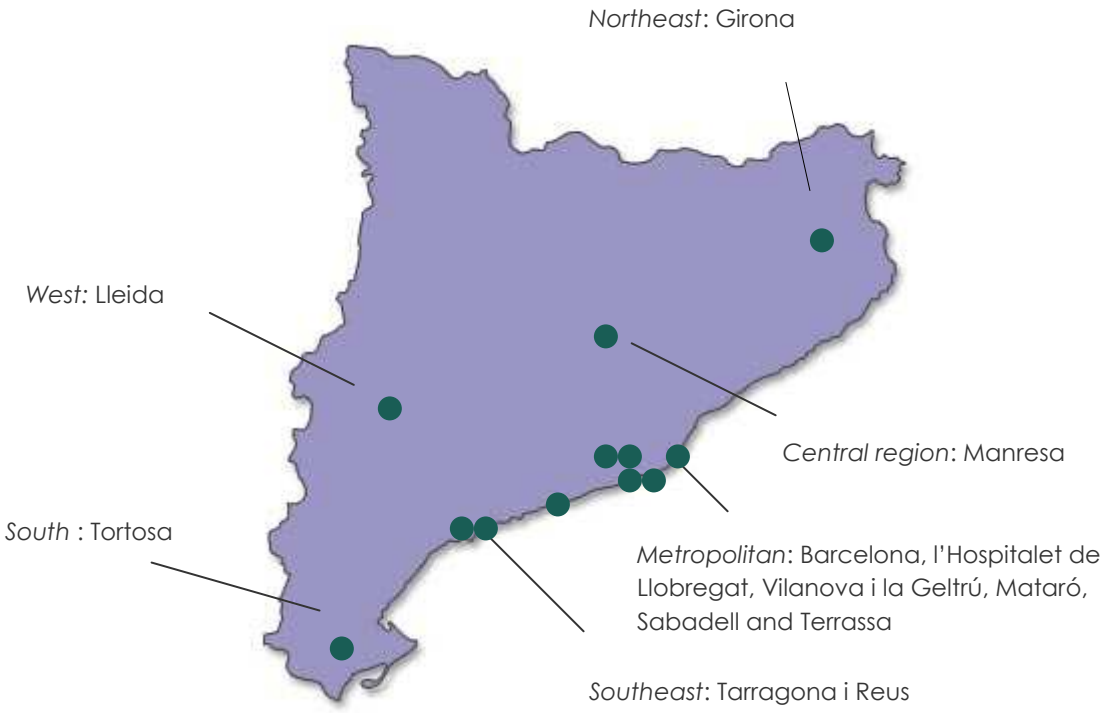

Fig. 4.2. Geographical distribution of sampled cities around Catalonia

From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of the mycotoxins was determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store if were available (12 cities x 6 stores / city = 72 samples / category). However, in some cases, 3 items were not available in the same store, thus, less than 72 composites could be obtained. In case of the dried fig samples, apple-based solid foods, gluten-free foods and ethnic food samples, were analysed individually. In case of pistachios and peanuts, only the edible fraction was analysed.

Regarding brands, finally a wide range of trade brands (n=770) were involved in the study, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of Spanish market (Table 4.2).

Table 4.2 Summary of the samples purchased, analyzed and the trade brands in each food category

Food categories	Individual samples	Analysed samples	Brands
Milk	211	72	36
Cheese	216	72	70
Yogurt	202	72	21
Peanuts	215	72	47
Pistachios	170	70	23
Dried figs	26	26	14
Sweet corn	182	71	30
Corn flakes	168	72	14
Wheat flakes	30	30	30
Corn snacks	216	72	74
Cereal baby foods	154	71	21
Bread	30	30	30
Sliced bread	145	71	43
Pasta	201	70	105
Red pepper	168	72	22
Beer	216	72	64
Apple juice	161	71	29
Solid Apple-based foods	77	77	18
Baby apple-based foods	124	124	26
Ethnic foods	35	35	35
Free-gluten foods	18	18	18
Total	**2.965**	**1.340**	**770**

4.2.3 Chemical analysis techniques

In this section the analytical techniques used in the chemical analysis of each mycotoxin in the different food matrices are exposed briefly. In each case, the methodologies were validated according to the performance criteria set by the European Commission in the Regulation (EC) Nº 401/2006 of 23th February of 2006, laying down the methods of sampling and analysis for the official control of the levels of mycotoxins

in foodstuffs.

In each chapter, each specific chemical analysis method is described more in depth. Extraction and clean-up of aflatoxins B_1, B_2, G_1 and G_2 were performed using immunoaffinity columns and they were detected and quantified by high performance liquid chromatography (HPLC) with post-column photochemical derivatisation and fluorescence detection. The limit of quantification (LOQ), was 0.1 μg kg^{-1} for AFB_1 and AFG_1, and 0.025 μg kg^{-1} for AFB_2 and AFG_2. AFM_1 content was analysed using an Enzyme-Linked Immunosorbent Assay commercial kit. The LOQ of AFM_1 in milk was 5 ng kg^{-1}, and 25 ng kg^{-1} in yogurt.

Immunoaffinity column extraction and cleanup of fumonisins from corn-based food samples, was coupled with detection and analysis by HPLC and *o*-phthaldialdehyde derivatization. The LOQ of fumonisins was between 11.7 and 15.6 μg kg^{-1}.

Patulin was analysed by a well-established validated method involving ethyl acetate extraction and direct analysis by HPLC with ultraviolet light detection. The LOQ was 6.25 μg kg^{-1}.

DON, T2 toxin and HT2 toxin were determined in breakfast cereals, snacks and pasta samples following extraction, clean–up, derivatization and finally analysis by gas chromatography with electrochemical detection (GC-ECD). Moreover, these mycotoxins were determined in sliced bread, sweet corn and beer by liquid chromatography with diode array detection (LC-DAD). The LOQ for DON was between 12-66 μg kg^{-1}, for HT2 toxin was between 9-61 μg kg^{-1}, and 12-153 μg kg^{-1} for T2 toxin.

Zearalenone was extracted and purified by means of immunoaffinity columns and determined by HPLC with fluorescence detector. The LOQ was between 1.95 and 0.70 μg kg^{-1}.

4.3 Nutritional study

Food-frequency methods should be chosen for chronic exposure assessment because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998). In this study, food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins, excluding those foods not consumed in the region. Five response options, ranging from never to annually, were considered to report frequencies. Quantities were assessed by portion size with the aid of a series of colour photograph models.

4.3.1 Population groups

According to World Health Organization (WHO) advices, studies to assess dietary intake of chemical contaminants, should show the significant intake among standard population, with all population groups that could have different dietary patterns.

Five different population groups of each sex were considered: babies (0-3 years), infants (4-9), teenagers (10-19 years), adults (20-65 years) and elders (>65 years). Finally, 76 elders, 720 adults, 235 teenagers, 69 infants and 164 baby parents were interviewed during 2008-2009 by trained interviewers. Moreover, 70 celiac sufferers and 56 individuals with an ethnic dietary pattern were included in the nutritional study (Table 4.3). Individuals were from 89 cities and towns from around of Catalonia.

Table 4.3 Age ranges and mean body weight of the population interviewed

Population groups	N	Age range (years)	Body weight (kg)*
Elders	76	>65	73.7
Immigrants	56	17-51	67.7
Celiac sufferers	70	16-75	58.7
Adult males	336	20-65	81.1
Adult females	384	20-65	66.6
Adolescents	235	10-19	54.4
Children	69	4-9	25.7
Infants	154	0-3	11.6

* Mean of the individuals from each interviewed population group

4.4 Exposure assessment

4.4.1 Left-censored data treatment

In the present study we have applied three different methods to obtain a mean level of contamination for each food dataset, taken into account the left-censored data.

1. Substitution method. Non detected samples were assumed to be the value of the LOD divided by 2. This is the most commonly used procedure in exposure assessment of populations to mycotoxins, thus we used this approach as starting point.

2. Parametric method: maximum likelihood estimation (MLE). A wide explanation about MLE is reported by Helsel (2005) and EFSA (2010). Briefly, the method is based on the assumption that data below and above the LOD follow a given statistical distribution. The parameters of the chosen distribution are estimated to best fit the distribution of the observed values above the detection limit,

compatibly with the percentage of data below the limit. The estimated parameters are those which maximize the likelihood function. Detailed information concerning the syntax of the NLMIXED procedure for SAS® used in the present work to fit the MLE model to obtain the mean and standard deviation of contamination datasets is included in the Appendix G in EFSA report (2010).

3. Non-parametric method: Kaplan-Meier estimation (KM). This is the standard non-parametric technique for censored data, based on the empirical cumulative distribution function. We address the readers to the report of Tressou (2006) to better understand the mathematic basis of the KM estimator, and its application in food risk assessment studies. With the KM method, the weight of the censored data is distributed over the different observed values below the censoring values. In order to obtain the mean and standard deviation of each contamination dataset, we have applied the syntax to fit the KM model based on the LIFETEST procedure from SAS® also included in the Appendix G in EFSA report (2010).

4.4.2 Direct method

The main limitation to estimate the normalised exposure distribution of consumer populations is derived from the complicated equation described by:

$$E_{\pi} = \sum_{j=1}^{p} C_{\pi,j} T_j \qquad (1)$$

where the random variable "normalised global exposure in a consumer population π" (E_{π}) is a function of the random variables: "normalised consumption of the foodstuff *j* in a consumer population π" ($C_{\pi,j}$) and "ZEA concentration level of the foodstuff *j*" (T_j). The difficulty is due to the probability density function (*pdf*) since these variables are generally unknown and even if they are known, they are generally different and not independent. Moreover, the *CT pdf* products are unknown as well.

If we assume independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, we can estimate the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j \qquad (2)$$

where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff j in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food.

Under these assumptions we can also calculate the variance estimate $\hat{V}(E_{\pi_0})$ using Eq. (5) and Eq. (6) in Gauchi and Leblanc (2002). We could consider this approach to be a theoretical approach, but because of the postulated assumptions, it would have to be a (very) simplified theoretical approach. For example, this simplified approach does not enable us to calculate complicated statistics such as high quantiles. Simulation methods would therefore be required to obtain estimates of these statistics. Finally, we will consider this approach to be a direct approach.

4.4.3 Probabilistic methods

In this section we recall some elements of the methodology used to calculate the exposure by means of the stochastic procedure, already given in Gauchi and Leblanc (2002).

The main advantage of the use of a parametric method is derived from the fact that NonParametric-NonParametric method could lead us to less reliable estimations, especially of the high quantiles (Gauchi and Leblanc, 2002). Taking the asymmetrical appearance of consumption and contamination histograms and our previous experience into account, we fitted the probability density functions, choosing the gamma distribution to be the best candidate. Therefore, for each consumption and contamination dataset, the gamma *pdf* was fitted with the method of maximum

likelihood using the CAPABILITY procedure of SAS software (SAS 2010). The Chi-Square statistic was used in the goodness-of-fit test, considering a significance level greater than 95%, to accept the distribution as a suitable candidate. Moreover, several graphical methods such as quantile-quantile plot exist, making it possible to quickly check if the fitted distribution is adapted to the selected hypothesis (Smout et al., 2000). Shape and scale parameters were estimated for each contamination set, in accordance with the methodology described above.

Concerning consumption datasets, the foodstuffs selected are commonly consumed within the Catalonian population, despite several exceptions related to age-group dietary patterns. The appearance of the histograms is therefore continuous and only one distribution was considered pooling all age groups data, fitting all data to gamma *pdf*. In case of gluten-free foods, the mean contamination was used in the simulations because few data were available.

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

$$\hat{E}_k^{[P-P]} = \sum_{j=1}^{p} \tilde{c}_{i(j)} \tilde{t}_j \tag{4}$$

where $\tilde{c}_{i(j)}$ is a random normalised consumption i for the foodstuff j, drawn from $\hat{C}_j$, the corresponding adjusted gamma *pdf*, and $\tilde{t}_j$ is a random contamination for the foodstuff j, drawn from $\hat{T}_j$, the corresponding adjusted gamma *pdf*.

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 (10,000 in the present study):

$$\hat{E}_S^{[P-P]} = \frac{1}{n} \sum_{k=1}^{n} \hat{E}_k^{[P-P]} \tag{5}$$

Other statistics were directly computed on the histogram built with the simulations of the S set, and statistics were also estimated from the lognormal and gamma fitted *pdf*s.

In some cases, the appearance of the consumption histograms was clearly irregular, especially for those foods whose consumption is seasonal or sporadic, such as pistachios or peanuts. Therefore, the histograms could be divided in two subclasses (non consumers and consumers), and fitting a mixed distribution as follows:

$$U_{\pi_0,j}^{[D]} = \left\{ \left[U\left(0, c_{i_{\min}(\pi_0),j}\right) \right] j, h; \left[\Gamma(r,\lambda,\theta)\right]_{\pi_{0,j}}, (1-h) \right\} \quad (6)$$

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 $c_{i_{\min}(\pi_0),j}$ the minimal consumption of the foodstuff j, in the sample π_0 (this part corresponds to the non consumer's class). $\left[\Gamma(r,\lambda,\theta)\right]_{\pi_{0,j}}$ is the Gamma 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* 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 $\left[\Gamma(r,\lambda,\theta)\right]_{\pi_{0,j}}$.

Method to build the bootstrap confidence intervals

To know the validity and accuracy of the high quantiles estimated for the simulation method, the confidence intervals are required. Therefore, this section focuses on building bootstrap confidence intervals in order to determine the reliability of the simulation methods. Several methods to build bootstrap confidence intervals (CI_b) were assessed by Gauchi and Leblanc (2002), revealing the difficulties involved in applying the nonparametric CI_b and highlighting the "*pseudo-parametric* CI_b" as the best choice from among the other parametric procedures and analytical methods proposed. We therefore built CI_b according to this method.

"P*seudo-parametric* CI_b", referred to as Type 1, was built by randomly drawing *B* samples of size n_{π_0} in the exposure simulation set *S*. Typically, *B* is equal to 10,000. The boundaries of the 95% confidence interval are calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution.

4.5 Risk characterization

In case of the mycotoxins that are not genotoxic and carcinogenic, the exposure estimates were compared with the guidance values (TDI, PTDI, PMTDI…) derived from the related no-observed-adverse-effect level (NOAEL) (Table 4.4).

In case of aflatoxins, following the EFSA advices to carry out the risk assessment of substances which are both genotoxic and carcinogenic, we have built the MoEs by division of the human $BMDL_{10}$ of 870 ng kg^{-1} bw day^{-1} (EFSA, 2007) by the exposure estimates for the population collectives from Catalonia.

The incidence of liver cancer in a population attributable to intake of AFs is derived by combining aflatoxin risk potency estimates and aflatoxin exposure estimates as follows:

Population risk = Exposure x Risk Potency

Taken into account that Catalonia is a region with a natural low occurrence of AFs and with a population having a small prevalence of carriers of hepatitis B, the risk potency was calculated as follows, assuming the 1% of population to be carriers of hepatitis B (JECFA, 1998):

0.01 x 99% + 0.3 x 1% = 0.013 cancers/year per 100,000 persons

Table 4.4. Safety levels used in the present study

Mycotoxin	Nomenclature	Safety level	Reference
Aflatoxin M_1	PSL*	1 ng kg^{-1} bw day^{-1}	Leblanc et al., 2005
Dexynivalenol	TDI	1000 ng kg^{-1} bw day^{-1}	SCF, 1999
Fumonisins	TDI	2000 ng kg^{-1} bw day^{-1}	SCF, 2003
Patulin	PMTDI	400 ng kg^{-1} bw day^{-1}	WHO, 1995
T2 toxin and HT2 toxin	TDI	60 ng kg^{-1} bw day^{-1}	SCF, 2001
	TDI	1000 ng kg^{-1} bw day^{-1}	EFSA, 2011b
Zearalenone	PMTDI	500 ng kg^{-1} bw day^{-1}	EFSA, 2004
	TDI	250 ng kg^{-1} bw day^{-1}	EFSA, 2011a

PSL* : provisional safety level set by the authors

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Chapter 5. Occurrence and exposure assessment to aflatoxins B and G in Catalonia

Food Chemical and Toxicology 2012 6, 188-193

5.1 Abstract

The main objective of this study was to assess the exposure of Catalonian (Spain) population to AFs. Thus, two sub-objectives were considered: 1) to assess the occurrence of AFs in food marketed in Catalonia, and 2) to assess the consumption of those foods susceptible to AFs contamination by Catalonian population. AFs were analysed in a total of 603 samples considering special commodities as free-gluten, ethnic or baby foods. Analytical method consisted of an extraction and clean-up of aflatoxins step using immunoaffinity columns, and determination by HPLC with post-column photochemical derivatization and fluorescence detection. Food dietary intake was assessed using a food frequency questionnaire, administered to 1387 individuals by trained interviewers. Contamination and consumption raw datasets were combined by means of a direct method and a stochastic method, building the pseudo-parametric bootstrap confidence intervals of the main outputs. Margins of exposure (MoE) and cancer incidence were estimated for the different collectives. The highest percentages of positive samples were found in red pepper, pistachios and peanuts. Considering our results, the most exposed group was the celiac sufferer collective followed by the adolescents; however health concern should not be expected in the population groups.

5.2 Introduction

Aflatoxins B_1, B_2, G_1 and G_2 are mycotoxins that can be mainly produced by moulds of *Aspergillus* species, like *A. flavus*, *A. parasiticus* and *A. nomius*, and can occur in a wide range of important raw food commodities including cereals, nuts, spices, figs and dried fruits (Malone et al., 2000; Otta et al., 2000).

Aflatoxin B_1 (AFB_1) is the most carcinogenic mycotoxin known and there is evidence from human studies that aflatoxins are major risk factors for hepatocellular carcinoma, therefore classified in group 1 by International Agency for Research on Cancer (IARC, 2002; Wang and Tang, 2004; World Health Organization, 1998).

The maximum level allowed in Europe for AFB_1 and total aflatoxins (AF_S) is 2 and 4 μg kg^{-1} respectively in processed cereals, groundnuts, dried figs and processed products intended for direct human consumption or as an ingredient in foodstuffs; 8 and 10 μg kg^{-1} in pistachios and almonds; 5 and 10 μg kg^{-1} in several spices, including paprika. Concerning baby food products, a maximum level of 0.1 μg kg^{-1} was established for AFB_1 (European Commission, 2010).

Occurrence of AFs in foods from Spanish market has been previously reported in several studies where corn-based products and other cereals, pulses, dried fruits and nuts, snacks, breakfast cereals, bread, herbs or spices were analysed (Sanchis et al., 1986, 1995; Santamarina et al., 1986; Jiménez et al., 1991; Blesa et al., 2004), nevertheless, exposure assessment of Spanish population has not been conducted yet.

The main problem to assess mycotoxins intake is related to few representative available dietary data regarding food bearing mycotoxins contamination. There are some methods developed to assess dietary intake overall known as market basket, 24-hour dietary recall and food record methods, food-frequency methods or dietary history. Given that corn-based products consumption is considered as sporadic or casual, food-frequency methods should be chosen because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998).

Risk characterization is the estimation of the severity and probable occurrence or absence of known and potential adverse health effects on an exposed population (Kuiper-Goodman, 1995). Evaluation of toxicological data carried out by the Scientific Committees of the European Community results commonly in the estimation of a tolerable daily intake (TDI) derived from the No-Observed-Adverse-Effect-Level (NOAEL) from an animal study, applying a 100-fold uncertainty factor. However, the NOAEL approach is not appropriate for genotoxic carcinogens, because no threshold can be assumed in this case. Alternatively, the margin of exposure (MoE), defined as the ratio between a point on the dose-response curve for the adverse effect and human intake, has been proposed to characterise the risk of these contaminants. Mathematical analysis of the dose-response data from animal bioassays can be used to define the intakes necessary to produce a given level of response, such as 10% or 25% cancer incidence. The most commonly used methods are the T25 approach (chronic daily dose which give tumours in 25% of the animals above background at a specific tissue site) and BMD_{10} approach (the 95% lower confidence interval on a benchmark dose (BMD) for a 10% increase in tumour incidence determined by fitting dose-response data to various mathematical models) (EFSA, 2005, 2007).

In the framework of the Project to Assess the Exposure of Catalonian Population to the Mycotoxins, and following the line of our previous studies (Cano-Sancho et al., 2009, 2010, 2011a,b), the main objective of this study was to assess the exposure of Catalonian population to aflatoxins combining raw contamination and consumption data. Thus, two sub-objectives were considered: 1) to assess the occurrence of aflatoxins in food marketed in Catalonia; and 2) to assess the consumption of those foods susceptible to aflatoxin contamination by Catalonian population.

5.3 Materials and methods

5.3.1 Samples

Food samples were selected to be the most susceptible commodities to aflatoxin contamination and to be commonly consumed in Catalonia. Between 2008 and 2009,

samples of peanuts (n=212), pistachios (n=169), dried figs (n=49), sweet corn (n=181), breakfast cereals (n=167), corn snacks (n=213), dried red pepper (n=165) and baby food (n=154) were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of AFs was determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store if were available (12 cities x 6 stores / city = 72 samples / category). However, in some cases, 3 items were not available in the same store, thus, less than 72 composites could be obtained. In case case of the 49 dried figs samples, 18 gluten-free foods (mainly maize-based bread and pasta) and 35 ethnic food samples, such as Mexican tortillas, corn flour or cuscus, were analysed individually. Regarding brands, we finally obtained forty-seven brands of peanut, twenty-three brands of pistachio, thirty-five of dried figs, thirty-one of sweet corn, sixty-two of breakfast cereals, seventy-nine of corn snacks, forty of red pepper and twenty-one of baby food, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of Spanish market. The samples were transported and stored in freeze at -20 ºC until analysis.

5.3.2 Analytical method

Easi-extract® Aflatoxin immunoaffinity cleanup columns (R-Biopharm, Rhône LTD Glasgow, UK) were used to extract AFB_1, AFB_2, AGB_1 and AFG_2 from all analytical samples. 10 g of homogenized composite was mixed with 15 mL of extractant solution (60 % acetonitrile, 40 % water) for 20 minutes and filtered with a Whatman Nº4 paper filter. 10 mL of filtered solution was diluted with 48 mL of phosphate buffer solution (PBS; 0.8 % NaCl, 0.12 % Na_2HPO_4, 0.02 % KH_2PO_4, 0.02 % KCl) and drained through the immunoaffinity column. After this, the columns were washed with 20 mL of PBS and AFs eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q

water. Fluorescent derivatives of AFs were obtained using a post-column photochemical derivatizator instrument (UVE™ Derivatizer LC Tech, Germany). Chromatography equipment consisted of a separations module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5μm ODS2, 4.6 x 250 mm, Multi λ Fluorescence Detector Waters 2475®. Excitation wavelength was 365 nm, and emission wavelength at 0-13 min and 13-25 min were 455 and 425 nm, respectively. Mobile phase consisted in a solution of water, methanol and acetonitrile (70:17:17). Aflatoxin concentration was expressed in μg of aflatoxin per kg of each assayed product.

5.3.2.1 Validation of the method

Recovery data, repeatability, limit of detection (LoD) and limit of quantification (LoQ) are shown in Table 5.1 for some matrices according to the performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission, 2006). The analytical method used for AFs was assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 100 μl of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations.

Precision and recovery were established by determination of AFB_1, AFB_2, AFG_1 and AFG_2 levels, spiked in each food category by triplicate. Recovery was determined by comparing the absolute responses of each AF, with the absolute responses of calibration standards. It ranked between 72±6 and 118±13 % for AFB_1, 70±1 and 100±6 % for AFB_2, 74±2 and 108±1 % for AFG_1 and between 61±9 and 100±6 % for AFG_2. Repeatability was expressed as relative standard deviation (RSDr), it ranged between 4 and 20 % in the cereal-based matrix and between 5 and 20 % in the dried fruit matrix. The limit of detection (LoD) was considered to be three fold the signal of blank noise, and the limit of quantification (LoQ) was considered equal to 3×LoD. The mean limit of detection (LoD) was 0.03 μg kg^{-1} for AFB_1 and AFG_1, and 0.008 for AFB_2 and AFG_2 μg kg^{-1}.

Table 5.1. Summary of the method performance characteristics for aflatoxin G_1, B_1, G_2 and B_2

	Matrix	LoD/LoQ	Spiking level	n	Recovery	RSDr
		μg kg^{-1}	μg kg^{-1}		(%)	(%)
AFG_1	Cereal-based foods	0.033/0.1	0.8-1.7	5-7	108±1-80±7	9-12
	Dried fruits		0.4-4	5-6	74±1-98±2	13-20
AFB_1	Cereal-based foods	0.033/0.1	0.8-3.3	5-6	72±6-84±7	8-11
	Dried fruits		0.4-4	5-6	96±2-118±1	5-15
AFG_2	Cereal-based foods	0.008/0.025	0.2-0.7	5-7	61±9-91±4	4-15
	Dried fruits		0.4-1	5-6	88±9-100±6	6-10
AFB_2	Cereal-based foods	0.008/0.025	0.2-0.8	5-7	70±1-100±2	9-20
	Dried fruits		0.4-1	5-6	88±9-100±6	6-10

5.3.3 Treatment of left-censored data

In the present study we have applied two different methods to obtain a mean level of contamination for each food dataset, taken into account the left-censored data.

4. Substitution method. Non detected samples were assumed to be the value of the LoD divided by 2. This is the most commonly used procedure in exposure assessment of populations to mycotoxins, thus we used this approach as starting point.

5. Non-parametric method: Kaplan-Meier estimation (KM). This is the standard non-parametric technique for censored data, based on the empirical cumulative distribution function. We address the readers to the report of Tressou (2006) to better understand the mathematic basis of the KM estimator, and its application in food risk assessment studies. With the KM method, the weight of the censored data is distributed over the different observed values below the censoring values. In order to obtain the mean and standard deviation of each contamination

dataset, we have applied the syntax to fit the KM model based on the LIFETEST procedure from SAS® also included in the Appendix G in EFSA report (2010).

5.3.4 Dietary intake assessment

In this study, food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. According to World Health Organization (WHO) advices, studies to assess dietary intake of chemical contaminants should show the significant intake among standard population, with all population groups that could have different dietary patterns. Therefore, the population groups considered were: infants (0-3 years), children (4-9), adolescents (10-19 years), adult males (20-65 years), adult females (20-65 years), elders (>65 years), immigrants (17-51 years), celiac sufferers (16-75 years). FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins, excluding those foods not consumed in the region. Concerning frequency of consumption, 5 response options were considered (never, annually, monthly, weekly and daily). Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 79 elders, 70 celiac sufferers, 56 immigrants, 336 adult males, 384 adult females, 235 adolescents, 69 children and 164 infant parents were interviewed during 2008 and 2009 in Lleida region (n=1393) by trained interviewers.

5.3.5 Exposure assessment

In this section we recall some general aspects concerning the exposure calculations, already given in Gauchi and Leblanc (2002).

If we assume independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, we can estimate the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j$$

where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff *j* in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food.

Under these assumptions we can also calculate the variance estimate $\hat{V}(E_{\pi_0})$ using Eq. (5) and Eq. (6) in Gauchi and Leblanc (2002). We could consider this approach to be a theoretical approach, but because of the postulated assumptions, it would have to be a (very) simplified theoretical approach. For example, this simplified approach does not enable us to calculate complicated statistics such as high quantiles. Simulation methods would therefore be required to obtain estimates of these statistics. Finally, we will consider this approach to be the direct approach.

Simulation method

Stochastic procedure used is adapted from the Mixed Parametric (MP) method reported extensively in Gauchi and Leblanc (2002), because some advantages against non parametric methods were clearly elucidated.

The appearance of the consumption histograms was clearly irregular, especially for those foods whose consumption is seasonal or sporadic, such as pistachios or peanuts. Therefore, the histograms could be divided in two subclasses (non consumers and consumers), and fitting a mixed distribution as follows:

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

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 $c_{i_{\min}(\pi_0),j}$ the minimal consumption of the foodstuff j, in the sample π_0 (this part corresponds to the non consumer's class). $\left[\Gamma(r,\lambda,\theta)\right]_{\pi_{0,j}}$ is the Gamma 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* 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 $\left[\Gamma(r,\lambda,\theta)\right]_{\pi_{0,j}}$.

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

$$\hat{E}_k = \sum_{j=1}^{p} \tilde{c}_{k(\hat{F}_{U,j}),j} \bar{t}_j$$

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 density function (*cdf*) adjusted of the distribution consumption defined above, and $\bar{t}_j$ is the mean of AFs contamination for the foodstuff *j*, computed through the substitution method or KM method.

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 (10,000 in the present study):

$$\hat{E}_S = \frac{1}{n} \sum_{k=1}^{n} \hat{E}_k$$

Other statistics were directly computed on the histogram built with the simulations of the *S* set, and statistics were also estimated from the lognormal and gamma fitted probability density functions (*pdf*s) (results not shown).

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 10,000). The boundaries of the 95% confidence interval are calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution.

5.3.6 Risk characterization

Following the EFSA advices to carry out the risk assessment of substances which are both genotoxic and carcinogenic, we have built the MoEs by division of the human $BMDL_{10}$ of 870 ng kg^{-1} bw day^{-1} (EFSA, 2007) by the exposure estimates for the population collectives from Catalonia.

The incidence of liver cancer in a population attributable to intake of AFs is derived by combining aflatoxin risk potency estimates and aflatoxin exposure estimates as follows:

Population risk = Exposure x Risk Potency

Taken into account that Catalonia is a region with a natural low occurrence of AFs and with a population having a small prevalence of carriers of hepatitis B, the risk potency was calculated as follows, assuming the 1% of population to be carriers of hepatitis B (JECFA, 1998):

0.01 x 99% + 0.3 x 1% = 0.013 cancers/years per 100,000 persons

5.4 Results

5.4.1 Occurrence of aflatoxins

Aflatoxins B_1, B_2, G_1 and G_2 were analysed in a total of 603 analytical samples of peanuts, pistachios, dried figs, sweet corn, breakfast cereals, corn snacks, red pepper, free-gluten food, ethnic food and baby food. Results of AF_S as sum of aflatoxin $B_1+B_2+G_1+G_2$ are shown in Table 2.

Table 5.2. Occurrence of total Aflatoxins (AFs) in food items marketed in Catalonia (Spain)

	n Individual	**n *analytical***	**positives**	**mean±sd*** **µg kg^{-1}**	**max** **µg kg^{-1}**
Peanuts	212	72	8	2.7±3.0	7.7
Pistachios	169	70	14	8.9±28.7	108.3
Dried figs	49	49	1	0.62	0.6
Sweet corn	181	71	2	0.9±0.1	1.0
Breakfast cereals	167	72	1	0.5	0.5
Corn snacks	213	72	1	0.8	0.8
Red pepper	165	72	41	2.2±1.9	8.8
Baby food	154	72	0	-	-
Ethnic food	35	35	3	8.2±5.4	14.2
Gluten-free food	18	18	0	-	-

* Mean±standard deviation of positive samples

Highest percentages of positive samples were found in red pepper, pistachios and peanuts (57, 20 and 11 %, respectively), but they were found in a low percentage of samples from the other categories, and not found in any sample of baby food nor gluten-free samples. Highest mean levels, calculated from positive samples, were found in pistachios and ethnic foods, followed by peanuts and red pepper. Three ethnic food samples, two samples of peanuts and one of pistachios were above the EU permitted levels of AF_S.

5.4.2 Exposure assessment

Results of exposure assessment obtained from the direct method, using the substitution and KM method, are shown in the Table 5.3 for each population group with the exception of infants and elders because not enough data was available to be computed.

Table 5.3. Results of exposure using the direct approach ($\hat{E}_{\pi_0} \pm \hat{V}(E_{\pi_0})$) (units in ng kg^{-1} bw day^{-1})

	Substitution	KM method
Children	0.105 ± 0.126	0.033 ± 0.044
Adolescents	0.178 ± 0.474	0.245 ± 0.822
Adult males	0.072 ± 0.167	0.098 ± 0.290
Adult females	0.077 ± 0.208	0.094 ± 0.330
Celiac sufferers	0.086 ± 0.085	0.086 ± 0.104
Immigrants	0.079 ± 0.102	0.276 ± 0.368

The highest estimations were commonly found by means of the KM method, with the exception of children and celiac sufferers. The most exposed groups were the adolescents and immigrants, estimated using the substitution and KM method, respectively. Widest differences among methods were found for immigrants, but narrow estimations were found for celiac sufferers or adult females.

Results of exposure estimates from the stochastic method are summarized in the Table 5.4. The parameters shown are the mean, standard deviation, skewness and kurtosis, and the percentiles 50, 90 and 95. Between brackets are the bootstrap confidence intervals for each parameter.

Table 5.4. Results of exposure assessment of the population groups from the simulation method. These results were obtained directly from the 10,000 simulation outputs without pdf fittings (units in ng kg^{-1} bw day^{-1})

Children

	Substitution	***Kaplan-Meier***
N	10,000	10,000
Mean	0.106 [0.090; 0.123]	0.033 [0.028; 0.386]
SD	0.113 [0.092; 0.137]	0.036 [0.028; 0.045]
Skewness	1.992	2.281
Kurtosis	5.588	7.840
Median	0.070 [0.054; 0.087]	0.022 [0.017; 0.027]
0.90^{th} quantile	0.255 [0.205; 0.307]	0.0 79 [0.062; 0.096]
0.95^{th} quantile	0.337 [0.265; 0.402]	0.106 [0.082; 0.129]

Adolescents

	Substitution	***Kaplan-Meier***
N	10,000	10,000
Mean	0.189 [0.146; 0.238]	0.252 [0.177; 0.337]
SD	0.316 [0.211; 0.434]	0.549 [0.357; 0.764]
Skewness	4.003	4.301
Kurtosis	23.092	25.869
Median	0.074 [0.057; 0.095]	0.042 [0.030; 0.059]
0.90^{th} quantile	0.488 [0.341; 0.674]	0.755 [0.452; 1.096]
0.95^{th} quantile	0.788 [0.518; 1.099]	1.314 [0.809; 1.865]

Adult male

	Substitution	***Kaplan-Meier***
N	10,000	10,000
Mean	0.135 [0.108; 0.164]	0.203 [0.073; 0.128]
SD	0.192 [0.148; 0.239]	0.226 [0.130; 0.257]
Skewness	2.562	2.246
Kurtosis	8.698	7.058
Median	0.056 [0.037; 0.078]	0.126 [0.017; 0.033]
0.90^{th} quantile	0.376 [0.281; 0.479]	0.497 [0.196; 0410]
0.95^{th} quantile	0.532 [0.393; 0.671]	0.675 [0.314; 0.641]

Adult female

	Substitution	***Kaplan-Meier***
N	10,000	10,000
Mean	0.078 [0.061; 0.097]	0.095 [0.068; 0.127]
SD	0.121 [0.084; 0.164]	0.201 [0.129; 0.280]
Skewness	3.680	4.401
Kurtosis	20.068	26.570
Median	0.036 [0.025; 0.047]	0.021 [0.014; 0.031]
0.90^{th} quantile	0.196 [0.145; 0.258]	0.269 [0.166; 0.381]
0.95^{th} quantile	0.299 [0.207; 0.417]	0.463 [0.284; 0.699]

Immigrants

	Substitution	***Kaplan- Meier***
N	10000	10000
Mean	0.048 [0.041; 0.055]	0.209 [0.173; 0.248]
SD	0.045 [0.038; 0.054]	0.260 [0.207; 0.316]
Skewness	1.675	2.155
Kurtosis	4.322	6.399
Median	0.036 [0.028; 0.044]	0.114 [0.778; 0.154]
0.90^{th} quantile	0.107 [0.089; 0.125]	0.552 [0.435; 0.666]
0.95^{th} quantile	0.136 [0.110; 0.163]	0.732 [0.573; 0.916]

Celiac sufferers

	Substitution	***Kaplan-Meier***
N	10000	10000
Mean	0.203 [0.171; 0.237]	0.295 [0.243; 0.351]
SD	0.226 [0.179; 0.277]	0.372 [0.293; 0.457]
Skewness	2.246	2.323
Kurtosis	7.058	7.396
Median	0.126 [0.099; 0.157]	0.158 [0.112; 0.208]
0.90^{th} quantile	0.497 [0.382; 0.601]	0.780 [0.591; 0.955]
0.95^{th} quantile	0.675 [0.514; 0.803]	1.076 [0.816; 1.294]

The results found through the simulation method were close to those obtained by the direct method for children, adolescents and adult females, but they were highest for adult males, celiac sufferers and immigrants. If we compare the methods to manage the left-censored data, the highest estimates were also found through the KM method, with exception of children. The most exposed group was the celiac sufferer collective, followed by the adolescents with respective mean estimates of 0.203 and 0.189 ng kg^{-1} bw day^{-1} from substitution method, and 0.295 and 0.252 ng kg^{-1} bw day^{-1} from the KM method. Pseudo-parametric bootstrap confidence intervals permit us to check the accuracy and reliability of related parameters. While each estimation was included between the upper and lower bound of the confidence interval, in case of adult males through KM method, several parameters where out of this range. Thus those parameter estimates should be considered unreliable. Another point to highlight is the increasing width of the intervals for the high percentiles, a well-known drawback to get accurate estimations of these parameters.

5.4.3 Risk characterization

The MoEs were calculated dividing the $BMDL_{10}$ of 870 ng kg^{-1} bw day^{-1} by the average and percentile 95 of the exposure estimates from substitution and KM method (Table 5). The lowest margins were estimated for celiac sufferers and adolescents from KM method (2,949 and 3,452 respectively). Highest estimations were computed for children through the KM method and immigrants by substitution with respective values of 26,364 and 18,125.

The cancer incidence attributable to AFs was calculated using the cancer potency of 0.013 cancers/year per 100,000 persons (Table 5.5). The incidence ranged between 0.000 and 0.004 cancers/year per 100,000 persons for children and celiac sufferers respectively, estimated from the KM method.

Table 5.5. Margins of exposure and cancer incidence attributable to aflatoxins estimated through the substitution method and Kaplan-Meier method (cancer/ year per 100,000 habitants). The MoEs were estimated by division of the $BMDL_{10}$ of 870 ng kg^{-1} bw day^{-1} by the exposure estimates

	Substitution			Kaplan-Meier		
	MoEs		Incidence	MoEs		Incidence
	mean	P95		mean	p95	
Children	8,208	2,582	0.001	26,364	8,208	0.000
Adolescents	4,603	1,104	0.002	3,452	662	0.003
Adult males	11,154	2,910	0.002	9,158	1,879	0.003
Adult females	6,444	1,635	0.001	4,286	1,289	0.001
Celiac sufferers	4,286	1,289	0.003	2,949	809	0.004
Immigrants	18,125	6,397	0.001	4,163	1,189	0.003

5.5 Discussion

5.5.1 Occurrence of aflatoxins

Earlier studies performed in peanuts from the Spanish market by thin layer chromatography showed similar incidence values to our study with percentages of positive samples ranging from 1.5 to 10.8 % and contamination levels close to 5 μg kg^{-1}, with the exception of several cases with highest contamination level of AFB_1, up to 120 μg kg^{-1} in peanuts in-shell (Sanchis et al., 1986). Subsequent surveys carried out in Spain with pistachios showed percentage of positives samples from 19 to 59 % and variable AFB_1 contamination levels: from 0.12-0.29 μg kg^{-1} as reported by Ariño et al. (2009), to 0.57-98.5 μg kg^{-1} closer to our results (Burdaspal and Legarda, 1998; Burdaspal et al., 2005; Fortuny et al., 2007).On the other hand, the AFs have not commonly been detected in cereal-based foods, like corn flakes or corn snacks (Sanchis et al., 1986, 1995; Santamarina et al., 1986; Blesa et al., 2004), with the exception of a recent study, where 12 out of 46 breakfast cereal samples were found above the LoQ (0.2 μg kg^{-1}), the maximum level being 0.13 μg kg^{-1} (Ibañez-Vea et al., 2011). One study has recently

reported the presence of AFs in paprika, with 59 % of positive samples (n=64) and a maximum value of 7.3 μg kg^{-1}, close to our results (57% and 8.8 μg kg^{-1}) found in red pepper (Santos et al., 2010).

When our results were compared with studies conducted out of Spain, very high levels have been found in pistachios, up to 2000 μg kg^{-1} in samples from Swedish market or 1450 μg kg^{-1} from Morocco (Thuvander et al., 2001; Juan et al., 2008). Also, highest levels of AFs in red pepper and paprika were found in other countries as Turkey or Morocco with quite high percentages of positive samples (20-100 %) and maximum contamination level of 154 μg kg^{-1} (Zinedine et al., 2006; Bircan et al., 2008). Concerning dried figs, previous studies have been conducted commonly in Turkey, as main producer and export country, or Brazil and Morocco. These studies showed variable occurrence of AF_S, up to 30 % of positive samples (Morocco) (Juan et al., 2008) with very high maximum values in exceptional cases (76,000 μg AFB_1 kg^{-1}) and >180,000 μg AFB_1 kg^{-1} in Turkey (Steiner et al., 1993) or 1,500 μg AFB_1 kg^{-1} in Brazil (Iamanaka et al., 2007). A four year survey (2003-2006) conducted in Turkey with dried figs intended to be exported to the European Union (EU), showed a percentage of samples above 4 μg AFB_S kg^{-1} of 2.6, 3.0, 5.1 and 2.7 %, respectively (Senyuva et al., 2007). AFs have commonly not been detected in corn flakes, despite of several exceptions at very low levels of contamination (Candlish et al., 2000) and an exception from Egypt market that reported high levels of contamination, 5-35 μg AFB_1 kg^{-1} (El-Sayed et al., 2003). Although very low levels of AFB_1 are permitted in baby foods by EU legislation (0.10 μg kg^{-1}), few surveys have reported the incidence of this mycotoxin in baby foods. Hernández-Martínez et al. (2010) found 66 out of 91 baby food samples from Spain to be above de LoD, and 7 samples exceeding the EU limit of 0.10 μg kg^{-1}, reaching values up to 3.11 μg kg^{-1}. In UK, AFB_1 was found in one sample of muesli baby food at 0.05 μg kg^{-1} (Food Standards Agency, 2004) and one sample (n=27) from Portugal at 0.009 μg kg^{-1} (Alvito et al., 2010). In The First French Total Diet Study, the authors reported

that AFs were not found in any composite sample analysed (n = 78), the LoQ being 0.1 μg kg^{-1} for each aflatoxin (Leblanc et al., 2005).

5.5.2 Exposure assessment

The low or sporadic consumption of those foods susceptible of AFs contamination, and the commonly low occurrence of AFs in the European countries, make the computation of exposure assessment studies more inaccurate and sometimes unreliable. Therefore, more sophisticated methods to manage left-censored data and simulation methods to combine contamination and consumption vectors, will be need to better estimate the exposure and measure its precision. Despite AFs are highly carcinogenic food contaminants, there are few studies accurately reporting the exposure of European population to this mycotoxin. These studies are mainly deterministic and dietary intake data was frequently provided by unspecific national surveys. The main contributors to the total aflatoxin intake should be expected peanuts and pistachios for adult population, while breakfast cereals and corn snacks were the most important contributors for adolescents and children. Despite the high incidence of aflatoxins in red pepper, this commodity was not considered in the exposure assessment because it is used in Spain as minor ingredient, and therefore not included in the dietary intake study.

Our results are in the line of those reported in The First French Total Diet Study where values of AFs dietary intake of 0.117 and 0.323 ng kg^{-1} bw day^{-1} for adults and children mean consumers, and 0.345 and 0.888 ng kg^{-1} bw day^{-1} for adults and children high consumers (Leblanc et al., 2005). Moreover, similar results were showed for Australians, 0.15 ng kg^{-1} bw day^{-1} or for Americans, 0.26 ng kg^{-1} bw day^{-1} (JECFA, 1998). Reports by members of European Union (SCOOP, 1996) showed estimated dietary exposure for Europeans to AFB_1 of 0.03 to 1.28 ng kg^{-1} bw day^{-1}, in the line of ours results, too. Nevertheless, Thuvander et al., (2001) estimated slightly higher values than ours for Swedish population, with estimates of 0.8 and 2.1 ng kg^{-1} bw day^{-1} for adults mean and percentile 95, respectively. The exposure of Spanish infant population to AFs through infant formulas was recently reported by Hernández-Martínez et al., (2010), identifying significant differences among organic and conventional food consumers.

Those values ranged between 0.08-0.94 ng kg^{-1} bw day^{-1} for conventional food consumers and 0.17-37.47 ng kg^{-1} bw $day^{-1}$for organic food consumers.

On the other hand, the highest values were estimated for Chinese population with a range from 0 to 91 ng kg^{-1} bw day^{-1} (JECFA, 1998) or Koreans with AFB_1 dietary intake of 1.19 to 5.79 ng kg^{-1} bw day^{-1} (Park et al., 2004).

5.5.3 Risk characterisation

The MoEs built in the present study are in the line of those estimations reported by EFSA (2007) for the European countries. Considering our worst scenario (percentile 95), the range was between 662 and 8,208 while the ranges provided by the EFSA were 1,266-2,472 and 450-1,038 for the cluster F and B, respectively.

The cancer incidence attributable to the AFs is also in the line of those estimations of EFSA who estimated the ranges of 0.004-0.007 and 0.002-0.009 cancer/year per 100,000 habitants, while our values were found between 0.001 and 0.004 for the adult populations. These values are higher than those estimates computed in Japan, where the incidence estimated was between 0.00004 and 0.00005 cancer/year per 100,000 habitants (Sugita-Konishi et al., 2010).

5.6 Conclusions

To sum up, the highest percentages of positive samples were found in red pepper, pistachios and peanuts (57, 20 and 11 %, respectively), but not found or found in few samples from the other categories. Highest mean levels, calculated from positive samples, were found in pistachios and ethnic foods, followed by peanuts and red pepper. Three ethnic food samples, two samples of peanuts and one of pistachios were above the EU permitted levels

Considering our results the most exposed group should be the celiac sufferer collective, followed by the adolescents. The MoEs built in the present study are in the

line of those estimations reported by EFSA (2007) for the European countries, the worst case scenario ranging between 662 and 8,208. The cancer incidence attributable to AFs was ranged from 0.000 to 0.004 cancers/year per 100,000 people for children and celiac sufferers respectively.

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Chapter 6. Occurrence and exposure assessment to aflatoxin M_1 in Catalonia

Revista Iberoamericana de Micología 2010 27, 130-5

6.1 Abstract

The objectives of this study were i) to determine the occurrence of AFM1 in the main dairy products consumed in Catalonia region (Spain), and ii) to assess the exposure of Catalonian population to aflatoxin M_1 through deterministic and probabilistic method. Occurrence of Aflatoxin M_1 (AFM_1) was determined in 72 composites of milk, 72 composites of cheese and composites of yoghourt from Catalonia. Composite samples consisted of 3 individual samples which came from same store. The samples were purchased from 6 supermarkets or hypermarkets from 12 different cities, which accounted for 72 % of the Catalonian population. AFM_1 content was analyzed using an Enzyme-Linked ImmunoSorbent Assay commercial kit. AFM_1 was quantified in 94.4 % (68/72) of whole UHT milk samples above the level of quantification (LOQ) of 5 ng/kg, in 2.8 % (2/72) of yoghourt samples, and not quantified in cheese samples above LOQ of 25 ng/kg. The maximum level was detected in one yoghurt sample with 51.58 ng/kg, only this sample being over the legal EU limit of 50 ng/kg. Milk, cheese and yoghourt mean concentrations were 9.29 ± 2.61, <12.5 and 13.22 ± 4.82 ng/kg, respectively. Three approach to exposure assessment was conducted: one deterministic method and two probabilistic models with Monte Carlo simulations (non parametric model and parametric model using gamma distributions). According to these values, should be expected Catalonian population is not exposed to a significant risk from aflatoxin M_1 including average and high consumers.

6.2 Introduction

Aspergillus flavus, A. parasiticus and the rare *A. nomius* (Rustom, 1997). Aflatoxin M_1 (AFM_1) is the main monohydroxylated derivative of aflatoxin B_1 (AFB_1) formed in liver by means of cytochrome p450-associated enzymes. Mammals that ingest AFB_1 contaminated diets excrete amounts of the principal 4-hydroxylated metabolite AFM_1 into milk (Prandini et al., 2009). The results of studies on heat processing and storage at low temperature of dairy products affecting the amount of AFM_1 indicate that such processes do not cause an appreciable change in the amount of AFM_1 in these products. Manufacture of fermented dairy products, concentration and drying of milk do not affect AFM_1 recovery, however manufacture of cheese can be an enrichment factor (JECFA, 2001; Yousef and Marth, 1989).

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic. Although AFM_1 is less toxic than AFB_1, it has been classified as a possible human carcinogen, Group 2B agent by International Agency for Research on Cancer (IARC) (IARC, 1993). The European Commission has determined the legal limit for AFM_1 in raw milk, treated milk and dairy products at 50 ng/kg, except for infant formulae, infant milk and special food products, which should be under 25 ng/kg (European Commission, 2006).

Occurrence of aflatoxin M_1 in milk and cheese samples from Spanish market has been previously reported in several studies, however, exposure assessment of Catalonian population to this contaminant had not been performed until now (Barrios et al., 1996; Blanco et al., 1988; Burdaspal et al., 1983; Díaz et al., 1995; López-Díaz et al., 1995; Rodríguez et al., 2002).

The current exposure assessment schemes, based on the combination of the mycotoxin occurrence data with dietary intake data of these products, are largely deterministic and uncertainty and/or variability issues are accounted for by means of cautionary measures which are implicitly embedded in calculation schemes and rules (Verdonck et al., 2006). Due to the difficulties of estimating exposure from punctual data, several techniques like probabilistic modelling have been developed to overcome the sporadic nature of consumption and variability in contamination levels. Probabilistic

modelling achieved through Monte-Carlo simulations accounts for every possible value that each variable could take and weighs each of them by its probability of occurrence (Council et al., 2005). Structure of a probabilistic model allows taking the variability of input data into account, which provides more realistic results than that produced by simple deterministic scenarios (Vose, 2000).

Risk characterization is the estimation of the severity and probable occurrence or absence of known and potential adverse health effects on an exposed population (Kuiper-Goodman, 2007). Evaluation of toxicological data carried out by Joint Expert Committee on Food Additives (JECFA) results commonly in the estimation of a tolerable daily intake (TDI). This hazard assessment approach does not apply for toxins where carcinogenicity is the basis for concern, as is the case with aflatoxins. In the risk management of genotoxic carcinogens, no threshold is presumed and it is recommended that levels of such substances should be as low as technologically feasible or, as JECFA recommends, as low as reasonably achievable (ALARA) (Egmond and Jonker, 2005; Kuiper-Goodman, 1995). Despite international expert committees (EFSA, 2004; JECFA, 1999, 2001; SCF, 1994) did not specify a numerical TDI for aflatoxins, it was established that a level of aflatoxins <1 ng/ kg bw/day does not contribute to the risk of liver cancer. This value was previously used as a TDI by Leblanc et al. (2004) in Total Diet Study of French population.

The objectives of this study were i) to determine the occurrence of AFM_1 in the main dairy products consumed in Catalonia, and ii) to assess the exposure of Catalonian population to aflatoxin M_1 through deterministic and probabilistic method.

6.3 Materials and methods

6.3.1 Samples

The main dairy products consumed in Spain are milk, cheese and yoghourt (MAPA, 2006). During the months of June and July 2008, two-hundred and eleven whole UHT milk samples, two-hundred and sixteen cheese samples and two-hundred

and two natural yoghourt samples, were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of AFM_1 was determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store if were available (12 cities x 6 stores / city = 72 samples / category). However, in some cases, 3 items were not available in the same store. Cheese composites were formed with grated, fresh and semi-dried cheese samples. Cheese was made with different types of milk (cow, sheep, goat or mixtures of these species). In total, thirty-six milk brands, seventy cheese brands and twenty-one yoghourt brands were purchased, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of the Spanish market. The samples were transported and stored under suitable conditions until analysis.

6.3.2 ELISA kit

AFM_1 was determined by competitive ELISA method RIDASCREEN ® Aflatoxin M_1 30/15 nº R1111 (Ridascreen®, R-Biopharm AG, Darmstadt, Germany) according to the procedure described by R-Biopharm GmbH. Reagents used were contained in the RIDASCREEN AFM_1 test kit, which included a microtiter plate coated with capture antibodies, AFM_1 standard solutions (1.3 ml each 0 ng/kg, 5 ng/kg, 10 ng/kg, 20 ng/kg, 40 ng/kg and 80 ng/kg), peroxidase conjugated AFM_1, substrate (urea peroxidase), chromogen (tetramethylbenzidine) and stop solution (1 N sulphuric acid). Methanol, n-heptane and dichloromethane used were provided by Merck. Phosphate Buffer Solution (PBS) was prepared by mixing 0.55 g sodium dihydrogen phosphate hydrate with 2.85 g disodium hydrogen phosphate-2-hydrate and 9 g sodium chloride and filling up to 1000 ml with distilled water.

Samples preparation for analysis

Milk samples were centrifuged for skimming at 3500 rpm, at 10ºC, during 10 min, then upper cream layer was removed completely by aspirating through a Pasteur pipette. The skimmed milk was used directly in the test.

Ten grams of triturated and homogenised composite samples of cheese or yoghourt were weighed and 40 mL dichloromethane was added and extracted by shaking for 15 min on a blender. The suspension was filtered and 10 ml of the extract was evaporated at 60 ºC under a weak nitrogen stream. The oily residue was redissolved in 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml heptane and was mixed thoroughly. After centrifugation for 15 min at 2700 rpm, the upper heptane-layer was completely removed. Aliquot of the lower methanolic-aqueous phase was carefully poured off using a Pasteur pipette. One-hundred microliters of this aliquot were brought up to 10 % methanol content by addition of 400 µl Ridascreen buffer 1. 100 µl were used per well in the test.

ELISA test procedure

A sufficient number of microtiter wells were inserted into the microwell holder for all standards and samples. One-hundred microliters of standard solution and prepared samples were added in separate wells and incubated for 60 min at room temperature (20 ºC) in the dark. The liquid was removed from the wells and the microwell holder was tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Then the wells were washed twice with 250 µL of distilled water. One-hundred microliters of the diluted enzyme conjugate (peroxidase conjugated AFM_1) were added and incubated for 60 min at room temperature in the dark. The wells were again washed with 250 µL of distilled water as described above. In the next stage 50 µL of substrate (urea peroxidase) and 50 µL of chromogen (tetramethylbenzidine) were added to each well and mixed thoroughly and incubated for 30 min at room temperature in the dark. Then 100 µL of the stop reagent (1 N H_2SO_4) were added to each well and mixed, and the absorbance was measured at 450 nm in an ELISA reader (ELX-800, Bio-Tek Instruments, Winooski, VT, USA).

AFM_1 quantification

The samples were evaluated according to the Rida Soft Win computer program prepared by R-Biopharm. The absorption is inversely proportional to the AFM_1 concentration. The lower quantification limit was 5 ng/kg for milk and 25 ng/kg for cheese and yoghourt.

6.3.3 Dietary intake assessment

Main problem to assess mycotoxins intake is related to few representative available dietary data regarding food bearing mycotoxins contamination. There are some methods developed to assess dietary intake overall known as market basket, 24-hour dietary recall and food record methods, food-frequency methods or dietary history. Food-frequency methods should be chosen because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998). In this study, dairy food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. According to World Health Organization (WHO) advices, studies to assess dietary intake of chemical contaminants should show the significant intake among standard population, with all population groups that could have different dietary patterns. Therefore, four different population groups were considered for each sex: infants (4-9), teenagers (10-19 years), adults (20-65 years) and elders (>65). FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins studied, excluding those foods not consumed in the region. Concerning frequency of consumption, 5 response options ranging from never to annually were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 70 elders, 720 adults, 236 teenagers and 68 infant parents were interviewed during 2008 in Lleida region (n = 1094) by trained interviewers.

6.3.4 Exposure assessment

Three approaches were conducted to estimate aflatoxin M_1 dietary intake. On the one hand, deterministic methods were performed combining normalised milk daily intake (per body weight) with mean concentration of AFM_1 in milk, as follows: individual AFM_1 exposure (ng AFM_1/ kg body weight per day) = (daily food intake/ body weight) x (mean concentration of AFM_1 in food). Statistics as means, standard deviation and percentiles, were applied for age and sex groups. On the other hand, two probabilistic models were designed to run exposure simulations using our experimental data through Monte Carlo method. Probabilistic models were built under the principles of the NonParametric (NP) proposed by Gauchi et al. (2002) and another essentially parametric method. The NP method is proposed as a natural method of exposure assessment when consumption and contamination data are available. It is a completely nonparametric method where each normalized consumption profile of the survey is taken into account and consumed food is attributed a value of contamination randomly drawn from the available contamination data. Monte Carlo simulations were conducted with Microsoft Office Excel 2003® and Statgraphics Plus 5.1® for Gamma distribution and NP approach. Kolmogorov-Smirnov statistic was applied in Goodness-of-Fit test of contamination and intake distributions. Two thousand iterations were considered, in which consumption data and contamination levels were produced at random from the proposed distributions. Statistics as means, standard deviation and percentiles were applied for age groups, considering both sex together.

6.4 Results

6.4.1 Occurrence of AFM1 in milk and dairy products

Results of the occurrence of AFM_1 in dairy samples is presented in Table 6.1.

Table 6.1. Occurrence of AFM_1 in milk, cheese and yoghourt from Catalonian (Spain) market

	Analytical Samples	Original Samples	Positives / Total	Mean[a] *ng/kg*	SD[a] *ng/kg*	Max *ng/kg*	<LOQ	LOQ -30	30-50	>50
Milk[b]	72	211	68/72	9.69	2.07	13.61	4	68	0	0
Cheese[c]	72	216	0/72	-	-	-	72	0	0	0
Yoghurt[c]	72	202	2/72	38.34	18.73	51.58	70	1	0	1

[a] Calculated from positives samples
[b] LOQ = 5 ng/kg
[c] LOQ = 25 ng/kg

AFM_1 was quantified in 94.4 % (68/72) of whole UHT milk samples above the level of quantification (5 ng/kg) while this mycotoxin was not quantified in cheese and only in 2 of 72 samples of yoghurt (LOQ = 25 ng/kg). Despite the high incidence of AFM_1 in milk, mean levels were 9.69 ± 2.07 ng/kg, far from EU limits (50 ng/kg).

6.4.2 Exposure assessment of Catalonian population to AFM_1

Occurrence of AFM_1 in milk data set shows a low percentage of left censored data within milk samples (5.6%). In cases where there is less than 60% of censored values among the data, it is recommended to replace left censored data by the corresponding LOD or LOQ divided by 2 (GEMs/Food-WHO, 1995). In the other hand, cheese and yoghourt contamination data from our study present a large percentage of left censored data (100 and 97.2 %, respectively). In these two cases, the use of these estimate values could induce important bias into exposure assessment. Thus, only milk consumption data and AFM_1 occurrence in milk data were considered in the exposure assessment, under assumption that those milk samples under LOQ was 0.5LOD.

Table 6.2. Exposure assessment of Catalonian population to AFM_1 through milk using deterministic method

				Mean Consumers*		High Consumers (percentile 95)	
		n cons	Mean Weight Kg	Milk Intake kg/day	AFM1 Intake ng/kg b.w./ day	Milk Intake kg/day	AFM1 Intake ng/kg b.w./ day
Infant 4-9	Male	30	27.24	0.487±0.298	0.182±0.109[a]	0.840	0.358
Infant 4-9	Female	34	23.45	0.572±0.227	0.236±0.103[b]	0.840	0.434
Teenager 10-19	Male	90	57.81	0.423±0.301	0.075±0.063[c]	1.120	0.175
Teenager 10-19	Female	119	51.30	0.390±0.344	0.074±0.071[c]	1.120	0.222
Adult 20-65	Male	201	80.83	0.305±0.216	0.036±0.027[cd]	0.750	0.081
Adult 20-65	Female	198	66.42	0.305±0.206	0.043±0.029[cd]	0.700	0.100
Elderly >65	Male	24	74.46	0.355±0.161	0.046±0.024[d]	0.560	0.079
Elderly >65	Female	25	72.20	0.457±0.188	0.060±0.028[d]	0.784	0.121

* Mean ± Standard Deviation

[a] Statistically significant differences (LSD test, $p<0.05$)

Values of exposition for consumer population through deterministic method are shown in table 6.2. Infants were the group with highest percentage of consumers (94.1 %) while adults were the group with lowest percentage of consumers (55.4 %). Adults (20-65) was the age group with lowest milk consumption and infants (4-9) was the group with highest milk consumption. Considering our results infants was the main risk group exposed to aflatoxin M_1, with statistically significant differences between sex (LSD test, $P<0.05$), being females the most exposed group. Highest values were found in high percentiles, for example percentile 95 of infants, who showed exposures of 0.358 and 0.434 ng/hg b.w./day by male and female, respectively. In any case, values were above the safe level of 1 ng/kg bw/day.

Table 6.3. Values of AFM_1 intake by Catalonian population estimated through and deterministic probabilistic model

		Mean± SD[a]	Var[b]	p50	p75	p90	p95	p99
4-9 years	Deterministic[c]	0.211±0.109	0.012	0.194	0.263	0.381	0.426	0.462
	NP-NP[d]	0.218±0.133	0.016	0.199	0.292	0.394	0.484	0.602
	Gamma	0.202±0.143	0.024	0.171	0.269	0.375	0.467	0.687
10-19 years	Deterministic[c]	0.074±0.068	0.005	0.053	0.094	0.150	0.202	0.359
	NP-NP[d]	0.072±0.073	0.005	0.055	0.084	0.154	0.208	0.369
	Gamma	0.075±0.083	0.007	0.049	0.099	0.170	0.229	0.405
20-65 years	Deterministic[c]	0.039±0.028	0.001	0.035	0.047	0.074	0.094	0.138
	NP-NP[d]	0.039±0.032	0.001	0.034	0.050	0.080	0.104	0.152
	Gamma	0.039±0.041	0.002	0.025	0.051	0.088	0.124	0.199
>65 years	Deterministic[c]	0.053±0.027	0.001	0.043	0.066	0.076	0.113	0.131
	NP-NP[d]	0.054±0.033	0.001	0.047	0.071	0.096	0.113	0.171
	Gamma	0.053±0.037	0.001	0.044	0.068	0.099	0.125	0.183

[a] Mean ± Standard Deviation
[b] Variance
[c] Deterministic approach considering males and females
[d] Non Parametric-Non Parametric approach

Consumption of AFM_1 was determined by JECFA in five regional diets. European regional consumption of milk and milk products was 340 g/person per day and AFM_1 intake was 6.8 ng/day in the European diet (JECFA, 2001). Similarly, in our study it ranged from 2.9 to 6.1 ng/person per day. The French Total Diet Study showed an estimated average intake of AFM_1 in the French population of 0.09 and 0.22 ng/kg bw/day for adults and children mean consumers, respectively. Despite the highest values of 0.21 and 0.55 ng/kg bw/day were reported for adults and children high consumers,

all of them were far from the safe level of 1 ng/kg bw/day. All ranges were very close to our results (Leblanc et al., 2004).

Monte Carlo simulations, either non-parametric and parametric, showed similar values than those results obtained through deterministic model (Table 6.3). Therefore, infant consumers, especially high consumers, were the most exposed group to AFM_1.

The most exposed group (infant, percentile 99th), showed highest variability of the results, being the values obtained by probabilistic method higher than obtained through deterministic method. This difference was less important in the other groups, probably due to the low variability in the milk intake patterns.

6.5 Discussion

An early study carried out in Spain showed low incidence of contamination of commercial milk by AFM_1 with 7.3% of positive samples (LOD = 20 ng/kg) and a range of 20-40 ng/kg (Burdaspal et al., 1983), however, a subsequent study reported higher contamination levels with a contamination range of 20-100 ng/kg (Blanco et al,m 1988). Later studies found 45.9-86 % of samples were below 10 ng/kg, and 4-6.6% among 10-20 ng/kg. AFM_1 mean levels reported were 10.5 and 17.3 ng/kg in UHT milk samples, in the line of our study (Díaz et al., 1995; Jalón et al., 1994; Rodríguez et al., 2002). The Commission of the European Union (1989–95) also provided a Scientific Cooperation (SCOOP) report, containing data for milk analysed between 1989 and 1995 in nine Member States. 3338 out of the 8791 samples (38%), contained concentrations of aflatoxin M_1 below the LOQ/LOD; 1017 samples (12%) contained concentrations below 50 ng/kg; six samples (0.07%) contained 50–100 ng/kg; and three samples (0.03%) contained >100 ng/kg. For the European regional diet calculated weighted mean was 23 ng/kg (JECFA, 2001). Regarding cheese samples, although we did not found detectable levels, previous studies found mean levels of 105.3, 13.8 and 42.6 ng/kg in ripened, semi-ripened and fresh cheese respectively; however, other study did not detect this mycotoxin in "manchego" and blue cheese samples from Spain (Barrios

et al., 1996; López-Díaz et al., 1995). Despite the low incidence of AFM_1 in yoghourt (2/72), these positive samples showed high levels of the toxin, with a mean and maximum level of 38.34±18.73 and 51.58 ng/kg, respectively. Concerning yoghurt, no studies carried out in Spain were found, however, a study conducted in Portugal showed levels of 43-45 and 19-68 ng/kg in natural yoghurt and yoghurt with strawberries respectively, similar to our range (Martins et al., 2004). Although our study was conducted in Catalonia, the main brands marketed in Spain have been purchased and analysed, therefore, results from our study could be extrapolated to the Spanish market.

Despite the exposure assessment methods are mainly deterministic, probabilistic models have been proposed to provide most realistic description of exposure. This methodology permits more accurate approximation to the risk characterization, considering associated variability and uncertainties. Many sources of uncertainty should be considered as well as left and right censored data of contamination or right censored data of dietary intake estimation that provides important bias to the exposure assessment process. Probabilistic methods applied to mycotoxin exposure assessment were previously reported in several cases. For example, Humphreys et al. (2001) performed a quantitative risk assessment for fumonisins in USA corn and Leblanc et al. (2002) simulated the exposure to DON of consumers of both organic and conventional wheat in cereal-based products, based on a probabilistic method. Previously cited quantitative assessment of exposure to ochratoxin A was conducted by Gauchi et al (2002), providing useful methodology to built quantitative models, with application for mycotoxins and other contaminants.

6.6 Conclusion

To sum up, the occurrence of AFM_1 in cheese and yoghourt from Catalonian market is very low. Although high occurrence in milk was observed, the contamination levels were very low, far from the safe limit established by EU. Moreover, results from all studied scenarios suggest that it should not be expected AFM_1 exposure to contribute to the risk of liver cancer among Catalonian population, including high consumers.

Considering that the latest nutritional study conducted in Catalonia reported that 17.4 % of population surveyed showed a risk calcium dietary intake, with calcium intakes below 2/3 of Recommended Daily Intake (RDI) (Serra-Majem et al., 2003), an increase in milk and dairy products consumption should be expected at long-term to supply suitable amounts of calcium, thus exposure assessment to this contaminant should be considered.

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Chapter 7. Occurrence and exposure assessment to fumonisins in Catalonia

Food Additives and Contaminants 2012 29, 799-808

7.1 Abstract

Fumonisin B_1 (FB_1) and B_2 (FB_2) are mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum* and common contaminants of cereal crops. The objectives of this study were to (1) study the occurrence of fumonisins in Catalonia (north-eastern region of Spain) and (2) assess the exposure of the Catalonian population to these mycotoxins. Contamination data was provided by a wide survey where 928 individual samples were pooled to analyse 370 composite samples. Fumonisins were extracted and purified using immunoaffinity columns and determined by HPLC with fluorescence detection. The raw consumption data came from a nutritional study specifically designed to assess the dietary intake of the main foodstuffs related to fumonisin contamination for all population age groups. In addition, two specific groups were selected with respect to maize consumption: immigrants and celiac sufferers. Contamination and consumption data were combined by simulation using an essentially parametric–parametric (*P-P*) method. The *P-P* method draws sampling values from distribution functions fitted to consumption and contamination datasets. Moreover, to quantify the accuracy and reliability of the statistical estimates, we built related confidence intervals using a Pseudo-Parametric bootstrap method. The results of this study show that fumonisins are commonly found in some commodities on the Catalonian market, such as beer, corn snacks and ethnic foods; however, the values were well below the permitted maximum EU levels. The most exposed group were infants followed by immigrants but, in all cases, they were below the TDI of 2 μg/kg bw/day.

7.2 Introduction

Fumonisin B_1 (FB_1) and B_2 (FB_2) are mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum* that commonly contaminate corn (Nelson et al., 1992). Fumonisins (FBs) occur mainly in maize and maize-based foods, therefore populations with high maize consumption could be exposed to significant amounts of these mycotoxins through the ingestion of fumonisin contaminated maize (Marasas, 1996; Shephard et al., 1996; Visconti et al., 1996; WHO, 2001).

FBs have a remarkable structural similarity to sphingolipids (Merrill and Sweeley, 1996; Riley et al., 2001). This group of mycotoxins, especially FB_1, potently inhibit the enzyme ceramide (CER) synthase which catalyzes the acylation of sphinganine (Sa) and reacylation of sphingosine (So). The inhibition of CER synthase by FBs increases the intracellular Sa concentration, main contributor to the toxicity and carcinogenicity of FB_1 (Wang et al., 1991; Merrill et al., 1993; Yoo et al., 1996; Riley et al., 2001). Based on this biological perturbation, elevation of Sa to So, or Sa 1-phosphate to So 1-phosphate, ratios in tissues, urine and blood, have been proposed as potential biomarkers of fumonisin exposure in various animal species (Wang et al., 1992; Riley et al., 1993; Wang et al., 1999; Van der Westhuizen et al., 2001; Kim et al., 2006; Tran et al., 2006; Cai et al., 2007; Cano-Sancho et al., 2011b).

Human exposure to fumonisin contaminated commodities has been linked to oesophageal and liver cancer in South Africa and China (Sydenham et al., 1990; Yoshizawa et al., 1994). Acute and chronic toxicity of FBs has been largely demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al., 1988, 1991). FB_1 is a cancer promoter, but a poor cancer initiator. It is not genotoxic because FB_1 does not induce unscheduled DNA synthesis in primary rat hepatocytes (Norred et al., 1992).

Based on toxicological evidence, the International Agency for Research on Cancer (IARC) has classified FB_1 as possibly carcinogenic (group 2B) to humans (IARC 2006). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated FBs and allocated a provisional maximum tolerable daily intake (PMTDI) of 2 μg/kg body

weight/day to FBs on the basis of the non observed effect level (NOEL) of 0.2 mg/kg body weight/day and a safety factor of 100 (WHO, 2001). This PMTDI for FBs has recently been confirmed by the Scientific Committee on Food (SCF, 2003). European Commission established a maximum FBs levels of 1000 μg/kg in maize and maize-based food intended for human consumption, 800 μg/kg in maize-based breakfast cereals and snacks and 200 μg/kg in maize-based infant food (European Commission, 2006b, 2007).

In the Mediterranean countries, maize is more widely used for animal nutrition than for human consumption, but some countries traditionally use the maize in regional cooking as *broa* (maize-based bread from Portugal) and polenta (from Italy). As a consequence of the use of maize grits as adjunct in brewing process, aflatoxins and FBs can be also found in beer (Torres et al., 1998; Pietri et al., 2009).

Natural occurrence of FBs in corn or corn-based foods has been studied largely (Sydenham et al., 1991; Shephard et al., 1996; SCOOP, 2003; Castelo et al., 1998; Hlywka et al., 1999), and surveys have been conducted in several cases in corn-based products marketed in Spain (Sanchis et al., 1994, 1995; Torres et al., 1998; Castellá et al., 1999; Velluti et al., 2001). Dietary intake of FBs has been previously assessed in population from France, USA, Netherlands, Canada or South Africa (Kuiper-Goodman et al., 1996; De Nijs et al., 1998; Humphreys et al., 2001; Leblanc et al., 2005; Shephard et al., 2007).

In the framework of the Project to Assess the Exposure of Catalonian Population to the Mycotoxins, and following the line of our previous studies (Cano-Sancho et al., 2009, 2010, 2011c), the objectives of this study were, in one hand, to study the occurrence of FBs in Catalonia (north-eastern region of Spain), and, in the other hand, to assess the exposure of Catalonian population to these mycotoxins.

7.3 Materials and methods

7.3.1 Samples

During 2008 and 2009, 185 samples of sweet-corn, 216 samples of corn-based snacks, 216 beer samples, 168 corn flakes, 90 baby food samples were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, representative of 72 % of the population. From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of FB_1 and FB_2 was determined in a total of 72 composite samples of each category by equally pooling the 2 or 3 items taken from each store (depending of availability) to obtain an analytical sample or *"composite"*: 12 cities x 6 stores/city = 72 samples/category. However, in some cases, 3 items or any items were not available in the store, thus, less than 72 composites could be obtained. Additionally, 18 gluten-free food samples (mainly maize-based bread and pasta) and 35 ethnic food samples, such as Mexican tortillas, corn flour or cuscus, were purchased in specialised stores to be analysed individually. Regarding brands, thirty-one sweet corn, seventy-nine corn-based snacks, sixty-four beers, thirty-two corn flakes different brands were purchased, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of Spanish market. The samples were transported and stored under suitable conditions until analysis.

7.3.2 Analytical procedure

Fumoniprep® immunoaffinity cleanup columns (IAC) (R-Biopharm, Rhône LTD Glasgow, UK) were used to extract FB_1 and FB_2 from beer samples. A volume of 5 mL of beer previously degassed in ultrasonic bath during 40 minutes was mixed with 15 mL of phosphate buffer solution (PBS; 0.8 % NaCl, 0.12% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl) and drained through the IAC. The column was washed with 20 mL of PBS solution and FBs were eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water. Regarding solid maize-based samples, 10 g of ground sample composite was mixed with 1 g NaCl, and 50 mL of extract solution (50% water, 25% methanol, 25%

acetonitrile) for 20 minutes and filtered. 10 mL of filtrate was diluted with 40 mL of PBS and drained through the IAC and follows as described previously.

Fluorescent derivatives of FB_1 and FB_2 were obtained using pre-column derivatization with an *o*-phthaldialdehyde (OPA) solution prepared diluting 40 mg of *o*-phthaldialdehyde with 1 mL of methanol HPLC grade and mixed with 5 mL of $Na_2B_4O_7 \cdot 10H_2O$ (0.1M) and 50 μL de 2-mercaptoethanol. Derivatization was conducted mixing 200 μL of eluate with 200 μL of OPA solution for 30 seconds in vortex. Chromatography equipment: Separations Module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5μm ODS2, 4.6 x 150 mm, Multi λ Fluorescence Detector Waters 2475®, kept at 35 ºC and a flow-rate maintained at 1 mL/min. Injection volume was 100 μl. Mobile phase was based on a methanol and 0.1M sodium dihydrogen phosphate (77:23, v/v) solution. Excitation and emission wavelength were 335 nm and 440 nm, respectively.

The analytical method used for FBs was assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 100 μl of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations.

Precision and recovery were established by determination of FB_1 and FB_2 levels, spiked in each food categories by triplicate. The concentrations of fumonisins fortified in tested samples were ranged from 25 μg/kg to 660 μg/kg. Recovery was determined by comparing the absolute responses of FBs, with the absolute responses of calibration standards. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was considered equal to 3×LOD.

Recovery ranges were 76.5 ± 11.5 - 109.7±12.3 % in corn snacks; 101.5±11.8 – 104.0±7.3 % in sweet corn; 91.6±8.4 - 107.5±5.0 in corn flakes, 93.0±14.0 - 108.0±10.0 % in beer and 90.7±15.1 – 115.5±27.9 % in baby food. Repeatability was expressed as relative standard deviation (RSDr), it ranged between 11.3 and 15.0 % in corn snacks;

between 7.0 and 11.6 % in sweet corn; between 4.6 and 9.1 % in corn flakes; between 10.0 and 15.0 % in beer; between 16.6 and 24.1 % in baby food. The mean limit of detection (LOD) was 3.9 μg/kg in beer and 5.2 in solid based foods. These values were in accordance to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission, 2006a).

7.3.3 Fumonisin dietary intake assessment

Main problem to assess mycotoxins intake is related to few representative available dietary data regarding food bearing mycotoxins contamination. There are some methods developed to assess dietary intake overall known as market basket, 24-hour dietary recall and food record methods, food-frequency methods or dietary history. Given that corn-based products consumption is considered as sporadic or casual among Catalonian population, food-frequency methods should be chosen because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998). In this study, food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. According to World Health Organization advices, studies to assess dietary intake of chemical contaminants, should show the significant intake among standard population, with all population groups that could have different dietary patterns. Therefore, five different population groups were considered: infants (0-3 years), children (4-9), adolescents (10-19 years), adults (20-65 years) and elders (> 65 years). Moreover, adult celiac sufferers and adults with ethnic dietary pattern were included in the nutritional study, and studied separately. FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins studied, excluding those foods not consumed in the region. Concerning frequency of consumption, 5 response options ranging from never to annually, were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 76 seniors, 720 adults,

235 adolescents, 69 parents of children, 164 parents of infants, 70 adult celiac sufferers and 56 adults with ethnic dietary pattern were interviewed in 2008-2009 by trained interviewers. In the present study, the adults were treated separately according to their sex, with a major population group of 336 adult males and another of 384 adult females. Individuals were from 89 cities and towns from Catalonia. Study level, dietary habits, sex, age, ethnics and anthropometric data were considered in the questionnaire.

Exposure Assessment through Direct Approach

If we assume independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, we can estimate the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j$$

Where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff *j* in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food. We could consider this approach to be a theoretical approach, but it would have to be a (very) simplified theoretical approach. For example, this simplified approach does not enable us to calculate complicated statistics such as high quantiles. Simulation methods would therefore be required to obtain estimates of these statistics. Finally, we will consider this approach to be a direct approach. Left-censored data (those samples below LOQ) was substituted by LOD/2 in order to calculate the mean toxin level used in the exposure assessment model.

Exposure Assessment through Stochastic Approach

Stochastic procedure used is based on the Parametric-Parametric (P-P) method reported extensively in Gauchi and Leblanc (2002) and Cano-Sancho et al. (2011b). In brief, for each consumption and contamination dataset, the gamma *pdf* was fitted with

the method of maximum likelihood using the CAPABILITY procedure of SAS software (SAS, 2010).

The appearance of the histograms was continuous and only one distribution was considered pooling all age groups data, fitting all data to gamma *pdf*. In case of gluten-free foods and sweet-corn, the mean contamination was used in the simulations because not enough data were available for computations. Left-censored was treated as the previous section, replacing non quantified samples by 0.5xLOD.

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

$$\hat{E}_k^{[P-P]} = \sum_{j=1}^{p} \tilde{c}_{i(j)} \tilde{t}_j$$

where $\tilde{c}_{i(j)}$ is a random normalised consumption *i* for the foodstuff *j*, drawn from $\hat{C}_j$, the corresponding adjusted gamma *pdf*, and $\tilde{t}_j$ is a random contamination for the foodstuff *j*, drawn from $\hat{T}_j$, the corresponding adjusted gamma *pdf*.

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 (10,000 in the present study):

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

Other statistics were directly computed on the histogram built with the simulations of the *S* set, and statistics were also estimated from the lognormal and gamma fitted *pdf*s (results not shown).

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 10,000). The boundaries of the 95% confidence interval are calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution.

7.4 Results

7.4.1 Occurrence of total fumonisins in food

FB_1 was the major fumonisin found in all food categories. In beer and corn snacks both mycotoxins were present simultaneously, and the mean ratios FB_1/FB_2 were 2.3 and 1.9, respectively. According to Table 7.1 and Figure 7.1, highest occurrence of total fumonisins (FB_1+FB_2) was found in beer samples with 90 % of positive samples, followed by corn snacks (61 %), ethnic foods (51 %) and corn flakes (39 %). Baby foods and gluten-free foods showed low levels of positive samples (23.3 and 5.6 %, respectively) and they were not detected in sweet corn.

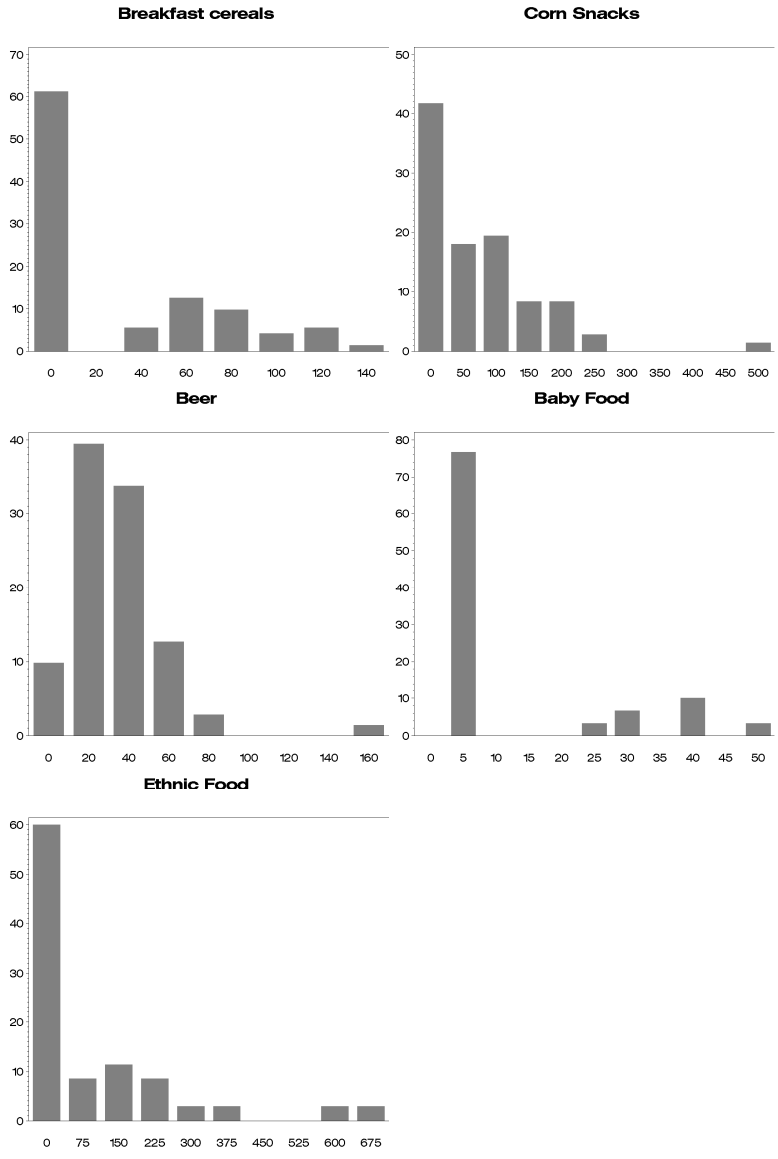

Fig 7.1. Contamination histograms (relative frequencies), in μg kg^{-1} for each food

Positive samples of corn snacks and corn flakes presented moderated mean values (119.1±83.1 μg/kg and 78.9±27.9 μg/kg, respectively). The highest values were found in ethnic food samples, with a mean of 196.7 ± 191.8 and a maximum level of 682.2 μg/kg. Despite the high maximum value, no sample exceeded the limit established by EU (European Commission, 2007). The gluten-free foods and baby food showed the lowest mean levels of positive samples.

Table 7.1. Occurrence of total fumonisins (FB_1+FB_2) in corn-based food marketed in Catalonia

Food category	n Individual samples	n *"Composite"*	Positives	Mean± SD μg/kg	Max μg/kg
Corn snacks	216	72	44	119.1 ± 83.1	475.5
Sweet corn	185	72	0	—	—
Corn Flakes	168	72	28	78.9 ± 27.9	139.5
Beer	216	72	64	36.9 ± 20.1	157.2
Baby foods	90	30	7	36.4 ± 8.5	50.7
Free-gluten food	18	18	1	22.5	22.5
Ethnic food	35	35	18	196.7 ± 191.8	682.2

Mean ± SD = Mean ± Standard Deviation

7.4.2 Fumonisins dietary intake assessment

Through the specific nutritional study performed with a sample of Catalonian inhabitants during 2008 and 2009 we could better estimate the dietary patterns of those commodities related with fumonisin contamination. As shown in Table 7.2, the corn flakes and corn snacks were mainly consumed by children and adolescents, while beer was most widely consumed by immigrants and adult males.

Table 7.2. Normalised consumption of the main foodstuff related to FB contamination (g kg^{-1} body weight day^{-1}) and percentage of consumers in each category (between parentheses, %).

	Corn flakes	Corn snacks	Beer	Sweet corn	Ethnic food	Gluten-free bread	Gluten-free pasta
Elders	0.01 (6.6)	0.00 (25)	0.53 (29)	0.00 (0)			
Adult females	0.19 (40)	0.02 (33)	0.76 (53)	0.19 (37)	-	-	-
Adult males	0.13 (33)	0.02 (32)	1.69 (73)	0.14 (39)	-	-	-
Adolescents	0.50 (65)	0.13 (82)	0.29 (25)	0.15 (45)	-	-	-
Children	1.41 (72)	0.18 (84)	0.00 (0)	0.33 (38)	-	-	-
Immigrants	0.23 (38)	0.11 (45)	2.27 (61)	0.14 (54)	0.39 (80)	-	-
Celiac sufferers	0.34 (64)	0.07 (72)	0.32 (40)	0.14 (52)	-	1.19 (94)	1.31 (96)

Celiac sufferers commonly consumed gluten-free products as bread and pasta, and other maize based food as breakfast cereals and corn snacks. Most of immigrants consumed food products imported from their origin countries, becoming a staple food in their current diet. Fumonisin dietary intake of the Catalonian population sample was assessed through a direct method and by means of the simulation method described above. The mean and variance of fumonisin dietary intake estimated through the direct approach for all population groups are presented in table 7.3.

Table 7.3. Exposure of each population group to fumonisins (units in ng kg^{-1} bw day^{-1}) using the direct approach. Where $\hat{E}_{\pi_0}$ is the exposure mean of the population π with the population sample π_0 and $\hat{V}(E_{\pi_0})$ the variance estimate

	$\hat{E}_{\pi_0}$	$\hat{V}(E_{\pi_0})$
Celiac sufferers	40.9	$(35.0)^2$
Immigrants	65.1	$(69.0)^2$
Adults females	34.5	$(55.5)^2$
Adults males	63.2	$(90.3)^2$
Adolescents	36.7	$(44.3)^2$
Children	63.0	$(64.0)^2$
Infants	156.0	$(152.4)^2$

(ng kg^{-1} bw day^{-1})

Considering these estimates, the most exposed population group was the infants, followed by immigrants, children and adult males. Differences were found between adult males and females.

Concerning the dietary intake estimated through the simulation procedure, mean values were commonly higher than those calculated using the direct method, with the exception of celiac sufferers (See Table 7.4 and Figure 7.2). Stochastic method was not applied to elders because few consumers of each item was found in this group and commonly those consumers reported a low level of consumption in each category.

Table 7.4. Results of exposure assessment of the population groups from P-P simulation method. These results were obtained directly from the 10,000 simulation outputs without pdf fittings. Bootstrap confidence intervals are presented between brackets (in ng kg^{-1} bw day^{-1}).

	Adult females	*Adult males*
Mean	103.02 [91.84 - 115.27]	100.41 [88.62 – 113.33]
SD	116.47 [91.82 - 147-31]	114.21 [89.78 – 143.31]
Skewness	3.55	3.28
Kurtosis	22.52	17.59
Median	66.97 [58.92 - 76.33]	64.17 [55.99 – 73.87]
0.90^{th} quantile	226.73 [194.24 - 268.01]	225.48 [187.84 -264.92]
0.95^{th} quantile	320.76 [263.42 - 394.07]	313.33 [258.01 – 394.49]
0.99^{th} quantile	563.08 [434.16 - 792.45]	577.19 [413.73 – 770.59]
	Adolescents	*Children*
Mean	79.25 [69.01 – 90.36]	85.30 [59.30 - 119.96]
SD	83.17 [64.81 – 103.93]	125.13 [62.71 - 250.98]
Skewness	2.78	4.99
Kurtosis	12.10	44.69
Median	53.09 [44.45 – 61.61]	44.57 [30.66 – 63.69]
0.90^{th} quantile	175.84 [143.52 – 211.80]	201.01 [128.33 – 314.25]
0.95^{th} quantile	241.05 [191.29 – 295.83]	293.66 [172.74 - 489.71]
0.99^{th} quantile	414.62 [282.63 – 559.11]	601.14 [280.30 – 1943.99]

	Immigrants	*Celiac sufferers*
Mean	130.99 [71.64 – 221.57]	33.16 [20.13 - 52.18]
SD	291.26 [106.22 – 548.57]	61.62 [24.78 - 125.43]
Skewness	7.97	6.16
Kurtosis	135.88	68.44
Median	37.07 [18.35 – 66.20]	12.93 [8.19 - 19.66]
0.90^{th} quantile	337.03 [171.85 – 631.48]	82.20 [43.72 - 153.27]
0.95^{th} quantile	560.64 [278.11 – 1264.82]	132.39 [68.71 - 276.57]
0.99^{th} quantile	1313.14 [479.93 – 3727.78]	294.88 [112.74 - 836.29]
	Infants	
Mean	195.19 [168.91 – 223.68]	
SD	161.90 [130.01 – 201.47]	
Skewness	1.73	
Kurtosis	5.06	
Median	152.33 [125.27 - 185.52]	
0.90^{th} quantile	402.98 [335.03 – 484.66]	
0.95^{th} quantile	508.20 [410.86 - 650.41]	
0.99^{th} quantile	748.56 [543.63 – 966.64]	

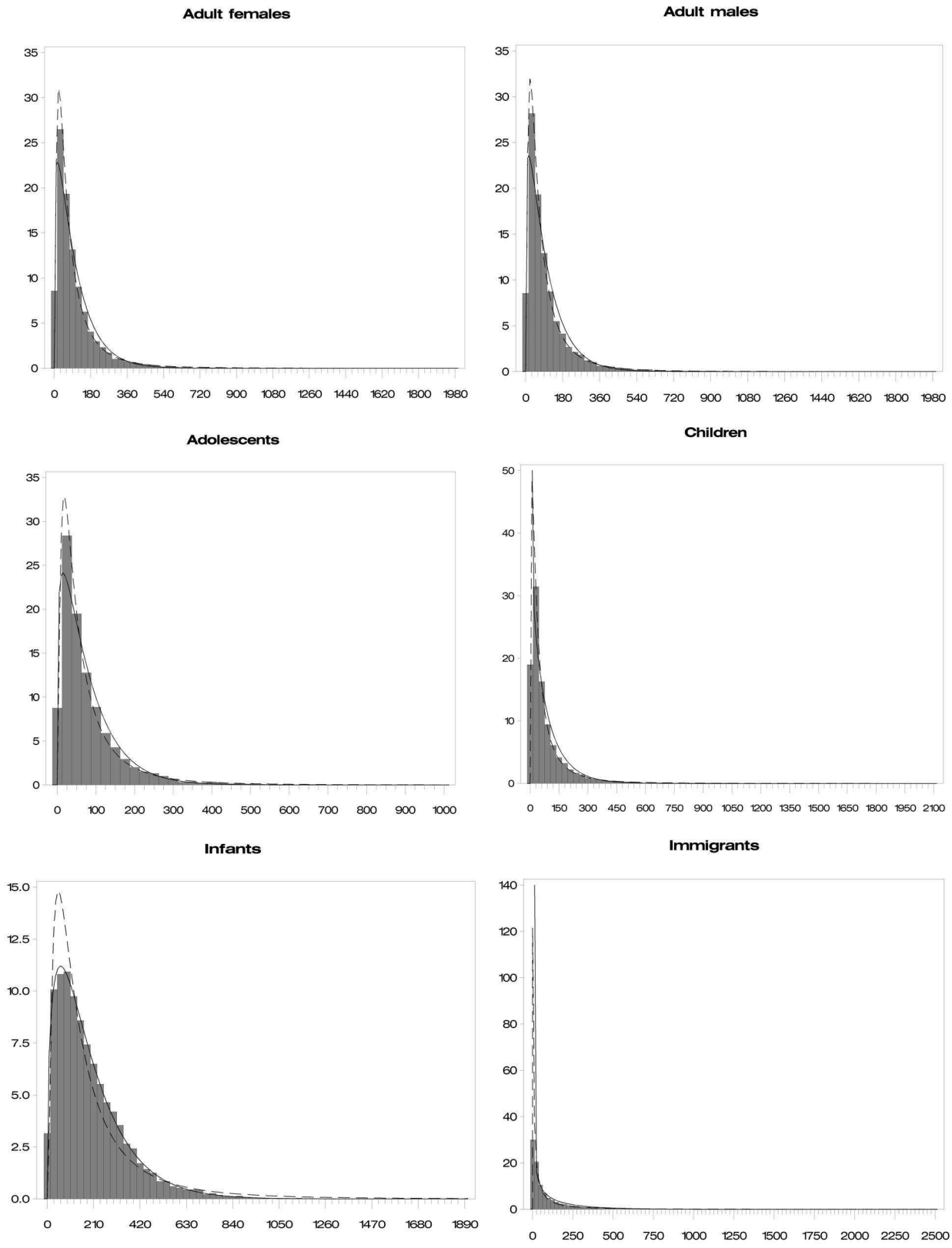

Fig. 7.2. Fitted gamma (solid line) and lognormal (broken line) *pdf*s for the population groups with exception of celiac sufferers. Exposure in ng kg^{-1} bw day^{-1}

The highest means were estimated for infants, followed by the immigrants and adults. Celiac sufferers showed the lowest exposure estimates. Pseudo-parametric bootstrap confidence intervals permitted to assess the reliability and accuracy of the computations. We observed that these intervals decreased when we estimated the highest quantiles. Extremely wide confidence intervals were built for the 95th and 99th quantiles, especially for immigrants and children. It is an accepted fact that it is very difficult to obtain good accuracy for the 99th-quantile, in particular (Breiman et al. 1990). All estimates were far from the TDI of 2,000 ng/kg body weight/day, including the highest quantiles of immigrants and infants (the most exposed groups).

7.5 Discussion

7.5.1 Occurrence of fumonisins

This is the first study conducted in Spain with a highly representative sampling method, using a high number of individual samples of the main corn-based food categories collected around the Catalonian geography to assess a wide pool of analytical samples, including baby foods, ethnic foods or gluten-free foods not reported until now. Although our study was conducted in Catalonia, the main brands marketed in Spain have been purchased and analysed, therefore, results from our study could be extrapolated to the Spanish market.

A previous study conducted in Spain showed smaller concentrations than our study for corn snacks and corn flakes but the authors found contaminated samples of sweet corn with respective mean values of 46.27, 17.03 and 55.96 μg/kg, all of them determined by HPLC (Velluti et al., 2001). D'Arco et al. (2008) compared organic and conventional maize-based food and they found higher percentage of contaminated samples in organic commodities (78 %) than in conventional food (16 %), moreover, the maximum and highest mean was found in the organic category (235 vs 51 μg/kg). In the present study, we analyzed mainly conventional food because the consumption of

organic maize foods is not widespread in Spain, and these products are supplied by a little number of retail stores. Regarding beer samples from Spain, a previous study performed by ELISA kit, showed similar values to our study in the range 85.53±0.34 – 4.76±1.21 μg/kg (Torres et al., 1998).

Previous studies carried out with corn-flakes, reported mean levels of contamination closest to our mean value of 78.9±27.9 μg/kg, being those means 76.0 μg/kg in France, 79.3 and 129.0 μg/kg in Canada (Kim et al., 2003; Molinié et al., 2005; Roscoe et al., 2008); however Martins et al. (2008) did not found any sample from Portugal exceeding the detection level. Lombaert et al. (2003) showed mean levels of 33 and 14 μg/kg for soy-based and multi-grain cereals for babies, respectively, in the line of our results. Moreover, FBs were previously analyzed in ethnic foods from United Kingdom, and only trace levels were found in cereal-based foods as noodles, rice, corn flour and pitta bread (Patel et al., 1996), quite different from our study where fumonisins were quantified in half of samples. In Spain, the ethnic food market started in the 90's, whilst the highest growth rates have occurred since 2000. Sales passed from 7.2 million dollars in 1999 to 40.6 million dollars in 2004 (Camarena and Sanjuán, 2008), therefore this commodities should be taken into account as potential mycotoxin carriers.

In the present study only FB_1 and FB_2 were determined in food, however, it is a well known that FBs can be conjugated when food is thermally treated. These bounded forms are not detectable using the conventional analytical techniques, but can be hydrolyzed in the gastrointestinal tract and produce the toxic effect (Dall'Asta et al., 2009). Therefore, in future exposure assessment works, powerful techniques focused on direct or indirect analysis of conjugated forms should be used to improve the accuracy of the estimates.

7.5.2 Fumonisin dietary intake assessment

The lack of accurate and representative dietary data of corn-based food intake from Spanish population was the main problem to assess the exposure of the population

to FBs. Through a specific FFQ administered to a population sample from the region, it has been possible to obtain a more accurate approximation to dietary intake habits from each population group.

Despite experts have recommended the use of stochastic approaches to provide a more realistic exposure assessment, to date, few studies have been published using this methodology to assess the exposure of populations to mycotoxins (Kroes et al., 2002; WHO, 2005; EFSA, 2006; Verger and Fabiansson, 2008), the direct or deterministic methods being the most commonly used.

Results of exposure assessment obtained through the direct method showed infants as the main risk group, followed by immigrants, male adults and children with respective daily dietary intake of FBs of 0.156±0.154, 0.065±0.069 and 0.063±0.090 and 0.063±0.064 μg/kg body weight/day. A previous study conducted in Spain on dietary intake of FBs was developed using corn dietary intake data of Spanish population supplied by Food and Agriculture Organization (FAO) combined with FBs levels in non processed corn obtained from their study (Ariño et al., 2007). Despite this fact could result in overestimation of FBs intake, that study showed lower levels of exposure than our results with mean intake for adults of 0.0038 μg/kg body weight/day. Leblanc et al. (2005) reported exposure of French population to FBs using dietary data from a strict epidemiological work (n=3003), showing results in the line of our study with values of 0.05 and 0.18 μg/kg body weight/day for children mean and high consumers (percentile 95) and 0.01 and 0.06 μg/kg body weight/day for adults mean and high consumers, respectively, our combined values (males and females) being: 0.048 and 0.16 μg/kg body weight/day for adult and baby means. Results from SCOOP (2003) showed variable levels, some of them slightly higher than our results, as for Italian consumers or Norwegian babies values of 0.52 and 0.86 μg/kg body weight/day were reported, respectively, while other results were in the line with ours, as those for population from Austria, Germany, Belgium or Netherlands. In all cases, these values are far from those estimated in other regions highly exposed to FBs as South-Africa, where mean daily

intake of FBs ranged from 3.43±0.15 to 8.67±0.18 μg/kg body weight/day (Shephard et al., 2007).

When we compared the direct method with stochastic method, we commonly found higher estimations through simulation. In the present study this was not crucial because in all cases the estimates were far from the safe level. However, this method can become mandatory in those cases when risk assessors need to accurately study the dietary intake of highly exposed populations.

To our knowledge, this is the first study to assess the exposure of special population groups to FBs. The special groups selected in the present study had dietary habits markedly different from the general population. In one hand, celiac sufferers substitute the wheat-based food for other gluten free cereals, and in the other hand, some ethnic groups partially maintain the dietary patterns from their country of origin. In Catalonia during 2009, the immigrant population represented 15.9 % of the total population (Migracat, 2010); therefore, their specificities should be taken into account to assess the exposure of this collective in other close regions.

7.6 Conclusion

Considering our results, despite FBs can be found regularly in Catalonian market, especially in ethnic food, beer and corn snacks, all levels remain far from EU maximum levels. Babies should be expected to be the main risk group to fumonisin intake; however these results suggest that no human risk derived from FBs should be expected in any Catalonian population group. The stochastic method provides more accurate estimations than the direct method, including the variability and uncertainties in the model, as well as the possibility to compute confidence intervals to validate the estimations.

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Chapter 8. Sphinganine and sphingosine levels and ratio in urine and blood samples from a Catalonian population, Spain

Food Additives and Contaminants 2011 28, 1055–1065

8.1 Abstract

Fumonisins occur mainly in maize and they produce alterations on sphingolipid metabolism, unbalancing the sphinganine (Sa)/sphingosine (So) ratio. This alteration has been proposed as a biomarker of fumonisin exposure. The objective of this study was to establish the urinary and plasmatic levels of Sa, So as well as the ratio Sa/So from a sample of the Catalonian (Spain) population exposed to fumonisins at low levels. Firstly, plasma and urinary Sa and So levels and the ratio Sa/So were compared between two population groups, and later urinary Sa and So levels from corn food consumers and a control group were monitored for 2 weeks under controlled intake of corn foods. Sa and So levels were determined in urine and blood samples using validated methods using HPLC with fluorescence detection. Significant differences were not found in urine samples when Sa/So ratios were compared from corn food consumers and non-consumers, while significant differences were found in urine and plasma samples, but evidence of the mechanism of action of fumonisins was not apparent. Through a time-course study we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans. This paper reports some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure.

8.2 Introduction

Fumonisin B_1 (FB_1) and B_2 (FB_2) are mycotoxins produced by *Fusarium verticillioides* and *F. proliferatum* that commonly contaminate maize (Nelson et al., 1992). Fumonisins occur mainly in maize and maize based foods, thus populations with a high maize consumption can be exposed to significant amounts of these mycotoxins via the ingestion of fumonisincontaminated maize (Marasas, 1996; Shephard et al., 1996; Visconti et al., 1996). Acute and chronic toxicity of fumonisin has been demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al., 1988, 1991; Howard et al., 2001; Shephard et al., 2007). FB_1 is a potent cancer promoter in rats after initiation with diethylnitrosamine and aflatoxin B_1 (Gelderblom et al., 1996). Human exposure to fumonisin-contaminated commodities has been correlated with high rates of oesophageal and liver cancer in South Africa and China (Sydenham et al., 1990; Yoshizawa et al., 1994) and, more recently, with neuronal tube defects on the Texas–Mexico border with other possible risk factors (Hendricks et al., 1999).

Based on toxicological evidence, the International Agency for Research on Cancer (IARC) classified FB_1 as a possible human carcinogen (group 2B) (IARC, 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated fumonisins and allocated a provisional maximum tolerable daily intake (PMTDI) of 2 $\mu g\ kg^{-1}$ body weight day^{-1} of fumonisins. This value was determined on the basis of the overall non-observed effect level (NOEL) of 0.2 mg kg^{-1} body weight day^{-1} for renal toxicity in rats; the safety factor was 100 (JECFA, 2001).

Natural occurrence of fumonisins in maize or maize-based foods has been widely studied worldwide (Castelo et al., 1998) and several surveys have been conducted in maize food for human consumption marketed in Spain (Sanchis et al., 1994, 1995; Torres et al., 1998; Castellá et al., 1999; Velluti et al., 2001; University of Lleida – Catalonian Food Security Agency (UdL-ACSA), 2009). In the latest study conducted in Catalonia to assess the incidence of mycotoxins in food for human consumption, 928 samples were purchased from the Catalonian market (in 2008 and 2009) and pooled in

370 composite samples to be analysed. The commodities analysed were beer, sweet corn, corn snacks, corn flakes, free-gluten pasta and bread, and ethnic food. The authors reported that the highest occurrence of fumonisins was found in beer (90% of positive samples), however those levels were low, while the incidence of these mycotoxins in other cereal-based foods was moderated. The mean values of positive samples of corn snacks and corn flakes were 119.1±83.1 and 78.9±27.9 μg kg^{-1}, respectively (UdL-ACSA, 2009). Fumonisins have a remarkable structural similarity to sphingolipids (Merrill and Sweeley, 1996; Riley et al., 2001). This group of mycotoxins, especially FB_1, potently inhibits the enzyme ceramide (CER) synthase which catalyses the acylation of sphinganine (Sa) and reacylation of sphingosine (So). The inhibition of CER synthase increases the intracellular Sa and other sphingoid bases, highly cytotoxic compounds. This imbalance has been proposed as the main responsible for the toxicity, and possibly carcinogenicity, of FBs, based on mechanistic studies with cells cultures and borne out by animal studies (Wang et al., 1991; Norred et al., 1992; Merrill et al., 1993, 2001; Yoo et al., 1996; Riley et al., 2001; Voss et al., 2006; Zitomer et al., 2009). Based on this biological perturbation, the elevations of Sa to So in tissues, urine and blood have been proposed as potential biomarkers of fumonisin exposure in various animal species (Wang et al., 1992, 1999; Riley et al., 1993; Morgan et al., 1997; van der Westhuizen et al., 2001; Kim et al., 2006; Tran et al., 2006; Cai et al., 2007). Several studies have been conducted to assess the effectiveness of this biomarker in humans, but the results did not allow an accurate validation (Hendricks et al., 1999; van der Westhuizen et al., 1999, 2008, 2010; Abnet et al., 2001; Qiu and Liu, 2001; Solfrizzo et al., 2004; Nikiema et al., 2008; Silva et al., 2009; Xu et al., 2010). The individual Sa and So basal levels, as well as the basal Sa/So ratio, vary depending on unknown parameters, being related to nutrition factors (Abnet et al., 2001; Shephard et al., 2007). The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (51 μg kg^{-1} bw day^{-1}). Considering that the PMTDI is 2 μg kg^{-1} bw day^{-1}, low sensitivity should be expected when we apply this biomarker in a human population (Kim et al., 2006; Voss et al. 2006; Cai et al., 2007).

The objective of the work reported here was to study the urinary and plasma levels of Sa, So as well as the ratio Sa/So from a sample of the Catalonian population, exposed to low levels of fumonisins, as a means to assess this ratio as a possible biomarker of fumonisin intake in the region. This work was structured in two experimental sections: in the first, plasma and urinary Sa and So levels and the ratio Sa/So were compared between the two population groups; in the second, urinary Sa and So levels from maize food consumers and a control group were monitored during 2 weeks under controlled intake of maize foods.

8.3 Materials and methods

8.3.1 Study design and sampling

This research project did not involve any risks for the volunteer donors; neither harmful modification of usual dietary habits nor administration was included in the methodology for the subjects. Each participant was informed about the study rules and a signed authorisation was requested individually.

Urinary and plasmatic Sa/So ratio point estimates. The first attempt to know the urinary and plasmatic Sa and So levels, and the ratio Sa/So from the Catalonian population was designed to compare Sa, So levels and the ratio Sa/So between high consumers of maize food and low/non-consumers.

Study of plasmatic sphingoid bases levels. Blood samples were collected from 136 healthy adult volunteers during 2008 from the Catalonian population following approval from the University of Lleida Ethical Council and with patients' informed consent. Blood was extracted and stored (less than 2 h) in Vacutainers with anticoagulant (EDTA) followed by centrifugation at 1000 *g* for 10 min, and finally the plasma was stored at 20C until analysis (in the same month). Maize food intake was requested with a Food Frequency Questionnaire (FFQ) in order to determine the approximate individual fumonisin intake.

Fumonisin intake was estimated through the combination of the consumption data with contamination data provided by UdL-ACSA (2009). Two population groups were made depending on their estimated fumonisin intake: high exposed and low or none exposed.

Urinary sphingoid base levels

First-morning urine from 89 volunteers was collected in sterile containers, during 2009, according to the Declaration of Helsinki. Urine samples were transported under refrigeration and stored at 20º C until analysis. In order to estimate the individual fumonisin intake, maize food intake was requested through a FFQ and a 3-day record (R3) during the time prior to the sample collection day. Fumonisin intake was estimated through the combination of the consumption data with contamination data provided by UdL-ACSA (2009). The population was grouped as high consumers or low consumers depending on their maize dietary estimates. Finally, seven urine samples were provided by oesophageal cancer sufferers from the University Hospital Arnau de Vilanova (Lleida). These samples were analysed to determine Sa and So levels and compared with the healthy group.

Study of the urinary Sa and So time-course

To know the changes of urinary sphingolipid levels over time, two groups of volunteers were monitored during 16 days. One group was composed of maize food consumers (exposed group, n=24), and the other, by non-consumers (control group, n=12). The exposed group was restricted of maize food consumption during 16 days, with the exception of the seventh day after restriction when the maize food intake was completely free. The food items consumed were home-made Mexican 'tortillas', corn snacks, maize-based cake, sweet corn and beer purchased from a Catalonian market. A representative sample of each maize food consumed during that day, was kept and analysed by a duplicated method to determine the FB levels. The control group was restricted to maize food during the entire study period (Figure 8.1).

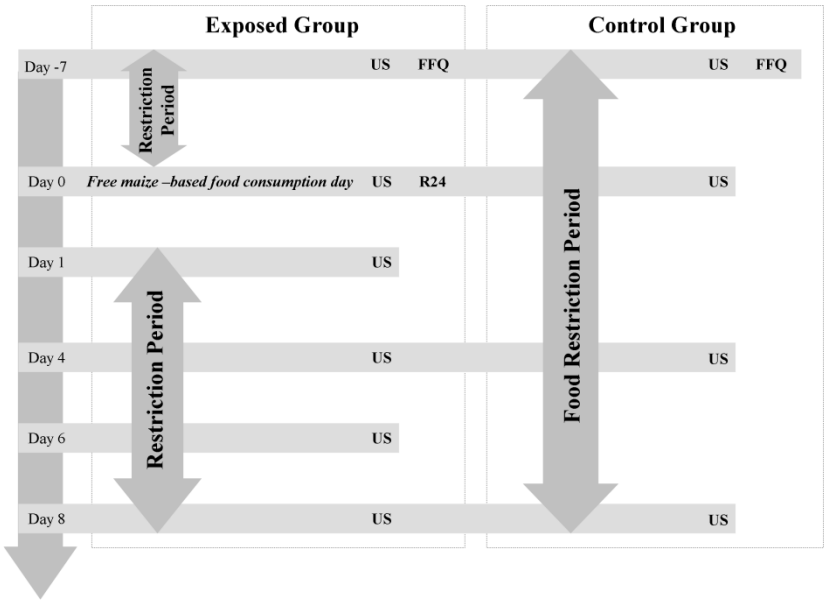

Figure 8.1. Urine sampling design and restriction periods performed to assess urinary Sa and So time-course. US, urine sample; R24, 24-h record; FFQ, food frequency questionnaire

At the beginning of the experiment, dietary habits of the individuals were requested via an FFQ. Maize food intake during the free consumption day (day 0) by the exposed group was requested with a 24-h dietary using household sizes previously standardised. Firstmorning urine was sampled according to the Declaration of Helsinki, from control group at days –7, 0, 4 and 8, while in the exposed group, 2 sampling days (1 and 6) were added in order to increase the accuracy. Urine samples were transported under refrigeration and stored at 20 ºC until analysis (during the same month). Sa and So levels were determined for each urine sample.

8.3.2 Fumonisin analysis in food

Fumoniprep® immunoaffinity clean-up columns (IAC) (R-Biopharm, Rhône Ltd, Glasgow, UK) were used to extract FB_1 and FB_2 from beer samples. A volume of 5ml of beer previously degassed in an ultrasonic bath for 40 min was mixed with 15 ml of phosphate buffer solution (PBS; 0.8% NaCl, 0.12% Na2HPO4, 0.02% KH2PO4, 0.02% KCl) and drained through the IAC. The column was washed with 20 ml of PBS solution

and fumonisins were eluted with 1.5 ml of methanolgrade HPLC and 1.5 ml of Milli-Q water. Regarding solid maize-based samples, 10 g of ground sample were mixed with 1 g NaCl, and 50 ml of extract solution (50% water, 25% methanol, 25% acetonitrile) for 20 min and filtered. A total of 10 ml of filtered solution was diluted with 40 ml of PBS and drained through the IAC and followed as described previously. Fluorescent derivatives of FB1 and FB2 were obtained using pre-column derivatisation with an ophthaldialdehyde (OPA) solution prepared diluting 40 mg of o-phthaldialdehyde with 1ml of methanol HPLC grade and mixed with 5ml of Na2B4O710H2O (0.1 M) and 50 ml de 2-mercaptoethanol. Derivatisation was conducted by mixing 200 ml of eluate with 200 ml of OPA solution for 30 s in a vortex. Chromatography equipment: separations, Module Alliance 2695 Waters®; analytical column, Waters Spherisorb® 5 mm ODS2, 4.6x150 mm; multi fluorescence detector, Waters® 2475, kept at 35C; and a flow-rate maintained at 1ml min^{-1}. The mobile phase was based on a methanol and 0.1M sodium dihydrogen phosphate (77 : 23, v/v) solution. Excitation and emission wavelengths were 335nm and 440 nm, respectively.

8.3.3 Sphinganine and Sphingosine analysis in plasma

Plasma (500 μL) was deproteinized with methanol (2 mL) and the protein precipitate was centrifuged down at 1200 g for 10 min at 10 ºC. An aliquot of the sobrenatant (1.5 mL) was mixed with 1.5 mL potassium chloride solution (0.8 %) and 50 μL potassium hydroxide (1 M). The mixture was extracted with 4 mL of ethyl acetate by gentle rotation in a blender for 20 min and the phases were separated by centrifugation at 1100 g for 15 min, as described by Castegnaro et al. (1998). The organic phase was evaporated to complete dryness at 55ºC under nitrogen. Dried samples were redissolved by vortex shaking in 275 μL methanol-water solution (88:12) and derivatized for 35 min by addition of 25 μL of OPA mixture. The derivatization mixture consisted of 50 mg OPA dissolved in 1 mL of ethanol and mixed with 50 μL of mercaptoethanol and 48.95 mL of boric acid solution (3 %) adjusted to pH 10.5 with potassium hydroxide (1 M) to

obtain a final volume of 50 mL. The derivatives were analyzed by HPLC with fluorescence detection (excitation wavelength of 340 nm, emission wavelength of 455 nm), using a Waters Spherisorb® 3 μm ODS2 4.5x250 mm column, kept at 35 ºC and a flow-rate maintained at 1 mL/min of methanol-water (88:12, v/v).

8.3.4 Sphinganine and sphingosine analysis in urine

Urinary samples were stored at -20ºC in the dark before the analysis. Extraction of Sa and So was performed using a method adapted from Castegnaro et al. (1996). To sum up, 20 mL of urine thawed sample were centrifuged at 2000 g for 15 minutes at 10 ºC, in order to isolate exfoliated cells. Cell pellets were re-suspended in 2 ml distilled water with 50 μl of potassium hydroxide (1 M). Following, 2 ml of ethyl acetate were added and mixed vigorously using the vortex for 1 minute. Then, the mixture was centrifuged at 2000 g for 15 minutes, and the upper solvent layer was kept, while the aqueous phase was extracted again. Finally, the mixed solvent layers containing sphingolipids were dried under a nitrogen stream. Dried samples were analyzed as described for plasma samples, under the same chromatographic conditions.

8.3.5 Validation of the analytical methods

The analytical methods used for fumonisins, Sa and So were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 50 μl of mycotoxin standard solutions three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations.

Accuracy and recovery were established by determination of FB_1 and FB_2 levels, spiked in samples of corn snacks, beer, and sweet corn; in the case of Sa and So, they were spiked in urine and blood samples. Recovery was determined by comparing the absolute responses of fumonisins, Sa and So, with the absolute responses of calibration

standards. The limit of detection (LOD) was considered as the mycotoxin and the sphingolipid concentration that provides a signal equal to b+3S*b*, where b is the intercept of the calibration curve and *Sb* is the standard error of the estimate assuming to be the blank, and the limit of quantification (LOQ) was considered equal to 3×LOD. Recovery data, repeatability, limit of detection (LOD) and limit of quantification (LOQ) of FB1 and FB2 in sweet corn, corn snacks and beer are shown in Table 8.1. These values are in accordance to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission 2006a).

Table 8.1. Method performance characteristics for fumonisin B_1 and B_2

		FB_1				FB_2			
Food Matrix	**n**	**LOD/LOQ µg/kg**	**Spiked level µg/kg**	**Recovery* %**	**RSDr %**	**LOD/LOD µg/kg**	**Spiked level µg/kg**	**Recovery %**	**RSDr %**
Sweet Corn	3	5.2/15.6	266	101.5±11.8	11.60	5.2/15.6	133	104.0±7.3	7.04
Corn Snacks	3	5.2/15.6	266	76.5±11.5	15.01	5.2/15.6	133	109.7±12.3	11.30
Beer	5	3.9/11.7	200	93.0±14.0	15.00	3.9/11.7	100	108.0±10.0	10.00

*: Mean ± Standard Deviation

Method performance characteristics for Sa and So in blood are shown in Table 8.2. This method showed recovery rates of So ranging from 92.2±19.7 to 104.0±12.8 %, while the recovery rates for Sa were between 93.1±13.4 and 98.3±11.2 %.

Table 8.2. Method performance characteristics for sphingosine and sphinganine in plasma

	LOQ/LOD ng/mL	**Spiked level ng/mL**	**n**	**Recovery* %**	**RSDr %**
Sphingosine	0.15/0.048	5	4	98.9±6.4	6.4
	0.15/0.048	20	4	92.2±19.7	21.3
	0.15/0.048	40	4	104.0±12.8	12.3
Sphinganine	0.14/0.047	2.5	4	96.8±9.7	9.3
	0.14/0.047	12	4	98.3±11.2	11.4
	0.14/0.047	24	4	93.1±13.4	14.3

*: Mean ± Standard Deviation

The method to determine the sphingoid bases in urinary samples was optimized in order to obtain a low detection limit, due to the low concentration of sphingolipids expected to be found in this matrix. Recovery rates, RSDr, LOQ and LOD are shown in Table 8.3.

Table 8.3. Method performance characteristics for sphingosine and sphinganine in urine

	LOD ng/mL	Spiked level ng/mL	n	Recovery* %	RSDr %
Sphingosine	0.04	5	5	122.9±5.5	4.5
	0.04	40	5	126.9±18.2	14.4
Sphinganine	0.02	5	5	107.6±5.6	5.2
	0.02	40	5	104.3±9.3	8.9

*: Mean ± Standard Deviation

8.3.6 Statistical Analysis

Sa/So ratios were calculated individually by division of Sa and So levels from each volunteer, and expressed as medians, means and standard deviations of ratios for each group. Mann-Whitney U test was used for two-group comparison and Kruskal-Wallis test was used to compare more samples. Principal Component Analysis was conducted to obtain matrix correlation from Sa/So ratio data and associated factors. Software SAS Enterprise guide v2.0.0.417® and SAS v9.0.® were used in statistical analysis.

8.4 Results

8.4.1 Study of sphingoid base levels and ratios in plasma

In this first study, 136 blood donors were grouped in high maize-based food consumers (68) and non consumers (68). The mean exposure to fumonisin estimated for the first group was 0.23±0.11 μg/kg bw/day. Medians were 0.53 and 0.46 for maize-based food consumers and non consumers, respectively. Although significant differences were observed when the Sa/So ratios were compared, non statistically significant differences were found between sphinganine levels (P>0.05), the sphingosine decrease

being the most probable responsible of ratio variation in the exposed group (See Table 4).

8.4.2 Study of the sphingoid base levels and ratios in urine

In this cross-sectional study, 78 volunteers were selected to assess the urinary Sa and So levels. Each volunteer was asked about dietary habits, through a FFQ and a R3. Considering the estimated fumonisin intake, the population was divided in high and low consumers in order to compare Sa and So levels and their ratios. The mean fumonisin intake estimated through the R3, was 0.013 and 0.046 μg/ kg bw/day for males and females, respectively, while these respective estimates were 0.089 and 0.057 μg/ kg bw/day when the estimation was made using the FFQ The most important bias sources were that males overestimated significantly the beer consumption in comparison with the R3 and the females underestimated the usual corn snacks consumption with this method. Levels of Sa, So and Sa/So ratio and fumonisin intake estimated through the R3 are shown in the Table 8.4.

Table 8.4. Sphinganine (Sa) and sphingosine (So) levels in urine and plasma, and the Sa/So ratio in population from Catalonia (Spain), from the cross-sectional studies

Group	n	Mean FB intake μg/kg bw/day	Sa* ng/mL	So* ng/mL	Ratio* Sa/So
Levels in urine					
Low and non consumers	43	0.02±0.02	0.38 (0.95±2.15)[A]	0.83 (2.57±5.02)[A]	0.40 (0.55±0.47)[A]
Consumers	35	0.14±0.83	0.26 (1.29±2.15)[A]	0.57 (2.59±0.85)[A]	0.56 (0.62±0.47)[B]
Levels in plasma					
Non consumers	68	0.00	4.12 (6.5±9.2) [A]	8.51 (14.3±16.5) [A]	0.46 (0.45±0.12) [A]
Consumers	68	0.23±0.11	3.14 (4.1±3.6) [A]	5.89 (7.8±6.8) [B]	0.53 (0.54±0.16) [B]

*Median (Mean ± Standard Deviation).
[(A)] Capital letter: different letters mean significant differences between groups, when we compare non consumers with consumers ($P < 0.05$; Mann-Whitney U test)

Significant differences were found between median ratios Sa/So from high and low consumers (p<0.05), but no differences were found in Sa and So levels. The urinary samples from esophageal cancer sufferers showed mean levels of Sa and So to be 0.376±471 and 0.208±0.484 ng/mL, respectively, while the mean Sa/So ratio was 0.363±0.458, no significantly different from that of healthy population.

8.4.3 Urinary Sa/So ratio time-course

Fumonisin dietary intake of individuals was assessed combining food consumption data with fumonisin levels on the maize-food consumed. Consumption data was recorded during the day 0 (free consumption day), using previously standardized portions. Mean levels of fumonisin contamination in corn snacks, Mexican "tortillas", corn-based cake, and sweet corn samples were 133.9, 99.3, 110.1 μg/kg and non detectable level, respectively. These values were far from EU limits of 400 μg/kg (European Commission 2006b). Volunteers were classified in three groups, depending on total fumonisin intake estimated during the "free maize-food consumption day": high consumers, H, (>0.6 μg/kg bw/day, mean 0.84±0.26 μg/kg bw/day); low consumers, L, (<0.6 μg/kg bw/day, mean 0.43±0.12 μg/kg bw/day) and non consumers, C, (control group, n=12). The high consumers did not exceed the tolerable daily intake of 2 μg/kg bw/day. The volunteer population was 18 males and 18 females. 55 % of volunteers presented a body mass index between 18.5 and 24.9 kg/m^2 (normal) and 45 % were overweight. Tobacco was consumed by 32 % of the individuals. Mean levels of Sa and So, and mean ratios in urine samples from volunteer donors collected during the restriction period, are shown in Table 8.5. The two volunteers excluded from the study showed So and Sa basal levels markedly higher than the rest of the group (40 fold greater than the mean group level).

Table 8.5. Time-course of median sphinganine and sphingosine levels, and Sa/So ratios (in ng/mL)

Ratio Sa/So	**Day -7**	**Day 0**	**Day 1**	**Day 4**	**Day 6**	**Day 8**
Control	0.25 ^{aA}	0.62 ^{aA}		0.52 ^{aA}		0.45 ^{aA}
Low exposed	0.70 ^{aAB}	0.26 ^{bA}	0.27 ^{aAB}	1.10 ^{abB}	0.43 ^{aA}	0.47 ^{aA}
High Exposed	0.51 ^{aA}	0.39 ^{abA}	0.80^{aA}	2.43 ^{bB}	0.98 ^{bAB}	0.45 ^{aAB}
Sphingosine						
Control	1.23 ^{aA}	0.53 ^{aA}		0.30 ^{aA}		0.21^{aA}
Low exposed	0.68 ^{aA}	0.45 ^{aA}	0.36 ^{aA}	0.19 ^{aA}	0.28 ^{aA}	0.32 ^{aA}
High Exposed	0.33 ^{aA}	0.20 ^{aA}	0.15 ^{aA}	0.08 ^{aA}	0.12 ^{aA}	0.31 ^{aA}
Sphinganine						
Control	0.27 ^{aA}	0.14 ^{aA}		0.12 ^{aA}		0.16 ^{aA}
Low Exposed	0.28 ^{aA}	0.13 ^{aA}	0.12 ^{aA}	0.24 ^{aA}	0.19 ^{aA}	0.15^{aA}
High Exposed	0.14 ^{aA}	0.10 ^{aA}	0.19 ^{aA}	0.19 ^{aA}	0.12 ^{aA}	0.16 ^{aA}

Control group (non consumers), Low exposed (< 0.6 μg/kg bw/day), High exposed (> 0.6 μg/kg bw/day).
$^{(A)}$ Capital letter: in each row, different letters mean significant differences among days ($P < 0.05$, Kruskal-Wallis test)
$^{(a)}$ Lower case letter: in each column, for each category, different letters mean significant differences between groups ($P < 0.05$, Kruskal-Wallis test)

Median Sa levels at the beginning of the study were 0.27, 0.28 and 0.14 ng/mL, for non consumers, low consumers and high consumers, respectively, and So median levels were 1.22, 0.68 and 0.33 ng/mL, respectively. The median Sa/So ratios were quite similar between exposition groups, without statistically significant differences for these values (0.25 to 0.70). During the first week of maize-food restriction, we did not observe significant differences in the Sa/So ratios for any group, however, after the free maize-

food consumption day, statistically significant differences were observed in exposed groups while no differences were observed in the control group with time. The maximum increase of the Sa/So ratio was observed the fourth day after the free consumption day, with mean values of 1.96±2.24 and 2.52±2.00 ng/mL for low and high consumers, respectively, while the mean ratio for the control group was 0.67±0.49 ng/mL (see figure 8.2). After the fourth day, the stabilization of the ratios was observed for these groups, recovering initial values, without statistical differences among all groups (See Table 8.5). This fact was confirmed by means of the correlation matrix of the ratios against the estimated daily intake during the free consumption day. Principal component analysis of Sa, Sa, Sa/So and fumonisin intake showed that the higher correlation should be expected between estimated fumonisin intake and Sa/So ratio from day 4 (r=0.3322; p<0.01) with low correlation with the other days. The mean Sa/So ratios through the time are represented in the figure 8.2.

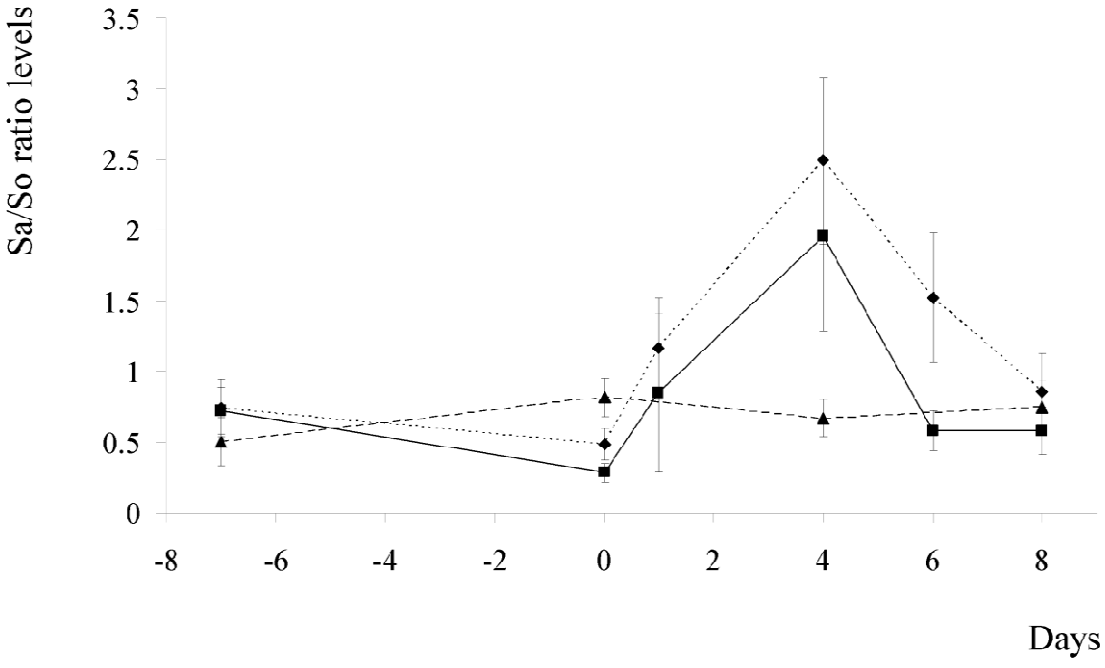

Figure. 8.2. Time-course of mean Sa/So ratio for high exposed (H,), low exposed (L,) and non exposed (C,)

Sa levels decreased during the first restriction week in each group, but increased after the free consumption day in exposed groups (high and low consumers), while the level slightly decreased in the control group. So levels decreased during the first week,

after day 0 the median values decreased slightly but no significant differences were found (See Table 8.5).

The absolute modification of the sphingoid bases, as well as of the ratio, after the free consumption day was quantified as the absolute difference (maximum – minimum) among day 0 and day 8 (Table 8.6). Sa and So levels were slightly modified during this period, without differences among exposure groups, neither significant differences were observed in the increase of the Sa/So ratio.

Table 8.6. Absolute variation of sphinganine and sphingosine urinary levels from volunteers under restricted conditions, variation was accounted between day 0 and day 8 (in ng/mL)

Group	Sa			So			Sa/So		
	max	min	median (mean±sd)	max	min	median (mean±sd)	max	min	median (mean±sd)
Control	1.30	0.02	0.17 (0.27±0.35) [a]	2.31	0.01	0.28 (0.56±0.67) [a]	1.76	0.09	0.96 (0.90±0.55) [a]
Low Exposed	0.62	0.11	0.23 (0.28±0.18) [a]	1.77	0.10	0.81 (0.83±0.51) [a]	7.56	0.12	0.98 (2.09±2.65) [a]
High Exposed	0.80	0.10	0.15 (0.26±0.22) [a]	1.69	0.08	0.27 (0.45±0.48) [a]	6.56	0.44	1.06 (2.73±2.32) [a]

Control group (non consumers), Low exposed (< 0.6 μg/kg bw/day), High exposed (> 0.6 μg/kg bw/day).

[(a)] Lower case letter: different letters mean significant differences when we compare categories ($P < 0.05$, Kruskal-Wallis test)

8.5 Discussion

Based on the mechanism of action, it has been observed that fumonisins inhibit CER synthase, a disruption that leads to an increase of Sa levels and Sa/So ratio (Riley et al., 2001). It is due to the rapid elimination and low bioavailability of fumonisins, that it is necessary to find an indirect indicator of human exposure to these toxins. Sa/So and Sa 1-phosphate / So 1-phosphate ratios in tissues, urine and blood, have been proposed as potential biomarkers in various animals (Wang et al., 1992, 1999; Morgan et al., 1997; van der Westhuizen et al., 2001; Tran et al., 2006), these ratios being validated in F344 rats by Cai et al. (2007), obtaining more sensitive results in urine than in serum

for acute and sub-chronic exposure to FB1. However, no successful results have been found when this biomarker has been assessed in human population, due to the low sensitivity when it is applied over individuals (van der Westhuizen et al., 1999, 2008, 2010; Abnet et al., 2001; Qiu and Liu, 2001; Solfrizzo et al., 2004; Nikiema et al., 2008; Silva et al., 2009; Xu et al., 2010).

In Catalonia, maize-based food is not highly consumed; therefore the exposure of the population to fumonisins is expected to be low, as reported in the Technical Report from UdL-ACSA (2009). In the present study, we have estimated that fumonisin intake of the volunteers from this region was in all cases below the PMTDI of 2 μg/kg bw/day, including the high consumers, who showed maximum estimates of 1.04 and 1.42 μg/kg bw/day. Other previous studies were conducted in regions where maize is highly consumed, and estimated fumonisin intake has been estimated to be quite high; for example, in some regions of South Africa the mean fumonisin intake was estimated to be between 5.8 and 3.8 μg/kg bw/day (van der Westhuizen et al., 1999, 2008, 2010), and the 93 % of 43 volunteers from Huian (China) had their daily FB intakes above the PMTDI of 2 μg/kg bw/day (Xu et al., 2010). Concerning our cross-sectional studies, the mean plasmatic Sa and So levels were higher than urinary levels, as reported previously (van der Westhuizen et al., 2008), while mean ratios were slightly higher in urinary samples.

In both cross-sectional studies we have found significant differences between ratios from exposed and non exposed groups, however no differences were found in sphinganine levels. In the study performed with plasma, the main responsible of ratio increase was elucidated through a decrease of So levels, with significant differences, therefore no evidences of mechanism of action of fumonisins were found. Esophageal cancer rates have been correlated with fumonisin exposure in China and South Africa, to regions highly exposed to fumonisins (Chu and Li, 1994; Zhang et al., 1999; Wang et al., 2000; Rheeder et al., 1992). In northern Italy region, maize consumption was correlated with higher rates of esophageal cancer than other regions (Rossi et al., 1982; Franceschi et al., 1990), and presence of fumonisin-producing Fusarium species in maize and

polenta was lately reported (Logrieco et al., 1995; Pascale et al., 1995). In this study seven urine samples from esophageal cancer sufferers were analyzed and compared with healthy groups, and non differences were found in any case.

In our latest study, we have monitored the expected alteration of Sa and So levels in urine from maize-food consumers after a free maize-food consumption day within a maize-food restriction period. Significant differences were observed for the ratio Sa/So after the free consumption day (day 0) for both exposed groups, while no differences were observed in the control group. The maximum values of the ratios were observed at day 4 after the free consumption day. Previous studies conducted with animal species dosed with fumonisins showed variable results concerning the day of maximum Sa/So ratio. For example, the maximum peak of Sa/So in weaned piglets dosed with 5 mg/kg bw/day showed the peak at 12 h (Dilkin et al., 2010), in rats dosed with 10 mg/kg bw/day the maximum was observed at day 3 and day 5 (Garren et al., 2001; Cai et al., 2007), while in vervet monkeys dosed with 1 mg/kg bw/day the maximum was found to be the day 3 (Van der Westhuizen et al., 2001). The time period between fumonisin intake and maximum peak of the ratio Sa/So is an important data to validate a human biomarker that will permit a better design of sampling and dietary exposure assessment.

To date, the cross-sectional studies have shown poor correlation between fumonisin dietary intakes and Sa/So ratio in humans, however, successful results have been found in several animal studies. Thus, there are several drawbacks which prevent this biomarker to be applied to humans for epidemiologic purposes:

1) The individual Sa and So basal levels, as well as, the basal Sa/So ratio vary depending on unknown parameters, being related with nutrition factors (Abnet et al., 2001; Shephard et al., 2007). Therefore, the absolute ratio could not be a good predictor of fumonisin intake.

2) The sensitivity of the correlation between fumonisin intake and Sa/So has been demonstrated to be poor at low and very low doses in animals (< 1 mg/kg bw/day). Considering that the PMTDI is 2 μg/kg bw/day, low sensitivity should be expected

when we apply this biomarker in human population. The Sa-P and So-P have been proposed to monitore the exposure of fumonisins, being more sensitive than the original sphingoid bases, therefore, could be suitable to use in human epidemiological studies for low-level exposed population (Kim et al., 2006; Voss et al., 2006; Cai et al., 2007).

3) To reach a realistic correlation between sphingoid base levels and fumonisin intake in human populations, it is required to use reliable analytical and consumption data (Willet, 1998). Improved analytical methods to determine Sa and So are reliable in urine and blood and likewise the methods to determine the FB levels in food. However, the dietary intake assessment methods used in previous studies do not report on their accuracy or reliability.

4) Finally, the most commonly used method to assess dietary intake has been the food frequency questionnaire (FFQ); it is the most comfortable method for researchers and volunteers. If we consider that the maximum Sa/So ratio has been closely correlated with a specific consumption day in animals (dose day) (Garren et al., 2001; van der Westhuizen et al., 2001; Dilkin et al., 2010), and reversible after that point, the dietary intake methods should assess rigorously those foods consumed 4-5 days before the urine sampling. Therefore, the dietary record could be a more accurate method to assess the fumonisin intake than de FFQ.

8.6 Conclusion

To our knowledge, this is the first study conducted in Spain to assess the sphingoid base levels and ratios in plasma and urine from a maize-food consumer population. We have proved that the volunteers were not exposed to high levels of fumonisins, in all cases below PMTDI of 2 μg/kg bw/day (maximum value of 1.4 μg/kg bw/day). The analytical method to determine Sa and So in urine and plasma was reliable, showing good recovery and reproducibility. The results showed higher Sa and So levels in plasma than in urine, and significant differences were shown when males

were compared to females. Concerning Sa/So ratios from maize-food consumers and non consumers, significant differences were found in urine and plasma samples but evidences of mechanism of action of fumonisins were not apparent. Through time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans.

In this paper we have reported some useful information to improve the design of studies to validate the ratio Sa/So as a possible biomarker of fumonisin exposure. However, more studies are required to better understand the use of this biomarker with human population, mainly, to improve the accuracy at low levels of exposure.

8.7 References

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Chapter 9. Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain)

Food Control 2011 22, 490-495

9.1 Abstract

The most important trichothecenes are HT-2 toxin (HT2) and T-2 toxin (T2) from type A and deoxynivalenol (DON) from type B. Thus, the aim of the current study was to assess the occurrence of these trichothecenes in the Catalonian market. 479 food samples were taken from the most susceptible to trichothecenes contamination and most commonly consumed in Catalonia commodities. DON, T2 and HT2 toxin were determined in breakfast cereals, snacks and pasta samples following extraction, clean-up, derivatization and finally analysis by GC-ECD. Moreover, these mycotoxins were determined in sliced bread, sweet corn and beer by LC-DAD. Our results showed that DON was the main trichothecene present in the cereal-based food from Catalonian market with percentages of positive samples ranging from 1.4 to 100.0%. Despite the high incidence of DON, only five samples were above EU limits. Concerning HT2 toxin, it was present in a low percentage of samples of sliced bread (15.3%), wheat flakes (14.8%), pasta (10.0%), corn snacks (8.5%), sweet corn (6.9%) and corn flakes (6.2%), while the T2 toxin only was quantified in 5 samples out of the total 479.

9.2 Introduction

Trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups, being the type A and B, the most common. Type A is represented by HT-2 toxin (HT2) and T-2 toxin (T2) and type B is most frequently represented by deoxynivalenol (DON). Trichothecenes are produced in several cereals by species of *Fusarium*, thus, a wide range of cereal-based food have been confirmed to be contaminated by these toxins (Lombaert et al., 2003; Schollenberger et al., 1999, 2005; Scott, 1997).

T2 is a potent inhibitor of protein synthesis and mitochondrial function both *in vivo* and *in vitro*, and shows immunosuppressive and cytotoxic effects. Moreover, it has been reported that the toxin has extremely toxic effects on skin and mucous (Eriksen and Pettersson, 2004; Sudakin, 2003; Visconti et al., 1991; Visconti, 2001). It has been shown that through deacetylation of T2, it is obtained HT2 as the major metabolite; however, little information is available regarding toxicity of HT2 alone (Sudakin, 2003; Visconti, 2001). Despite T2 toxic effects have been widely studied in animals, the toxicology has never been assessed in humans. Although DON is not as toxic as other trichothecenes such as T2 or HT2, this mycotoxin is one of the most common contaminants of cereals worldwide (Jelinek et al., 1989; Scott, 1989). Upon ingestion it can cause severe toxicosis in humans and farm animals. Acute effects of food poisoning in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in the stool (Rotter et al., 1996).

Maximum level of DON permitted in Europe is 0.750 μg/g in pasta and 0.500 μg/g in bread, pastries, biscuits, cereal snacks and breakfast cereals (European Commission, 2006b). The Joint FAO/WHO Expert Committee on Food Additives (JECFA), after assessing the toxic effect of T2 and HT2, concluded that the toxic effects of these mycotoxins could not be differentiated. Thus, the provisional maximum tolerable daily intake (PMTDI) for the combination of these toxins or alone was set at 0.06 μg/kg body weight/day (JECFA, 2001). Concerning DON, a tolerable daily intake

(TDI) of 1 μg/kg body weight based on a reduction of body weight gain was established by the EC SCF (SCF, 2002).

Unfavourable weather conditions in Western Europe have recently led to a high level of *Fusarium* infection in wheat and correspondingly high trichothecene contents (Larsen et al., 2004). Incidence of DON, T2 and HT2 toxin, have been widely reported in raw food and foodstuff, around of the European region (Cirillo et al., 2003; JECFA, 2001; Leblanc et al., 2005; Martins et al., 2008; Martins and Martins, 2001; Schollenberger et al., 1999, 2005), confirming that food processing methods do not completely remove the mycotoxins in the matrix (Hazel and Patel, 2004). Nevertheless, the occurrence of these trichothecenes in corn-based food from Spain, has been partialy addressed in two previous studies, where the breakfast cereals and corn snacks, were the main foodstuffs analysed (Castillo et al., 2008; Cerveró et al., 2007), thereby, currently there is a lack of raw contamination data of foodstuffs for human consumption, in order to be used in accurate exposure assessment studies, specially concerning T2 and HT2.

The aim of the present study was to study the incidence of trichothecenes DON, T2 and HT2 toxin in Catalonian market.

9.3 Materials and methods

9.3.1 Samples

Food samples were taken from the most susceptible to trichothecenes contamination and most commonly consumed in Catalonia commodities. During the months of June and July 2008, corn flakes (n=168), wheat flakes (n=27), sweet corn (n=185), corn snacks (n=213), pasta (n=201), beer (n=213), sliced bread (n=147) and bread (n=31) were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 items (if present) of each product were randomly taken. The level of trichothecenes was

determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store if were available (12 cities x 6 stores / city = 72 samples / category). However, in some cases, 3 items were not available in the same store, thus, less than 72 composites could be obtained. Regarding brands, we finally obtained 62 of corn flakes, 29 of wheat flakes, 31 of sweet corn, 79 of corn snacks, 105 of pasta, 64 of beer, 43 of sliced bread, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of Spanish market. The samples were transported and stored under suitable conditions until analysis.

9.3.2 Chemicals and reagents

Trichothecene standards, including DON, HT2 and T2 were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Toluene was purchased from Sigma. Acetonitrile, methanol, were purchased from J.T. Baker (Deventer, The Netherlands). Bencene and n-hexane were purchase from Merck (Darmstadt, Germany). All solvents were LC grade. Standardized 70–230 mesh aluminium oxide 90 (0.063–0.2 mm particle size) and ammonium hydroxide solution (32%, v/v) were purchased from Merck. C18 silica was purchased from Waters (Milford, MA, USA). Activated charcoal (Norit) was purchased from Fluka (Sigma–Aldrich, Alcobendas, Spain). Glass microfibre filters (GF/C) and filter papers (Whatman No 4) were from Whatman (Maidstone, UK). Pentafluoropropionic anhydride (PFPA), 4–dimethylaminopyridine (DMAP), sodium hydrogen carbonate, zinc acetate dihydrate (33% w/v) and potassium hexacyanoferrate (II) were also purchased from Sigma. T2/HT2 HPLC and DONTEST HPLC monoclonal antibody-based immunoaffinity columns were from Vicam (Waters Business, Milford, MA, USA). Mycosep # 225 and Multisep # 216 columns (Romer Laboratories), imidazole (Merck), sodium sulfate anhydrous for analysis (Merck). Pure water was obtained from a Milli–Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required. Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen

phosphate (0.2 g) (Sigma), disodium phosphate anhydrous (1.16 g) (Panreac), sodium chloride (8.0 g) (J.T Baker) in 1 liter of pure water; the pH was brought to 7.4.

9.3.3 Preparation of standard solutions

Each standard of DON, HT2 and T2 was dissolved in acetonitrile at a concentration of 1.0 mg/ml and stored at −20 ºC in a sealed vial until use. Working standards (10.0, 2.0, 1.0, 0.5, 0.25, 0.1, 0.05 and 0.02 μg/ml) were prepared by appropriate dilution of known volumes of the stock solution with acetonitrile and used to obtain calibration curves after derivatization (when it was necessary) and injection in the appropriated chromatographic system.

9.3.4 Equipment

The GC system was composed of a HP–6890 plus gas chromatograph, equipped with a ^{63}Ni ECD (Hewlett–Packard, Avondale, PA, USA) and an Agilent 7683 Series injector (Agilent Technologies, Waldbronn, Germany). Signals were processed by HP GC ChemStation software Version A.10:02(1757) (Hewlett–Packard).

To analyse trichothecenes in bread a GC system, 6890N-5973 equipped with a mass spetrometer was used. An HP-5MS fused silica capillary column (30m x 0.25 mm, film thickness 0.25 μm) from Agilent Technologies was used.

The LC system consisted of a Waters 600 pump, a Waters 717 automatic injector and a Waters 996 UV diode–array detector (DAD) (Waters Corporation, Manchester, UK). Millennium 32 software, version 3.05.01 (Waters Co., Milford, MA, USA) was used to control the system.

9.3.5 Trichothecene determination in the studied samples

Development and optimization of the analytic method for the trichothecene

determination in breakfast cereals, corn snacks and pasta samples, was carried out by means of various steps: firstly, a mycotoxin extraction phase, then a clean–up procedure, after a derivatization step and finally analysis by GC–ECD, while the bread samples were analysed by GC-MS. Concerning sliced bread, sweet corn and beer samples, the procedure for the determination of trichothecenes was carried out by means of extraction, clean–up and LC–DAD steps, because, this method showed the best accuracy and recovery rates for these matrices.

9.3.5.1 Extraction procedure

Bread, sliced bread and sweet corn samples were previously dried for 48 hours at 50ºC. Fifty grams of composites of breakfast cereals, corn snacks, pasta, sweet corn and sliced bread were finely ground with a laboratory mill and 2 g of flour was poured into a 50–ml screw top tube. After adding 15 ml of acetonitrile–water (84:16, v/v), the mixture was blended in an orbital blender for 90 min. The procedure with bread was slightly modified, 5 g of dried sample was extracted with 20 ml of acetonitrile-water (84:16, v/v) by shaking with an ultraturrax for 3 minutes. After filtering through Whatman No. 4 filter, the extraction mixture was stored in a tightly closed glass bottle at −20 ºC until use.

9.3.5.2 Clean-up procedures

Breakfast cereals, corn snacks and pasta samples

'Made–in–laboratory' cartridges were prepared using 5–ml sterile plastic syringes. A glass microfibre filter was placed at the bottom. Then, a mixture of packing bed was poured on it. The packed material consisted of 1.16 g of alumina–charcoal–C18 silica (75:1:40, w/w/w). Another glass microfibre filter was placed on the bed top. Then, it was pressed tightly but carefully with a plunger. Three milliliters of sample extract was passed through the prepared cartridge and collected in a vial. The cartridge was rinsed with 2 ml of acetonitrile–water (84:16, v/v). The eluate was collected in the same vial

and the purified extract was concentrated to dryness at 45 ºC under gentle stream of nitrogen. The samples of breakfast cereals, corn snacks and pasta continued the process with the derivatization of the extracts.

Sliced bread and sweet corn samples

Firstly, the clean-up procedure is the same that for breakfast cereals, corn snacks and pasta samples. Then, 10 ml of PBS was added to the dried extract and were shaken for 30 s with the help of a Vortex. Diluted solution was loaded into the immunoaffinity columns (both columns were consecutively coupled) and passed at a flow rate at one to two drops per second. The columns were washed with 5 ml of pure water. Then, it was eluted with 2 ml of methanol at a rate of about one drop per second in a 4–ml vial. The purified extract was concentrated to dryness at 45 ºC under gentle stream of nitrogen and solved in 0.25 ml of initial LC mobile phase.

Bread

A 5 ml aliquot of the extract was transferred to the tube that follows the Mycosep 225 columns. The Mycosep column was put on the top of the tube (as a cap) and mix carefully. The extract was pressed slowly through the Mycosep column (about 25-30 sec. per column). 3 ml of the purified extract were loaded into the Mycosep 216 column previously conditioned with 10 ml acetonitrile/ water (84:16). Then it was eluted with 10 ml acetonitrile/ water (9:1). The purified extract was evaporated to dryness under a gentle stream of nitrogen at 60ºC.

Beer

In the case of beer samples, the process of clean–up was as following: about 50 ml of cool beer was thoroughly degassed in ultrasonic bath for 1 hour in 500 ml Erlenmeyer flask. Ten milliliters of degassed beer was transferred to a screw top tube. Then, 0.1 ml of ammonium hydroxide solution (32%, v/v) was added to alkalinize the sample and hence to precipitate proteins and other matrix components. The mixture was shaken and let stand for 10 min. Then, 0.4 ml of a 25% aqueous solution of zinc acetate dihydrate was added to eliminate dyes without affecting trichothecene levels. Zn^{2+} excess

was controlled with 0.4 ml of potassium hexacyanoferrate (II) (10%, w/v) which provided a voluminous precipitate of zinc hexacyanoferrate (II). The mixture was vigorously shaken for 1 min, centrifuged at 6840 g for 10 min and the supernatant was collected to another tube. The supernatant was loaded into the immunoaffinity columns and passed at a flow rate at one to two drops per second. The columns were washed with 5 ml of pure water. Then, it was eluted with 2 ml of methanol at a rate of about one drop per second in a 4–ml vial. The purified extract was concentrated to dryness at 45 ºC under a gentle stream of nitrogen and solved in 0.25 ml of initial LC mobile phase.

9.3.5.3 Trichothecene derivatization by GC–ECD determination in samples of corn snacks, pasta and breakfast cereals

One hundred microliters of a 2 mg/l solution of DMAP in toluene–acetonitrile (80:20, v/v) and 50 µl of pentafluoropropionic anhydride (PFPA) were added to each dry extract in a screw cap vial. After capping tightly, the reaction mixture was heated at 60 ºC for 60 min in an aluminium heater block. After the mixture had cooled, 1 ml of a 3% (w/v) aqueous solution of NaHCO3 was added and the vial was vortexed for 15 s. The two layers were allowed to separate. The top (organic phase) layer was transferred to a GC autoinjector vial and analyzed by GC–ECD as pointed out below.

9.3.5.4 Trichothecene derivatization by GC–MS determination in samples of bread

1 ml benzene was added to the evaporated extract and mixed for 1 min. The extract was evaporated once again to dryness and 500 µl 0.4 M imidazole (as catalyst) in toluene-acetonitrile (85+15) and 100 µl PFPA were added to each derivatization vial and mixed for 1 min. The reaction was left for derivatization at 60ºC for 1 hour and cooled afterwards for about 10 min and washed (to remove the excess reagent) by adding 500 µl hexane and 1 ml 5% sodium hydrogen carbonate solution. The derivatives were mixed carefully and the vials were opened carefully to get rid off the pressure and mixed 1 min more. The aqueous phase was removed and 1 ml water was added and mixed 1 min.

Then the aqueous phase was removed completely. A small amount of sodium sulphate was added to remove any water left. Finally, about 200 µl of the derivatized extract were transferred to a GC vial.

9.3.5.5 GC–ECD analysis in the samples of corn snacks, pasta and breakfast cereals

The GC–ECD determination was carried out using the following chromatographic conditions. One microliter of solution was injected in splitless mode. The temperatures of the injection port and the detector were 250 and 300 ºC, respectively. The procedure used a fused silica capillary column HP–5 [5% methyl phenyl siloxane (30 m × 0.32 mm, 0.25 µm film thickness, Agilent Technologies)]. The oven temperature program was: 90 ºC held for 1 min, 40 ºC/min to 160 ºC, 1.5 ºC/min to 182 ºC, 5 ºC/min to 240 ºC, and then 40 ºC/min to 275 ºC, held for 8 min. Helium at a constant pressure of 42.1 kPa was used as carrier gas.

9.3.5.6 GC–MS analysis in bread samples

The GC-MS conditions were as follows: 1 µl of extract was injected in splitless mode. The temperature of the injection port was 250ºC and the temperatures of the detector were 230ºC (ion source) and 150ºC (quadrupole). The oven temperature program was: 120ºC held for 2 min, 30ºC/ min to 175ºC (held for 3 min), 1ºC/min to 180ºC and finally 25ºC/min to 265ºC (held for 15 min). Helium at a constant flow of 0.7 ml/ min was used as carrier gas. The transfer line temperature was held at 260ºC and selected ion monitoring at m/z 309 and 543 for DON, 377 and 555 for HT2 and 377, 451 for T2 were scanned for GC-MS analysis.

9.3.5.7 LC–DAD analysis in the samples of sweet corn, sliced bread and beer

Fifty–µl dissolved extract were injected in the chromatographic system. The elution was carried out at a flow of 1 ml/min with a mixture of acetonitrile (solvent A) and water (solvent B) applying the following gradient program: (i) initial solvent: 94% B,

maintained for 6 min, (ii) lineal variation of B from 94% to 70% for 4 min, (iii) lineal variation of B from 70% to 50% for 12 min, (iv) 50% of B for 3 min, and (v) return to the initial conditions in 0.2 min. Separation was performed on a Zorbax Eclipse Plus C18 column (150 mm x 4.6 mm, 3.5 mm particle size) connected to an Eclipse Plus C18 guard column (12.5 mm x 4.6 mm, 3.5 mm particle size) (Agilent Technologies Inc, Santa Clara, USA) filled with the same phase. The column was kept at 35 ºC. Quantification of trichothecenes was performed by measuring its peak area with the help of a calibration curve calculated by regression from standard solutions. Detection wavelengths were 200 nm for HT2 and T2, and 220 nm for DON. The UV spectrum of compounds in the sample was compared with the standard UV spectrum for the mycotoxin identification.

9.3.6 Validation of analytical methods

The analytical methods used for DON, HT2 and T2 were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting three times 50 µl of mycotoxin standard solutions before injecting extracted samples and comparing the peak retention times and the UV spectra of the substances that produce these peaks.

Linearity was assessed by performing triplicate injections of standard solutions whose concentrations were 0.02, 0.05, 0.1, 0.25, 0.5 and 1.0 µg each of mycotoxin/ml. Standard curves were generated by linear regression of peak areas against concentrations.

Accuracy and recovery were established for each method by determination of DON, HT2 and T2 in samples of pasta, corn snack, sliced bread, beer, breakfast cereal and sweet corn, covering the range of the method (range between 0.05 and 1.0 µg/g). Recovery was determined by comparing the absolute responses of trichothecenes obtained from the studied samples with the absolute responses of calibration standards.

The limit of detection (LOD) was considered as the mycotoxin concentration that provides a signal equal to b+3Sb, where b is the intercept of the calibration curve and Sb is the standard error of the estimate assuming to be the blank, and the limit of

quantification (LOQ) was considered equal to 3×LOD.

9.4 Results and Discussion

9.4.1 Method validation

Samples of pasta, corn snacks, sweet corn, sliced bread, beer, corn flakes and wheat flakes were spiked at levels of 1, 0.2 and 0.05 μg/g of DON, HT2 and T2 for triplicated. Concerning bread, these samples were spiked with each mycotoxin at LOQ level and the concentration of 0.473, 0.437 and 0.612 μg/g with DON, HT2 and T2, respectively. Analytical procedures were carried out three times. Calibration curves showed good linearity with correlations coefficients R^2 ranging from 0.9799 to 0.9994. Percentage of recovery and relative standard deviation (RSDr) for each food matrix and spiking level are shown in Table 9.1.

Recovery ranges between 77-143.1, 78.3-115.6 and 74-129.7 % were found for DON, T2 and HT2, respectively, according to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission, 2006a), with the exception of recovery value of 143.1%, found in pasta samples spiked at 0.05 μg/g. Maximum relative standard deviation (RSDr) levels for DON, T2 and HT2 were 19.8, 24.7 and 26.7 %. Quantification limits of DON, T2 and HT2 toxin ranged from 0.012 μg/g (beer) to 0.066 μg/g (corn snacks), 0.042 μg/g (wheat flakes) to 0.135 μg/g (sweet corn) and 0.009 μg/g (beer) to 0.061 μg/g (corn snacks), respectively.

Table 9.1. Method performance characteristics for DON, HT2 and T2

		DON			HT2			T2		
Sample =3	Spiking level (µg/g)	LOQ (µg/g)	Recovery (%)	RSDr (%)	LOQ (µg/g)	Recovery (%)	RSDr (%)	LOQ (µg/g)	Recovery (%)	RSDr (%)
Pasta	0.05	0.042	143.1	19.2	0.030	113.2	12.3	0.063	90.9	7.8
	0.2		78.1	17.9		99.3	11.2		80.9	24.7
	1		77	17.9		88.4	18.9		86.2	13.5
Corn snacks	0.05	0.066	105.2	17.4	0.061	112.7	24.2	0.054	115.6	8.8
	0.2		91.6	16.5		93.9	10.1		94.7	21.5
	1		77.8	8.9		82.4	14.7		92.4	11.7
Sweet corn	0.05	0.027	110.5	16.8	0.024	88.8	6.7	0.135	-	-
	0.2		89.3	5.1		74	14.9		69.8	4.0
	1		94.7	12.3		83.4	9.2		101.1	5.2
Sliced bread	0.05	0.030	109.6	10.0	0.030	98.9	8.2	0.108	-	-
	0.2		109.9	12.2		107.1	6.6		90.0	14.0
	1		86.4	5.7		104.8	10.3		94.4	8.6
Beer	0.05	0.012	110.2	10.0	0.009	125	16.9	0.057	-	-
	0.2		101.5	6.1		121.7	7.7		90.3	5.8
	1		93.6	5.4		73.9	14.4		83.3	5.9
Corn flakes	0.05	0.045	95.6	19.8	0.030	129.7	26.7	0.057	119.6	14.4
	0.2		99.8	7.6		87.3	5.2		102.8	18.9
	1		70.9	8.1		79.8	10.5		78.3	7.9
Wheat flakes	0.05	0.041	89.3	14.0	0.036	109.5	17.5	0.042	66.6	19.7
	0.2		84.5	11.9		93.6	5.5		96.5	6.0
	1		85.8	18.4		86	8.2		85.5	3.1
Bread	[a]	0.018	80.3	5.1	0.050	88.2	5.9	0.070	95.0	4.6
	[b]		76.6	6.4		87.0	3.9		90.7	5.4

[a] Spiking level DON: 0.018 µg/g, HT2: 0.050 µg/g, T2: 0.070 µg/g.

[b] Spiking level DON: 0.473 µg/g, HT2: 0.437 µg/g, T2: 0.612 µg/g.

9.4.2 Incidence of DON

In this study 479 "composite" samples pooled from 1147 individual samples from Catalonian market, were analyzed to detect and quantify DON, T2 and HT2. Previous studies conducted in Spain were carried out with fewer samples than the current work, in

the order of 25 (Cerveró et al., 2007) and 175 (Castillo et al., 2008), thus, this work, represents the widest survey conducted in Spain to assess the incidence of trichothecenes in foodstuffs for human consumption, to date.

As shown in Table 2, the mycotoxin DON was found in all cereal-based food items analyzed in this study. The lowest percentage of positive samples (quantified) was found in beer (1.4%), sweet corn (2.8%) and sliced bread (16.7%). In contrast, high percentage of positive samples was found in breakfast cereals (74.1 and 73.4%), corn snacks (78.9%), pasta (74.3%) and specially in bread, where DON was found in all samples. Mean values of positive samples ranged from 0.012 μg/g, found in beer, to 0.246±0.158 μg/g found in bread. Despite the high percentage of positive samples in foodstuffs from Catalonian market, only two samples were above the limit of 0.750 μg/g in pasta (0.946 μg/g), while one sample of corn flakes (0.580 μg/g) and two of bread (0.523 and 0.739 μg/g), were above the limit of 0.500 μg/g, established by European Commission (European Commission, 2006a).

Table 9.2. Occurrence of DON in foodstuff from Catalonian market

	N individual	N composite	Samples >LOQ	Mean±sd	Max	<LOD	LOD-LOQ	LOQ-0.2	0.2-0.4	>0.5
				μg/g	μg/g					
Wheat flakes	27	27	20/27	0.190±0.117	0.437	3	4	13	7	0
Corn flakes	168	65	49/65	0.109±0.078	0.580	15	1	48	0	1
Beer	213	71	1/70	0.012	0.012	70	0	1	0	0
Sweet corn	185	72	2/72	0.114±0.036	0.139	70	0	2	0	0
Corn snacks	213	71	56/71	0.153±0.058	0.304	9	6	45	11	0
Pasta	201	70	52/70	0.226±0.177	0.946	14	4	40	9	3
Sliced bread	147	72	12/72	0.068±0.018	0.098	54	6	12	0	0
Bread	31	31	31/31	0.246±0.158	0.739	0	0	12	17	2

The previous studies, carried out to assess the incidence of DON in foodstuff from Spanish market, commonly found lower levels of positive samples, mean and maximum values than our study. For example, in the earliest study the range of 0.038-

0.195 μg/g was reported for corn flakes, 0.035-0.061 μg/g for sweet corn and 0.028-0.109 μg/g for fried corn snacks (Cerveró et al., 2007). Moreover, in other recent study, concentrations of 0.030-0.121 and 0.026-0.080 μg/g, were found in breakfast cereals and fried snacks, respectively (Castillo et al., 2008). However, we found a wide range of contamination for these foodstuffs, with maximum values of 0.580, 0.139 and 0.304 μg/g in corn flakes, sweet corn and corn snacks, respectively. In the line with our results, there is a study conducted in Canada with breakfast cereals where the authors showed a maximum level and mean of positive samples of 0.420 and 0.070 μg/g, respectively (Roscoe et al., 2008), while in Italy, a lowest range, between 0.012-0.047 μg/g was found (Cirillo et al., 2003). Highest levels were stated in other study performed in Portugal, where the range 0.103-6.040 μg/g and the mean 0.754 μg/g were found in wheat-based breakfast cereals (Martins and Martins, 2001).

Regarding baked products, in the The First French Total Diet Study the mean of the positive samples of bread and rusk was estimated to be 0.109 μg/g (Leblanc et al., 2005). While in a German study, the authors reported a high percentage of positive samples in conventional and organic bread, with ranges of 0.015-0.690 μg/g and 0.015-0.224 μg/g, and means of 0.184 and 0.062 μg/g, respectively (Schollenberger et al., 2005). In Italy, the level of DON in bread and related food was observed between 0.007 and 0.270 μg/g (Cirillo et al., 2003), all these values being much lower than our range of 0.027-0.739 μg/g.

Few data is available about the levels of DON in pasta. This mycotoxin was not detected in France (Leblanc et al., 2005) and the levels ranged from 0.009 to 0.077 μg/g in Italy (Cirillo et al., 2003), while, the level in noodles from Germany was between 0.015-1.670 μg/g, with a mean of 0.158±0.334 μg/g (Schollenberger et al., 1999), closest to our study with a respective mean and maximum value of 0.226 and 0.946 μg/g.

Concerning beer, to our knowledge, few studies have been conducted to assess the level of DON in this alcoholic beverage from European markets. In an early study

that reported the incidence of 176 beer samples collected in European and North American markets, the authors found that 64% of samples were above the LOQ of 0.003 μg/g, and the mean ranged from 0.002 to 0.011 μg/g; the maximum value was 0.036 μg/g (Kostelanska et al., 2009). The higher incidence in this previous study could be explained by the lower LOQ of this study (0.012 μg/g).

9.4.3 Incidence of T2 and HT2 toxin

Percentage of positive samples of T2 and HT2 toxin and contamination levels in food marketed in Catalonia, are shown in Table 9.3. Low percentage of positive samples was found for these type-A trichothecenes, specially for T2, which was quantified only in 5 samples of 479 (wheat flakes, sweet corn and corn flakes). HT2 was present in a low percentage of sliced bread samples (15.3 %), wheat flakes (14.8%), pasta (10 %), corn snacks (8.4%), sweet corn (6.9 %) and corn flakes (6.2%). Mean levels of HT2 in positive samples was between 0.041±0.017 μg/g (corn flakes) and 0.214±0.334 μg/g (corn snacks), being the maximum level 0.895 μg/g in a corn snack sample.

In a previous study conducted in Spain, the authors did not find T2 in any sample out of the 25 samples analyzed, above the LOD of 0.030 μg/g neither in corn flakes, sweet corn nor in corn snacks (Cerveró et al., 2007). Leblanc et al., (2005) quantified HT2 in one sample out of 238 composite samples with a level of 0.270 μg/g. In Germany, HT2 was the main type-A trichothecene found in cereal-based food, with ranges of 0.012-0.032 μg/g in bread and related products, 0.012-0.025 μg/g in noodles and 0.012-0.022 μg/g in breakfast cereals, slightly lower values than our results (Schollenberger et al., 1999).

Table 9.3. Occurrence of T2 and HT2 toxin in food from Catalonian market

T2	N individual	N composite	Samples >LOQ	Mean±sd µg/g	Max µg/g	<LOD	LOD-LOQ	LOQ-0.2	0.2-0.4	>0.5
Wheat flakes	27	27	2/27	0.072±0.005	0.075	20	5	2	0	0
Corn flakes	156	65	0/65	-	-	58	7	0	0	0
Beer	216	71	0/71	-	-	71	0	0	0	0
Sliced bread	147	72	0/72	-	-	71	1	0	0	0
Sweet corn	185	72	2/72	0.215±0.058	0.256	66	4	1	1	0
Corn snacks	213	71	1/71	0.070	0.070	63	7	1	0	0
Pasta	201	70	0/70	-	-	56	14	0	0	0
Bread	31	31	0/31	-	-	31	0	0	0	0
HT-2										
Wheat flakes	27	27	4/27	0.087±0.066	0.183	13	10	4	0	0
Corn flakes	156	65	4/65	0.041±0.017	0.065	30	31	4	0	0
Beer	216	71	0/71	-	-	71	0	0	0	0
Sliced bread	147	72	11/72	0.047±0.012	0.075	56	5	11	0	0
Sweet corn	185	72	5/72	0.043±0.023	0.084	64	3	5	0	0
Corn snacks	213	71	6/71	0.214±0.334	0.895	46	19	5	0	1
Pasta	201	70	7/70	0.051±0.022	0.080	38	25	7	0	0
Bread	31	31	0/31	-	-	31	0	0	0	0

9.4.4 Co-occurrence of trichothecenes in the samples

Several studies have suggested that the mycotoxins could have synergistic effect in vivo (Bacon et al., 1996; Javed et al., 1993), therefore the knowledge of the occurrence of all mycotoxins should be considered a challenge in exposure assessment studies. In our work, the co-occurrence of different mycotoxins in the same sample, was studied considering positive those samples above LOD (Table 9.4).

Table 9.4. Co-occurrence of trichothecenes in foodstuff from Catalonian market

	DON-T2	DON-HT2	T2-HT2	DON-T2-HT2
Wheat flakes	2	8	0	4
Corn flakes	1	17	0	4
Beer	0	0	0	0
Pan loaf	1	4	0	0
Sweet-corn	0	0	3	0
Corn snacks	2	20	0	4
Pasta	2	16	0	12
Bread	0	0	0	0
Total	8	55	3	24

Note: Co-occurrence was determined considering samples above LOD, and expressed as the number of samples with simultaneous contamination the mycotoxins

The results suggest us that the most common case of co-occurrence with trichothecenes can be drawn by the simultaneous incidence of DON with HT2 toxin, specially in corn snacks, corn flakes and pasta. The co-occurrence of DON, HT2 and T2 toxin, was mainly observed in pasta, and few cases of simultaneousness were found for the combination DON-T2 or T2-HT2. This fact was elucidated previously by Schollenberger et al., (1999), where the authors found that DON was present in each co-occurring sample except for 3 samples, due to the high incidence of this mycotoxin.

9.5 Conclusions

With 479 analytical samples, this is the widest study to assess the incidence of trichothecenes in food for human consumption until now in Spain. We have confirmed the high incidence of DON in cereal-based foodstuff from Catalonian market, specially in wheat flakes, corn flakes, corn snacks, pasta and bread, with percentage of positive samples ranging from 73.4 to 100.0%. Despite the high percentage of positive samples in foodstuff from Catalonian market, only five samples were above the limit established by European Commission (European Commission, 2006a). In the other hand, T2 toxin was only quantified in 5 samples out of total samples of cereal-based food. HT2 was present in a low percentage of the samples (from 6.2% in corn flakes to 15.3% in sliced bread), and it was not detected in samples of bread and beer. Although no worrying

values have been found in this study for DON, T2 and HT2, the annual variability of the levels of trichothecenes in the crops make necessary the monitoring of these mycotoxins, specially in our temperate regions. The high consumption of cereal-based food in Catalonia, requires that we carry out an accurate exposure assessment study for all population groups, currently in course, where an stochastic methodology have been applied to combine contamination and consumption raw data.

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Chapter 10. Quantitative Dietary Exposure Assessment of the Catalonian Population (Spain) to the Mycotoxin Deoxynivalenol

Food Additives and Contaminants 2011 28, 1098–1109

10.1 Abstract

The mycotoxin deoxynivalenol (DON) is one of the most common contaminants of cereals worldwide, and its occurrence has been widely reported in raw foods and foodstuffs, around the European region, including Catalonia. In the present work, a stochastic methodology has been applied to accurately assess the exposure of the Catalonian population (Spain) to DON through food consumption. Raw contamination data was provided by a large survey conducted in this region, in addition to the raw consumption data from a nutritional study specifically designed to assess the dietary intake of the main foodstuffs related to DON contamination for all population age groups. Contamination and consumption data were combined by simulation using an essentially parametric (*P-P*) method. The *P-P* method draws sampling values from distribution functions fitted to consumption and contamination data sets. Moreover, to quantify the accuracy and reliability of the statistics estimates, we built the related confidence intervals using a pseudo-parametric bootstrap method. Considering the results drawn from the *P-P* simulation method, the Catalonian population should be expected to be exposed at moderated levels of deoxynivalenol, the infants and individuals with ethnic dietary patterns being the most exposed population groups.

10.2 Introduction

Trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups, the type A and B being the most common. Type A is represented by HT-2 toxin (HT2) and T-2 toxin (T2) and type B is most frequently represented by deoxynivalenol (DON). Trichothecenes are produced in several cereals by species of *Fusarium*, thus a wide range of cereal-based foods have been reported to be contaminated by these toxins (JECFA, 2001), DON being one of the most common contaminants of cereals worldwide (Jelinek et al., 1989; Scott, 1989).

Acute effects of food poisoning by DON in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in stool (Rotter et al., 1996). A tolerable daily intake (TDI) of 1 μg/kg body weight based on a reduction of body weight gain was established by the EC SCF (SCF, 2002).

Occurrence of DON has been widely reported in raw foods and foodstuffs in European countries (JECFA, 2001), confirming that food processing methods do not completely remove mycotoxins (Hazel and Patel, 2004). The presence of trichothecenes in Catalonia (Spain) has been recently studied by means of a thorough methodology involving a large sampling and accurate chemical analysis. That study concluded that the occurrence of DON was high in cereal-based foodstuffs from Catalonian market, especially in wheat flakes, corn flakes, corn snacks, pasta and bread (Cano-Sancho et al., 2011). Cereal-based foods are the base of the energy intake in Catalonia, as well as in the other Mediterranean countries (Serra-Majem et al., 2003). Therefore, in the food safety framework, to assess the exposure of Catalonian population to DON is a priority.

To our knowledge, few studies have been published to assess the exposure to DON, and the ones that have been published have generally used deterministic approaches (SCOOP, 2003). Experts have recommended the use of a stochastic approach to provide a more realistic exposure assessment, taking model uncertainties and variability into consideration. Nevertheless, there is no consensus on which specific

methodology should be applied in each case (Kroes, 2002; WHO, 2005; EFSA, 2006; Verger and Fabiansson, 2008). A stochastic methodology was developed by Gauchi and Leblanc (2002) to assess the exposure of human populations to food contaminants, as in the case of the exposure of the French population to ochratoxin A (OTA). The authors proposed two simulation approaches to estimate the exposure, based on Monte Carlo simulations (using NonParametric-NonParametric and Mixed Parametric-Parametric methods), leading to the assessment of four types of bootstrap confidence intervals.

The aim of this paper is to quantitatively assess the exposure of the Catalonian population (Spain) to DON.

10.3 Materials and methods

10.3.1 Raw contamination data

Raw contamination data was mainly taken from the study of Cano-Sancho et al. (2011), and completed with some data from the Project to Assess the Exposure of the Catalonian Population to Mycotoxins (UdL-ACSA, 2010).

In the first work, during the months of June and July 2008, corn flakes (n=168), wheat flakes (n=27), sweet corn (n=185), corn snacks (n=213), pasta (n=201), beer (n=213), sliced bread (n=147), bread (n=31), gluten-free foods (n=12), ethnic foods (n=35) and baby foods (n=90) were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 items (if present) of each product were randomly taken. The level of trichothecenes was determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store if available (12 cities x 6 stores / city = 72 samples / category). However, in some cases, no items were available in the store, thus, less than 72 composites could be obtained. Finally, a wide range of brands was obtained, which can be considered the majority of market share in Catalonia of these products, as well as

in the rest of the Spanish market. Moreover, some commodities were selected because they are highly consumed by some population groups with different dietary patterns: baby foods, ethnic foods and gluten-free foods. DON was determined in breakfast cereals, snacks and pasta samples following extraction, clean–up, derivatization and final analysis by GC-ECD. Moreover, this mycotoxin was determined in sliced bread, sweet corn, beer, baby food, ethnic food and gluten-free food by LC-DAD. In this study, non-detected samples were assumed to be equal to the limit of detection (LOD) divided by 2, an assumption widely recognised to reduce the uncertainty of values between 0 and the LOD (GEMs/Food-WHO, 1995). Some statistics from contamination data and the related histograms are shown in Table 10.1 and Fig. 10.1, respectively.

Table 10.1. Occurrence of deoxynivalenol in food products available in the Catalonian market.

	N individual	N composite	Samples >LOQ	Mean±sd µg/g	Max µg/g
Wheat flakes[1]	27	27	20/27	0.190±0.117	0.437
Corn flakes[1]	168	65	49/65	0.109±0.078	0.580
Beer[1]	213	71	1/70	0.012	0.012
Sweet corn[1]	185	72	2/72	0.114±0.036	0.139
Corn snacks[1]	213	71	56/71	0.153±0.058	0.304
Pasta[1]	201	70	52/70	0.226±0.177	0.946
Sliced bread[1]	147	72	12/72	0.068±0.018	0.098
Bread[1]	31	31	31/31	0.246±0.158	0.739
Ethnic foods[2]	35	35	20/35	0.406±0.272	1.080
Gluten-free Bread[2]	5	5	1/5	0.270	0.270
Gluten-free pasta[2]	7	7	1/7	0.163	0.163
Baby food[x]	30	30	12/30	0.131±0.054	0.286

[1] Contamination data provided by Cano-Sancho et al. (2010).
[2] Contamination data provided by UdL-ACSA et al. (2010).

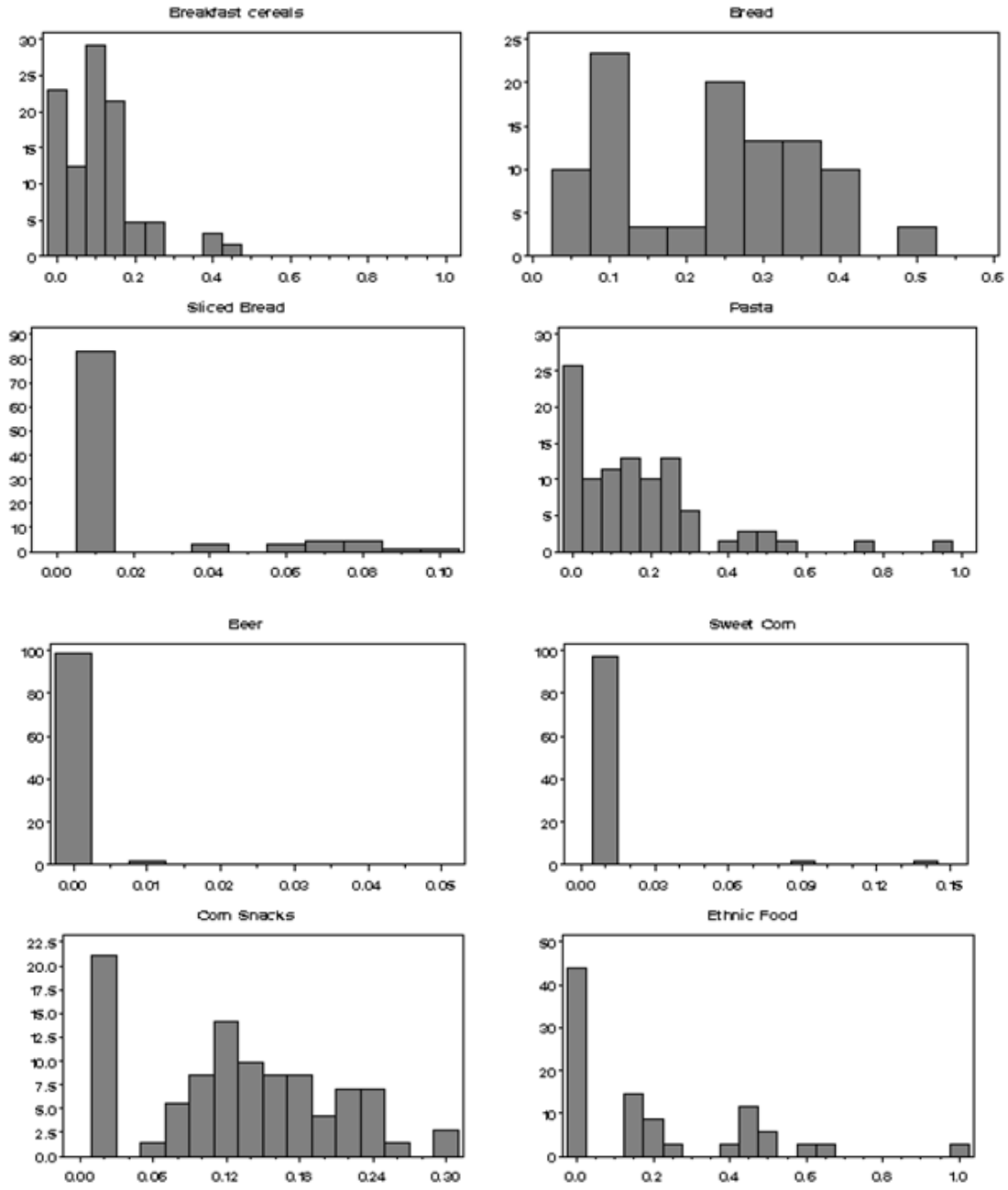


Fig 10.1. Contamination histograms (relative frequencies), in μg g^{-1} for each food

10.3.2 Raw consumption data

Consumption data were taken from the Project to Assess the Exposure of the Catalonian Population to Mycotoxins (UdL-ACSA, 2010). This study involved a wide nutritional survey designed to specifically identify the dietary patterns of the Catalonian population in relation to the main foodstuffs susceptible to mycotoxins contamination.

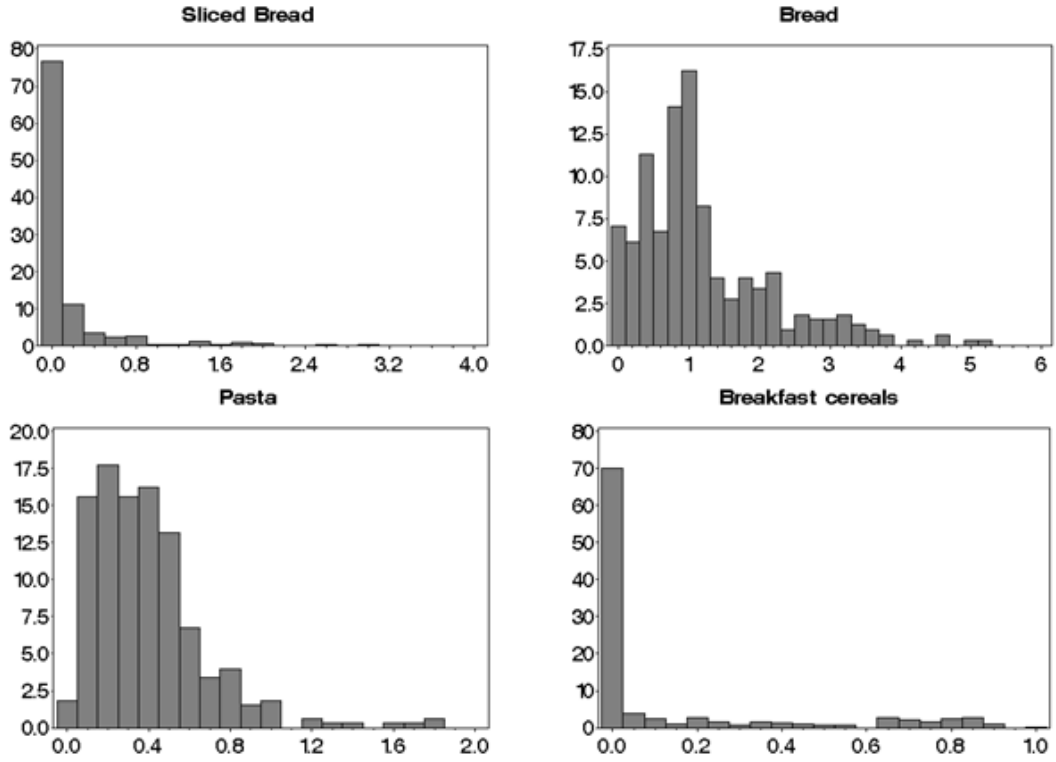
Food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ), developed for the Catalonian population including those foods typically

consumed in the region that may be potentially contaminated with these mycotoxins. According to World Health Organization (WHO) recommendations, studies to assess the dietary intake of chemical contaminants should show the significant intake within the standard population, as well as within all population groups that could have different dietary patterns. Therefore, five different age population groups were considered: infants (0-3 years), children (4-9 years), adolescents (10-19 years), adults (20-65 years) and seniors (> 65 years). Moreover, adult celiac sufferers and adults with ethnic dietary pattern were included in the nutritional study, and studied separately. The FFQ included 38 specific food items known to be the major foods contaminated by mycotoxins worldwide, excluding those foods not consumed in the region. Concerning consumption frequency, five response options, ranging from 'never' to 'annually', were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 76 seniors, 720 adults, 235 adolescents, 69 children, 164 parents of infants, 70 adult celiac sufferers and 56 adults with ethnic dietary pattern were interviewed in 2008-2009 by trained interviewers. In the present study, the adults were treated separately according to their sex, with a major population group of 336 adult males and another of 384 adult females. Individuals were from 89 cities and towns from Catalonia. An example of some statistics from the consumption data of adult females and the related histograms is shown in Table 10.2 and Fig. 10.2, respectively. They are in the line of those obtained for males.

Dependencies on consumption patterns can be quantified by Spearman correlation coefficients and can be taken into account by the Iman and Conover method (Iman and Conover 1982). However, Gauchi and Leblanc (2002) did not report significant differences in the results, regardless of whether or not dependencies were taken into account. We therefore did not consider dependencies in the present work.

Table 10.2. Normalised consumption of the main foodstuffs related to DON contamination by the 384 adult females (g/kg body weight/day)

	Consumers	% Consumers	Mean	*SD*	Max
Breakfast cereals	153	39.8	0.48	0.46	2.35
Sliced Bread	161	41.9	0.22	0.32	1.74
Bread	365	95.1	0.91	0.72	4.78
Pasta	378	98.4	0.34	0.29	3.51
Corn Snacks	114	29.7	0.07	0.11	0.91
Beer	204	53.1	1.43	1.97	10.14
Sweet Corn	143	37.2	0.50	1.08	8.33



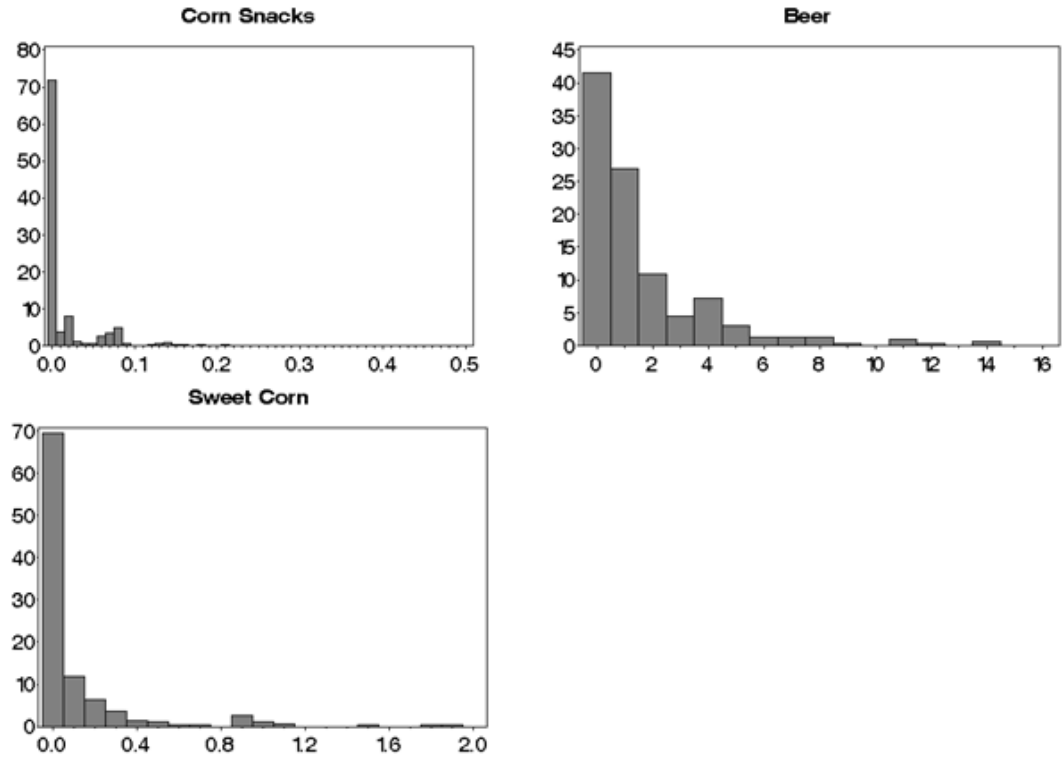


Fig 10.2. Consumption histograms (relative frequencies) for adult female consumers, in μg kg^{-1} bw day^{-1}

10.3.3 Methodology used to calculate the exposure

In this section we recall some general aspects concerning the exposure calculations, already given in Gauchi and Leblanc (2002).

The main limitation to estimate the normalised exposure distribution of consumer populations is derived from the equation:

$$E_{\pi} = \sum_{j=1}^{p} C_{\pi,j} T_j \qquad (1)$$

where the random variable "normalised global exposure in a consumer population π" (E_{π}) is a function of the random variables: "normalised consumption of the foodstuff j in a consumer population π" ($C_{\pi,j}$) and "DON concentration level of the foodstuff j" (T_j). The difficulty is due to the probability density functions (*pdf*) since these variables

are generally unknown and even if they are known, they are generally different and not independent. Moreover, the $C_{\pi,j}$ x T_j *pdf* products are unknown, too.

If we assume independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, we can estimate the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j \qquad (2)$$

Where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff j in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food.

Under these assumptions we can also calculate the variance estimate $\hat{V}(E_{\pi_0})$ using Eq. (5) and Eq. (6) in Gauchi and Leblanc (2002). We could consider this approach to be a theoretical approach, but because of the postulated assumptions, it would have to be a (very) simplified theoretical approach. For example, this simplified approach does not enable us to calculate complicated statistics such as high quantiles. Simulation methods would therefore be required to obtain estimates of these statistics. Finally, we will consider this approach to be a direct approach.

10.3.4 Stochastic procedure to calculate the exposure: Parametric-Parametric (*P-P*) method

In this section we recall some elements of the methodology used to calculate the exposure by means of the stochastic procedure, already given in Gauchi and Leblanc (2002).

The main advantage of the use of a parametric method is derived from the fact that NonParametric-NonParametric method could lead us to less reliable estimations, especially of the high quantiles (Gauchi and Leblanc, 2002). Taking the asymmetrical

appearance of consumption and contamination histograms and our previous experience into account, we fitted the probability density functions, choosing the gamma distribution to be the best candidate. Therefore, for each consumption and contamination dataset, the gamma *pdf* was fitted with the method of maximum likelihood using the CAPABILITY procedure of SAS software (SAS 2010). The Chi-Square statistic was used in the goodness-of-fit test, considering a significance level greater than 95%, to accept the distribution as a suitable candidate. Moreover, several graphical methods such as quantile-quantile plot exist, making it possible to quickly check if the fitted distribution is adapted to the selected hypothesis (Smout et al. 2000). Shape and scale parameters were estimated for each contamination set, in accordance with the methodology described above.

Concerning consumption datasets, the foodstuffs selected are commonly consumed within the Catalonian population, despite several exceptions related to age-group dietary patterns. The appearance of the histograms is therefore continuous and only one distribution was considered pooling all age groups data, fitting all data to gamma *pdf*. In case of gluten-free foods, the mean contamination was used in the simulations because few data were available.

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

$$\hat{E}_k^{[P-P]} = \sum_{j=1}^{p} \tilde{c}_{i(j)} \tilde{t}_j \qquad (4)$$

where $\tilde{c}_{i(j)}$ is a random normalised consumption *i* for the foodstuff *j*, drawn from $\hat{C}_j$, the corresponding adjusted gamma *pdf*, and $\tilde{t}_j$ is a random contamination for the foodstuff *j*, drawn from $\hat{T}_j$, the corresponding adjusted gamma *pdf*. The density parameter estimates for $\hat{C}_j$ and $\hat{T}_j$ are shown in Table V.

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 (10,000 in the present study):

$$\hat{E}_S^{[P-P]} = \frac{1}{n}\sum_{k=1}^{n} \hat{E}_k^{[P-P]} \quad (5)$$

Other statistics were directly computed on the histogram built with the simulations of the *S* set, and statistics were also estimated from the lognormal and gamma fitted *pdf*s.

10.3.5 Method to build the bootstrap confidence intervals

To know the validity and accuracy of the high quantiles estimated for the simulation method, the confidence intervals are required. Therefore, this section focuses on building bootstrap confidence intervals in order to determine the reliability of the simulation methods. Several methods to build bootstrap (Efron 1993) confidence intervals (CI_b) were assessed by Gauchi and Leblanc (2002), revealing the difficulties involved in applying the nonparametric CI_b and highlighting the "*pseudo-parametric* CI_b" as the best choice from among the other parametric procedures and analytical methods proposed. We therefore built CI_b according to this method.

"P*seudo-parametric* CI_b", referred to as Type 1, was built by randomly drawing *B* samples of size n_{π_0} in the exposure simulation set *S*. Typically, *B* is equal to 10,000

The boundaries of the 95% confidence interval are calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution.

10.4 Results

10.4.1 Result direct approach

The parameter estimates of this direct approach, $\hat{E}_{\pi_0}$ and $\hat{V}(E_{\pi_0})$, for each population group are shown in Table 10.3. They should be compared with the results of the subsequent tables. We observed that they were quite different; the infants and ethnics being the most exposed groups.

Table 10.3. Results of exposure using the direct approach

	$\hat{E}_{\pi_0}$	$\hat{V}(E_{\pi_0})$
Seniors	0.04	$(0.04)^2$
Celiac sufferers	0.13	$(0.09)^2$
Ethnics	0.57	$(0.47)^2$
Adults females	0.09	$(0.07)^2$
Adults males	0.10	$(0.08)^2$
Adolescents	0.15	$(0.13)^2$
Children	0.36	$(0.27)^2$
Infants	0.74	$(0.71)^2$

($\mu g\ kg^{-1}\ bw\ day^{-1}$)

10.4.2 Results of the *P-P* method

The scale and shape parameters estimated from the gamma *pdf* fitted to consumption and contamination datasets are shown in Table 10.4.

Table V provides statistics calculated from the simulation outputs. The estimated statistics presented are the mean, standard deviation, skewness and kurtosis, as well as the median and the main high quantiles (90^{th}, 95^{th} and 99^{th}). The highest values of skewness and kurtosis were found in the celiac sufferers group, while it was assumed that the most exposed group would be the infants and ethnics, with the highest mean and quantiles, especially the related 99th quantile. An example of exposure output histograms of relative frequencies, obtained for adult females is given in Fig. 10.3. When the estimated means from the simulation method are compared to those obtained through the direct approach, it is observed that they are quite higher.

Table 10.4. Parameters of the gamma *pdf* fitted to the subclasses of normalized consumption

Foodstuff[a]	Elders ($\hat{r}$, $\hat{\lambda}$)	Adult females ($\hat{r}$, $\hat{\lambda}$)	Adult males ($\hat{r}$, $\hat{\lambda}$)	Teenagers ($\hat{r}$, $\hat{\lambda}$)
Breakfast cereals (0.457; 1.437)	not enough data	0.895 ; 0.542	1.229 ; 0.354	0.873 ; 0.891
Bread (0.458; 1.436)	1.999; 0.381	0.765; 1.089	0.705; 1.701	0.554; 2.218
Sliced bread (0.824; 52.632)	2.840 ; 0.0384	0.762 ; 0.294	0.611 ; 0.624	0.674 ; 0.785
Pasta (0.747; 4.386)	1.423 ; 0.1616	2.263 ; 0.151	2.149 ; 0.195	1.770 ; 0.292
Corn snacks (1.410; 11.364)	not enough data	0.635 ; 0.787	1.396 ; 0.044	0.790 ; 0.211
Beer (not enough data)	0.606 ; 3.039	0.735 ; 1.945	0.937 ; 2.471	0.759 ; 1.532
Sweet corn (not enough data)	not enough data	0.635 ; 0.787	0.777 ; 0.475	0.653 ; 0.543
Ethnic foods (0.892; 0.535)	-	-	-	-
Baby food (0.458; 1.436)	-	-	-	-

Foodstuff[a]	Infants ($\hat{r}$, $\hat{\lambda}$)	Babies ($\hat{r}$, $\hat{\lambda}$)	Ethnics ($\hat{r}$, $\hat{\lambda}$)	Celiacs ($\hat{r}$, $\hat{\lambda}$)
Breakfast cereals (0.457; 1.437)	1.106 ; 1.772	-	0.130; 1.739	0.193; 2.020
Bread (0.458; 1.436)	0.741; 5.707		0.892; 1.869	0.550; 2.096
Sliced bread (0.824; 52.632)	0.853 ; 1.102	-	not enough data	not enough data
Pasta (0.747; 4.386)	2.256 ; 0.449	-	1.231; 0.242	0.786; 0.236
Corn snacks (1.410; 11.364)	1.074 ; 0.212	-	0.152; 0.716	0.258; 0.272
Beer (not enough data)	-	-	0.157; 14.485	0.133; 2.413
Sweet corn (not enough data)	0.484 ; 1.77	-	0.140; 0.461	0.167; 0.908
Ethnic foods (0.892; 0.535)	-	-	0.295; 1.297	-
Baby food (0.458; 1.436)	-	1.474 ; 10.579	-	-

Table 10.5. Results of exposure assessment of the population groups from *P-P* simulation method. These results were obtained directly from the N simulation outputs without *pdf* fittings; they must be compared to the results of Table VI (in μg kg^{-1} bw day^{-1})

***P-P* Method**

	Seniors	*Adult females*	*Adult males*
N	10000	10000	10000
Mean	0.28	0.56	0.37
SD	0.48	0.77	0.48
Skewness	5.58	4.97	4.79
Kurtosis	59.47	41.65	41.08
Median	0.12	0.32	0.22
0.90^{th} quantile	0.71	1.26	0.82
0.95^{th} quantile	1.08	1.84	1.18
0.99^{th} quantile	2.27	3.70	2.33
	Adolescents	*Children*	*Infants*
N	10000	10000	10000
Mean	0.43	0.68	0.90
SD	0.55	1.83	1.51
Skewness	3.26	10.16	4.80
Kurtosis	14.39	156.74	42.15
Median	0.25	0.24	0.36
0.90^{th} quantile	0.99	1.38	2.33
0.95^{th} quantile	1.46	2.49	3.57
0.99^{th} quantile	2.86	8.17	7.15
	Ethnics	*Celiac sufferers*	
N	10000	10000	
Mean	0.96	0.15	
SD	1.98	0.55	
Skewness	6.95	19.20	
Kurtosis	79.96	724.87	
Median	0.36	0.04	
0.90^{th} quantile	2.24	0.29	
0.95^{th} quantile	3.82	0.64	
0.99^{th} quantile	9.38	2.18	

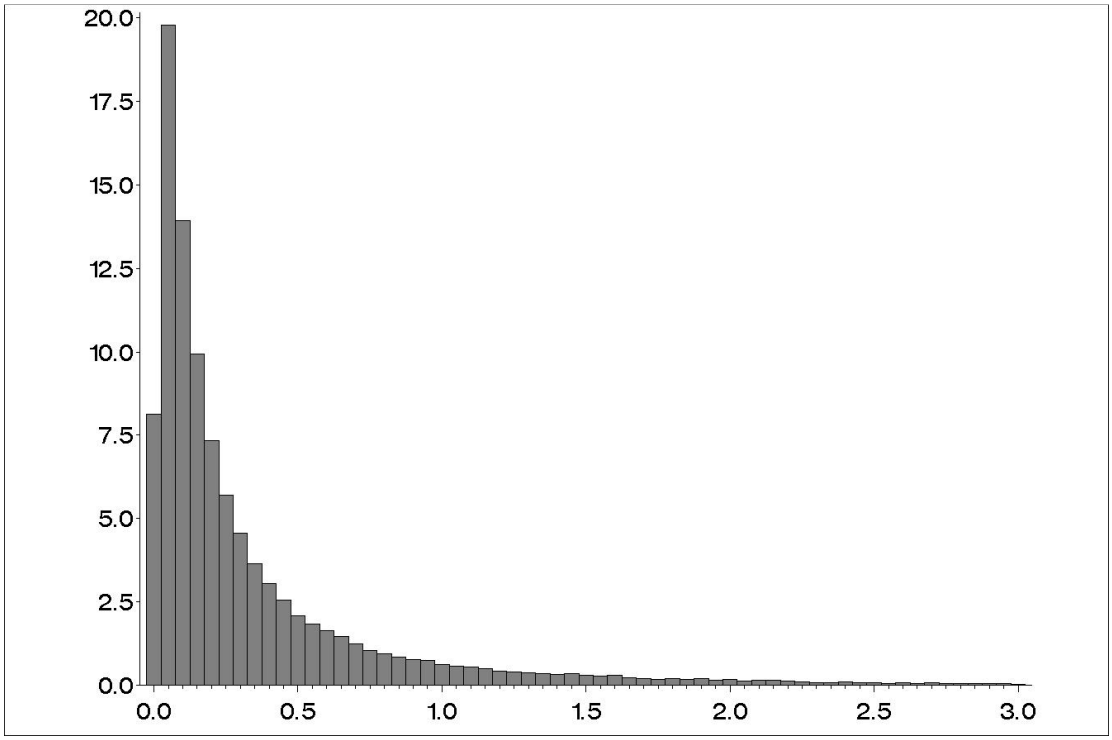


Fig. 10.3. Exposure output histograms (relative frequencies) obtained with the P-P method, for adult females. Exposure in μg kg^{-1} bw day^{-1}

Table VI shows the estimated statistics of exposure to DON by lognormal and gamma *pdf* fitted to the outputs of the simulation method. An example of the exposure histograms is given in Fig. 4 where the fitted gamma and lognormal *pdf*s for adolescents are given. The Anderson-Darling and Chi-Square statistics showed that lognormal and gamma distributions can be acceptable, with the exception of fitted lognormal *pdf* for adult females and ethnics. When comparing the means from lognormal and gamma *pdf* fittings to the simulation outputs, the values were close for all age groups with the exception of infants, the highest value of which was estimated for lognormal *pdf* fittings. On the other hand, large differences were found for the high quantiles (95th and 99th quantiles), with the biggest values observed again for infants.

Table 10.6. Estimated statistics of exposure to DON by lognormal and gamma *pdf* fitted to the outputs of the *P-P* method. See the rigorous definitions of the shape and scale parameters in the appendix. (in μg kg^{-1} bw day^{-1})

***P-P* Method**

	Seniors	***Adult Females***	***Adult Males***
Lognormal	$\hat{r}$ ***=1.614;*** $\hat{\lambda}$ ***=-2.302***	$\hat{r}$ ***=1.061;*** $\hat{\lambda}$ ***=-1.146***	$\hat{r}$ ***=1.036;*** $\hat{\lambda}$ ***=-1.526***
Mean	0.37	0.56	0.37
SD	1.30	0.80	0.52
Median	0.10	0.32	0.22
0.90^{th} quantile	0.79	1.24	0.82
0.95^{th} quantile	1.42	1.82	1.19
0.99^{th} quantile	4.27	3.75	2.42
AD (p-value)	31.88 (<0.005)	0.25 (>0.5)	0.79 (<0.042)
Gamma	$\hat{r}$ ***=0.607;*** $\hat{\lambda}$ ***=0.456***	$\hat{r}$ ***=1.027;*** $\hat{\lambda}$ ***=0.542***	$\hat{r}$ ***=1.083;*** $\hat{\lambda}$ ***=0.340***
Mean	0.28	0.56	0.37
SD	0.36	0.55	0.35
Median	0.15	0.39	0.26
0.90^{th} quantile	0.72	1.27	0.83
0.95^{th} quantile	0.99	1.65	1.07
0.99^{th} quantile	1.65	2.53	1.63
Chi-Sq (p-value)	15402.96 (<0.001)	133.80 (<0.001)	108.08 (<0.001)
	Adolescents	***Children***	***Infants***
Lognormal	$\hat{r}$ ***=1.066;*** $\hat{\lambda}$ ***=-1.389***	$\hat{r}$ ***=1.397;*** $\hat{\lambda}$ ***=-1.439***	$\hat{r}$ ***=1.841;*** $\hat{\lambda}$ ***=-1.240***
Mean	0.44	0.63	1.58
SD	0.64	1.55	8.44
Median	0.25	0.24	0.29
0.90^{th} quantile	0.98	1.42	3.06
0.95^{th} quantile	1.44	2.36	5.98
0.99^{th} quantile	2.98	6.12	20.98
AD (p-value)	4.37 (<0.005)	3.79 (<0.005)	80.97 (<0.005)
Gamma	$\hat{r}$ ***=1.039;*** $\hat{\lambda}$ ***=0.417***	$\hat{r}$ ***=0.591;*** $\hat{\lambda}$ ***=-1.146***	$\hat{r}$ ***=0.550;*** $\hat{\lambda}$ ***=1.644***
Mean	0.43	0.68	0.90
SD	0.43	0.88	1.22
Median	0.31	0.35	0.45
0.90^{th} quantile	0.99	1.77	2.40
0.95^{th} quantile	1.28	2.45	3.36
0.99^{th} quantile	0.96	4.10	5.69
Chi-Sq (p-value)	1239.06 (<0.001)	190006.68 (<0.001)	14077.63 (<0.001)

AD: Anderson Darling statistic; Chi-Sq: Chi Square statistic

	Ethnics	***Celiacs***
Lognormal	$\hat{r}$ ***=1.434;*** $\hat{\lambda}$ ***=-1.088***	$\hat{r}$ ***=1.374;*** $\hat{\lambda}$ ***=-3.089***
Mean	1.00	0.12
SD	2.61	0.28
Median	0.36	0.05
0.90th quantile	2.25	0.27
0.95th quantile	3.78	0.44
0.99th quantile	10.06	1.11
AD (p-value)	0.46 (0.267)	66.40 (<0.005)
Gamma	$\hat{r}$ ***=0.622;*** $\hat{\lambda}$ ***=1.546***	$\hat{r}$ ***=0.517;*** $\hat{\lambda}$ ***=0.299***
Mean	0.96	0.16
SD	1.22	0.22
Median	0.52	0.07
0.90th quantile	2.48	0.42
0.95th quantile	3.51	0.59
0.99th quantile	5.67	1.01
Chi-Sq (p-value)	52310.98 (<0.001)	154786.81 (<0.001)

AD: Anderson Darling statistic; Chi-Sq : Chi Square statistic

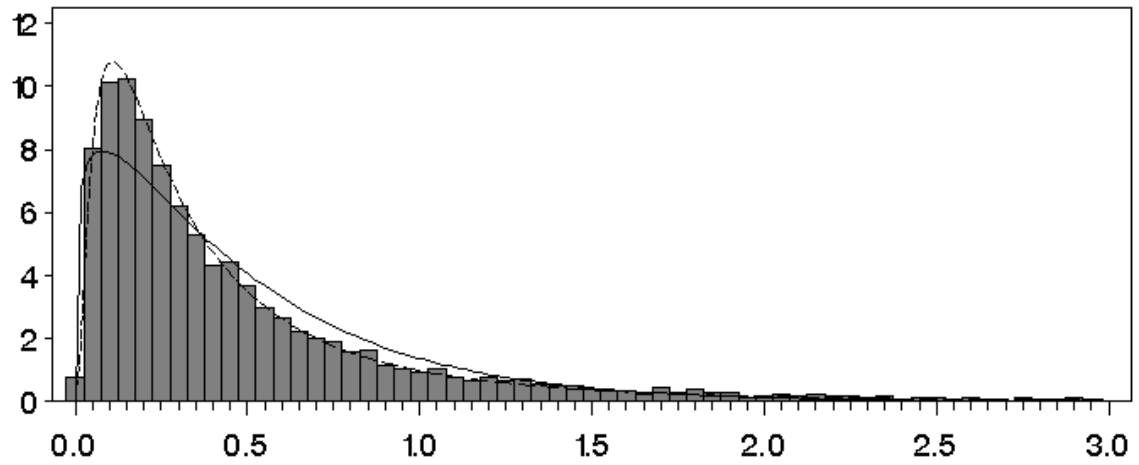


Fig. 10.4. Fitted gamma (solid line) and lognormal (broken line) pdfs for adult females. Relative parameters are given in Table 10.6. Exposure in μg kg^{-1} bw day^{-1}

10.4.3 Results of the Bootstrap Confidence Intervals

Table 10.8. Bootstrap confidence intervals obtained from the results of the P-P method. (in μg kg^{-1} bw day^{-1})

P-P Method 95% Bootstrap Confidence Interval

	Seniors	***Adult Females***	***Adult Males***
Mean	[0.19; 0.40]	[0.48; 0.64]	[0.32; 0.42]
SD	[0.24; 0.88]	[0.55; 1.05]	[0.35; 0.66]
Median	[0.07; 0.18]	[0.27; 0.36]	[0.19; 0.25]
0.90th quantile	[0.43; 1.12]	[1.03; 1.47]	[0.68; 0.98]
0.95th quantile	[0.66; 1.95]	[1.43; 2.26]	[0.94; 1.45]
0.99th quantile	[1.11; 6.51]	[2.60; 5.89]	[1.61; 3.46]

	Adolescents	***Children***	***Infants***
Mean	[0.37; 0.51]	[0.37; 1.23]	[0.67; 1.19]
SD	[0.41; 0.70]	[0.48; 4.47]	[0.93; 2.56]
Median	[0.21; 0.29]	[0.16; 0.35]	[0.25; 0.53]
0.90th quantile	[0.80; 1.28]	[0.80; 2.86]	[1.62; 3.21]
0.95th quantile	[1.12; 1.98]	[1.16; 5.60]	[2.40; 5.46]
0.99th quantile	[1.88; 3.99]	[2.41; 36.42]	[3.95; 12.29]

	Ethnics	***Celiacs***
Mean	[0.56; 1.58]	[0.07; 0.33]
SD	[0.70; 4.09]	[0.10; 1.21]
Median	[0.22; 0.55]	[0.03; 0.06]
0.90th quantile	[1.23; 4.33]	[0.12; 0.81]
0.95th quantile	[1.89; 8.79]	[0.22; 2.07]
0.99th quantile	[3.21; 28.40]	[0.50; 8.23]

Confidence intervals built using the bootstrap pseudo-parametric method for the outputs of *P-P* method are given in Table 10.7. The estimates given in Tables 10.4 and 10.6 were always are contained in the confidence intervals. It was therefore considered that the *P-P* method provided reliable estimates, and that this hypothesis was valid for both those statistics estimated from lognormal and gamma *pdf* fitted to the outputs of this simulation method.

Another point to highlight in this section is the progressive decrease in accuracy when we estimate the CI_b of high quantiles, particularly excessive in the case of the 95th and 99th quantiles. It is an accepted fact that it is very difficult to obtain good accuracy for the 99th-quantile, in particular (Breiman et al., 1990; Beirlant et al., 1996, 1999).

10.5 Discussion

In the present work the stochastic methodology developed by Gauchi and Leblanc (2002) was applied to quantify the exposure of Catalonian population to deoxynivalenol. A raw contamination data set obtained by means of thorough chemical analysis of many foodstuff samples was used. Moreover, consumption data was taken from a specific nutritional study to assess the consumption of those commodities susceptible to mycotoxin contamination, by Catalonian population. The exposure was estimated, in one hand, through a direct method, the most commonly method used in the previous studies, and in the other hand, by means of a simulation method (*P-P*). Finally, the pseudo-parametric bootstrap confidence intervals were calculated for the parameters obtained through the simulation method.

In both cases, direct method and simulation method, the most exposed groups to DON were infants and ethnics. The means estimated through the simulation method were 0.15 and 0.96 μg kg^{-1} bw day^{-1}, for celiac sufferers and ethnics, respectively. The median was quite low in all cases (0.04-0.36 μg kg^{-1} bw day^{-1}), but the high percentiles increased these estimated intakes until worrying values, for example 3.82 μg kg^{-1} bw day^{-1} (percentile 95 for ethnic group). When the estimates were compared to the TDI of 1 μg kg^{-1} body weight (SCF, 2002), it was observed that the mean values were between 15% and 96% of TDI, and the high percentiles commonly exceeded it.

Other studies have already estimated a high DON consumption worldwide, for example, JECFA estimated human dietary intake of DON in five regional diets, the highest one occurring in the Middle Eastern region (2.4 μg kg^{-1} bw day^{-1}), followed by

Far Eastern and European (1.6 and 1.4 μg kg^{-1} bw day^{-1}, respectively) (Canady et al., 2001). In the First French Total Diet Study, Leblanc et al. (2005) estimated DON daily intakes of 0.28 and 0.57 μg kg^{-1} bw day^{-1}, for mean and percentile 95 for adults, respectively; while 0.45 and 0.93 μg kg^{-1} bw day^{-1}, were estimated for children. A probabilistic methodology to assess the exposure to DON was conducted in Netherlands, concluding that 1-year-old children was the most exposed group, with estimated intakes of 0.46 and 1.00 μg kg^{-1} bw day^{-1}, for the median and percentile 95, respectively (Pieters et al., 2004).

To our knowledge, this is the first study to assess the exposure of special population groups to deoxynivalenol. The special groups selected in the present study had dietary habits markedly different from the general population. In one hand, celiac sufferers substitute the wheat-based food for other gluten free cereals, and in the other hand, some ethnic groups partially maintain the dietary patterns from their country of origin. In Catalonia during 2009, the immigrant population represented 15.9 % of the total population (Migracat, 2010), therefore, their specificities should be taken into account to assess the exposure of this collective in other close regions.

10.6 Conclusion

To sum up, considering our results from the *P-P* simulation method, the Catalonian population should be expected to be exposed at moderated levels of deoxynivalenol, being the infants and individuals with ethnic dietary patterns, the most exposed population groups. Although the majority of the population do not exceed the TDI of 1 μg kg^{-1} bw day^{-1}, there is still a large population exceeding this safety value. A reduction of DON levels in foodstuffs would entail to reach safety levels on global DON intake.

10.7 Appendix

The lognormal *pdf* for the continuous random variable Y defined in [0,+∞[is:

$$g(y) = \frac{1}{\sqrt{2\pi}y\sigma_X} \exp\left(-\frac{1}{2}\left(\frac{Log(y) - m_X}{\sigma_x} \right)^2 \right)$$

where m_X and σ_X are λ and r, the scale and the shape parameters, respectively. X then follows a normal distribution with mean m_X and variance σ_X^2 . The mean and the variance of Y are defined by $E(Y) = \exp\left(m_X + \sigma_X^2/2\right)$ and $V(Y) = \left[\exp\left(2m_X + \sigma_X^2\right)\right]\left[\exp\left(\sigma_X^2\right) - 1\right]$, respectively.

The gamma *pdf* for a continuous random variable X defined in [0, +∞[is:

$$f(x) = \frac{1}{\lambda\Gamma(r)}\left(\frac{x-\theta}{\lambda} \right)^{r-1} \exp\left(-\frac{x-\theta}{\lambda} \right)$$

where r, λ and θ are the shape, scale, and the threshold parameters, respectively, and $\Gamma(r)$ is the usual Euler's integral. The mean and variance of the gamma distribution are related to r and λ in the following way: $E(X) = r\lambda$ and $V(X) = r\lambda^2$.

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Chapter 11. Effect of food processing on exposure assessment studies with mycotoxins

Food Additives and Contaminants (in Process)

11.1 Abstract

The goals of the present work were, in one hand, to assess the effect of baking on stability of ZEA and DON, as well as the transference of DON from pasta to the boiling water. In the other hand, to quantify the impact of DON depletion, during cooking of pasta, on the global exposure estimations. Therefore, bread making process was emulated at pilot plant scale using naturally contaminated flour with DON and ZEA. Transference of DON from pasta to water was evaluated at different boiling times. Pasta was elaborated at pilot plant scale using naturally contaminated durum wheat flour; subsequently it was boiled simulating in-house cooking process. Results showed stability of DON and ZEA during the bread making process, including fermentation with *Saccharomyces cerevisiae* and baking at 200 ºC. Our results showed a high transference of DON from pasta to boiling water reaching depletion levels of almost 75 %, which correlated with those ones in water. Accordingly, these cooking depletion rates were computed through a stochastic exposure model to weight its impact on the final exposure estimates. Finally, statistically significant differences were found in most of parameters and population assessed, but to be moderately protective in the highest risk groups.

11.2 Introduction

Trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups, the type A and B being the most common. Type A is represented by HT-2 toxin (HT2) and T-2 toxin (T2) and type B is most frequently represented by deoxynivalenol (DON). Trichothecenes are produced in several cereals by species of *Fusarium*, thus a wide range of cereal-based foods have been reported to be contaminated by these toxins (JECFA, 2001), DON being one of the most common contaminants of cereals worldwide (Jelinek et al., 1989; Scott, 1989). Acute effects of food poisoning by DON in humans are abdominal pains, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in stool (Rotter et al., 1996). A tolerable daily intake (TDI) of 1 μg/kg body weight based on a reduction of body weight gain was established by the EC SCF (SCF, 2002). Plants can metabolize DON to a variable extend through enzymatic conjugation to glucose (Berthiller et al., 2009). The resulting "masked" mycotoxin deoxynivalenol-3-β-d-glucoside (D3G) affects protein biosynthesis to a far lower extent than DON in vitro and is therefore regarded as a detoxification product of DON in plants (Poppenberger et al., 2003). Although there is some preliminary evidence on possible release of DON from its conjugated form by action of hydrolytic enzymes catalyzing the cleavage of β-O-glucosidic bonds, the bioavailability of D3G is not fully understood yet.

Zearalenone (ZEA) is a non-steroidal oestrogenic mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals, including maize and wheat, in temperate and warm regions around the world (Jelinek et al., 1989; Jiménez et al., 1996; Kuiper-Goodman et al., 1987; Patel et al., 1996). Absorption of ZEA has been reported as rapid and extensive in rats and rabbits (Kuiper-Goodman et al., 1987; Ramos et al., 1996), being estimated in the range of 80–85% in pigs (Biehl et al., 1993). ZEA and some of its metabolites competitively bind to oestrogen receptors. Thus, the toxicity is associated with reproductive problems in specific animal species and possibly in humans (Gromadzka et al., 2009; Wood, 1992). Fertility problems have been observed in animals

such as swine and sheep (Krska et al., 2003). ZEA may be an important etiologic agent of intoxication in infants or foetuses exposed to this mycotoxin, resulting in premature thelarche, pubarche, and breast enlargement (SCOOP, 2003).

Occurrence of DON and ZEA has been widely reported in raw foods and foodstuffs in European countries (JECFA, 2001), confirming that food processing methods do not completely remove mycotoxins (Hazel and Patel, 2004). The presence of trichothecenes in Catalonia (Spain) has been recently studied by means of a thorough methodology involving a large sampling and accurate chemical analysis. That study concluded that the occurrence of DON was high in cereal-based foodstuffs from Catalonian market, especially in wheat flakes, corn flakes, corn snacks, pasta and bread (Cano-Sancho et al., 2011a). Cereal-based foods are the base of the energy intake in Catalonia, as well as in the other Mediterranean countries (Serra-Majem et al., 2003). Cano-Sancho et al. (2011b) has reported a thorough study on the exposure of Catalonian population to DON, considering special collectives with particular dietary requirements as case of celiac sufferers and immigrants. Considering the simulation outputs, the authors concluded that Catalonian population could be exposed at moderated levels of DON, being the infants and individuals with ethnic dietary patterns, the most exposed population groups. Although the majority of the population do not exceed the TDI of 1 $\mu g\ kg^{-1}$ bw day^{-1}, there is still population exceeding this safety value. Most of commodities included in that study were analysed as commonly consumed, following the harmonisation towards for Total Diet Studies produced by EFSA (2011). However, pasta was a commodity not analysed as consumed. Cooking of pasta samples entailed arduous and expensive additional steps in the chemical analysis, thus cooking was assumed to do not affect final concentration of mycotoxins.

Food processing can impact on mycotoxins levels but details on the effects remains unclear. Cleaning removes broken and mouldy grain kernels. The milling processes dilute and distribute mycotoxins into certain fractions that most commonly become animal feed. Most of studies reporting thermal treatments and cooking were focused in aflatoxins, fumonisins or ochratoxin A, but few studies have been published

to check the stability of DON and ZEA during food processes. Several studies have reported reduction of DON in pasta or noodles ranged between 40-70% (Nowicki et al., 1988; Sugita-Koonishi et al., 2006). The treatment at 190°C and 200°C temperature resulted in minor reduction of DON level, between 1.7 and 4.1%. The influence of baking at 230°C conducted at more relevant reducing of deoxynivalenol level 7.6 - 9.9% (Israel-Roming and Avram, 2010). Reduction in bread has resulted from a combination of DON 'loss' and dilution by recipe ingredients (Voss and Snock, 2010). An interesting observation describing increase of DON levels within the fermentation phase of dough preparation and also during the bread baking has recently been reported. Authors assumed that enzymatic release of DON from its unknown bound forms takes place (Bergamini et al., 2010).

The goals of the present work were, in one hand, to assess the effect of bread-making on stability of ZEA and DON, as well as the transference of DON from pasta to the boiling water, and in the other hand, to quantify the impact of DON depletion, during cooking of pasta, on the global exposure estimations.

11.3 Materials and methods

11.3.1 Study Design

In the first section of the present study we have evaluated the fate of the mycotoxins DON and ZEA during the bread-making process. Bread was prepared under pilot plant conditions reproducing the common traditional manufacture steps. Samples were taken in most relevant points during food processing as detailed in the following sections. Transference of DON from pasta to water during boiling was also evaluated following the common in-house cooking steps. Pasta was first prepared at pilot plant scale, and then boiled and sampled at different times. Subsequently, the samples were analysed by HPLC to determine the amount of each toxin across the process. The late step consisted to weight the impact of the transference of DON from pasta to the boiling water in the final exposure estimates through a stochastic exposure model developed for Catalonian (Spain) population.

11.3.1.1 Effect of bread-making on ZEA and DON fate

Traditional bread-making consists essentially in three steps: mixing and kneading, fermentation and baking. Wheat flour, yeast and water are the most common raw materials used in traditional bakeries from Catalonia. Thus, wheat flour and yeast (based on *Saccharomyces cerevisiae*) were purchased from local manufacturers for the present work. Detailed summary of bread-making process and sampling is shown in the Figure 11.1.

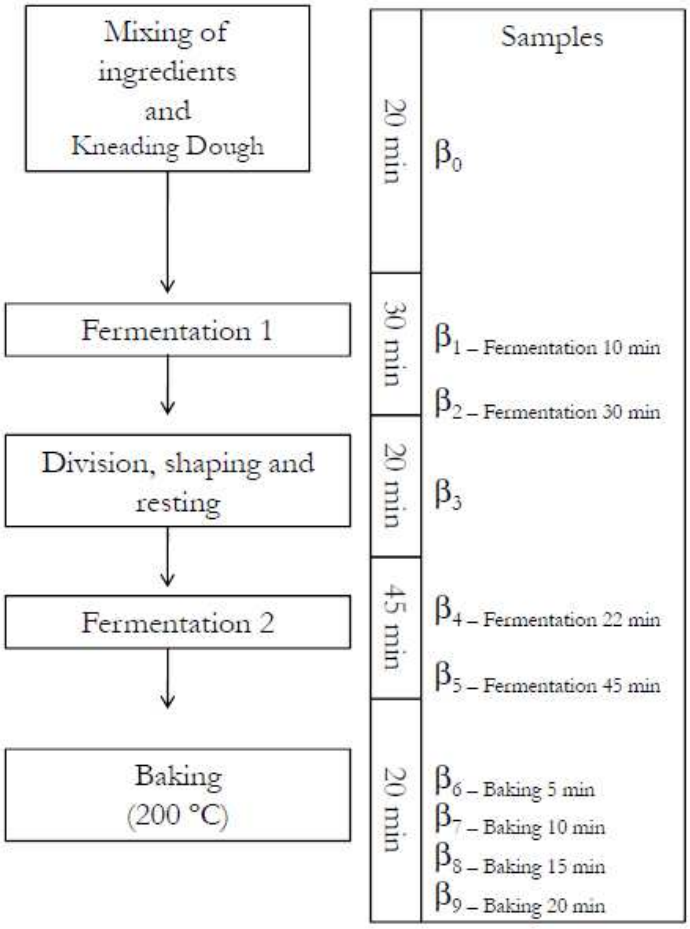

Fig. 11.1. Bread-making process and sampling plan

The full process and sampling was performed completely by triplicate. In each triplicate, 450 g of wheat flour were mixed with 150 mL of water and 24 g of yeast, and subsequently it was kneaded until the dough was consistent and elastic. Next step was the first fermentation at 25 ºC during 30 min. At the end of the first fermentation, the dough was divided in equal portions of 50 g, shaped and allowed to rest for 20 min. Following, the dough was introduced in the fermention chamber for 45 min at 25 ºC. Finally, bread was baked in the oven at 200 ºC during 20 min. In order to evaluate the

fate of the mycotoxins, the DON and ZEA levels were determined in the flour and in the knead dough, as starting point. During the process, an individual portion of 50 g of dough was sampled in the most critical points as specified in the Figure 1. In order to stop any change in sample conditions, each of them were immediately frozen at -20ºC. Solid frozen samples were lyophilised and fine ground prior the analysis. Determination of the water content was performed by weighting the samples before and after lyophilisation.

11.3.1.2 Effect of production and boiling of pasta on ZEA and DON fate

In order to assess the fate of ZEA and DON during boiling of pasta, we followed the artisanal process of production and the common boiling used in domestic conditions. The experiment was completely repeated by triplicate. The pool of durum wheat flour was divided in three batches of 310 g and each one mixed with 200 mL of water. Following, it was kneaded and shaped by means of an artisanal machine. Durum flour and dough were initially analysed, thereafter, during the boiling process of pasta, aliquots (50 g or mL) of both pasta and boiling water were sampled at different times (0, 4, 6, 8 and 10 min). The samples were immediately frozen at -20ºC. Each solid frozen sample was lyophilised and fine ground prior the analysis. Determination of the water content was performed by weighting the samples before and after of lyophilisation.

11.3.2 Chemical analysis

11.3.2.1 Chemical and reagents

DON and ZEA standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile and methanol, reagent and HPLC grade, were purchased from Merck (Darmstadt, Germany). Filter papers (Whatman Nº 4) and glass microfiber filters (Whatman GF/A) were purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography (IAC) columns for extracts clean-up in deoxynivalenol (DONPREP®) and zearalenone (Easi-extract Zearalenone®) analysis were purchased from R-Biopharm

(Rhône LTD Glasgow, UK). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer was prepared with NaCl (0.8 %), Na_2HPO4 (0.12%), KH_2PO4 (0.02%) and KCl (0.02%), from Panreac (Panreac Química SA, Castellar del Valles, Spain) brought to pH 7.4. o-phthaldialdehyde (OPA) solution was prepared diluting 40 mg of o-phthaldialdehyde (Merck) with 1 mL of methanol HPLC grade and mixed with 5 mL of $Na_2B_4O_710H_2O$ (0.1M, Panreac) and 50 µL de 2-mercaptoethanol (Merck).

11.3.2.2 Deoxynivalenol

Five mL of ground sample was mixed with 40 mL of distilled water for 10 minutes and filtered with glass microfiber filter. 4 mL of filtered sample were drained through the IAC column and washed with 10 mL of distilled water. DON was eluted with 3 mL of methanol grade HPLC. The purified samples were dried under nitrogen stream. Each dried sample was resuspended with the mobile phase solution based on milli-Q water, acetonitrile and methanol at proportion 920:40:40 (all reagents were HPLC grade). DON was determined in purified samples by HPLC coupled with a UV/Visible Dual λ absorbance Detector Waters 2487. Results are presented on wet product.

11.3.2.3 Zearalenone

Five g of ground sample were mixed with 25 mL of extractant solution (75% acetonitrile, 25% water) for 10 minutes and filtered. 10 mL of filtered solution were diluted with 40 mL of PBS and drained through the IAC column. After this, the columns were washed with 20 mL of PBS and ZEA eluted with 1.5 mL of acetonitrile grade HPLC and 1.5 mL of milli-Q water. Finally, ZEA was determined, using HPLC with the fluorescence detector and mobile phase of acetonitrile-water (60:40), adjusted at pH 3.2 with acetic acid. Excitation and emission wavelengths were 274 nm and 455 nm, respectively. Results are presented on wet product.

11.3.2.4 Validation of analytical methods

The analytical methods used were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 100 μL of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations. Precision and recovery were established by determination of DON and ZEA levels, spiked in the food matrices (flour, bread and pasta) by triplicate at least. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was considered equal to 3×LOD. LOD of 10, 2 and 0.7 μg/kg were established for DON and ZEA, respectively. Recoveries ranged between 83 and 123 %, in accordance to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission, 2006).

11.3.3 Impact of DON depletion during boiling of pasta on global exposure assessment estimates

In order to check the effect of boiling on global exposure assessment of DON, we have introduced the new variable "boiling reduction" in the simulation exposure model. Thus, we have derived a reducing factor, of DON during boiling of pasta, from our experimental studies and then we have run the simulation models. Finally, the results were compared with the previous outputs without reducing factor, in order to quantify the final relative effect.

11.3.3.1 Raw contamination data

Raw contamination data was mainly taken from the study of Cano-Sancho et al. (2011a,b), and completed with some data from the Project to Assess the Exposure of the Catalonian Population to Mycotoxins (UdL-ACSA, 2010). Contamination data

comes from a thorough study carried out in Catalonia to assess the levels of trichotecenes on foodstuffs intended for human consumption purchased from the market. Almost 500 food "composite" samples were taken from the most susceptible to trichothecenes contamination and most commonly consumed in Catalonia commodities. DON was determined in breakfast cereals, snacks and pasta samples following extraction, clean-up, derivatization and finally analysis by GC-ECD. Moreover, this mycotoxin was determined in sliced bread, sweet corn, beer and ethnic foods by LC-DAD.

11.3.3.2 Raw consumption data

Consumption data was also taken from Cano-Sancho et al. (2011) and the Project to Assess the Exposure of the Catalonian Population to Mycotoxins (UdL-ACSA, 2010). This study involved a wide nutritional survey designed to specifically identify the dietary patterns of the Catalonian population in relation to the main foodstuffs susceptible to mycotoxins contamination. Food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ), developed for the Catalonian population including those foods typically consumed in the region that may be potentially contaminated with these mycotoxins. Finally, 720 adults, 235 adolescents, 69 children and 56 adults with ethnic dietary pattern were interviewed in 2008-2009 by trained interviewers.

11.3.4 Stochastic procedure to calculate the exposure: Parametric-Parametric (P-P) method

In this section we recall some elements of the methodology used to calculate the exposure by means of the stochastic procedure, already given in Cano-Sancho et al. (2011b). The main advantage of the use of a parametric method is derived from the fact that NonParametric-NonParametric method could lead us to less reliable estimations, especially of the high quantiles (Gauchi and Leblanc, 2002). For each consumption and contamination dataset, the gamma *pdf* was fitted with the method of maximum

likelihood using the CAPABILITY procedure of SAS software (SAS 2010). Shape and scale parameters were estimated for each contamination set, in accordance with the methodology described above. The *P-P* method used to estimate the normalised exposure *k* of the *S* simulation set was built as follows:

$$\hat{E}_k^{[P-P]} = \sum_{j=1}^{p} \tilde{c}_{i(j)} \tilde{t}_j$$

where $\tilde{c}_{i(j)}$ is a random normalised consumption *i* for the foodstuff *j*, drawn from $\hat{C}_j$, the corresponding adjusted gamma *pdf*, and $\tilde{t}_j$ is a random contamination for the foodstuff *j*, drawn from $\hat{T}_j$, the corresponding adjusted gamma *pdf*. The density parameter estimates for $\hat{C}_j$ and $\hat{T}_j$ are shown in Table V from Cano-Sancho et al. (2011b). 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 (10,000 in the present study):

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

Other statistics were directly computed on the histogram built with the simulations of the *S* set, and statistics were also estimated from the lognormal and gamma fitted *pdf*s.

11.3.5 Method to build the bootstrap confidence intervals

We built confidence intervals (*CI*) according to the "pseudo-parametric bootstraping" method highlighted by Gauchi and Leblanc (2002). "Pseudo-parametric CI_b", referred to as Type 1, was built by randomly drawing B samples of size in the exposure simulation set S. Typically, B is equal to 10,000. The boundaries of the 95% confidence interval are calculated taking the 0.025th and 0.975th empirical quantiles of the final bootstrap distribution.

11.3.6 Statistical analysis

Both approximations (with and without reduction effect of boiling in pasta) have been compared in order to statistically assess the differences. Means were compared through a t-test considering a *p*-value equal or less than 0.05 to reject the null hypothesis that both means are equals. *F*-test and Mann-Whitney *W* test were computed to compare the variances and medians from both approaches, respectively. Finally Kolmogorov-Smirnov test was run to compare both distributions, performed by computing the maximum distances between the cumulative distributions of the two samples. Quantile plot and quantile-quantile representation were plotted to better describe differences between approaches. Statgraphics® plus v9.1 and SAS® program were used in statistics.

11.4 Results

11.4.1 Fate of ZEA and DON during baking of bread

Wheat flour was naturally contaminated with moderate levels of DON and ZEA (2.807±0.757 and 0.319±0.281 μg/g, respectively). Mixed and knead dough prior bakery process presented initial concentrations of DON and ZEA of 0.744±0.057 and 0.241±0.103 μg/g, respectively. Levels of DON during the bread-making process were very stable, without differences.

Despite no statistical significant difference was found during the process in the levels of ZEA, an important decrease was achieved in the levels of ZEA after the first fermentation (Table 11.1). Therefore these mycotoxins were stable after fermentation and baking under these experimental conditions.

Table 11.1. Fate of DON and ZEA during bread-making process

Sampling code	Processing step	DON levels*	ZEA levels*
β_0	Dough	0.744±0.057[a]	0.241±0.103[a]
β_1	Fermentation 1 (10 min)	0.875±0.158[a]	NA
β_2	Fermentation 2 (30 min)	0.626±0.021[a]	0.130±0.049[a]
β_3	Final dough rest	0.852±0.138[a]	NA
β_4	Fermentation 2 (20 min)	0.865±0.131[a]	NA
β_5	Fermentation 2 (45 min)	0.801±0.038[a]	NA
β_6	Baking (5 min)	0.734±0.155[a]	0.139±0.026[a]
β_7	Baking (10 min)	0.858±0.044[a]	0.146±0.003[a]
β_8	Baking (15 min)	0.741±0.437[a]	0.173±0.041[a]
β_9	Baking (20 min)	1.041±0.030[a]	0.207±0.027[a]

*Mean±SD units in μg/g

[a] Different letter means statistically significant differences between steps (α =0.05)

NA – No analysed samples

11.4.2 Fate of DON during boiling of pasta

DON in pasta showed a clear decrease during boiling, reaching reduction values up to 75 % of the initial content after 10 min of boiling (Table 11.2). In contrast, a direct increase of DON levels was reached in boiling water, from 0 to 0.459±0.042 μg/g after 10 min (almost 75 % of initial concentration in pasta). Therefore a direct transference of DON from pasta to boiling water was elucidated in these empirical conditions.

Table 11.2. Fate of DON during boiling of pasta

Sampling code	Processing step	DON levels pasta	DON levels boiling water	Sum of DON in pasta and water
		Mean±SD	*Mean±SD*	
β_0	Dough	0.621±0.120[a]	<LOD	0.621±0.120[a]
β_6	Boiling (2 min)	0.379±0.120[ab]	0.137±0.050[a]	0.516±0.170[a]
β_7	Boiling (4 min)	0.308±0.038[ab]	NA	NA
β_8	Boiling (6 min)	0.270±0.035[ab]	0.363±0.047[b]	0.633±0.082[a]
β_9	Boiling (8 min)	0.249±0.055[b]	NA	NA
β_9	Boiling (10 min)	0.158±0.104[c]	0.459±0.042[b]	0.617±0.146[a]

[a] Different letter means statistically significant differences between steps (α =0.05)
NA – No analysed samples

11.4.3 Impact of DON depletion during boiling of pasta on global exposure assessment estimates

Mean reduction of pasta after 10 min of boiling was found to be almost 75 %, therefore we derived a reduction factor of 0.255, to estimate the DON dietary intake through the pasta considering the boiling water is rejected. Results of both approximations are simultaneously presented in the table 11.3 from approximation without the reduction factor derived from boiling of pasta and the second approximation with this factor.

Statistically significant differences were found when we compared the mean exposures from each collective, with the exception of immigrants, who presented the narrowest estimates (*t*-test, α=0.05). When we compared the medians of both approximations, we found significant differences in each case. Widest differences were estimated for children, who showed the major contribution of pasta to the global exposure to DON. In any case, when we compare the exposure estimates with the TDI of 1 μg/kg bw/day, we did not found relevant reductions to consider this effect as protective in most exposed collectives.

Table 11.3. Results of exposure assessment of the population groups from *P-P* simulation method. These results were obtained directly from the N simulation outputs without *pdf* fittings, for model without reduction factor (No RF) and reduction factor (RF) (in μg kg^{-1} bw day^{-1})

Adult females	No *RF*	*RF*	
N	10000	10000	
Mean	0.56 [0.48; 0.64]	0.51 [0.44; 0.59]	t=3.9822 (p<0.001)
SD	0.77 [0.55; 1.05]	0.77 [0.54; 1.04]	F= 1.0137 (p=0.497)
Median	0.32 [0.27; 0.36]	0.27 [0.23; 0.31]	W=4.5x10^7 (p<0.05)
0.90th quantile	1.26 [1.03; 1.47]	1.20 [0.97; 1.43]	KS=5.3538 (p<0.05)
0.95th quantile	1.84 [1.43; 2.26]	1.79 [1.38; 2.22]	
0.99th quantile	3.70 [2.60; 5.89]	3.68 [2.55; 5.88]	
Adult males	No *RF*	*RF*	
N	10000	10000	
Mean	0.37 [0.37; 0.42]	0.32 [0.26; 0.37]	t=-7.9037 (p<0.05)
SD	0.48 [0.35; 0.66]	0.47 [0.32; 0.71]	F=0.94914 (p<0.05)
Median	0.22 [0.19; 0.66]	0.16 [0.14; 0.19]	W=5.7x10^7 (p<0.05)
0.90th quantile	0.82 [0.68; 0.98]	0.73 [0.57; 0.84]	KS=8.1176 (p<0.05)
0.95th quantile	1.18 [0.94; 1.45]	1.09 [0.83; 1.26]	
0.99th quantile	2.33 [1.61; 3.46]	2.26 [1.36; 4.13]	

Adolescents	No *RF*	*RF*	
N	10000	10000	
Mean	0.43 [0.37; 0.51]	0.39 [0.31; 0.49]	t=-8.7695 (p<0.05)
SD	0.55 [0.41; 0.70]	0.69 [0.41; 1.31]	F=1.089 (p<0.001)
Median	0.25 [0.21; 0.29]	0.18 [0.15; 0.22]	W=5.7x10^7 (p<0.05)
0.90th quantile	0.99 [0.80; 1.28]	0.91 [0.69; 1.18]	KS=11.1603 (p<0.05)
0.95th quantile	1.46 [1.12; 1.98]	1.42 [1.04; 2.01]	
0.99th quantile	2.86 [1.88; 3.99]	3.09 [1.88; 5.17]	
Children	No *RF*	*RF*	
N	10000	10000	
Mean	0.68 [0.37; 1.23]	0.55 [0.27; 1.03]	t=-4.6758 (p<0.001)
SD	1.83 [0.48; 4.47]	1.80 [0.40; 4.47]	F=0.96757 (p<0.099)
Median	0.24 [0.16; 0.35]	0.15 [0.10; 0.22]	W=5.9x10^7 (p<0.05)
0.90th quantile	1.38 [0.80; 2.86]	1.08 [0.54; 2.60]	KS=10.1753 (p<0.05)
0.95th quantile	2.49 [1.16; 5.60]	2.21 [0.85; 5.53]	
0.99th quantile	8.17 [2.41; 36.42]	7.96 [2.10; 36.39]	
Immigrants	*No RF*	*RF*	
N	10000	10000	
Mean	0.96 [0.56; 1.58]	0.92 [0.52; 1.55]	t=-1.3701 (p<0.171)
SD	1.98 [0.70; 4.09]	1.98 [0.69; 4.09]	F=0.9981 (p<0.926)
Median	0.36 [0.22; 0.55]	0.31 [0.18; 0.50]	W=5.2x107 (p<0.05)
0.90th quantile	2.24 [1.23; 4.33]	2.20 [1.18; 4.27]	KS=4.009 (p<0.05)
0.95th quantile	3.82 [1.89; 8.79]	3.76 [1.85; 8.78]	
0.99th quantile	9.38 [3.21; 28.40]	9.38 [3.17; 28.40]	

11.5 Discussion

The interest to know the effect of food processing on mycotoxin levels has increased during the last decade. Most of the publications have described the effect of

processes such as cleaning and milling of grains, microbiological fermentation or thermal processes (cooking, boiling, extrusion….). The published studies mainly were focused on to measure the effects on aflatoxins, fumonisins and ochratoxin, but fewer studies have been performed to assess the effect on DON and ZEA. Cleaning and milling of grains have shown to reduce the mycotoxin because these processes are able to eliminate the mouldy bran as a main source of myctoxin in raw food. In the wet milling of corn, mycotoxins may be dissolved into the steep water or distributed among the products of the process, but not destroyed.

Considering our results, DON was stable during the bread-making process, from kneading and fermentation with Saccharomyces cerevisiae, until the end of baking at 200ºC. The effect of baking on DON was hardly reported with controversial effects among previous studies. Head treatment at 190°C and 200°C temperature resulted in minor reduction of DON level, between 1.7 and 4.1%, and major effects at 230°C (7.6 - 9.9%) (Israel-Roming and Avram 2010). Young et al. (1984) observed that the production of yeast containing products resulted in an increase in DON, which they attributed to the enzymatic conversion of DON precursors. Neira et al. (1997) found reductions in stages of fermentation and oven baking, with mean reduction rates of 21.6 and 28.9 %, respectively, and final reductions ranged between 16.8 and 96.6 %. A significant decrease occurred of approximately 38–46% of the original content during fermentation, but these rates were reverted after baking without any final reduction (Lancova et al. 2008). Authors suggested transformation of DON to conjugated forms as a possible mechanism of reversible mycotoxin depletion. Boyaciouglu et al. (1993) examined the effects of bread additives on DON level post-bake and showed that potassium bromate and L-ascorbic acid had no effect, but sodium bisulphite, L-cysteine and ammonium phosphate resulted in drops of up to 40%. Greenhalgh et al. (1984) identified a DON isomer in baked bread at levels of 3–13% of DON originally present. Further information has recently been produced by Kostelanska et al. (2011) about fate of DON and it masked form D3G during baking. The authors found a moderate decrease on the final content of both DON and D3G (87 and 90 %, respectively), finding significant increase of D3G (145 %) when enzymatic bakery improvers were

used in the process.

ZEA is relatively heat stable and in the absence of reaction to form conjugates, ZEA cannot be expected to substantially degrade during moderate thermal processing. Baking and roasting, can substantially decrease ZEA, although given the thermal stability of the molecule much often survives the treatment as well (Maragos 2010). Fermentation by lactic acid bacteria has also been shown to reduce ZEA (Mokoena et al. 2005). However, we have not found any effect on ZEA content during baking, neither, after fermentation with Saccharomyces cerevisiae, nor after baking at 200 ºC during 20 min in the line of the previous reports. We have not assessed the effect of additional factors as different levels of toxins or addition of bakery improvers, reported as modulators of the final response.

The high solubility of DON in water provides the suitable conditions for its depletion during wet food processing. Despite this chemical property, reduction of DON through common cooking processes such us boiling of pasta, has been poorly studied to date. We found high reduction rates, almost 75 %, where DON is mainly transferred to the boiling water without other eventual losses. Previous studies found moderate depletion rates of 50-60% (Nowicki et al. 1988; Sugita-Konishi et al. 2006).

To our knowledge, this is the first study to assess the impact of any processing effect to the global exposure assessment outputs of a mycotoxin. A simulation model was used to compare both approximations, with and without boiling reduction effect. Considering that large reduction rates were found in boiling experiments, significant decreases were expected when using the derived reduction factor in the exposure models. Despite of statistically significant differences were found in most of parameters and collectives, the impact on risk characterization was not so marked to avoid health concern estimation in the most exposed groups. The reduction effect was counteracted by the low contribution of pasta to the global DON intake. Total diet studies are the gold standard method recommended in chemical exposure assessment studies. This methodology involves the chemical analysis of samples prepared as consumed, but this issue not always is easily feasible. In the framework of large epidemiological studies,

most of times the risk managers need use contamination raw datasets from routinely analysis, unspecific for risk assessment. Therefore, the use of several foods uncooked is extended for methodological reasons, but the derived bias can be alleviated by means of the food processing effect knowledge. We have proposed a statistical approximation were the reduction factor is computed in the model as a tool to quantify the related bias.

Further researches are need to better understanding the complex and dynamic system involving the dietary intake of mycotoxins from diet, taking into account each food processing, cooking steps, mixing with other matrices, transformation into mycotoxin isomers or "masked" forms…

11.6 Conclusion

To sum up, DON was stable during bread-making process, considering fermentation and baking. ZEA showed depletion during the first fermentation but without enough statistical differences. By contrast, major decreases were found for DON during boiling of pasta, with almost 75 % of transference from pasta to water.

When we applied the reduction factor in the simulation exposure models, the final reduction was statistically significant but not enough to consider the process as protective because the contribution of pasta to the global DON intake was commonly low.

11.7 References

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Chapter 12. Exposure assessment of T2 and HT2 toxins in Catalonia (Spain)

Food and Chemical Toxicology 2012 50, 511–517

12.1 Abstract

HT-2 toxin (HT2) and T-2 toxin (T2) are mycotoxins produced in several cereals by species of *Fusarium*. The aim of the present study was to assess the exposure of Catalonian population to the combined mycotoxins T2 and HT2. Three different approaches were considered to handle the left censored data: (1) a substitution method, (2) a parametric method using the maximum likelihood estimation (MLE) and (3) a non-parametric method using the Kaplan–Meier (KM) estimator. Raw contamination data was provided by a large survey conducted in this region, in addition to the raw consumption data from a nutritional study specifically designed to assess the dietary intake of the main foodstuffs related to T2 and HT2 contamination for all population age groups. Contamination and consumption data were combined by simulation using a stochastic method. Accuracy and reliability of the statistic estimates were assessed building the related confidence intervals using a pseudo-parametric bootstrap method. Considering the results from the present study, the most exposed group population to the toxin T2 and HT2 should be expected to be the children.

12.2 Introduction

Trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups. Type A and B are the most common groups. Type A is represented by HT-2 toxin (HT2) and T-2 toxin (T2) and type B is most frequently represented by deoxynivalenol (DON). Trichothecenes are produced in several cereals by species of *Fusarium*, thus, a wide range of cereal-based foods have been confirmed to be contaminated by these toxins (JECFA, 2001).

T2 is a potent inhibitor of protein synthesis and mitochondrial function both *in vivo* and *in vitro*, and shows immunosuppressive and cytotoxic effects. Moreover, it has been reported to have extremely toxic effects on skin and mucous membrane (Visconti et al., 1991; Visconti, 2001; Sudakin, 2003; Eriksen and Pettersson, 2004). It has been shown that through T2 deacetylation, HT2 is obtained as the major metabolite; however, little information is available regarding the single effect of HT2 (Sudakin, 2003; Visconti, 2001). Despite T2 toxic effects having been widely studied in animals, its toxicology has never been assessed in humans.

The occurrence of trichothecenes in the Catalonian (Spain) market was thoroughly studied in a previous work (Cano-Sancho et al., 2011b). The authors concluded that DON was the main mycotoxin in the analyzed cereal-based food samples, while T2 and HT2 were quantified only in 5 and 37 samples out of 479 composite samples, respectively. Apparently, the dietary intake of T2 and HT2 toxin from food should not be a problem to human health due to its low occurrence. However, if we consider the high toxicity of these trichothecenes and also, that cereal food is the base of the dietary energy intake in Catalonia as well as in other Mediterranean countries, an accurate assessment should be carried out in order to characterize the population exposure. The Scientific Committee on Food established a tolerable daily intake (TDI) of 0.06 μg/kg bw/day for both toxins combined (SCF, 2002).

Two kinds of mathematical methods have been developed to assess the exposure to food contaminants: 1) the point estimates, also known as deterministic methods and 2) the stochastic or probabilistic methods. Although the most commonly methods used to date are deterministic; the expert panels have recommended the use of stochastic methods to obtain more accurate estimates that take into account uncertainties and variability (Kroes et al., 2002; World Health Organization (WHO), 2005; European Food Safety Authority (EFSA), 2006). One of the critical points in dietary exposure assessment comes from those data below the limit of detection (LOD) or quantification (LOQ) also known as left-censored data, determined by the performance of the analytical methods. The most commonly used method in food risk assessment is to substitute the non-detects by LOD, LOD/2 or LOD/√2. However, improved mathematical approaches have been developed to better manage these uncertainties such as maximum likelihood estimation methods, log-probit regression methods or non-parametric methods.

To our knowledge, few data concerning occurrence of T2 and HT2, as well as the exposure of human population to these mycotoxins, have been published to date. In the framework of the Project to Assess the Exposure of Catalonian Population to the Mycotoxins, and following the line of our previous studies (Cano-Sancho et al., 2009, 2010, 2011a,b), the aim of the present study was to assess the exposure of Catalonian population to the combined mycotoxins T2 and HT2. Three different approaches were considered to handle the left censored data: 1) a substitution method, 2) a parametric method using the maximum likelihood estimation (MLE) and 3) a non parametric method using the Kaplan-Meier (KM) estimator.

12.3 Materials and methods

12.3.1 Raw datasets

The present work is included in the Project to Assess the Exposure of Catalonian Population to Mycotoxins (2008-2009) who provided all raw datasets (UdL-ACSA, 2010). That project included a large and thorough survey to determine the

contamination levels of T2 and HT2 in the main cereal-based food, previously published by Cano-Sancho et al., (2011b). Moreover, the project included a specific nutritional study to assess the consumption patterns of Catalonian population concerning those foods related with mycotoxin contamination.

12.3.1.1 Contamination data

Briefly, during 2008, corn flakes (n=168) wheat flakes (n=27), sweet corn (n=185), corn snacks (n=213), pasta (n=201), beer (n=213), sliced bread (n=147) and bread (n=31) were obtained in six hypermarkets and supermarkets from twelve cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 items of each product were randomly taken (if present). The level of trichothecenes was determined in a total of 72 composite samples obtained by pooling the items taken from each store (12 cities x 6 stores / city = 72 samples / category). However, in some cases, no items were available in the store, thus, less than 72 composites were obtained. Finally, a wide range of brands was purchased, which can be considered the majority of market share in Catalonia of these products, as well as in the rest of the Spanish market. T2 and HT2 toxins were determined in breakfast cereals, snacks and pasta samples following extraction, clean–up, derivatization and final analysis by GC-ECD or GC-MS in case of bread samples. Furthermore, these mycotoxins were determined in sliced bread, sweet corn and beer by LC-DAD (Cano-Sancho et al., 2011b).

On the first attempt to check the contamination data we observed that a high percentage of samples were below the LOQ, therefore we assessed those values between LOD and LOQ, and we found a significant quantity of samples in this range. Contamination levels and percentage of samples that exceed the LOD are summarized in the Table 12.1 and Figure 12.1.

Table 12.1. Occurrence of T2 and HT2 toxin in food from Catalonian market. The mean and standard deviation (sd) were calculated considering only positive samples (> LOD)

T2	N composite	LOQ µg/g	Samples >LOD	Samples LOD-LOQ	Mean±sd µg/g	Max µg/g
Wheat flakes	27	0.014	6	4	0.041±0.024	0.075
Corn flakes	65	0.019	7	7	0.024±0.012	-
Beer	71	0.019	0	0	-	-
Sliced bread	72	0.036	1	1	0.088	0.088
Sweet corn	72	0.045	6	4	0.145±0.063	0.256
Corn snacks	71	0.018	8	7	0.033±0.021	0.070
Pasta	70	0.021	14	14	0.027±0.010	-
Bread	31	0.023	0	0	-	-
HT-2						
Wheat flakes	27	0.012	14	10	0.040±0.044	0.183
Corn flakes	65	0.010	35	31	0.023±0.010	0.065
Beer	71	0.003	0	0	-	-
Sliced bread	72	0.010	16	5	0.039±0.016	0.075
Sweet corn	72	0.008	8	3	0.032±0.024	0.084
Corn snacks	71	0.020	24	18	0.083±0.170	0.895
Pasta	70	0.010	32	25	0.025±0.018	0.080
Bread	31	0.017	0	0	-	-

In order to reduce the uncertainties related with those samples under the LOQ, we used the samples between LOQ and LOD in the statistics. Those foods with less than 20 % of positive samples (more than 80 % censoring) were excluded from statistics as recommended by EFSA (2010) to include these data in a probabilistic exposure assessment model. Risk assessment guidelines take into account the coupled toxic effect of T2 and HT2 toxin to derive the safety levels. Therefore we used the contamination datasets of HT2 in wheat flakes, corn flakes, corn snacks, pasta and sliced bread, and also, the level of T2 in pasta.

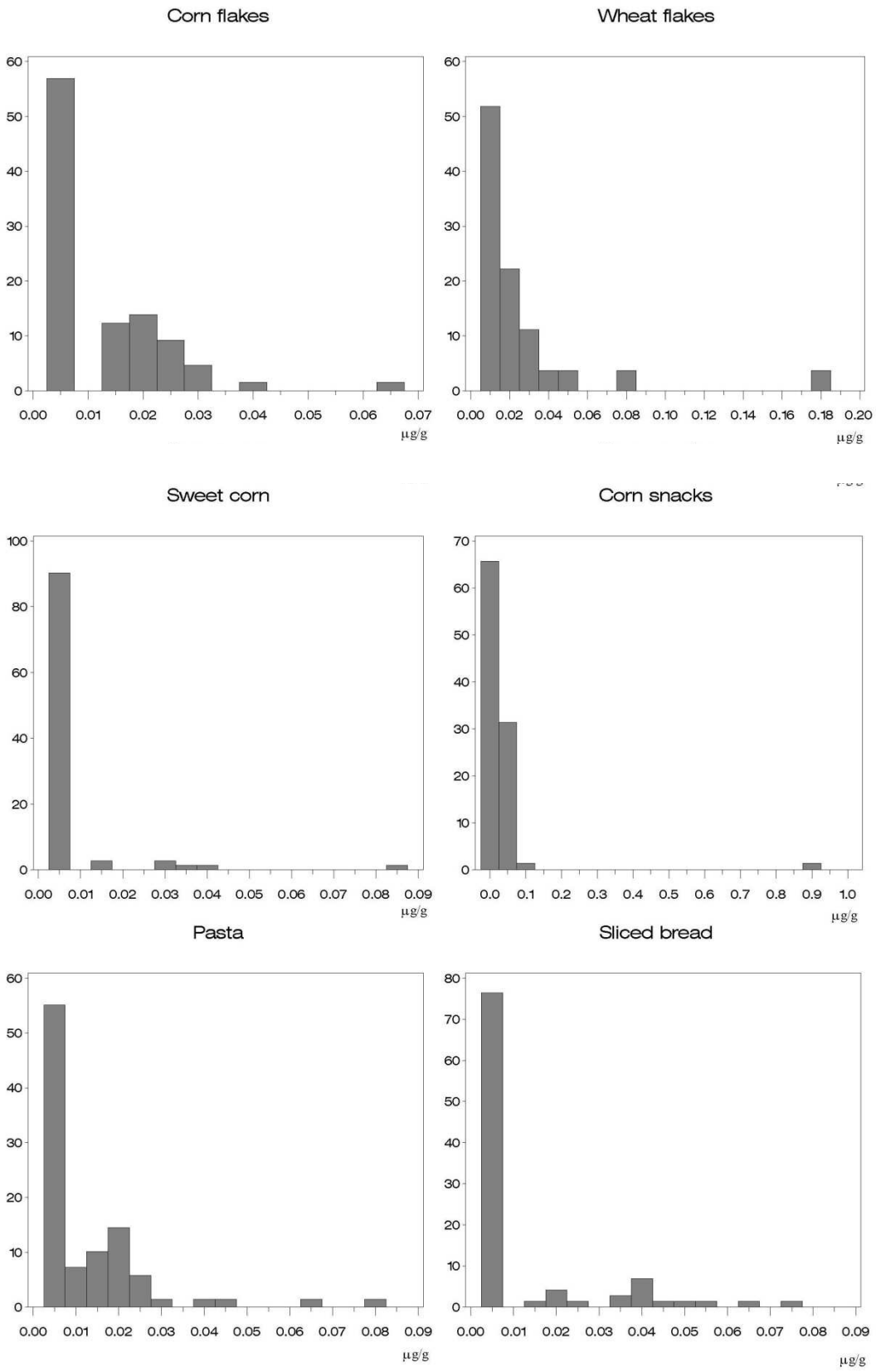

Fig 12.1. Contamination histograms of HT2 toxin (relative frequencies), in µg g^{-1} for each food

12.3.1.2 Treatment of left-censored data

In the present study we have applied three different methods to obtain a mean level of contamination for each food dataset, taken into account the left-censored data.

1. Substitution method. Non detected samples were assumed to be the value of the LOD divided by 2. This is the most commonly used procedure in exposure assessment of populations to mycotoxins, thus we used this approach as starting point.

2. Parametric method: maximum likelihood estimation (MLE). A wide explanation about MLE is reported by Helsel (2005) and EFSA (2010). Briefly, the method is based on the assumption that data below and above the LOD follow a given statistical distribution. The parameters of the chosen distribution are estimated to best fit the distribution of the observed values above the detection limit, compatibly with the percentage of data below the limit. The estimated parameters are those which maximize the likelihood function. Detailed information concerning the syntax of the NLMIXED procedure for SAS® used in the present work to fit the MLE model to obtain the mean and standard deviation of contamination datasets is included in the Appendix G in EFSA report (2010).

3. Non-parametric method: Kaplan-Meier estimation (KM). This is the standard non-parametric technique for censored data, based on the empirical cumulative distribution function. We address the readers to the report of Tressou (2006) to better understand the mathematic basis of the KM estimator, and its application in food risk assessment studies. With the KM method, the weight of the censored data is distributed over the different observed values below the censoring values. In order to obtain the mean and standard deviation of each contamination dataset, we have applied the syntax to fit the KM model based on the LIFETEST procedure from SAS® also included in the Appendix G in EFSA report (2010).

12.3.1.3 Consumption data

Consumption data were also taken from the Project to Assess the Exposure of the Catalonian Population to Mycotoxins (UdL-ACSA, 2010). This study involved a wide nutritional survey designed to specifically identify the dietary patterns of the Catalonian population in relation to the main foodstuffs susceptible to mycotoxins contamination. Food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ), developed for the Catalonian population including those foods typically consumed in the region that may be potentially contaminated with these mycotoxins. According to the World Health Organization (WHO) recommendations, studies to assess the dietary intake of chemical contaminants should show the significant intake within the standard population, as well as within all population groups that could have different dietary patterns. Therefore, five different population groups were considered: children (4-9 years), adolescents (10-19 years), adult males (20-65 years), adult females (20-65 years) and seniors (> 65 years). The FFQ included 38 specific food items known to be the major foods contaminated by mycotoxins worldwide, excluding those foods not consumed in the region. Concerning consumption frequency, five response options, ranging from 'never' to 'annually', were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 76 seniors, 336 adult males, 384 adult females, 235 adolescents and 69 children, were interviewed in 2008-2009 by trained interviewers. Seniors were excluded from the study because there was a little number of consumers in each food category. Individuals were from 89 cities and towns from Catalonia.

12.3.2 Exposure assessment

In this section we recall some general aspects concerning the exposure calculations, already given in Gauchi and Leblanc (2002).

The main limitation to estimate the normalised exposure distribution of consumer populations is derived from the equation:

$$E_{\pi} = \sum_{j=1}^{p} C_{\pi,j} T_j$$

where the random variable "normalised global exposure in a consumer population π" (E_{π}) is a function of the random variables: "normalised consumption of the foodstuff *j* in a consumer population π " ($C_{\pi,j}$) and "T2 or HT2 concentration level of the foodstuff *j*" (T_j). The difficulty is due to the probability density functions (*pdf*) since these variables are generally unknown and even if they are known, they are generally different and not independent. Additionally, the $C_{\pi,j}$ x T_j *pdf* products are also unknown.

If we assume independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, we can estimate the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j \qquad (2)$$

where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff *j* in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food.

Under these assumptions we can also calculate the variance estimate $\hat{V}(E_{\pi_0})$ using Eq. (5) and Eq. (6) in Gauchi and Leblanc (2002). We could consider this approach to be a theoretical approach, but because of the postulated assumptions, it would have to be a (very) simplified theoretical approach. For example, this simplified approach does not enable us to calculate complicated statistics such as high quantiles. Simulation methods would therefore be required to obtain estimates of these statistics. Finally, we will consider this approach to be a direct approach.

Stochastic methodology to assess the exposure

In this section we recall some elements of the methodology used to calculate the exposure by means of the stochastic procedure, already given in Gauchi and Leblanc (2002) and Cano-Sancho et al. (2011a) with several modifications.

The main advantage of the use of a parametric method is derived from the fact that NonParametric-NonParametric method could lead us to less reliable estimations, especially of the high quantiles (Gauchi and Leblanc, 2002). Taking the asymmetrical appearance of consumption histograms and our previous experience into account, we fitted the probability density functions, choosing the gamma distribution to be the best candidate. Therefore, for each consumption dataset, the gamma *pdf* was fitted with the method of maximum likelihood using the CAPABILITY procedure of SAS software (SAS, 2010). The Chi-Square statistic was used in the goodness-of-fit test, considering a significance level greater than 95%, to accept the distribution as a suitable candidate.

The foodstuff categories selected are commonly consumed within the Catalonian population, despite several exceptions related to age-group dietary patterns. The appearance of the histograms is therefore continuous and only one distribution was considered pooling all age groups data, fitting all data to gamma *pdf*. Contamination datasets were computed obtaining the mean value estimated as described above, considering three methods to handle the left censored data.

The method used to estimate the normalised exposure k of the S simulation set was built as follows:

$$\hat{E}_k = \sum_{j=1}^{p} \tilde{c}_{i(j)} \dot{t}_j$$

where $\tilde{c}_{i(j)}$ is a random normalised consumption i for the foodstuff j, drawn from $\hat{C}_j$, the corresponding adjusted gamma *pdf*, and $\dot{t}_j$ is the mean of T2 or HT2 contamination for the foodstuff j, computed through the substitution method, MLE or KM method. The density parameter estimates for $\hat{C}_j$ are shown in Table V from Cano-Sancho et al. (2011a).

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 (10,000 in the present study):

$$\hat{E}_S = \frac{1}{n}\sum_{k=1}^{n} \hat{E}_k$$

Other statistics were directly computed on the histogram built with the simulations of the *S* set, and statistics were also estimated from the lognormal and gamma fitted *pdf*s. 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 10,000). The boundaries of the 95% confidence interval are calculated taking the 0.025^{th} and 0.975^{th} empirical quantiles of the final bootstrap distribution. The statistic software SAS ® v9.1 was used in these computations.

We referred the exposure to T2-HT2 considering the sum of both toxins coming from the specified foodstuffs: HT2 from wheat flakes, corn flakes, corn snacks, pasta and sliced bread, and also, T2 from pasta.

12.4 Results

12.4.1 Contamination estimates

The mean and standard deviation from each contamination dataset used in the exposure assessment model, and estimated by substitution, MLE and KM method are summarized in the Table 12.2.

Table 12.2. Mean and standard deviation from each contamination vector included in the simulation model computed through the substitution method (replacing non detected samples by LOD/2), MLE and KM estimation

		Substitution	MLE	KM
		µg/g	µg/g	µg/g
Wheat Flakes				
	HT2	0.013±0.011	0.015±0.050	0.011±0.012
Corn Flakes				
	HT2	0.024±0.036	0.029±0.103	0.026±0.044
Corn Snacks				
	HT2	0.0351±0.106	0.027±0.131	0.042±0.145
Pasta				
	HT2	0.013±0.014	0.013±0.033	0.012±0.016
	T2	0.014±0.008	0.009±0.021	0.006±0.011
Sliced Bread				
	HT2	0.013±0.017	0.042±2.725	0.009±0.017

Several differences were observed between mean contamination estimates after managing left censored data. The mean estimated for the corn snacks through the KM method was higher than those estimated for MLE and substitution. The MLE method produced higher estimates for sliced bread and cereal flakes datasets than KM or substitution. Wide differences were found in some case as HT2 in sliced bread or T2 in pasta.

12.4.2 Direct method

The parameters $\hat{E}_{\pi_0}$ and $\hat{V}(E_{\pi_0})$ estimated through the Direct Method are shown in the Table 12.3, for the three methods managing censored data. These results showed that children were the most exposed population group for the three approaches. MLE was the method that showed the highest estimation values, while KM presented the lowest estimates.

Table 12.3. Results of exposure using the direct approach ($\hat{E}_{\pi_0} \pm \hat{V}(E_{\pi_0})$), units in μg kg^{-1} bw day^{-1}

	ND =LOD/2	MLE	Kaplan Meier
Children	0.065±0.045	0.079±0.056	0.056±0.041
Adolescents	0.032±0.025	0.040±0.036	0.027±0.022
Adult males	0.016±0.014	0.019±0.023	0.012±0.011
Adult females	0.014±0.012	0.016±0.015	0.011±0.010

12.4.3 Stochastic procedure

More complex statistics as mean, standard deviation, skewness and kurtosis, as well as the median and the main high quantiles (90th, 95th and 99th) were obtained through the simulation methods and presented here in the Table 4 for the three approaches (Substitution, MLE and KM).

Significant differences were obtained when the three censoring handle approaches were compared. MLE approach showed the highest estimates in all population groups, while we obtained the lowest estimates by means of the KM approach.

Considering our results, the children were the most exposed group in each situation, and adult males presented the lowest values of exposure. Median estimates of exposure in children ranged from 0.077 to 0.036 μg/kg bw/day computed through MLE and KM approach, respectively, therefore the probability to exceed the safety level of 0.06 μg/kg bw/day could be cause of concern for high consumers.

Table 12.4. Results of exposure assessment of the population groups from simulation method. These results were obtained directly from the N simulation outputs without pdf fittings (in μg kg^{-1} bw day^{-1})

Children

	ND =LOD/2	MLE	Kaplan Meier
N	10000	10000	10000
Mean	0.059 [0.054; 0.065]	0.091 [0.082; 0.099]	0.041 [0.038; 0.045]
SD	0.038 [0.032; 0.044]	0.059 [0.050; 0.069]	0.024 [0.020; 0.028]
Skewness	1.559	1.393	1.371
Kurtosis	3.632	2.749	2.898
Median	0.050 [0.044; 0.056]	0.077 [0.068; 0.087]	0.036 [0.032; 0.040]
0.90th quantile	0.109 [0.094; 0.123]	0.170 [0.146; 0.191]	0.073 [0.064; 0.082]
0.95th quantile	0.131[0.111; 0.152]	0.204 [0.174; 0.238]	0.086 [0.074; 0.101]
0.99th quantile	0.189 [0.148; 0.255]	0.288 [0.232; 0.368]	0.121 [0.098; 0.159]

Adolescents

	ND =LOD/2	MLE	Kaplan Meier
N	10000	10000	10000
Mean	0.041 [0.038; 0.044]	0.055 [0.050; 0.060]	0.029 [0.027; 0.031]
SD	0.021 [0.018; 0.025]	0.034 [0.028; 0.040]	0.015 [0.013; 0.017]
Skewness	1.134	1.490	1.021
Kurtosis	2.004	3.461	1.430
Median	0.037 [0.033; 0.040]	0.047 [0.042; 0.052]	0.026 [0.024; 0.029]
0.90th quantile	0.069 [0.062; 0.077]	0.100 [0.086; 0.112]	0.049 [0.044; 0.054]
0.95th quantile	0.081 [0.070; 0.092]	0.121 [0.102; 0.138]	0.057 [0.050; 0.065]
0.99th quantile	0.107 [0.090; 0.137]	0.169 [0.136; 0.223]	0.075 [0.063; 0.093]

Adult male

	ND =LOD/2	MLE	Kaplan Meier
N	10000	10000	10000
Mean	0.027 [0.0025; 0.028]	0.036 [0.033; 0.040]	0.018 [0.017; 0.019]
SD	0.013 [0.011; 0.014]	0.020 [0.019; 0.028]	0.008 [0.007; 0.010]
Skewness	0.977	1.857	0.956
Kurtosis	1.271	5.606	1.274
Median	0.025 [0.022; 0.027]	0.031 [0.028; 0.034]	0.017 [0.016; 0.018]
0.90^{th} quantile	0.044 [0.039; 0.048]	0.066 [0.056; 0.076]	0.030 [0.026; 0.032]
0.95^{th} quantile	0.050 [0.044; 0.056]	0.082 [0.067; 0.097]	0.034 [0.030; 0.038]
0.99^{th} quantile	0.065 [0.055; 0.077]	0.119 [0.093; 0.158]	0.043 [0.037; 0.053]

Adult female

	ND =LOD/2	MLE	Kaplan Meier
N	10000	10000	10000
Mean	0.038 [0.035; 0.042]	0.041 [0.038; 0.045]	0.034 [0.030; 0.038]
SD	0.025 [0.020; 0.031]	0.024 [0.020; 0.028]	0.028 [0.022; 0.035]
Skewness	1.923	1.429	2.356
Kurtosis	6.268	3.346	0.028
Median	0.032 [0.029; 0.036]	0.036 [0.032; 0.040]	0.026 [0.023; 0.029]
0.90^{th} quantile	0.070 [0.060; 0.081]	0.072 [0.063; 0.082]	0.068 [[0.056; 0.080]
0.95^{th} quantile	0.087 [0.072; 0.103]	0.0.87 [0.074; 0.100]	0.089 [[0.070; 0.108]
0.99^{th} quantile	0.128 [0.100; 0.180]	0.120 [0.098; 0.153]	0.139 [0.104; 0.197]

Pseudo-parametric bootstrap confidence intervals are also presented in the Table 12.4 in squared brackets. All simulation estimates are contained in the confidence interval, therefore we considered that the stochastic method provided reliable estimates, including those statistics estimated from lognormal and gamma *pdf* fitted to the outputs of this simulation method. The confidence intervals increased progressively for the high percentiles, especially excessive in case of 95^{th} and 99^{th} quantiles. It is an accepted fact that it is very difficult to obtain good accuracy for the 99^{th}-quantile, in particular (Beirlant et al., 1996, 1999; Breiman et al., 1990).

The outputs of the simulation method were fitted to the lognormal and gamma distributions, using the Anderson-Darling statistic to assess the goodness-of-fit, considering a significance level greater than 95%, to accept the distribution as a suitable candidate. When we compare the statistics estimated directly with those statistics obtained through fitting, we did not find wide differences. In the Table 12.5 an example is shown of different statistics computed; the estimated statistics of exposure of children to T2-HT2 by lognormal and gamma *pdf* fitted to the outputs of the simulation method. In the Figure 12.2 we show the typical histograms from simulation outputs of children using the three censored data approaches.

Table 12.5. Estimated statistics of exposure to T2-HT2 by lognormal and gamma pdf fitted to the outputs of the stochastic method for the group of children (in μg kg^{-1} bw day^{-1})

	Substitution	MLE	KM
Lognormal	$\hat{r}$ ***=0.6374*** $\hat{\lambda}$ ***=-3.0234***	$\hat{r}$ ***=0.6810*** $\hat{\lambda}$ ***=-2.6135***	$\hat{r}$ ***=0.5968*** $\hat{\lambda}$ ***=-3.3587***
Mean	0.060	0.092	0.042
SD	0.042	0.071	0.027
Median	0.049	0.073	0.035
0.90th quantile	0.110	0.175	0.075
0.95th quantile	0.139	0.225	0.093
0.99th quantile	0.214	0.357	0.140
AD (*p*-value)	7.9975 (<0.005)	21.7108 (<0.005)	14.9011 (<0.005)
Gamma	$\hat{r}$ ***=2.7587*** $\hat{\lambda}$ ***=0.0211***	$\hat{r}$ ***=2.5139*** $\hat{\lambda}$ ***=0.0360***	$\hat{r}$ ***=3.1594*** $\hat{\lambda}$ ***=0.0130***
Mean	0.059	0.091	0.041
SD	0.035	0.057	0.023
Median	0.052	0.079	0.037
0.90th quantile	0.107	0.167	0.072
0.95th quantile	0.127	0.200	0.085
0.99th quantile	0.171	0.272	0.113
AD (*p*-value)	13.3567 (<0.001)	5.0894 (<0.001)	4.8604 (<0.001

AD: Anderson Darling statistic

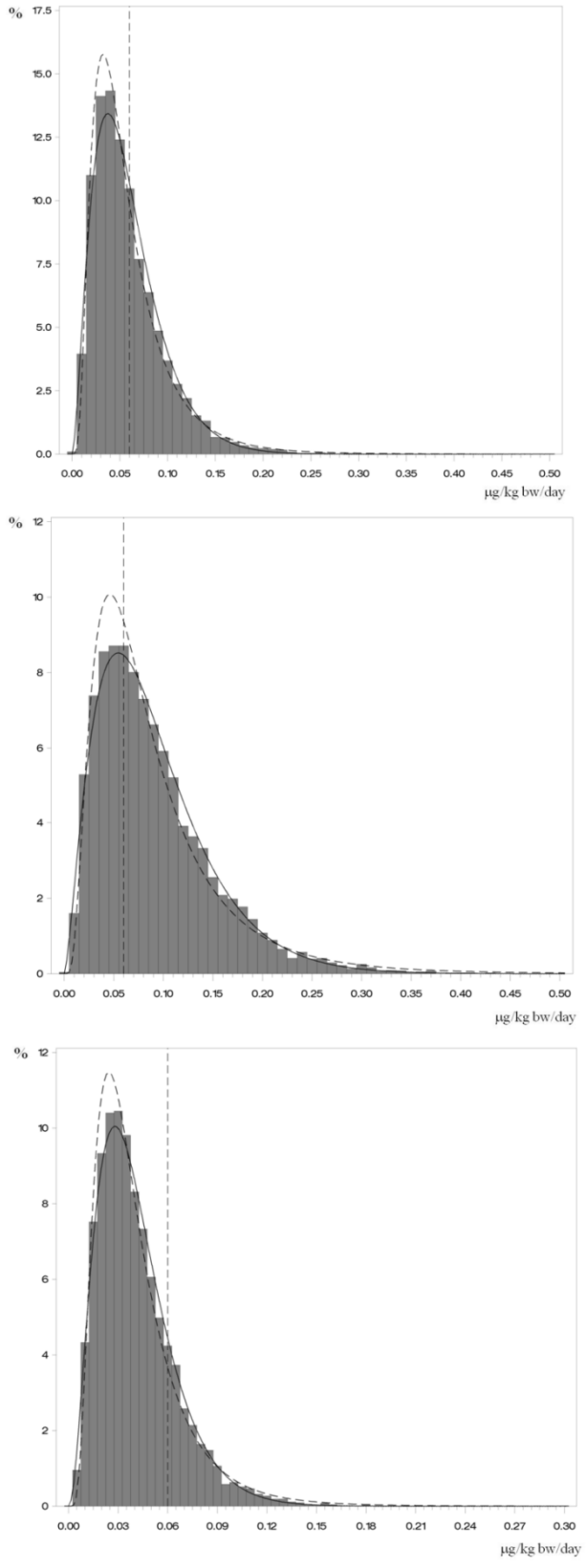

Fig. 12.2. Fitted gamma (solid line) and lognormal (broken line) pdfs for children; using the substitution method (top), MLE method (middle) and KM method (bottom). Reference line (vertical broken line) indicates the TDI of 0.06 µg kg^{-1} bw day^{-1}. Relative parameters are given in Table 5. Exposure in µg kg^{-1} bw day^{-1}

12.5 Discussion

In the present work we attempted to assess the exposure of Catalonian population to the mycotoxins T2 and HT2 comparing three methods to handle left censored data. To reach this objective, we computed accurately large contamination datasets provided from a thorough survey carried out around the region involving a sturdy sampling and analytical method. We carried out three approaches to handle the left censored data: a substitution method, a parametric method and a non-parametric method. Moreover we combined the contamination data with consumption datasets provided by a specific nutritional study conducted in Catalonia, applying a stochastic method to take into account the variability and uncertainties in the model.

Risk assessment on T2 and HT2 toxins has not been done yet by EFSA, therefore it is a priority to provide scientific information about the current situation to risk managers. Maximum limits have not been stated in food for human consumption while waiting for the technical reports. Estimated intakes of T2 and HT2 toxins were reported by JECFA (2001) only for the European regional diet due to the limited information available in the other GEMS/Food regional diets. The average intakes of the two toxins were calculated by multiplying the weighted mean concentration of each commodity times the corresponding amount consumed in the GEMS/Food European diet, converted to intake per kilogram of body weight per day, assuming a body weight of 60 kg. The average intake of T2 in the European diet was estimated to be 0.008 μg/kg bw per day, while that of HT2 was estimated to be 0.009 μg/kg bw per day.

At the national level, JECFA (2001) reported dietary intake estimations by UK and Norwegian populations, calculated by multiplying the national food consumption values by the weighted mean concentrations of T2 in samples from the European diet. In both cases, the group of children was the most exposed population group, but that study reported the mean dietary from each raw cereal (barley, maize, oats, rye and wheat) , therefore it was not possible to compare with our results. The First French Total Diet Study attempted previously to assess the exposure to HT2 toxin in France. They found

only one out of 235 composite samples above the LoQ of 0.080 μg/g, therefore they did not carry out any exposure assessment study for this mycotoxin (Leblanc et al., 2005).

To our knowledge, this is the first study that reports the exposure to T2 and HT2 toxins, to date. Despite Cano-Sancho et al. (2011b) reported low presence of HT2 and very low presence of T2 (exceeding LoQ), we have found a considerable number of samples between LoD and LoQ. Therefore, we were motivated to accurately handle the left-censored data in order to compute the exposure assessment estimates.

In the present study, we have found that treatment of censored data has a wide impact in the final exposure estimate when the percentage of non-detects is high. EFSA (2010) reported an analysis of different methods to handle left censored data in dietary exposure assessment of chemical substances, but there is no consensus about the method to apply in each case. The most commonly used methods are based on the substitution of those non-detected samples by LOD, LOD/2 or LOD/√2. The drawbacks of this approach were elucidated by Helsel (2006), concluding that the resulting estimates of correlation coefficients, regression slopes, hypothesis tests, or simple means or standard deviations are inaccurate and irreproducible, being far from their true values, and the amount and type of deviation is unknown. The different methods to handle censored data were also reviewed by Helsel (2005) and offered the recommendations: for <50 % censored use the KM method (for all samples sizes); for 50-80 % censored and sample sizes >50 use the MLE. Therefore, considering the high percentage of non-detected samples and Helsel (2005) recommendations, MLE estimates should provide us the best estimation, being also, the most conservative scenario.

12.6 Conclusion

Concluding remarks: considering the results from the present study, the most exposed group population to the toxin T2 and HT2 should be expected to be the children. Despite of the drawbacks to set the exposure outputs derived from the large

censored data, we have found that a group of child population could exceed the safety limits of 0.06 μg/kg bw/day. New analytical methods should be developed in order to decrease the LOD and better characterize the presence of these mycotoxins in the market. Left-censored data should be accurately handled, by means of the statistical methods advised by EFSA, to provide more accurate estimates of exposure when non-detects are widely present in the exposure assessment model.

12.7 References

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Chapter 13. Occurrence of zearalenone, an oestrogenic mycotoxin, in Catalonia (Spain) and exposure assessment

Food and Chemical Toxicology 2012 50, 835–839

13.1 Abstract

The objectives of this study were to assess the occurrence of zearalenone (ZEA) in food in Catalonia (Spain); to assess the consumption of the main food items contaminated with ZEA; and finally, to estimate the dietary exposure of the Catalonian population to the mycotoxin. Therefore 1187 samples were collected from the Catalonian market and pooled to obtain 485 analytical samples (composites) of the commodities most susceptible to ZEA contamination and commonly consumed in the region. Extraction and clean-up was performed using immunoaffinity columns and ZEA was detected and quantified by high performance liquid chromatography (HPLC) and fluorescence detection. A specific food frequency questionnaire (FFQ) was administered to 1264 individuals by trained interviewers to estimate the consumption of the relevant foods. The results showed that the occurrence of ZEA in food marketed in Catalonia was infrequent. Sliced bread, corn snacks and sweet corn were the commodities where it was most often present. In considering the contamination levels and food consumption estimates, the dietary intake of ZEA by the Catalonian population was estimated to be considerably lower than the temporary tolerable daily intake (TDI) of 200 ng ZEA kg^{-1} bw day^{-1}.

13.2 Introduction

Zearalenone (ZEA) is a non-steroidal oestrogenic mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals, including maize and wheat, in temperate and warm regions around the world (Jelinek et al., 1989; Jiménez et al., 1996; Kuiper-Goodman et al., 1987; Patel et al., 1996).

Absorption of ZEA has been reported as rapid and extensive in rats and rabbits (Kuiper-Goodman et al., 1987; Ramos et al., 1996), being estimated in the range of 80–85% in pigs (Biehl et al., 1993). ZEA and some of its metabolites competitively bind to oestrogen receptors. Thus, the toxicity is associated with reproductive problems in specific animal species and possibly in humans (Gromadzka et al., 2009; Wood, 1992). Fertility problems have been observed in animals such as swine and sheep (Krska et al., 2003). ZEA may be an important etiologic agent of intoxication in infants or foetuses exposed to this mycotoxin, resulting in premature thelarche, pubarche, and breast enlargement (CAST, 2003). Risk assessment of ZEA performed by the EC SCF concluded on a temporary tolerable daily intake (TDI) of 200 ng kg^{-1} body weight day^{-1}, based on a short-term study in pigs (SCF, 2000), whereas a provisional maximum tolerable daily intake (PMTDI) of 500 ng kg^{-1} body weight day^{-1} was established by JECFA (EFSA, 2004). The European Commission established maximum levels of zearalenone in bread, pastries, biscuits, cereal snacks, and breakfast cereals at 50 μg kg^{-1}, and 20 μg kg^{-1} in processed cereal-based foods and baby foods for infants and young children (European Commission, 2006b), and later established the limits in maize for direct consumption and maize-based snacks and breakfast cereals at 100 μg kg^{-1} (European Commission, 2007).

Occurrence of ZEA in processed food for human consumption in Spain has been previously studied at one occasion with 25 samples (Cerveró et al., 2007), in which raw food was most widely reported matrix containing ZEA (Jaimez et al., 2004; Muñoz et al., 1990; Viñas et al., 1985). To our knowledge, no studies have been published yet

assessing the exposure of the Spanish population to this mycotoxin.

The objectives of this study were to assess the occurrence of zearalenone in foods in Catalonia (Spain), and to estimate the dietary intake of this mycotoxin among the Catalonian population.

13.3 Materials and methods

13.3.1 Samples

Food samples were selected by considering its susceptibility to ZEA contamination and its consumption in Catalonia. Between the months of July and December 2008, samples of sweet corn (n = 185), corn flakes (n = 168), wheat flakes (n = 29), corn snacks (n = 216), pasta (n = 201), sliced bread (n = 145), beer (n = 213) and baby food (n = 30) were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain. These represent 72% of the market populations. From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of zearalenone was determined in a total of 72 composite samples obtained by pooling the 3 items taken from each store, if available (12 cities 6 stores/city = 72 samples/category). However, in some cases, 3 items in the same store were not available, thus, less than 72 composites could be obtained. In case of wheat flakes and baby food minor sampling was conducted. The samples were chosen to have the highest presence in the shop shelves, considering a wide range of brands. We finally obtained, 31 brands of sweet corn, 62 of corn flakes, 29 of wheat flakes, 79 of corn snacks, 105 of pasta, 43 of sliced bread, 64 of beer and 21 of baby food, which can be considered the majority of the market share in Catalonia of these products, as well as in the rest of the Spanish market. The samples were transported and stored under suitable conditions until analysed.

13.3.2 Analytical method

Easi-extract Zearalenone® immunoaffinity cleanup columns (R-Biopharm, Rhône Ltd., Glasgow, UK) were used to extract ZEA from all analytical samples. A volume of 5 mL of beer, previously degassed in an ultrasonic bath during 40 min were mixed with 15 mL of phosphate buffer solution (PBS: 0.8% NaCl, 0.12% Na2HPO4, 0.02% KH2PO4, 0.02% KCl), and drained through the column. The column was washed with 20 mL of PBS solution and ZEA was eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water. Regarding solid maize-based samples, 5 g of mixed composite were mixed with 25 mL of extractant solution (75% acetonitrile; 25% water) for 10 min and filtered. 10 mL of filtered solution were diluted with 40 mL of PBS and drained through the Zearalenone immunoaffinity columns. After this, the columns were washed with 20 mL of PBS and zearalenone eluted with 1.5 mL of acetonitrile grade HPLC and 1.5 mL of milli-Q water. Finally, ZEA was determined, in purified samples, using HPLC with fluorescence detector and mobile phase of acetonitrile–water (60:40), adjusted at pH 3.2 with acetic acid.

Chromatography equipment: Separations Module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5 μm ODS2, 4.6 x150 mm, Multi λ Fluorescence Detector Waters 2475. Excitation and emission wavelength was 274 nm and 455 nm, respectively. To validate the analytical method, recoveries from the different matrices were determined by spiking triplicates with ZEA at a level of 20 $\mu g\ kg^{-1}$. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal-to-noise ratio as more than 3:1 and 9:1, respectively. Finally, the concentrations of ZEA in each food were not corrected for recovery rate.

13.3.3 Dietary intake assessment

The main problem in assessing the intake of mycotoxins is related to the fact that there are only few representative data available on consumption of foods with mycotoxins contamination. Some methods have been developed to assess overall dietary

intake known as market basket, 24-h dietary recall and food record methods, food-frequency methods or dietary history. Given that consumption of related products is considered as sporadic or casual, food-frequency methods should be chosen because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998). In this study, food dietary intake was assessed through a specific food frequency questionnaire (FFQ) developed for the Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. According to the World Health Organization (WHO), studies to assess dietary intake of chemical contaminants should show the significant intake among the standard population, together with all population groups that could have different dietary patterns. Therefore, five different population groups of each sex were considered: infants and toddlers (0–3 years), children (4–12 years), adolescents (13–18 years), adults (20–65 years) and elderly (>65 years). The FFQ consisted of 38 items of specific foods known worldwide to be the most important foods contaminated by mycotoxins, excluding those foods not consumed in the region. Concerning frequency of consumption, five response options, ranging from never to annually, were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 76 elders, 744 adults, 181 adolescents, 99 children and 164 infants and toddler parents were interviewed during 2008–2009 (n = 1264) by trained interviewers. The individuals were from 89 cities and towns from Catalonia. This population was considered as representative of Catalonia due to the homogeneous dietary habits around the region.

13.3.4 Exposure assessment to zearalenone

Considering the limitations to carry out an accurate exposure assessment study, in the current work, we have estimated the exposure in a sample of Catalonian population through a direct approach as follows. If we assume independency between consumptions ($C_{\pi,j}$) and contaminations (T_j), and also between their products, we can

estimate the exposure mean of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0, j} \overline{T}_j$$

where $\overline{C}_{\pi_0, j}$ is the arithmetical mean of the normalized consumption of the foodstuff *j* in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of related food (Gauchi and Leblanc, 2002).

13.3.5 Statistical analysis

Analysis of variance, one-way ANOVA, was applied to compare contamination and estimated ZEA daily intake average results, using Least Significant Differences (LSD) at confidance level of 95%. Software used in statistical analysis was Statgraphics Plus 5.1®.

13.4 Results

13.4.1 Method validation

Recovery data, repeatability, limit of detection (LOD) and limit of quantification (LOQ) are shown in Table 13.1. The LOD defined as the mass of the analyte which produces a signal three times the standard deviation of the baseline noise, was 0.65 μg kg^{-1}, in solid matrices. This value is in the line with those reported by Briones-Reyes et al. (2007), 0.7 μg kg^{-1}, and Roscoe et al. (2008), 1 μg kg^{-1}. The average recoveries of ZEA were in the range of 83 ± 2–116 ± 5% for pasta and beer, respectively, with corn snacks being the matrix with the best recovery values (98 ± 2%) at the spiking level of 20 μg kg^{-1}. The precision was estimated by the relative standard deviation (RSDr) of the recovery, being in the range of 2–33%, where the worst cases were related with the spiking level of 6 μg kg^{-1}. According to performance criteria established by Commission Regulation

(EC) Nº 401/2006 (European Commission, 2006a) this method can be qualified as acceptable. This performance criterion declared that recoveries from samples at a spiked level below 50 μg kg^{-1} should range between 60–120%, and the RSDr should be below 40%.

Table 13.1. Method performance characteristics for zearalenone

Matrix	LOD/LOQ[a] (μg kg^{-1})	Spiked level (μg kg^{-1})	n	Recovery[b] (%)	RSDr[c] (%)
Pasta	0.65/1.95	20	3	83±2	18
		6		86±3	33
Breakfast cereals	0.65/1.95	20	3	87±1	2
		6		85±2	19
Corn snacks	0.65/1.95	20	3	98±2	2
		6		108±5	4
Sweet corn	0.65/1.95	20	3	88±7	7
		6		104±2	19
Sliced Bread	0.65/1.95	20	3	87±2	2
		6		108±9	8
Beer	0.23/0.70	20	3	116±5	5
		6		88±3	27
Baby food	0.65/1.95	20	3	113±1	8
		6		85±5	5

[a] LOD = Limit of detection / LOQ = Limit of quantification
[b] Mean ± standard deviation
[c] RSDr = relative standard deviaton

13.4.2 Occurrence of zearalenone

Results for contamination of foods marketed in Catalonia (Spain) are summarized in Table 13.2. The highest percentage of positive samples was found in sliced bread (43.7%), while ZEA was not quantified in corn flake samples. The mean levels of ZEA in the positive samples ranged from 3.1 ± 1.4 to 5.9 ± 6.8 μg kg^{-1}, in the case of baby food the mean value was 4.1 ± 0.6 μg kg^{-1}. The maximum values were found in sliced bread (20.9 μg kg^{-1}) and corn snacks (22.8 μg kg^{-1}) well below the EU limits of 50 μg kg^{-1}.

Table 13.2. Occurrence of zearalenone in food from Catalonian (Spain) market

Matrices	n individual	n *"composite"*	Positives/ total	Mean±sd μg kg^{-1}	Max μg kg^{-1}	<LOQ	LOQ-10	>10
Pasta[a]	201	70	10/70	3.8±1.8	5.9	60	10	0
Corn flakes[a]	168	71	0/71	-	-	71	0	0
Wheat flakes[a]	29	29	4/29	6.3±5.4	12.1	25	3	1
Corn snacks[a]	216	72	17/72	5.9±6.8	22.8	55	13	4
Sweet corn[a]	185	72	13/72	4.9±0.7	5.9	59	13	0
Sliced bread[a]	145	71	31/71	3.7±4.5	20.9	40	27	4
Beer [b]	213	71	8/71	3.1±1.4	5.1	63	8	0
Baby food[a]	30	30	7/30	4.1±0.6	5.4	23	7	0
Total	1187	485	90/485	-	-	-	-	-

[a]LOQ = 1.95 μg kg^{-1}
[b]LOQ = 0.70 μg kg^{-1}

13.4.3 Dietary intake assessment

Due to a low percentage of positive samples (<44%), the uncertainty derived from left censored data, becomes an important source of bias in the exposure assessment. Thus, advices from the Report on aWorkshop in the frame of GEMS/Food-EURO on Reliable Evaluation of Low-Level Contamination of Food (GEMs/Food-WHO, 1995) were followed in this section. These advices recommended to produce two estimates using 0 and LOD for all results less than 1.6 x LOD to obtain a simple estimate of mean, where there are more than 60% of non quantified results. Ranges of estimated zearalenone intakes are shown in Table 13.3 for different age groups.

Table 13.3. Estimates of zearalenone dietary intake among Catalonian (Spain) population.

	Percentage of contribution to ZEA exposure (%)						Ranges of ZEA exposure* ng kg^{-1} bw day^{-1}	
	Corn snacks	Breakfast cereals	Sliced bread	Pasta	Beer	Sweet corn	mean	p95
Elders	1.1	2.3	9.7	44.4	32.4	9.9	0.3-0.5[a]	0.9-2.0
Adults	3.2	10.4	23.5	24.1	21.4	17.4	0.8-1.4[ab]	2.5-4.2
Teenagers	12.5	18.5	37.2	19.1	3.4	9.3	1.4-2.2[b]	4.3-6.6
Infants	9.9	27.9	32.2	18.4	0.0	11.5	2.7-4.2[c]	6.0-10.3
Babies	-	-	-	-	-	-	12.2-17.9[d]	35.4-51.9

* Lower bound of the range was estimated considering non detected samples to be 0, and the upper bound, considering non detected samples to be the LOD.
[a] Different letter means statistically significant differences (p>0.05; LSD test).
TDI = 200 ng kg^{-1} bw day^{-1}(SCF 2000).

No differences were found between sexes ($p > 0.05$), therefore, results for males and females were shown together. The age group with the highest estimated intake of ZEA was infants and toddler, where high consumers had estimated intakes in the range of 35.4–51.9 ng kg^{-1} bw day^{-1}. The elderly was the group with the lowest exposure values. In all cases, exposure values were below the temporary TDI of 200 ng kg^{-1} bw day^{-1} (SCF, 2000). Several differences were observed in dietary patterns between the age groups. For example, the adult population consumed the highest amounts of beer and the lowest amount of breakfast cereals, while the contrary case was found for children (see Table 13.4). Pasta and sliced bread were the main contributors to ZEA intake within the age groups.

Table 13.4. Consumption of the main commodities related with ZEA contamination among Catalonia (Spain) population

	n	Age ranges (years)	Mean height (m)	Mean weight (Kg)	Daily mean consumption of the main commodities related with ZEA contamination (expressed in g/day)					
					Corn snacks	Breakfast cereals	Sliced bread	Pasta	Beer	Sweet corn
Elders	76	>75	1.64	74.17	0.2	0.9	1.2	0.2	43.1	2.2
Adults	720	18-65	1.69	73.34	1.6	11.4	9.1	26.8	87.6	11.4
Teenagers	235	13-17	1.63	54.41	7.1	25.8	18.3	25.4	14.5	9.2
Infants	69	4-12	1.28	25.74	4.4	33.6	14.2	24.3	0	7.0
Babies	164	0-3	0.83	11.60	-	-	-	-	-	-

13.5 Discussion

Occurrence of ZEA has been mainly studied in feed and raw food from Spain (Muñoz et al., 1990; Jaimez et al., 2004) and other countries as was reported in previous reviews (Oldenburg, 1993; Zinedine et al., 2007; Gromadzka et al., 2008). To our knowledge, only one study was carried out to assess the occurrence of ZEA in maize-based food for human consumption from the Spanish market (Cerveró et al., 2007). That study was conducted with few samples (five of each item) and showed higher occurrence and mean contamination values than in the present study. The authors reported percentages of positive samples ranging between 40% and 80% (LOD = 3 lg kg^{-1}), with mean values of 114.0 ± 10.6 lg kg^{-1} in corn flakes, 11.1 ± 6.5 lg kg^{-1} in sweet corn and 91.2 ± 45.2 μg kg^{-1} in corn snacks, while in our study, ZEA was not quantified in corn flakes samples and low ZEA amounts were found in sweet corn and corn snacks (mean of 4.9 ± 0.7 and 5.9 ± 6.8 μg kg^{-1}, respectively). In the line with our results, no samples of corn flakes were found above the limit of detection of 2 μg kg^{-1} in Argentina (Solovey et al., 1999). Mean concentrations in positive samples of 6.1 and 2.4 μg kg^{-1} were found, respectively, in maize and wheat-based breakfast cereals from Canada

(Roscoe et al., 2008) and 22.7 lg kg^{-1} in breakfast cereals from France (Leblanc et al., 2005). ZEA occurrence in beer samples were previously studied in Korea, where 54 imported beers were analysed using GC–MS without finding any sample above the limit of detection of 1.5 ng mL^{-1} (Shim et al., 1997). Concerning contamination of infant food with ZEA, Lombaert et al. (2003) found a similar percentage of positive samples of multi-grain cereals as in this study (30/71), and the mean contamination value of positive samples of 10.7 μg kg^{-1} was slightly higher than our result (4.1 ± 0.6 μg kg^{-1}). Both results were different from the early study carried out by Warner and Pestka (1987) using ELISA kit, who found all samples to be contaminated.

Until now only few data have been available on the exposure assessment of the Spanish population to ZEA. In this study, a wide range of the Catalonian population was interviewed to assess their dietary habits related to those foods which could be probable carriers of this mycotoxin. We have compared the result from our study with the SCOOP (2003) study and The First French Total Diet Study (Leblanc et al., 2005). Estimates of ZEA intake by the French population were significantly higher than our estimates; the authors reported an average intake of 33 ng kg^{-1} bw day^{-1} in adults and 66 ng kg^{-1} bw day^{-1} in infants, and 70 and 132 ng kg^{-1} bw day^{-1} respectively, for the 95 percentile. Several estimates were found that were in line with our results which ranged from the mean of 0.3 ng kg^{-1} bw day^{-1} for the elderly to 51.9 ng kg^{-1} bw day^{-1} for babies at the 95 percentile., For example, adults from Italy and Portugal, showed estimates of 0.8 and 4.05 ng kg^{-1} bw day^{-1} respectively and the stimates for infants and babies from Austria, Finland, Germany, Norway, Austria or UK ranged between 8 to 31.6 ng kg^{-1} bw day^{-1} (SCOOP, 2003). The daily intake of ZEA by Canadians was estimated to be in the range between 50 and 100 ng kg^{-1} bw day^{-1} in a study to assess the risk of the population to this mycotoxin (Kuiper-Goodman et al., 1987). Despite the high variability in the estimation of exposure to ZEA between studies, all these values were lower than the TDI of 200 ng kg^{-1} bw day^{-1}.

Several aspects should be taken into account in future works to improve the accuracy of exposure assessment studies with zearalenone. There are some mycotoxin

forms that cannot be detected using conventional analytical methods, but can be converted to the toxic form in the intestine. *Fusarium* mycotoxins are susceptible to be bound, frequently by conjugation, forming conjugates of mycotoxins, also known as "masked mycotoxins". The main natural conjugated form of ZEA is zearalenone 4-sulfate, which can be released easily in the intestine as ZEA through the action of sulphates (Berthiller et al., 2009). Therefore, in future works, "masked" mycotoxins should be considered in order to assess a most realistic amount of mycotoxin released in the intestine. Concerning the food consumption data, we have used a FFQ because it is widely accepted to assess the individual's usual dietary habits. However, the need for a validation against a more reliable method has been stressed (Walter, 1998). Another point to take into account is to identify population groups with different dietary habits in order to assess the exposure individually. In our latest nutritional study, we have found different nutritional trends among immigrants and celiac sufferers (unpublished data). Finally, in order to improve the assessment of the exposure to food contaminants, the use of probabilistic models has been advised to provide more realistic estimates. The main advantages of probabilistic models are, on one hand, they permit to consider the whole distribution of exposure, from minimum to maximum, with all modes of percentiles, and on the other hand, and they include a comprehensive analysis of the sensitivities of the resulting exposure with respect to uncertainties in parameters (Kroes et al., 2002).

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Chapter 14. Quantitative Exposure Assessment of Catalonian (Spain) population and special groups to the zearalenone mycotoxin

Food and Chemical Toxicology (In Process)

14.1 Abstract

In the present work, stochastic methodology has been applied to assess accurately the exposure of Catalonian (Spain) population to the oestrogenic mycotoxin zearalenone (ZEA) through food. Raw contamination data was provided by a large survey conducted around of this region, as well as, the raw consumption data, that came from a nutritional study specifically designed to assess the dietary intake of the main foodstuff related with ZEA contamination, for all age population groups. Contamination and consumption data was combined by simulation, using a NonParametric-NonParametric (NP-NP) method and another essentially parametric (P-P). In general terms, the NP-NP method combine consumptions and contaminations values exclusively from the raw data, and the P-P method, draw the sampling values from distribution functions fitted to consumption and contamination datasets. Moreover, to quantify the accuracy and reliability of the statistics estimates, we built the related confidence intervals using a Pseudo-Parametric bootstrap method.

14.2 Introduction

Zearalenone (ZEA) is a non-steroidal oestrogenic mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals, including corn and wheat, in temperate and warm regions around the world (Kuiper-Goodman et al., 1987; Jelinek et al., 1989; Jiménez et al., 1996; Patel et al., 1994).

ZEA and some of its metabolites have been shown to competitively bind to oestrogen receptors. Thus, the toxicity is associated with reproductive problems in specific animals and possibly in humans (Wood, 1992; Gromadzka et al., 2009). Fertility problems have been observed in animals such as swine and sheep (Krska et al., 2003). ZEA may be an important etiologic agent of intoxication in children or foetuses exposed to this mycotoxin, with results in premature thelarche, pubarche, and breast enlargement (CAST, 2003). Based on recent data in the most sensitive animal species, the pig, and taking into account comparisons between pigs and humans, the Panel on Contaminants in the Food Chain established a new tolerable daily intake (TDI) for zearalenone of 0.25 μg^{-1} kg b.w. (EFSA, 2011).

Occurrence of ZEA in processed food from Spain for human consumption has been previously studied in one occasion with 25 samples (Cerveró et al., 2007), being the raw materials the most widely reported matrix (Viñas et al., 1985; Muñoz et al., 1990; Jaimez et al, 2004). Occurrence of ZEA in cereal-based food was found to be moderate in European countries, however, to our knowledge, few studies have been published until now to assess the exposure of these populations to ZEA, being mainly deterministic approaches (SCOOP, 2003; Leblanc et al., 2005). A recent study carried out in Spain found the occurrence of ZEA to be low, reporting a percentage of positive samples of almost 19 % of total samples (n=485). A gross exposure assessment study was performed by means of a deterministic method without finding of population groups under health risk. However, the authors concluded that, in one hand, more refined methods like probabilistic approaches are required to get refined assessments. In the other hand, they also highlighted several population groups to have different dietary

habits such us immigrants or celiac sufferers, thus, with specific computation requirements (Cano-Sancho et al., 2011,2012b).

Expertise panels have advised the use of stochastic approach to provide a more realistic exposure assessment, taken in consideration, uncertainties and variability in the model. Nevertheless, there is no consensus on which specific methodology should be applied in each case (Kroes et al., 2002; WHO, 2005; EFSA, 2006; Verger et al., 2008). Stochastic methodology has been developed by Gauchi and Leblanc (2002) to assess the exposure of the population to food contaminants, applying to assess the exposure of French population to ochratoxin A (OTA). The authors proposed two simulation approaches to estimate the exposure, based on Monte Carlo simulations (NonParametric-NonParametric and Mixed Parametric-Parametric method), and finally they assessed four types of bootstrap of confidence intervals.

The aim of this paper is to assess quantitatively the exposure of Catalonian (Spain) population to ZEA. Therefore we have computed, contamination and consumption raw datasets through a parametric and non-parametric approaches, building pseudo-parametric bootstrap confidence.

14.3 Materials and methods

14.3.1 Description of the data

The contamination and consumption raw data was provided by the Project to Assess the Exposure of Catalonian Population to Mycotoxins (UdL-ACSA, 2009; Cano-Sancho et al., 2012a). This study carried out, in one hand, a wide survey to assess the occurrence of ZEA in Catalonian market, and in the other hand, a specific nutritional study to know dietary patterns of Catalonian population concerning the main related foodstuffs.

14.3.1.1 The Contamination Data

Food samples were selected to be the most susceptible commodities to ZEA contamination and to be commonly consumed in Catalonia. Between 2008 and 2009, samples of sweet corn (n=185), breakfast cereals (n=168), corn snacks (n=216), pasta (n=201), bread (n=147), beer (n=213) and baby food (n=30) were obtained in six hypermarkets and supermarkets from twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72 % of the population. From each supermarket or hypermarket, 3 samples (if present) of each product were randomly taken. The level of zearalenone was determined in a total of 72 composite samples obtained by pooling 2 or 3 items taken from each store if available (12 cities x 6 stores / city = 72 samples / category). In some cases, all 72 composites were not available and in baby food case, the samples were analyzed individually. Moreover, the study was strengthened with ethnic and gluten-free samples. Ethnic food samples were purchased in grocery stores specialised in imported (ethnic and regional) food products, and hypermarkets or supermarkets with an international-food section. The ethnic foods samples purchased were cuscus, corn flour, wheat flour, corn cream and corn grits. Gluten-free food samples were purchased from health food stores and hypermarkets or supermarkets with a health food section, being mainly bread, pasta and pastries without gluten intended for celiac sufferers. Finally, 35 ethnic food and 18 gluten-free food samples were collected in Lleida (Spain). The samples were ground and freeze stored (-20ºC) until analysis.

Extraction and clean-up was performed using immunoaffinity columns and ZEA was detected and quantified by high performance liquid chromatography (HPLC) and fluorescence detection. In this study was assumed that non-detected samples were limit of detection (LOD) divided by 2, assumption widely recognized to reduce the uncertainty of those values between 0 and the LOD. Some statistics from contamination data and the corresponding histograms are shown in Table 14.1 and Fig. 14.1, respectively (Cano-Sancho et al. 2012a,b).

Table 14.1. Occurrence of zearalenone in food matrices from Catalonian market

Matrices	n individual	n *"composite"*	Positives/total	Mean µg/kg	*SD*	Max µg/kg
Pasta	201	70	10/70	3.8	1.8	5.9
Breakfast cereals	168	71	4/71	6.3	5.4	12.1
Corn snacks	216	72	17/72	5.9	6.8	22.8
Sweet corn	185	72	13/72	4.9	0.7	5.9
Bread	147	71	31/71	3.7	4.5	20.9
Beer	213	71	8/71	3.1	1.4	5.1
Baby food	30	30	7/30	4.1	0.6	5.4
Ethnic food	35	35	3/30			12.4
Gluten-free food	18	18	0	-	-	-

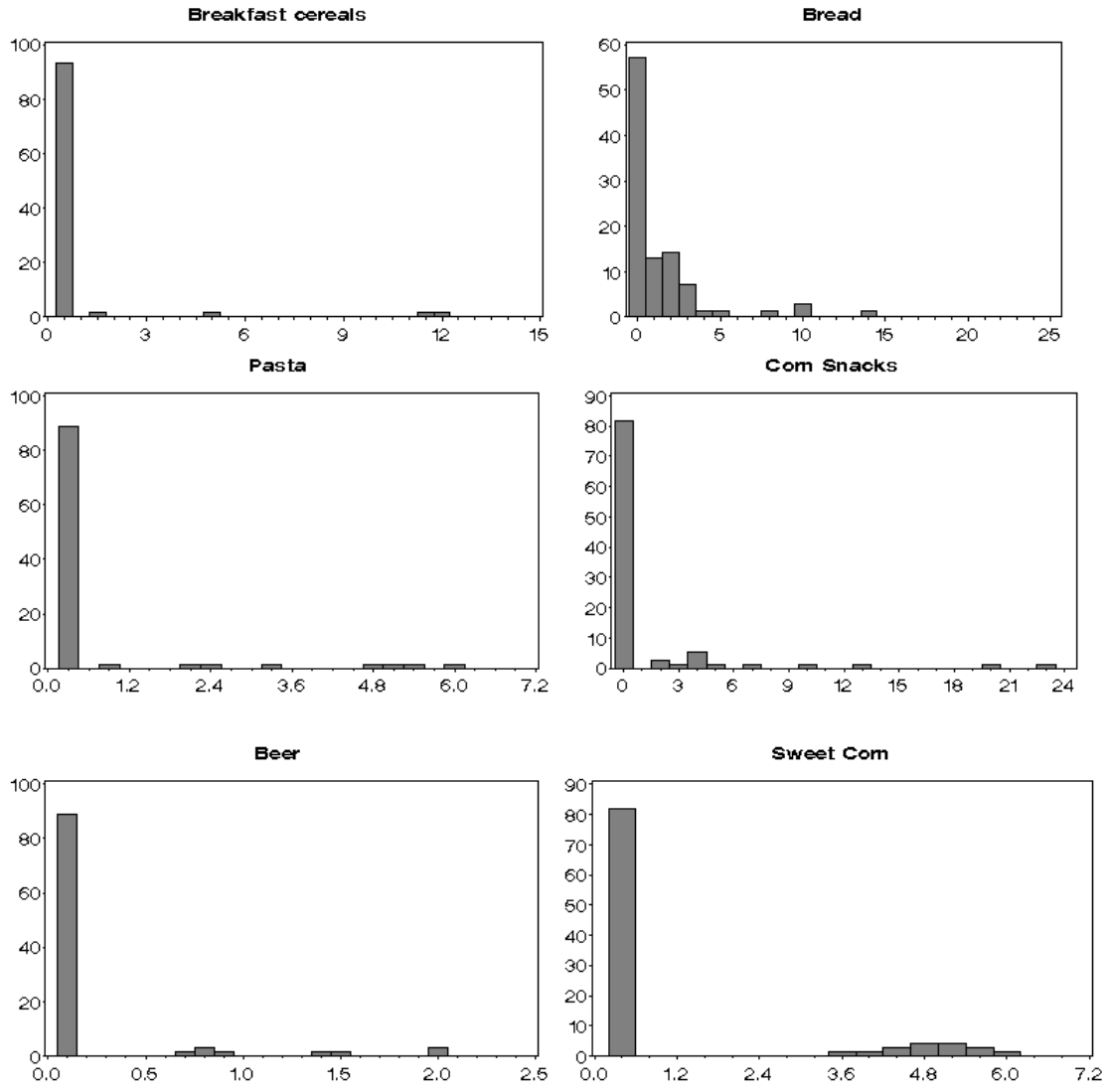

Fig 14.1. Contamination histograms (relative frequencies), in µg/kg for each food

14.3.1.2 The Consumption Data

Food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for Catalonian population including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. According to World Health Organization (WHO) advices, studies to assess dietary intake of chemical contaminants, should show the significant intake among standard population, with all population groups that could have different dietary patterns. Therefore, five different population groups of each sex were considered: infants (0-3 years), children (4-9), adolescents (10-19 years), adults (20-65 years), elders (>65 years), immigrants (17-51 years) and celiac sufferers (16-75 years). FFQ consisted of 38 items of specific foods worldwide known to be the most important food contaminated by mycotoxins, excluding those foods not consumed in the region.

Table 14.2. Normalized consumption of the main foodstuff related with ZEA contamination by the 384 adult females (g/kg body weight/day)

	Consumers	% Consumers	Mean	*SD*	Max
Breakfast cereals	153	39.8	0.48	0.46	2.35
Bread	161	41.9	0.22	0.32	1.74
Pasta	378	98.4	0.34	0.29	3.51
Corn Snacks	114	29.7	0.07	0.11	0.91
Beer	204	53.1	1.43	1.97	10.14
Sweet Corn	143	37.2	0.50	1.08	8.33

Concerning frequency of consumption, 5 response options, ranging from never to annually, were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 56 immigrants, 70 celiac sufferers, 76 elders, 720 adults, 235 adolescents, 69 children and 164 baby parents were interviewed during 2008-2009 (n=1393) by trained interviewers. In the present study, the adults were treated separately by sexes to be a great population group being 336 adult males and 384 adult females. Individuals were from 89 cities and towns from Catalonia. An example of

some statistics from consumption data of adult females and the corresponding histograms are shown in Table 14.2 and Fig. 14.2, respectively, in the line of those obtained for males.

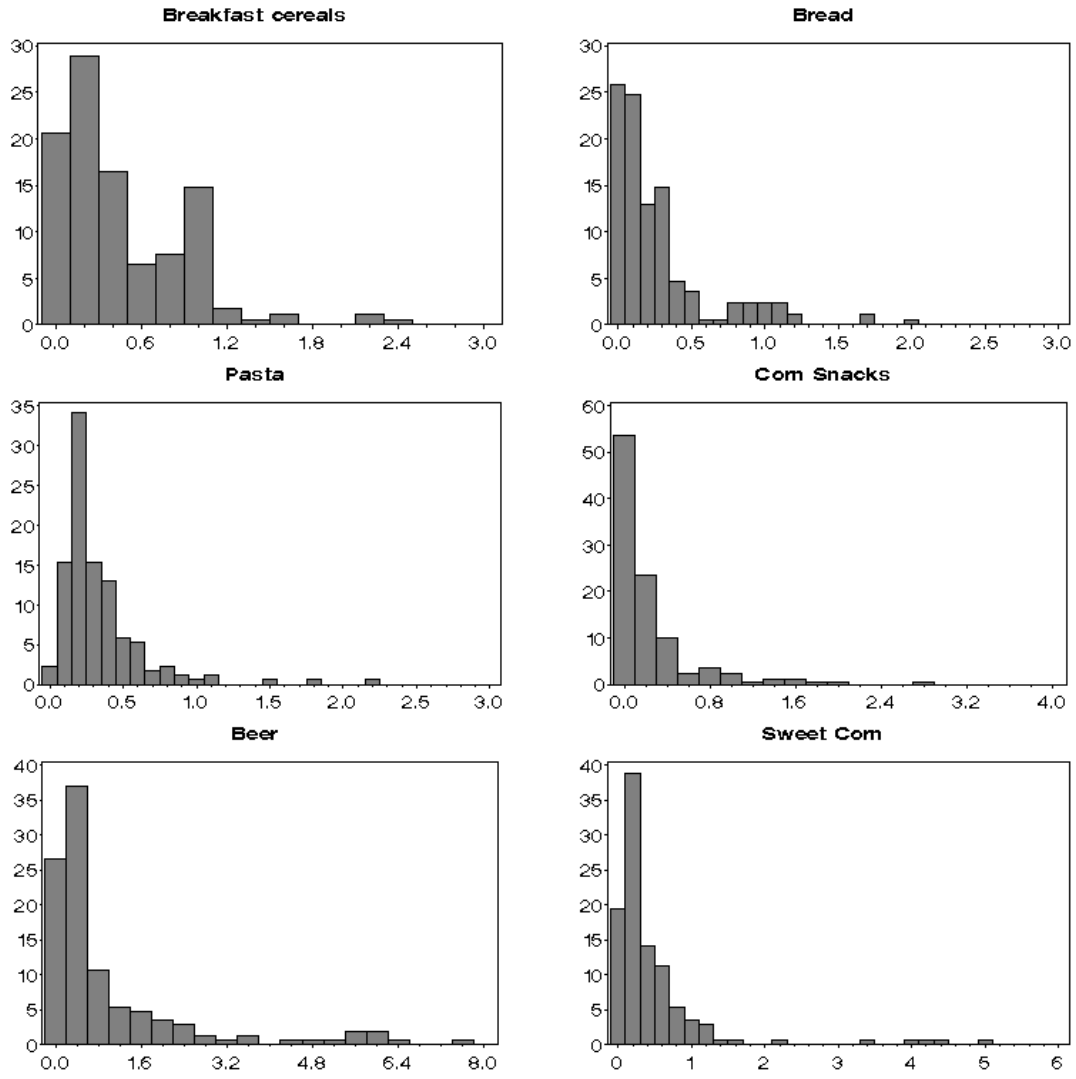

Fig 14.2. Consumption histograms (relative frequencies) for adult female consumers, in g/kg bw/day

Dependencies on consumption patterns can be quantified by Spearman correlation coefficients and can be taken into account by the Iman and Conover method (Iman and Conover, 1982). However, Gauchi and Leblanc (2002) did not reported significant differences in the results, if dependencies are taken into account or not, therefore we have not considered dependencies in the present work.

14.3.2 Exposure assessment methodology

The main limitation to estimate the normalized exposure distribution of consumer population is derived from the complicated equation described by:

$$E_{\pi} = \sum_{j=1}^{p} C_{\pi,j} T_j \qquad (1)$$

where the random variable "normalized global exposure in a consumer population π" (E_{π}) is function of the random variables: "normalized consumption of the foodstuff *j* in a consumer population π " ($C_{\pi,j}$) and "ZEA concentration level of the foodstuff *j*" (T_j). The difficulty is due to the probability density function (*pdf*) of these variables are commonly unknown and even if they are known they are generally different and not independent, and also the products *CT pdf* are unknown.

If we assume independency between consumptions ($C_{\pi,j}$) and contaminations (T_j), and also between their products, we could estimate the exposure mean of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j \qquad (2)$$

where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalized consumption of the foodstuff *j* in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of related food.

Under these assumptions we could also calculate the variance estimate $\hat{V}(E_{\pi_0})$ by eq (5) and eq(6) given in Gauchi and Leblanc (2002). Finally we could refer this approach to as a theoretical approach but because the postulated assumptions it should be a (very) simplified theoretical approach. For instance this simplified approach does not enables us to calculate complicated statistics such as high quantiles; then simulation methods will be required to obtain estimates of these statistics. Finally this approach will be referred to as a direct approach.

14.3.2.1 The *NP-NP* method

This method was proposed as a natural method to assess the exposure when raw data, either of consumption and contamination, is available, without made *pdf* hypothesis of respective distributions on these consumption and contamination data. Therefore, each normalized consumption profile of the survey is taken into account and each type of consumed food is attributed a value of contamination drawn from the available contamination data.

For an individual i belonging to the π_0 sample the estimated normalized exposure for *p* foodstuff consumed was estimated as follows:

$$\hat{E}_{i(\pi_0)}^{[NP-NP]} = \sum_{j=1}^{p} c_{i(\pi_0),j}\, \tilde{t}_{F_j^0} \qquad (3)$$

where, $c_{i(\pi_0),j}$ was the daily normalized consumption of the foodstuff *j* by this individual *i*, and $\tilde{t}_{F_j^0}$, a random deviate drawn from the observed cumulative frequency F_j^0 of ZEA contamination level of the respective foodstuff *j*.

For obtaining an exposure simulation histogram corresponding to this sample π_0, *N* simulation exposure outputs were generated with formula (3). From this histogram several statistics were calculated. Then, Lognormal *pdf* and Gamma *pdf* were estimated by fitting the *N* simulation outputs as explained by Gauchi and Leblanc (2002, subsection 3.2.2). We recall in Appendix the formulas for the Lognormal and Gamma distributions. Finally from these fitted *pdf's* several simulation statistics were calculated to compare with those statistics calculated directly from the *N* outputs.

14.3.2.2 The *P-P* method

The main advantage of the use of a parametric method is derived from the fact that NP-NP method could lead us to less reliable estimations, especially of the high

quantiles.

Taken into account the asymmetrical appearance of consumption and contamination histograms and our previous experience, we fitted the probability density functions choosing the distribution Gamma to be the best candidate.

Therefore, for each consumption and contamination dataset the *pdf* of Gamma was fitted through the method of maximum likelihood using CAPABILITY procedure of SAS software (SAS, 2010). The Chi-Square statistic was used in the goodness-of-fit test, considering a significance level greater than 99%, to accept the distribution as an suitable candidate. Moreover, there are several graphical methods as quantile-quantile plot, that permit us to check quickly if the fitted distribution is adequate to the selected hypothesis (Smout et al., 2000). Following the explained methodology, shape and scale parameters were estimated of each contamination sets.

Concerning consumption datasets, the foodstuffs selected are commonly consumed among Catalonian population despite of several exceptions properly of age dietary patterns. Thus, the histograms appearance is continuous and only one distribution was considered, fitting all data to Gamma *pdf*.

Thus, *P-P* method, to estimate the normalized exposure k of the *S* simulation set, was built as follows:

$$\hat{E}_k^{[P-P]} = \sum_{j=1}^{p} \tilde{c}_{i(j)} \tilde{t}_j \qquad (4)$$

where $\tilde{c}_{i(j)}$ is a random normalized consumption *i* for the foodstuff *j*, drawn from $\hat{C}_j$ the corresponding adjusted Gamma *pdf*; and $\tilde{t}_j$ is a random contamination for the foodstuff *j*, drawn from $\hat{T}_j$ the corresponding adjusted Gamma *pdf*. The density parameter estimates for $\hat{C}_j$ and $\hat{T}_j$ are shown in Table V.

Then, the mean of normalized exposures over the simulation set *S* was estimated using the follow equation, where n was the number of random deviates drawn (in the present study 10,000) :

$$\hat{E}_S^{[P-P]} = \frac{1}{n}\sum_{k=1}^{n} \hat{E}_k^{[P-P]} \quad (5)$$

As for the *NP-NP* method other statistics were computed directly on the histogram built with the simulations of the *S* set, and also statistics were estimated from the Lognormal and Gamma fitted *pdf*'s.

14.3.2.3 Quantiles calculations

These quantiles were calculated for both methods. Theoretical exposure quantiles were defined by $F_{E_\pi}(Q_\pi^\alpha) = \alpha$ where, F_{E_π} means the theoretical cumulative density function (*cdf*) and Q_π^α the α^{th} theoretical exposure quantile. While, the empirical exposure quantile ($\hat{Q}_S^\alpha$) were defined as follows:

$$\#\left(\hat{E}_k \le (\hat{Q}_S^\alpha)\right)/N = \alpha \quad (6)$$

where the notation # ($x_i \le$ K) is the number of xi less than or equal to *K*.

14.3.2.4 Building confidence intervals

Several methods to built the bootstrap (Efron and Tibshirani, 1993) confidence intervals (CI_b) were assessed by Gauchi and Leblanc (2002), exposing the difficulties to apply the nonparametric CI_b and highlighting the "pseudo-parametric CI_b" to be the best choice among the other parametric procedures and analytical methods proposed. Thus, in the present study we have built CI_b following this method.

"Pseudo-parametric CI_b" named as Type 1, was built drawing randomly *B* samples of size in the exposure simulation set S, typically B equals 10,000. The size of *S* is for example 235,000 for the adolescents' class with the *NP-NP* method and 100,000 for the classes with P-P method.

For each type, the bounds of 95%-confidence interval are calculated taking the 0.025th and 0.975th empirical quantiles of the final bootstrap distribution.

14.4 Results and Discussion

14.4.1 Direct Approach

The parameter estimates of this direct approach and for each population groups are shown in Table 14.3. They have to be compared with results of next Tables. Celiac sufferers were excluded of the analysis because not enough data was available for the computations. Most exposed collectives were infants, followed by children and immigrants.

Table14.3. Results of Exposure with the Direct Approach

	$\hat{E}_{\pi_0}$	$\hat{V}(E_{\pi_0})$
Elders	0.40	$(0.48)^2$
Adults females	0.99	$(1.06)^2$
Adults males	1.29	$(1.26)^2$
Adolescents	1.80	$(1.62)^2$
Children	3.45	$(2.57)^2$
Infants	15.05	$(14.52)^2$
Immigrants	2.33	$(2.37)^2$

(ng kg^{-1} bw day^{-1})

14.4.2 *NP-NP* method

The results of exposure of the different population groups calculated with *NP-NP* method are shown in Table 14.4. The estimated statistics presented were the mean, standard deviation, skewness and kurtosis, as well as, the median and the main high quantiles (90th, 95th and 99th). The highest value of skewness and kurtosis was found in the teenager class, while the most exposed group should be expected to be the infants, with the highest mean and quantiles, especially the related 99th quantile.

The second part of Table 14.6. shows the estimated statistics of exposure to ZEA by Lognormal and Gamma *pdf* fitted to the outputs of both simulations methods. An example of the exposure histograms is given in the Fig. 14.3 where are drawn the fitted

Gamma and Lognormal *pdf*s for adolescents. The Anderson-Darling and Chi-Square statistics showed that Lognormal and Gamma distribution can be accepted, however, unrealistic values were generated for children and infants through Lognormal. In the other hand, estimated statistics obtained from Gamma *pdf* fitted, were close to those means obtained directly from the empirical simulation, while the high quantiles were underestimated through Gamma hypothesis.

Table 14.4. Results of exposure assessment of the age groups from *P-P* and *NP-NP* simulation methods. These results were obtained directly from the N simulation outputs without *pdf* fittings; they must be compared to the results of Table 14.6. (in ng kg^{-1} bw day^{-1}).

***P-P* Method**

	Elders	*Adult females*	*Adult males*	*Immigrants*
N	10000	10000	10000	10000
Mean	0.63	2.40	1.82	14.64
SD	0.94	2.71	1.57	46.83
Skewness	4.88	5.12	2.91	8.31
Kurtosis	41.06	62.60	15.76	100.26
Median	0.33	1.61	1.39	1.84
0.90th quantile	1.05	5.12	3.57	33.68
0.95th quantile	2.20	6.99	4.66	72.58
0.99th quantile	4.48	13.29	7.74	216.16

***NP-NP* Method**

N	76000	384000	336000	56000
Mean	0.38	0.99	1.28	2.19
SD	1.03	2.28	2.67	3.94
Skewness	8.25	8.04	8.49	4.39
Kurtosis	94.11	97.53	135.68	27.44
Median	0.09	0.38	0.51	0.76
0.90th quantile	0.93	2.16	2.91	5.43
0.95th quantile	1.54	3.68	4.90	9.43
0.99th quantile	4.81	11.24	11.79	19.64

***P-P* Method**

	Adolescents	Children	Infants
N	10000	10000	10000
Mean	2.01	3.75	18.61
SD	1.78	3.99	33.11
Skewness	2.81	3.46	6.71
Kurtosis	14.00	20.07	92.88
Median	1.50	2.54	7.45
0.90^{th} quantile	4.08	7.94	49.22
0.95^{th} quantile	5.36	10.92	73.38
0.99^{th} quantile	8.74	19.97	145.05
***NP-NP* Method**			
N	235000	69000	164000
Mean	2.00	3.42	15.37
SD	6.77	7.00	32.84
Skewness	31.19	5.27	3.39
Kurtosis	1566.98	36.15	13.65
Median	0.66	1.33	4.19
0.90^{th} quantile	4.05	6.85	58.19
0.95^{th} quantile	7.90	13.16	91.35
0.99^{th} quantile	20.49	39.83	160.28

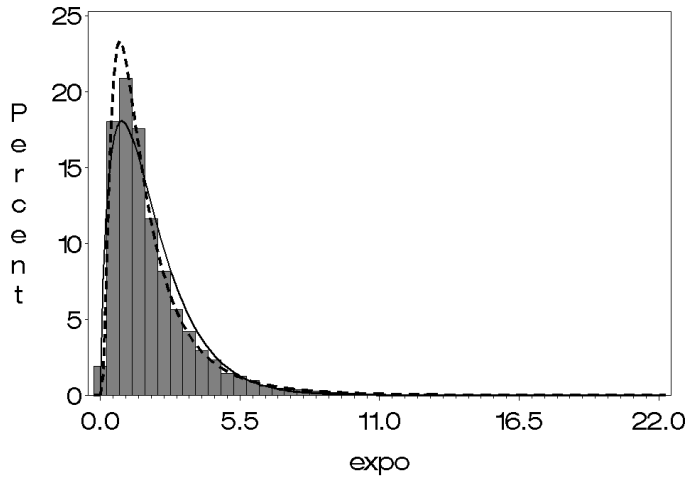

Fig. 14.3. Fitted Gamma (solid line) and Lognormal (broken line) *pdf*s for adolescents. Relative parameters are given in Table 14.6. Exposure in ng/kg bw/day

14.4.3 *P-P* method

The scale and shape parameters estimated from the Gamma pdf fitted to consumption and contamination datasets are shown in Table 14.5. In Table 14.4. are given statistics calculated from the simulation outputs. Related results obtained through *P-P* simulation method can be observed in Table 14.6. Exposure output histograms of relative frequencies are similar than those obtained through *NP-NP* method, an example obtained for infants is given in the Fig. 14.4.

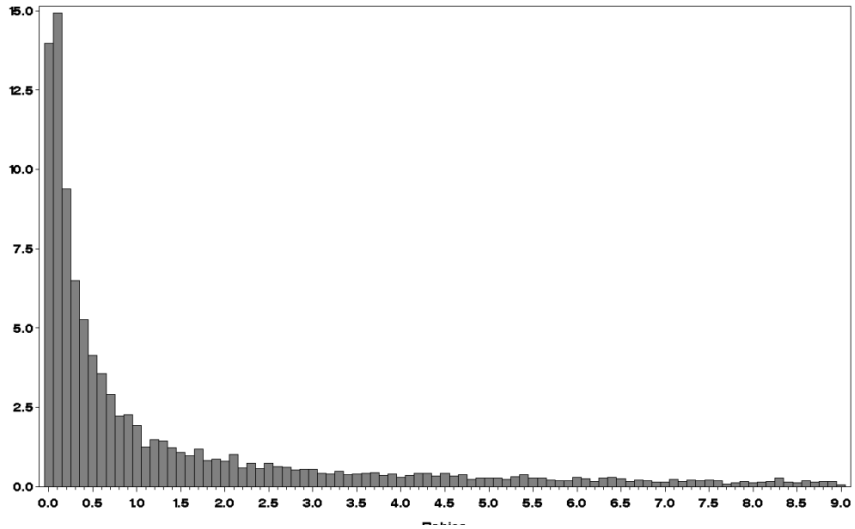

Fig. 14.4. Exposure output histograms (relative frequencies) with the *P-P* method, for infants (Exposure in ng/kg bw/day).

Table 14.5. Parameters of the Gamma *pdf* fitted to the Subclasses of normalized consumption

Foodstuff[a]	Elders ($\hat{r}, \hat{\lambda}$)	Adult females ($\hat{r}$, $\hat{\lambda}$)	Adult males ($\hat{r}$, $\hat{\lambda}$)	Adolescents ($\hat{r}$, $\hat{\lambda}$)
Breakfast cereals (0.753; 1.048)	not enough data	0.895 ; 0.542	1.229 ; 0.354	0.873 ; 0.891
Pan loaf (0.679; 2.592)	2.840 ; 0.0384	0.762 ; 0.294	0.611 ; 0.624	0.674 ; 0.785
Pasta (0.990; 0.691)	1.423 ; 0.1616	2.263 ; 0.151	2.149 ; 0.195	1.770 ; 0.292
Corn snacks (0.526; 3.102)	not enough data	0.635 ; 0.787	1.396 ; 0.044	0.790 ; 0.211
Beer (1.205; 0.205)	0.606 ; 3.039	0.735 ; 1.945	0.937 ; 2.471	0.759 ; 1.532
Sweet corn (0.715; 1.566)	not enough data	0.635 ; 0.787	0.777 ; 0.475	0.653 ; 0.543

Foodstuff[a]	Children ($\hat{r}$, $\hat{\lambda}$)	Infants ($\hat{r}, \hat{\lambda}$)	Ethnics ($\hat{r}, \hat{\lambda}$)	Celiacs ($\hat{r}, \hat{\lambda}$)
Breakfast cereals (0.753; 1.048)	1.106 ; 1.772	-	0.130; 1.739	0.193; 2.020
Pan loaf (0.679; 2.592)	0.853 ; 1.102	-	not enough data	not enough data
Pasta (0.990; 0.691)	2.256 ; 0.449	-	1.231; 0.242	0.786; 0.236
Corn snacks (0.526; 3.102)	1.074 ; 0.212	-	0.152; 0.716	0.258; 0.272
Beer (1.205; 0.205)	-	-	0.157; 14.485	0.133; 2.413
Sweet corn (0.715; 1.566)	0.484 ; 1.77	-	0.140; 0.461	0.167; 0.908
Baby food (0.756; 1.572)	-	1.474 ; 10.579	-	-
Ethnic foods (0.892; 0.535)	-	-	0.295; 1.297	-

[a] Values between parentheses are ($\hat{r}, \hat{\lambda}$) of the Gamma *pdf* fitted to the contamination data.

Those means values estimates through *P-P* method were commonly higher than those values estimates using *NP-NP* method, whereas several exceptions were found for the highest quantiles.

Another aspect observed was that the skewness and kurtosis were lower than those estimated through *NP-NP* method; however for infants the contrary case was found. If we compare the means from Lognormal and Gamma *pdf* fittings to the simulation outputs, we found that values are close for all age groups with the exception of infants, which highest value was estimated for Lognormal *pdf* fittings. In the other hand we found large differences for the high quantiles (95th and 99th quantiles), being most important in infants again.

In spite of the high quantiles have been estimated for both methods, we do not know still the validity and accuracy of these values, thereby confidence intervals are required. Thus, the next sections are focused to build the bootstrap confidence intervals to know the reliability of these simulation methods.

Table 14.6. Estimated statistics of exposure to ZEA by Lognormal and Gamma *pdf* fitted to the outputs of the *P-P* method and the *NP-NP* method. See the rigorous definitions of the shape and scale parameters in Appendix. (in ng kg^{-1} bw day^{-1})

***P-P* Method**	***Elders***	***Adult Females***	***Adult Males***	**Immigrants**
Lognormal	$\hat{r}$ *=1.201;* $\hat{\lambda}$ *= -1.126*	$\hat{r}$ *=0.912;* $\hat{\lambda}$ *=0.465*	$\hat{r}$ *=0.787;* $\hat{\lambda}$ *= 0.300*	$\hat{r}$ *=1.848;* $\hat{\lambda}$ *=0.8577*
Mean	0.67	2.41	1.84	12.99
SD	1.20	2.75	1.71	70.45
Median	0.32	1.59	1.35	2.358
0.90th quantile	1.51	5.13	3.70	25.17
0.95th quantile	2.34	7.14	4.93	49.25
0.99th quantile	5.30	13.30	8.43	173.47
AD (p-value)	2.83 (<0.005)	1.37 (<0.005)	4.36 (<0.005)	57.2433 (<0.005)
Gamma	$\hat{r}$ *=0.872;* $\hat{\lambda}$ *= 0.730*	$\hat{r}$ *=1.361;* $\hat{\lambda}$ *=1.764*	$\hat{r}$ *=1.836;* $\hat{\lambda}$ *=0.989*	$\hat{r}$ **=0.3657;** $\hat{\lambda}$ **=40.031**
Mean	0.64	2.40	1.82	14.64
SD	0.68	2.06	1.34	24.21
Median	0.42	1.85	1.50	4.76
0.90th quantile	1.52	5.12	3.60	41.98
0.95th quantile	2.00	6.46	4.43	62.72
0.99th quantile	3.14	9.50	6.26	115.38
Chi-Sq (p-value)	19607.73 (<0.001)	7858.80 (<0.001)	19183.68 (<0.001)	89927.64 (<0.005)

***NP-NP* Method**	***Elders***	***Adult Females***	***Adult Males***	**Immigrants**
Lognormal	$\hat{r}$ ***=2.316;*** $\hat{\lambda}$ ***=-2.469***	$\hat{r}$ ***=1.389;*** $\hat{\lambda}$ ***=-0.961***	$\hat{r}$ ***=1.244;*** $\hat{\lambda}$ ***=-0.597***	$\hat{r}$**=1.391;** $\hat{\lambda}$**=-0.181**
Mean	1.24	1.00	1.19	2.20
SD	18.06	2.43	2.30	5.35
Median	0.09	0.38	0.55	0.84
0.90th quantile	1.65	2.27	2.71	4.96
0.95th quantile	3.82	3.76	4.26	8.23
0.99th quantile	18.53	9.68	9.95	21.23
AD (p-value)	3633.95(<0.005)	945.13(<0.001)	5226.36(<0.005)	227.69 (<0.005)
Gamma	$\hat{r}$ ***=0.433;*** $\hat{\lambda}$ ***=0.876***	$\hat{r}$ ***=0.645;*** $\hat{\lambda}$ ***=1.531***	$\hat{r}$ ***=0.716;*** $\hat{\lambda}$ ***=1.782***	$\hat{r}$**=0.635;** $\hat{\lambda}$**=3.447**
Mean	0.38	0.99	1.28	2.19
SD	0.58	1.23	1.51	2.75
Median	0.15	0.55	0.75	1.20
0.90th quantile	1.06	2.53	3.19	5.62
0.95th quantile	1.53	3.46	4.31	7.72
0.99th quantile	2.72	5.71	6.98	12.77
AD (p-value)	1008316.74(<0.001)	2884359.14(<0.001)	2287483.46(<0.001)	37153.11 (<0.005)

***P-P* Method**	***Adolescents***	***Children***	***Infants***
Lognormal	$\hat{r}$**=0.809;** $\hat{\lambda}$**=0.383**	$\hat{r}$**=0.917;** $\hat{\lambda}$**= 0.977**	$\hat{r}$**=1.887;** $\hat{\lambda}$**=1.741**
Mean	2.03	3.81	33.85
SD	1.96	4.37	197.96
Median	1.47	2.50	5.71
0.90th quantile	4.14	8.09	64.06
0.95th quantile	5.55	11.29	127.15
0.99th quantile	9.63	21.09	460.09
AD (p-value)	3.06 (<0.005)	1.64 (<0.005)	88.35(<0.005)
Gamma	$\hat{r}$**=1.748;** $\hat{\lambda}$**=1.151**	$\hat{r}$**=1.375;** $\hat{\lambda}$**= 2.727**	$\hat{r}$**=0.532;** $\hat{\lambda}$**= 34.966**
Mean	2.01	3.75	18.61
SD	1.52	3.20	25.51
Median	1.64	2.89	8.93
0.90th quantile	4.03	7.99	49.69
0.95th quantile	4.97	10.06	69.91
0.99th quantile	7.08	14.78	119.34
Chi-Sq (p-value)	12081.80 (<0.001)	12113.72 (<0.001)	25809.03 (<0.001)

***NP-NP* Method**			
Lognormal	$\hat{r}$**=1.314;** $\hat{\lambda}$**=-0.306**	$\hat{r}$**=2.353;** $\hat{\lambda}$**=0.090**	$\hat{r}$**=5.386 ;** $\hat{\lambda}$**=-0.773**
Mean	1.75	17.41	920850.90
SD	3.76	276.63	$1.84^{E}12$
Median	0.74	1.10	0.12
0.90^{th} quantile	3.97	22.31	459.30
0.95^{th} quantile	6.40	52.45	3251.00
0.99^{th} quantile	15.66	260.63	127700.00
AD (p-value)	554.46 (<0.005)	5635.94 (<0.005)	21225.34(<0.005)
Gamma	$\hat{r}$**=0.616;** $\hat{\lambda}$**=3.252**	$\hat{r}$**=0.549;** $\hat{\lambda}$**=6.234**	$\hat{r}$**=0.209;** $\hat{\lambda}$**=73.711**
Mean	2.00	3.42	15.37
SD	2.55	4.62	33.66
Median	1.07	1.69	1.78
0.90^{th} quantile	2.18	9.08	46.49
0.95^{th} quantile	7.14	12.72	78.34
0.99^{th} quantile	11.87	21.58	165.43
AD (p-value)	488722.67 (<0.001)	126206.05 (<0.001)	57974.85 (<0.001)

14.4.4 Confidence intervals

Confidence intervals built using the bootstrap pseudo-parametric method for the outputs of *P-P* and *NP-NP* methods are given in Table 14.7 and 14.8, respectively. These values suggest us that, in general cases, *NP-NP* method provides us worse estimates than those values obtained through *P-P* method. This fact becomes more evident when we check if the estimates from the Table 14.4 and 14.6 are contained in the range described by the related confidence interval. Therefore, we assert this hypothesis for those statistics estimated from Lognormal *pdf* fitted to the outputs of the *NP-NP* method of elders, children and infants.

Another point to highlight in this section is the progressive reduction of the accuracy when we estimate the CI of high quantiles, excessive in case of the 95th and 99th-quantiles. Especially for the 99th-quantile it is well-known that it is very difficult to obtain a good accuracy (Breiman et al., 1990; Beirlant and Devroye, 1999; Albert and Gauchi, 2002 ; Beirlant et al., 2006).

For this reason, sensitivity analysis of the fitted *pdf* parameters is in progress to assess the stability of the high quantiles as advised in previous several studies.

Table 14.7. Bootstrap confidence intervals obtained from the results of NP-NP method. (in ng kg^{-1} bw day^{-1})

NP-NP Method 95% Bootstrap Confidence Interval	***Elders***	***Adult Females***	***Adult Males***	***Immigrants***
Mean	[0.19; 0.55]	[0.73; 1.07]	[0.98; 1.42]	[1.32; 3.46]
SD	[0.27; 1.63]	[1.19; 2.33]	[1.52; 2.92]	[1.74; 7.03]
Median	[0.06; 0.14]	[0.30; 0.42]	[0.43; 0.56]	[0.44; 1.44]
0.90^{th} quantile	[0.47; 1.19]	[1.485; 2.56]	[2.15; 3.64]	[2.92; 10.98]
0.95^{th} quantile	[0.72; 2.38]	[2.55; 5.22]	[3.58; 6.54]	[4.60; 20.05]
0.99^{th} quantile	[1.20; 11.98]	[5.69; 12.17]	[7.31; 15.67]	[8.093;44.652]

	Adolescents	***Children***	***Infants***
Mean	[1.42; 2.48]	[1.92; 5.36]	[10.97; 20.40]
SD	[2.45; 6.42]	[2.14; 12.80]	[22.76; 38.20]
Median	[0.55; 0.76]	[1.06; 1.73]	[3.02; 5.80]
0.90^{th} quantile	[2.77; 6.16]	[3.76; 13.22]	[21.58; 87.95]
0.95^{th} quantile	[4.84; 13.87]	[5.18; 34.68]	[66.16; 109.88]
0.99^{th} quantile	[12.74; 30.56]	[10.56; 83.85]	[100.51; 197.76]

Table 14.8. Bootstrap confidence intervals obtained from the results of P-P method. (in ng kg^{-1} bw day^{-1})

P-P Method 95% Bootstrap Confidence Interval	***Elders***	***Adult Females***	***Adult Males***	***Immigrants***
Mean	[0.45; 0.88]	[2.14; 2.69]	[1.65; 1.99]	[5.8; 29.80]
SD	[0.48; 1.70]	[2.02; 4.22]	[1.26; 1.94]	[11.30; 108.63]
Median	[0.23; 0.46]	[1.44; 1.79]	[1.24; 1.53]	[1.06; 3.189]
0.90^{th} quantile	[0.94; 2.23]	[4.35 ; 5.91]	[3.16; 4.07]	[12.19; 86.01]
0.95^{th} quantile	[1.36; 3.78]	[5.86; 8.63]	[3.99; 5.55]	[25.49; 192.86]
0.99^{th} quantile	[2.19; 13.25]	[9.61; 19.70]	[5.88; 10.30]	[56.11; 765.79]

	Adolescents	***Children***	***Infants***
Mean	[1.79; 2.24]	[2.88; 4.82]	[13.72; 24.96]
SD	[1.39; 2.27]	[2.31; 6.47]	[19.09; 68.89]
Median	[1.33; 1.68]	[1.93; 3.33]	[5.08; 10.53]
0.90^{th} quantile	[3.45; 4.75]	[5.59; 11.44]	[32.95; 68.34]
0.95^{th} quantile	[4.44 ; 6.52]	[6.96; 16.66]	[51.07; 109.36]
0.99^{th} quantile	[6.26; 12.80]	[10.46; 47.71]	[80.31; 258.17]

14.5 Discussion

In this work, we have applied the stochastic methodology proposed by Gauchi and Leblanc (2002) to assess the exposure of Catalonian population to zearalenone. Thus, to reach this objective, the next steps were followed:

- In the first place, we calculated the mean and variance through the direct approach, using the equations 2 and 6 from Gauchi and Leblanc (2002).
- More complicated statistics were calculated from the outputs of the *NP-NP* and P-P methods, in order to validate them each other, if the results are close.
- Pseudo-parametric bootstrap confidence intervals for the parameters obtained in the previous step.

Finally, we have obtained close results in each category with the exception of statistics of children and infants estimated by lognormal *pdf* fitted to the outputs of *NP-*

NP method. Confidence intervals suggest us that, in general cases, *NP-NP* method provides us worse estimates than those values obtained through *P-P* method.

Although probabilistic methods have been proposed to be the most suitable tool in exposure assessment to food contaminants, it has not been widely applied in toxicological studies. Concerning ZEA dietary intake estimation, previous works published were conducted using essentially a deterministic method, thus, reliability and accuracy of the results was not provided. In the present work, we presented two probabilistic approaches and a direct estimation through deterministic method.

Considering our results, either from *NP-NP* and *P-P* method, the most exposed group should be expected to be the infants, especially for the high quantiles. Means estimated from NP-NP and P-P method were 15.37 and 18.61 ng kg^{-1} of body weight per day, respectively, while, the mean estimated through the Direct Approach was 15.05 ng kg^{-1} of body weight per day. This last value in the line of that estimated from *NP-NP* method and slightly lower than P-P method. Confidence intervals of high quantiles were extremately wide, however, in all cases the estimates were far from the TDI of 250 ng kg^{-1} body weight, established by the EFSA (EFSA, 2011).

Despite our approach was based on a stochastic method, these results were compared with the study SCOOP (2003) and The First French Total Diet Study (Leblanc et al., 2005). Estimates of ZEA intake by French population were significantly higher than our estimates. The authors reported an average intake of 33 ng kg^{-1} bw day^{-1} in adults and 66 ng kg^{-1} bw day^{-1} in children, being 70 and 132 ng kg^{-1} bw day^{-1} respectively, for percentile 95. Lowest values were estimated in other European countries, for example adults from Italy and Portugal, showed estimates of 0.8 and 4.05 ng kg^{-1} bw day^{-1} respectively; moreover children and infants from Austria, Finland, Germany, Norway, Austria or UK with range between 8 to 31.6 ng kg^{-1} bw day^{-1} (SCOOP, 2003).

14.6 Acknowledgements

The authors would like to acknowledge Exposure Assessment of Spanish Population to *Fusarium* Toxins Project, National Plan of Spanish Government (AGL2008-05030-C02-01), Catalonian Food Safety Agency of 'Generalitat de Catalunya' Health Department and University of Lleida for their financial support, and to Mathematics and Informatics Applied Group, INRA (Jouy-en-Josas center), for their technical support.

14.7 Appendix

The Lognormal *pdf* for the continuous random variable *Y* defined in [0,+∞[is:

$$g(y) = \frac{1}{\sqrt{2\pi}\, y \sigma_X} \exp\left(-\frac{1}{2}\left(\frac{Log(y) - m_X}{\sigma_x} \right)^2 \right)$$

where m_X and σ_X are λ and *r*, the scale and the shape parameters, respectively. Then *X* follows a Normal distribution with mean m_X and variance σ_X^2. The mean and the variance of *Y* are defined by $E(Y) = \exp\left(m_X + \sigma_X^2/2\right)$ and $V(Y) = \left[\exp\left(2m_X + \sigma_X^2\right)\right]\left[\exp\left(\sigma_X^2\right) - 1\right]$, respectively.

The Gamma *pdf* for a continuous random variable *X* defined in [0, +∞[is:

$$f(x) = \frac{1}{\lambda \Gamma(r)} \left(\frac{x - \theta}{\lambda} \right)^{r-1} \exp\left(-\frac{x - \theta}{\lambda} \right)$$

where r, λ, θ are the shape, scale, and the threshold parameters, respectively, and Γ (*r*) is the usual Euler's integral. The mean and variance of the Gamma distribution are connected to *r* and λ in the following way: $E(X) = r\lambda$ and $V(X) = r\lambda^2$.

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Chapter 15. Presence and co-occurrence of aflatoxins, deoxynivalenol, fumonisins and zearalenone in gluten-free and ethnic foods

Food Control 2012 26, 282-286

15.1 Abstract

The mycotoxins aflatoxins (AFs), deoxynivalenol (DON), fumonisins (FBs) and zearalenone (ZEA) are produced by fungal species can contaminate cereal-based food and therefore can be found alone or simultaneously in cereal-based commodities. The objectives of this study were, to study the occurrence of AFs, DON, FBs and ZEA in ethnic and gluten-free food samples from Lleida (Spain), and also to assess the exposure of immigrant and celiac population to the selected mycotoxins. Ethnic food and gluten-free samples were purchased in specialized grocery stores from Lleida (Spain). Mycotoxins were extracted and purified from the samples using immunoaffinity chromatography (IAC) columns. Determination and quantification of AFs, ZEA and FBs was performed with high performance liquid chromatography (HPLC) with fluorescence detector, and coupled to absorbance detector in case of DON. The results showed that FBs were widely found in corn-based foods purchased from special shops specialized on imported foods. DON was mainly found in cuscus and several corn-based ethnic foods. Several gluten-free food samples were contaminated by FBs (6 out of 18) and DON (2 out 18), while AFs and ZEA only were found in 6 ethnic foods samples. Considering these results, these special commodities should not be a health concern for these collectives; however, special attention should be focused on bulk imported foods distributed in special retail shops.

15.2 Introduction

Mycotoxins are produced by fungi and can contaminate various agricultural commodities either before harvest or under post-harvest conditions (FAO, 1991). One of the most important mycotoxins produced by the genera *Aspergillus* are the aflatoxins (AFs), which can occur in a wide range of important raw food commodities including cereals, nuts, spices, figs and dried fruits (Malone et al., 2000; Otta et al., 2000). Aflatoxin B_1 (AFB_1) is the most carcinogenic mycotoxin known and there is evidence from human studies that aflatoxins are major risk factors for hepatocellular carcinoma, therefore classified in the group 1 by International Agency for Research on Cancer (IARC, 2002).

The most reported *Fusarium* toxins in foods are the trichothecenes, zearalenone (ZEA) and fumonisins (FBs), that can be found alone or simultaneously, as well as cooccurring with other mycotoxins such as AFs, in cereals and thus in cereal-based foods (Jestoi, 2008). Trichothecenes are a family of related cyclic sesquiterpenoids, which are divided into four groups (types A–D) according to their characteristic functional groups. The type-B trichothecenes are most frequently represented by deoxynivalenol (DON) (Scott, 1997), the most frequent trichothecene found in the Catalonian (Spain) market (Cano-Sancho et al. 2011b). Fumonisin B_1 (FB_1) and B_2 (FB_2) are mycotoxins produced by *F. verticillioides* and *F. proliferatum* that can commonly contaminate corn (Nelson et al., 1992). FBs occur mainly in maize and maize-based foods, therefore populations with high maize consumption can be exposed to significant amounts of these mycotoxins through the ingestion of fumonisin contaminated maize (Marasas, 1996; Shephard et al., 1996; Visconti et al., 1996; WHO, 2001). ZEA is a non-steroidal estrogenic mycotoxin produced by *Fusarium graminearum* and other *Fusarium* species, which are plant pathogenic fungi that infect a wide variety of cereals, including corn and wheat, in temperate and warm regions around the world (Jelinek et al., 1989; Jiménez et al., 1996; Kuiper-Goodman et al., 1987; Patel et al., 1996).

Maximum levels of mycotoxins in raw materials and processed food for human consumption were set in the European Regulation 1881/2006 of 19 December 2006,

and subsequently extended in the Commission Regulation 1126/2007 of 28 September 2007 where finally the maximum levels of the *Fusarium* toxins DON, ZEA and FBs were set in maize and maize products (European Commission, 2006b, 2007). Based on toxicological studies, the European Food Safety Agency derived safety dietary exposure levels of 2000 ng/kg bw/day for FBs (SCF, 2003), 200 ng/kg bw/day for ZEA (EFSA, 2004) and 1000 ng/kg bw/day for DON (SCF, 2002). Concerning risk assessment of AFs, despite international expert committees did not specify a numerical threshold for AFs whose carcinogenicity is the basis for concern, it was established that a level of AFs below 1 ng/ kg bw/day did not contribute to the risk of liver cancer.

The presence of these mycotoxins was thoroughly assessed in foodstuffs from Catalonian (Spain) market, by means of a wide survey of samples purchased around the region. The sample categories were selected to be the main foodstuffs susceptible to mycotoxins contamination and also commonly consumed by Catalonian population. However, that selection did not consider several food categories typically consumed by special population groups with dietary habits markedly different from the general population. Among these population groups we can highlight, in one hand, the celiac sufferers, who substitute wheat-based foods by other gluten-free foods, and in the other hand, some immigrant collectives that partially maintain the dietary patterns from their country of origin. In Catalonia, during 2009, the immigrant population represented 15.9 % of the total population (Migracat, 2010); therefore, their dietary specificities should be taken into account to assess the exposure of this collective.

The main problem to assess mycotoxins intake is related to few representative available dietary data regarding food bearing mycotoxins contamination. There are some methods developed to assess dietary intake overall known as market basket, 24-hour dietary recall and food record methods, food-frequency methods or dietary history. Food-frequency methods are largely used in food chemical exposure assessment studies because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter, 1998).

In the framework of the Project to Assess the Exposure of Catalonian Population to the Mycotoxins, and following the line of our previous studies (Cano-Sancho et al., 2009, 2010, 2011a,b), the objectives of this study were, to study the occurrence of AFs, DON, FBs and ZEA in ethnic and gluten-free food samples from Lleida (Spain), and also to assess the exposure of immigrant and celiac population to the selected mycotoxins.

15.3 Materials and methods

15.3.1 Samples

Ethnic food samples were purchased in grocery stores specialised in imported (ethnic and regional) food products, and hypermarkets or supermarkets with an international-food section. The ethnic foods samples purchased were cuscus, corn flour, wheat flour, corn cream and corn grits. Gluten-free food samples were purchased from health food stores and hypermarkets or supermarkets with a health food section, being mainly bread, pasta and pastries without gluten intended for celiac sufferers. Finally, 35 ethnic food and 18 gluten-free food samples were collected in Lleida (Spain) during 2009. The samples were ground and freeze stored (-20ºC) until analysis.

15.3.2 Chemical and reagents

AFB_1, AFB_2, AFG_1 and AFG_2, DON, FB_1 and FB_2, and ZEA standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile and methanol, reagent and HPLC grade, were purchased from Merck (Darmstadt, Germany). Filter papers (Whatman Nº 4) and glass microfiber filters (Whatman GF/A) were purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography (IAC) columns to clean-up aflatoxins (Easi-extract ® Aflatoxin), deoxynivalenol (DONPREP®), fumonisins (Fumoniprep®) and zearalenone (Easi-extract Zearalenone®) were purchased from R-Biopharm (Rhône LTD Glasgow, UK). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer was prepared with NaCl

(0.8 %), Na_2HPO_4 (0.12%), KH_2PO_4 (0.02%) and KCl (0.02%), from Panreac (Panreac Química SA, Castellar del Valles, Spain) brought to pH 7.4. *o*-phthaldialdehyde (OPA) solution was prepared diluting 40 mg of *o*-phthaldialdehyde (Merck) with 1 mL of methanol HPLC grade and mixed with 5 mL of $Na_2B_4O_7 \cdot 10H_2O$ (0.1M, Panreac) and 50 μL de 2-mercaptoethanol (Merck).

15.3.3 Analytical methods

15.3.3.1 Aflatoxins

Five g of ground sample was mixed with 15 mL of extractant solution (60% acetonitrile, 40% water) for 10 minutes and filtered. 2 mL of filtered solution was diluted with 48 mL of phosphate buffer solution and drained through the IAC column. After this, the columns were washed with 20 mL of PBS and AFs were eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water. The chromatography equipment used was a separations Module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5μm ODS2, 4.6 x 250 mm, Multi λ Fluorescence Detector Waters 2475®. Excitation wavelength was 365 nm, and emission wavelength at 0-13 min and 13-25 min were 455 and 425 nm, respectively. Derivatization of aflatoxins was obtained using a post-column photochemical derivatization device (UVE™ Derivatizer LC Tech). Mobile phase consisted of a solution of water, methanol and acetonitrile (70:17:17).

15.3.3.2 Deoxynivalenol

Five mL of ground sample was mixed with 40 mL of distilled water for 10 minutes and filtered with glass microfiber filter. 4 mL of filtered sample were drained through the IAC column and washed with 10 mL of distilled water. DON was eluted with 3 mL of methanol grade HPLC. The purified samples were dried under nitrogen stream. Each dried sample was resuspended with the mobile phase solution based on milli-Q water, acetonitrile and methanol at proportion 920:40:40 (all reagents were

HPLC grade). DON was determined in purified samples by HPLC coupled with a UV/Visible Dual λ absorbance Detector Waters 2487.

15.3.3.3 Fumonisins

Ten g of ground sample were mixed with 1 g NaCl, and 50 mL of extract solution (50% water, 25% methanol, 25% acetonitrile) for 20 minutes and filtered. 10 mL of filtrate was diluted with 40 mL of PBS and drained through the IAC column. The column was washed with 20 mL of PBS solution and FBs were eluted with 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water. Fluorescent derivatives of FB_1 and FB_2 were obtained using pre-column derivatization with *o*-phthaldialdehyde (OPA) solution. Derivatization was conducted mixing 200 μL of eluate with 200 μL of OPA solution for 30 seconds in vortex. Finally, FBs were determined using HPLC with fluorescence detector. Mobile phase was based on a methanol and 0.1M sodium dihydrogen phosphate (77:23, v/v) solution. Excitation and emission wavelength were 335 nm and 440 nm, respectively.

15.3.3.4 Zearaleone

Five g of ground sample was mixed with 25 mL of extractant solution (75% acetonitrile, 25% water) for 10 minutes and filtered. 10 mL of filtered solution was diluted with 40 mL of PBS and drained through the IAC column. After this, the columns were washed with 20 mL of PBS and ZEA eluted with 1.5 mL of acetonitrile grade HPLC and 1.5 mL of milli-Q water. Finally, ZEA was determined, using HPLC with the fluorescence detector and mobile phase of acetonitrile-water (60:40), adjusted at pH 3.2 with acetic acid. Excitation and emission wavelengths were 274 nm and 455 nm, respectively.

15.3.4 Validation of analytical methods

The analytical methods used were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 100 μL of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations.

Table 15.1. Method performance characteristics for AFs, DON, FBs and ZEA

		LoD/LoQ μg/kg	n	Spiking level μg/kg	Recovery (%)	RSDr (%)
Aflatoxins						
	B_1	0.033/0.100	3-7	0.4-4.0	86±29-119±11	5-33
	B_2	0.008/0.025	3-7	0.1-0.7	70±13-120±6	5-28
	G_1	0.033/0.100	4-7	0.4-4.0	80±7-120±7	2-25
	G_2	0.008/0.025	3-6	0.2-1.0	72±17-100±6	4-25
DON		10.0/30.0	3	30-500	87±7-123±44	7-20
Fumonisins						
	B_1	5.2/15.6	3-7	266-660	77±12-116±28	5-24
	B_2	5.2/15.6	3-7	133-340	91±15-110±12	7-17
ZEA		0.65/1.95	3	6-20	85±5-116±5	2-27

[a] LoD = Limit of detection / LoQ = Limit of quantification
[b] Mean ± standard deviation
[c] RSDr = relative standard deviation

Precision and recovery were established by determination of AFB_1, AFB_2, AFG_1 and AFG_2, DON, FB_1 and FB_2, and ZEA levels, spiked in the food samples by triplicate at least. Recovery was determined by comparing the absolute responses of the mycotoxins, with the absolute responses of calibration standards. Cereal-based food matrices such as pasta, bread, flour and breakfast cereals were considered in the validation study. Samples from each category were purchased from Catalonian market, ground and analyzed following the procedure described above. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was considered equal to 3×LOD. Method performance characteristics for AFs, DON, FBs and ZEA are summarized in Table 15.1. These

values are in accordance to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission, 2006a).

15.3.5 Dietary intake estimation

In the present study, food dietary intake was assessed through a specific Food Frequency Questionnaire (FFQ) developed for celiac-sufferer and ethnic population, including those foods typically consumed in the region which may be potentially contaminated with these mycotoxins. Concerning frequency of consumption, 5 response options were considered: never, daily, weekly, monthly and annually. Quantities were assessed by portion size with the aid of a series of colour photograph models. Finally, 70 adult celiac sufferers and 56 adults with ethnic dietary pattern were interviewed in 2009 by trained and native speaker interviewers.

Advices from the Report on a Workshop in the frame of GEMS/Food–EURO on Reliable Evaluation of Low-Level Contamination of Food (GEMs/Food-WHO, 1995) were given to handle left-censored data. These advices induced to produce two estimates using 0 and LOD for all results less than 1.6xLOD to obtain a simple estimate of mean, where there are more than 60 % of non quantified results.

We assumed independency between consumption ($C_{\pi,j}$) and contamination (T_j), as well as between their products, therefore, we estimated the mean exposure of the population π with the population sample π_0 as follows:

$$\hat{E}_{\pi_0} = \sum_{j=1}^{p} \overline{C}_{\pi_0,j} \overline{T}_j$$

where $\overline{C}_{\pi_0,j}$ is the arithmetical mean of the normalised consumption of the foodstuff j in the population group π_0, and $\overline{T}_j$ is the arithmetical mean of the available contamination data of the related food.

The exposure was calculated for each mycotoxin group considering AF_T to be the sum of AFB_1, AFB_2, AFG_1 and AFG_2, and FB_T the sum of FB_1 and FB_2. Moreover we estimated the global exposure considering complementary sources of exposure from those foods also commonly consumed and susceptible to be contaminated by mycotoxins. The food categories considered were corn flakes, bread, sliced bread, pasta, corn snacks, sweet corn, beer or pistachios and peanuts in case of AFs. The contamination data of these categories was provided by UdL-ACSA (2010), which widely surveyed these products with the exception of DON, thoroughly reported in Cano-Sancho et al. (2011b). Considering the substitution of the censored data, we summarize the exposure results as a range, with a lower bound (ND=0 means that all contamination results less than 1.6xLOD were substituted by 0) and upper bound (ND=LOD means that all contamination results less than 1.6xLOD were substituted by LoD).

15.4 Results

The analysed gluten-free samples were mainly composed with corn or rice flour in order to avoid gluten in their composition. A wide variety of products were purchased from health food stores because these establishments are the most important distribution channel. The analysed categories were pasta (spaghetti, macaroni, etc.), bread and pastries (croissant, cake, etc.). AFs and ZEA were not detected in any gluten-free sample. DON was found in two samples of pasta and bread with respective levels of 163 and 270 μg/kg. Fumonisins were detected in 6 out of the 18 samples analysed, FB_1 being the most present fumonisin with a range of concentration between 5.7 and 18.9 μg/kg (see Table 15.2).

Table 15.2. Summary of presence and co-presence deoxynivalenol and fumonisins in gluten-free food positive samples (μg/kg)

Product Category	DON	FB_1	FB_2
Gluten-free Pasta	163	-	4.43
	-	6.16	-
	-	18.9	3.57
	-	15.3	-
	-	8.38	-
Gluten-free Bread	270	5.70	-

FB_1 and FB_2 were the most common mycotoxins found in the ethnic food category, with a percentage of contamination of 51 and 40 %, respectively, mainly detected in corn-based food samples as corn flour or corn grits intended for human consumption. Concentration of FB_1 ranged from 16.1 to 547 μg/kg, while the range of FB_2 was set between 25.7 and 135 μg/kg. DON was detected in 15 out of 35 samples, but in this case, the wheat-based foods were the most contaminated samples by this type-B trichothecene. The range of concentration was between 125 and 657 μg/kg. The presence of ZEA and AFs was so low, detected only in three samples, in the first case (from 2.4 to 12.4 μg/kg), and three in the second (from 3.9 to 14.3 μg/kg).

FB_1 and FB_2 were found simultaneously in most of the samples. Other combinations were found in the present study as FBs-DON (4 samples), FBs-AFs (2 samples), FBs-DON-ZEA (1) and FBS-AFs-DON (1).

Table 15.3. Summary of presence and co-presence of AFs, DON, FBs and ZEA in ethnic food positive samples (μg/kg)

Product Category	DON	FB_1	FB_2
Corn-based food			
Corn flour	189	18.0	-
Corn flour	-	21.7	-
Corn flour	151	26.3	25.7
Corn flour	169	189	53.0
Corn flour	-	59.8	35.0
Corn flour	-	160	62.3
White corn flour	-	130	41.9
Spiced corn flour	-	255	30.8
Flaked corn flour	-	105	54.7
Pre-cooked yellow corn flour	-	297	48.5
Pre-cooked white corn flour	-	55.9	31.6
Corn grits	162	-	-
White corn grits	-	511	109
Fine corn grits	-	155	52.2
Great corn grits	510	98.3	34.6
Corn cream	-	113	53.3
Corn cream	-	16.2	-
Wheat-based food			
Cuscus	430	-	-
Cuscus	-	16.1	-
Cuscus	432	-	-
Cuscus	657	-	-
Cuscus	153	-	-
Cuscus	524	-	-
Cuscus	214	-	-
Cuscus with dried fruits	-	547	135
Wheat flour	443	-	-
Wheat grits	459	-	-
Pre-boiled wheat grits	125	-	-
Hard-wheat grits	581	-	-

We also estimated the dietary intake of each mycotoxin through the ethnic food samples using a simplified method, also known as a point estimate or deterministic (see Table 15.4). In case of celiac sufferers, we do not report the relative results because there was a wide range of non-quantifiable samples, and therefore the exposure assessment became widely inaccurate.

Table 15.4. Exposure assessment of immigrants to deoxynivalenol and fumonisins through ethnic food samples (mean ± standard deviation, units in ng/kg bw/ day)

Exposure assessment from special foods

	Cuscus (ng/kg bw/ day)				***Corn flour (ng/kg bw/ day)***			
	AFs	**FBs**	**DON**	**ZEA**	**AFs**	**FBs**	**DON**	**ZEA**
ND=0	0.00±0.00	2.84±1.90	9.79±2.28	0.01±0.46	0.31±81.3	53.9±36.1	16.1±3.74	0.14±7.14
ND=LOD	0.00±0.64	3.24±2.16	9.99±2.31	0.04±1.66	0.35±49.6	54.1±36.1	18.6±4.31	0.31±14.6

Global exposure assessment

	Total intake (ng/kg bw/ day)				**Percentage of TDI[1] (%)**			
	AFs	**FBs**	**DON**	**ZEA**	**AFs**	**FBs**	**DON**	**ZEA**
ND=0	0.38±0.55	149±163	429±387	1.95±2.03	38±55	7±8	43±39	1±1
ND=LOD	0.70±0.78	150±162	432±386	2.15±2.11	70±78	7±8	43±39	1±1

[1] Safety levels: 1 ng/kg bw/ day (AFs), 2000 ng/kg bw/ day (FBs), 1000 ng/kg bw/ day (DON) and 200 ng/kg bw/ day (ZEA). % of TDI was computed individually and summarized as mean ± standard deviation.

These results showed a wide variability depending on the assumption applied to handle left censored data, widest in case of AFs and ZEA. Dietary patterns were strongly related with country of origin of the individuals interviewed; cuscus being mainly consumed by immigrants from Maghreb, and corn flour mainly consumed by the collective from Latin America.

In order to know the relative contribution of these special foods to the global exposure, we have included in the model other foods commonly consumed by the interviewed population. Absolute results and the relative percentage of the TDI are also shown in the table 15.4, for both scenarios. The contribution of special foods was most

important in case of AFs and FBs, especially from corn flour. In any case, these results were far from the respective safety reference level; 2000 ng/kg bw/day for FBs (SCF, 2003), 200 ng/kg bw/day for ZEA (EFSA, 2004), 1000 ng/kg bw/day for DON (SCF, 2002) and 1 ng/ kg bw/day for AFs.

15.5 Discussion

In Catalonia during 2009, the immigrant population represented 15.9 % of the total population. The most important origins of immigrants were Morocco, Rumania, Ecuador, Bolivia and Colombia, who represented 19, 8, 7, 5 and 4 % of total immigrant population, respectively (Migracat, 2010). Health Department from Catalonian Government published a report about dietary habits in origin, and also, the new dietary patterns adopted by the immigrants in Catalonia. The report highlighted the maintaining of origin patterns, with use of cuscus, spices, bread and pastries among immigrants from Maghreb; in case of citizens from Colombia and Ecuador, the most commonly consumed cereal is corn, its flour is the basis to cook the homemade "tortas" or "tortillas"; Romanians use also the corn flour in traditional cooking to make some dishes as "mamaliga" or soups (Vidal-Ibáñez and De la Cruz, 2007).

In the other hand, according to the Catalonian Celiac-sufferers Association, the population who suffers this anomaly could be around 1 %, only the 10 % of patients being diagnosed. This collective need to change their dietary habits in order to substitute cereal-based foods by gluten-free foods, commonly manufactured with rice and corn flour.

It is widely known that wide range of fungal species can grow in the same substrate at similar environmental or storage conditions. Moreover, several research studies reported the simultaneous presence of different mycotoxins in cereals or cereal-based feed or foods. However, to our knowledge, few articles have been published to date, reporting the presence of AFs, DON, FBs and ZEA in gluten-free foods and ethnic foods.

Patel et al. (1996) surveyed in UK a wide range of ethnic food samples and found trace levels of AFs, OTA, ZEA and FBs in cereal based foods, such as rice, noodles, corn flour, pitta bread, chapatti, nan bread and poppadoms. Aflatoxin levels was ranged from 5.2 to 40.8 μg/kg, ZEA between 5.2 and 40.8 μg/kg, FBs between 26 and 218 μg/kg, and DON between 4 and 92 μg/kg.

More recently, gluten-free products were analyzed in order to determine free and bound FBs, also known as masked forms, by means of an analytical methodology involving an alkaline hydrolysis. The results showed that free FBs were detected in 33 out of 40 samples. Pasta and bread and also extruded products had a very low contamination, while corn flours were highly contaminated (median of 1020 μg/kg, n=7). An important point to highlight was the fraction of bounded FBs found in these foods. After alkaline hydrolysis, the occurrence of bound FBs was higher, and at higher levels than the free forms (Dall'Asta et al., 2009).

In the framework of exposure assessment studies, the food categories surveyed to assess the contamination are frequently those products consumed by the general population, those foods typically purchased by reduced and special collectives being ignored. In the present case we suspected of celiac sufferers and immigrants to be risk groups of exposition to several mycotoxins as FBs and DON, as well as their combinations. This hypothesis was confirmed in the case of immigrants, however, in the case of celiac sufferers, the gluten-free foods commonly found in the marked presented very low levels of contamination or they were below the LOQ. Despite these special collectives did not present worrying exposure levels, accurate exposure assessment studies should be carried out in order to reach more realistic values using stochastic methods which permit including uncertainties and variability in the computation model.

Moreover, a novel challenge for risk assessors is to better understand the interaction between mycotoxins as well as with nutritional matrices, and its modulation of final toxic effects. Grenier and Oswald (2011) have deeply reported the state-of-the art of the toxicological interaction of mycotoxins. Grenier et al. (2011) studied the combined

effects of subclinical doses of DON and FBs in piglets, highlighting the high complexity of their interactions, some effects being not enhanced by the combination, while others became potentiated, as liver lesions, cytokines expressions or specific IgG content.

15.6 Conclusion

To summarize, FBs were widely found in corn-based foods purchased from special shops specialized on imported foods. DON was mainly found in cuscus and several corn-based ethnic foods. Several gluten-free food samples were contaminated by FBs (6 out of 18) and DON (2 out 18), while AFs and ZEA only were found in 6 ethnic foods samples. Considering these results, these special commodities should not be a health concern for these collectives; however, special attention should be focused on bulk imported foods distributed in special retail shops.

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Chapter 16. Survey of patulin occurrence in apple juice and apple products in Catalonia, Spain, and an estimate of dietary intake

Food Additives and Contaminants: Part B 2009 2, 59–65

16.1 Abstract

This study was conducted to assess patulin exposure in the Catalonian population. Patulin levels were determined in 161 apple juice samples, 77 solid apple-based food samples and 146 apple-based baby food samples obtained from six hypermarkets and supermarkets from twelve main cities of Catalonia, Spain. Patulin was analysed by a well-established validated method involving ethyl acetate extraction and direct analysis by high-performance liquid chromatography (HPLC) with ultraviolet light detection. Mean patulin levels for positive samples in apple juice, solid apple-based food and apple-based baby food were 8.05, 13.54 and 7.12 μg kg^{-1}, respectively. No samples exceeded the maximum permitted levels established by European Union regulation. Dietary intake was separately assessed for babies, infants and adults through a Food Frequency Questionnaire developed from 1056 individuals from Catalonia. Babies were the main group exposed to patulin, however no risk was detected at these levels of contamination. Adults and infants consumers were far from risk levels. Another approach to determine estimated exposure was conducted through Monte Carlo simulation that distinguishes variability in exposures from uncertainty of distributional parameter estimates.

16.2 Introduction

Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin produced by a wide range of fungal species of the *Penicillium*, *Aspergillus*, *Byssochlamys*, *Eupenicillium* and *Paecilomcyes* genera of which *Penicillium expansum*, a common contaminant of damaged fruits, is the most important (Fuchs et al., 2008). *Penicillium expansum*, known as the causal agent of the blue mould rot in pome and stone fruits, attacks apples, pears, plums, peaches, apricots, cherries, blackcurrants, grapes, melons, and strawberries (Snowdon, 1990; Larsen et al., 1998). However, the most common fruits in which patulin has been detected are apples and pears, usually cold stored under refrigeration (Barkai-Golan et al., 2008).

A study performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that this mycotoxin has neither teratogenic nor reproductive effects, but shows embryotoxicity combined with maternal toxicity (JECFA, 2002). However, there is insufficient evidence that patulin has a carcinogenic effect in experimental animals and it has not yet been tested in humans. Therefore, the International Agency for Research on Cancer (IARC) classified patulin in group 3 or as 'not carcinogenic to humans' (IARC, 1993). Joint JECFA has established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 mg kg^{-1} body weight day^{-1} (World Health Organisation (WHO), 1995). Based on this PMTDI, patulin is regulated in the European Union at levels of 50 mg kg^{-1} in fruit juices and fruit nectar, 25 μg kg^{-1} in solid apple products, and 10 μg kg^{-1} in apple-based products for infants and young children (European Commission, 2006).

The occurrence of patulin in apple-based products, mainly apple juice, has been reported by several researchers (Burda, 1992; Rovira et al., 1993; Prieta et al., 1994; Sylos and Rodríguez-Amaya, 1999; Armentia et al., 2000; Beretta et al., 2000; Yurdun et al., 2001; Ritieni, 2003; Tangni et al., 2003; Boonzaaijer et al., 2005; Piemontese et al., 2005; Iha et al., 2007; Li et al., 2007; Spadaro et al., 2007; Karimi et al., 2008), but few studies on the occurrence of patulin in apple-based baby food products have been reported (Lewit et al., 1995; Leggott and Shephaed, 2001; Ritieni, 2003; Legarda et al., 2005;

Mhadhbi et al., 2007). Infants and children are considered to be more susceptible to different toxins than adults because of their lower body weight, higher metabolic rate, lower ability to detoxify, and because of the incomplete development of some organs and tissues (Sherif et al., 2008), and they may be expected to be a risk group due to their highest dietary intake of apple-based food. Exposure assessment of the Spanish population to patulin from the consumption of apple-based products has been estimated using hypothetical intake data (González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009). Dietary intake of patulin was assessed in the Swedish and French populations through representative consumption data (Thuvander et al., 2001; Leblanc et al., 2004). Due to the difficulties of estimating exposure from punctual data, several techniques such as probabilistic modelling have been developed to overcome the sporadic nature of consumption and variability in contamination levels. Probabilistic modelling achieved through Monte Carlo simulations accounts for every possible value that each variable could take and weighs each of them by its probability of occurrence (Council et al., 2005). The structure of a probabilistic model allows taking the variability of input data into account, which provides far more realistic results than those produced by simple deterministic scenarios (Vose, 2000).

The aim of this study was to determine the contamination by patulin of the most important apple-based products marketed in Catalonia and to assess the exposure of different Catalonian population groups to this important mycotoxin, especially in babies as the main population group exposed.

16.3 Materials and methods

16.3.1 Samples

During June and July 2008, 161 clear apple juice samples, 77 solid apple-based food samples (apple purees, compotes and jam), and 124 apple-based baby food samples (baby apple-based compote, baby multifruit compote and baby apple juice) from conventional agriculture were obtained from six hypermarkets and supermarkets from

twelve main cities (Tortosa, Tarragona, Reus, Vilanova i la Geltrú, l'Hospitalet de Llobregat, Barcelona, Terrassa, Sabadell, Mataró, Girona, Manresa and Lleida) of Catalonia, Spain, representative of 72% of the region population. From each supermarket or hypermarket, three samples (if present) of each product were randomly taken. The level of patulin was determined in a total of 71 composite samples of apple juice obtained by pooling the three items taken from each store (twelve cities x six stores/city=72 samples/category, except one of them), 77 individual solid apple-based products, and 124 apple-based baby products. There were three different categories of baby products: baby apple juice (n=12), baby apple compote (n=36), and baby multifruit compote with apple (n=76). Twenty-nine apple juice brands, 18 apple-based food brands and 26 applebased baby food brands were purchased, which can be considered the total market share in Catalonia of apple-based products. The samples were transported and stored under suitable conditions until analysis.

16.3.2 Patulin analysis

For solid apple-based products, distilled water was added to the solid portion in 1:1 w/w relation (the same weight in water than portion weight). The purée obtained was enzymatically treated (at 40ºC for 1 h) with pectinase for the removal of pectin and centrifuged at 6000 rpm for 5 min. Supernatant (5 ml) was taken to analyse patulin with the same procedure used for apple juice. Following AOAC method 995.10 (Brause et al. 1996), patulin was extracted from apple juices with ethyl acetate and then cleaned by extraction with sodium carbonate solution. Extracted samples were dehydrated with anhydrous sodium sulphate. After evaporation of ethyl acetate, patulin was resuspended in water adjusted to pH 4 with glacial acetic acid and determined by reversed-phase LC with ultraviolet light (UV) detection. A solution of water and tetrahydrofuran (100+0.8) was used as the mobile phase. Patulin was detected at UV at 276nm wavelength. The chromatography equipment was: Separations Module Alliance 2695 Waters®, analytical column Waters Spherisorb® 5 μm ODS2, 4.6 x 250 mm, Dual Absorbance Detector UV Waters 2487®. Patulin was expressed in $\mu g\ kg^{-1}$ of each assayed apple product. Recovery data, repeatability, limit of detection (LOD) and limit of quantification (LOQ) are

shown in Table 16.1. Results were not corrected for recovery. External analytical quality assurance measures were not conducted.

Table 16.1. Method performance characteristics

Analyte	Matrix	LOD	LOQ	Fortification	Recovery (n=5)	RSD_r	Accreditation
		µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	%	%	
Patulin	Apple compote	2.08	6.25	10, 40	96.2, 88.5	12.3, 8.4	No
Patulin	Apple juice	2.08	6.25	6.25, 10, 25, 50	90.9, 72.7, 81.5, 80.9	15.6, 16.4, 14.6, 7.5	No

16.3.3 Dietary intake assessment

The main problem in assessing the intake of mycotoxins is related to the fact that there is little representative available dietary data regarding mycotoxin contamination of foods. There are some methods developed to assess dietary intake overall known as market basket, 24-h dietary recall and food record methods, food-frequency methods or dietary history. Given that consumption of apple-based products is considered as sporadic or casual, food frequency methods should be chosen because it may be advantageous to sacrifice precise intake measurements in exchange for more crude information related to an extended period of time (Walter 1998). In this study, apple-based food dietary intake was obtained through a specific food frequency questionnaire (FFQ) developed for the Catalonian population including those foods typically consumed in the region that may be potentially contaminated with mycotoxins. Three different population groups were considered: babies (0–3 years), infants and teenagers (4–18 years), and adults (19–66 years). FFQ for babies consisted of four items and FFQ for infants and adults consisted of 38 items of specific foods worldwide known to be the most important food contaminated with mycotoxins studied, excluding those foods not consumed in the region. Apple juice was assumed as the main contributor to patulin intake for adults and people aged 12–18, for babies, baby apple juice, baby apple compote and baby multi-fruit compote were considered. Concerning frequency of

consumption, five response options ranging from never to annually were considered. Quantities were assessed by portion size with the aid of a series of colour photograph models. A total of 598 adults, 303 infants and teenagers, and 164 parents of babies were interviewed during 2008 in Lleida region (n=1056) by trained interviewers. This population has been considered as representative of Catalonia due to the homogeneous dietary habits around the region. Study level, dietary habits, sex, age, ethnicity and anthropometric data were considered in the questionnaire. For the deterministic approach, the mean toxin level was used in the intake calculations because this methodology is internationally recognized as providing a satisfactory estimate of long-term exposure and is considered suitable for comparison with tolerable daily intake (TDI) or provisional TWI (PTWI) values (Food and Agricultural Organization/World Health Organization (FAO/WHO), 1997). The mean toxin level was calculated considering that those samples containing patulin levels below the LOQ have a level of 0.5 LOQ. Provisional maximum tolerable daily intake (PMTDI) of 0.4 μg kg^{-1} body weight (b.w.) day^{-1} was used to assess consumer exposition (WHO, 1995).

16.3.4 Probabilistic exposure modelling

The method used to build the probabilistic model was based on the principle of the non-parametric–nonparamentric method (NP-NP) proposed by Gauchi and Leblanc (2002). The NP-NP method is proposed as a natural method of exposure assessment when consumption and contamination data are available. It is a completely non-parametric method where each normalized consumption profile of the survey is taken

into account and consumed food is attributed a value of contamination randomly drawn from the available contamination data (Cullen and Frey, 1999). Monte Carlo simulations were conducted with Microsoft Office Excel 2003® and Statgraphics Plus 5.1® for Log-normal, Gamma, Weibull and Chi-square distributions either to daily apple juice intake and apple juice intake contamination as described by Faulín (2002). One thousand iterations were considered in which both consumption data and contamination levels were produced at random from the proposed distributions. Mean patulin intake levels and percentiles 50, 95, 97.5 and 99 were calculated. Both in the deterministic and

probabilistic approaches only consumption data from the apple product-consuming population were taken into account.

16.4 Results and discussion

16.4.1 Occurrence of patulin in apple-based food

The patulin levels obtained in the 272 apple-based food samples analysed are shown in Table 16.2. Patulin was rarely detected in solid apple-based products (5.2%) compared with apple juice or baby foods (42.3% and 33.9%, respectively). However, a maximum level of 17.6 μg kg^{-1} and a highest mean level of positive samples (13.5 μg kg^{-1}) was found in this category.

Table 16.2. Occurrence of patulin in apple-based products marketed in Catalonia

	Mean[a] positives	Max	SD[a]	Positive/ total	% positive	<6.25	6.25-10	10-25	>25	n
	(μg kg^{-1})					(μg kg^{-1})				
Apple juice	8.1	15.0	1.9	30/71	42.3	41	25	5	0	71
Solid apple-based products	13.5	17.6	4.9	4/77	5.2	73	1	3	0	77
Baby apple-based food[b]	7.1	9.6	0.9	42/124	33.9	82	42	0	0	124
Baby apple-based food categories										
Apple juice	7.5	9.2	1.5	3/12	25.0	9	3	0	0	12
Apple compote	7.4	9.6	1.2	15/36	41.7	31	15	0	0	36
Multifruit compote	6.9	8.6	1.8	24/76	31.6	52	24	0	0	76

[a] Considering positive samples.
[b] Considering all baby categories.

Previous studies conducted with apple juice marketed in Spain showed higher levels of patulin than this study: mean levels ranged from 13.8 to 19.4 μg kg^{-1}, maximum levels of 118.7 and 170 μg kg^{-1}, and values over the limit established by the European

Union in a range of one to eleven samples per study (Rovira et al., 1993; Prieta et al., 1994; González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009). The highest levels of patulin in apple juice were found in Italy, Australia and Iran at levels of 1150, 646 and 285 μg l^{-1}, respectively (Burda, 1992; Beretta et al., 2000; Cheraghali et al., 2005). Overall, there is a wide variability in the percentage of positive samples among studies. However, these values are too complex to be used because every study has a different LOQ. In this study, there was an average level of positive samples (42.3%) in comparison with other European studies. There are few previous studies conducted in solid apple-based food that involved a reduced number of samples (six to 45) (Leggott and Shephard, 2001; Ritieni, 2003; González-Osnaya et al., 2007; Funes and Resnik, 2008). Higher percentages of positive samples but similar levels of contamination as in this study were shown in a study conducted in Spain with apple purees and compotes, where the range of concentration was 7.7–28.4 μg kg^{-1} (González-Osnaya et al., 2007). Maximum values were found in Argentina and Italy, with levels of 221 and 74.2 μg kg^{-1}, respectively, and the percentage of positive samples between 22.2% and 50.0% (Ritieni, 2003; Funes and Resnik, 2008). Concerning baby food samples, three subcategories were considered: baby apple juice, baby apple compote and baby multi-fruit compote. All mean values of positive samples were higher the 50% of European Union limits, with a maximum level of 9.6 μg kg^{-1} in baby apple compote, close to the 10 μg kg^{-1} permitted by law. Baby apple compote was the category with more positive samples, but similar levels of patulin were found in the three categories. Although babies are the main consumers of apple-based products, there are fewer references of baby food products than of adult products. A previous study carried out with twelve baby food samples from the Spanish market showed no positive samples (Prieta et al., 1994). Other studies related to apple-based baby food have shown very low levels of patulin too, with undetectable levels in baby fruit puree from Tunisia or all baby food categories from Italy and an average value of 5 μg kg^{-1} in baby apple juice from South Africa. However, there is a case in South Africa with a mean level of patulin of 15.4 μg kg^{-1}, exceeding the maximum permitted level in the European Union of 10 μg kg^{-1} (Leggott and Shephard, 2001; Ritieni, 2003;

Mhadhbi et al., 2007).

This is the first study conducted with a wide variety and number of apple-based food products to assess occurrence of patulin. Results from this study showed low occurrence of patulin in all apple-based products from the Spanish market. Lower levels shown in this study compared with previous ones may be explained by the efforts made by the apple-based product industry to decrease the incidence of samples with patulin.

16.4.2 Consumer exposure assessment

Exposure to patulin of population from Catalonia is shown in Tables 16.3 and 16.4. Regarding the surveyed population (n=1056), people aged 4–18 years and babies showed the highest percentage of consumers, 41.3% and 36.6%, respectively, in contrast to adult consumers (13.4%). Patulin intakes of 0.04, 0.008 and 0.005 μg kg^{-1} b.w. day^{-1} were estimated for babies, infants and adult average consumers, respectively.

Table 16.3. Exposure to patulin (PAT) of Catalonian population (Babies)

	n *	% *	Weight	Apple compote		Multifruit compote		Apple juice		Total PAT b.w.	% TDI
			(kg)	Intake	PAT	Intake	PAT	Intake	PAT		
				(mg day^{-1})	(μg day^{-1})	(mg day^{-1})	(μg day^{-1})	(mg day^{-1})	(μg day^{-1})	(μg kg^{-1} b.w. day^{-1})	
Babies mean	60/164	36.6	11.3	22.2	0.1	58.3	0.6	20.4	0.1	0.04	10.3
Babies p 95	60/164	36.6	11.3	130.0	0.6	130.0	0.6	135.5	0.6	0.13	32.4

[a] Mean levels were calculated considering that those samples with quantities of patulin below the LOQ, have a level of 0.5LOQ (LOQ = 6.25 μg kg^{-1}, 0.5LOQ = 3.13 μg kg^{-1}).
* Consumers out of total and percentatge of consumers

Babies were the most exposed with values of 10.3 % and 32.4 % of TDI for average and high consumers (95^{th} percentile), respectively. Multi-fruit compote was the main baby food contributor to patulin intake, with over 55 % of total toxin intake, followed by apple compote and apple juice with values of 24 % and 20 %, respectively.

The rest of the population assessed is far from the PMTDI of 0.4 μg kg^{-1} b.w. day^{-1} (WHO, 1995), with negligible differences between adults and infants.

Table 16.4. Exposure to patulin (PAT) of Catalonian population (Infants and adults)

	n consumers	% consumers	Weight (kg)	Apple juice intake (mL day^{-1})	PAT intake Mean consumers[b] (μg day^{-1})	PAT intake Percentile 95 (μg day^{-1})	PAT intake b.w. (μg kg^{-1} bw day^{-1})[a]	% TDI
Infants (4-18)	126/303	41.3	48.2	64.53	0.15	1.042	0.008	1.49
Adults (19-66)	80/598	13.4	74.6	63.95	0.33	1.042	0.005	1.20

[a] Mean consumers.
[b] Mean levels were calculated considering that those samples with quantities of patulin below the LOQ, have a level of 0.5LOQ (LOQ = 6.25 μg kg^{-1}, 0.5LOQ = 3.13 μg kg^{-1}).

Previous studies carried out in Spain estimated ranges of patulin daily intake of 0.015–0.055 μg kg^{-1} b.w. day^{-1} for adults, 0.012–0.155 μg kg^{-1} b.w. day^{-1} for children, and 0.086–0.252 μg kg^{-1} b.w. day^{-1} for babies, which are higher values than in this study (González-Osnaya et al., 2007; Murillo-Arbizu et al., 2009). The reason for these higher values might be the use of hypothetical intake data, which may not be representative of the Spanish population. Highest intake levels were estimated in a French total diet study too, which showed an average patulin intake of the French population of 0.018 μg kg^{-1} b.w. day^{-1} for adults and 0.03 μg kg^{-1} b.w. day^{-1} for children aged 3–14 years (Leblanc et al., 2004). However, similar results to ours were shown by Thuvander et al. (2001) who assessed the Swedish population exposure to patulin through dietary data from a national dietary study: an intake for mean consumers of 0.008 μg kg^{-1} b.w. day^{-1} for children and 0.004 μg kg^{-1} b.w. day^{-1} for adults was calculated with intakes for high consumers of 0.024 and 0.011 μg kg^{-1} b.w. day^{-1}, respectively.

Table 16.5. Estimated statistics of exposure to Patulin by Lognormal, Gamma, Weibull and Chi-Square from Monte Carlo simulation

Distribution model		Mean	P50	P95	P97.5	P99
Apple juice intake	**Contamination**	**(μg kg^{-1} b.w. day^{-1})**				
Lognormal	Lognormal	0.0040	0.0000	0.0019	0.0092	0.0337
Lognormal	Gamma	0.0036	0.0000	0.00209	0.0089	0.0471
Lognormal	Weibull	0.0035	0.0000	0.00189	0.0090	0.0331
Lognormal	Chi-Square	0.0034	0.0000	0.00149	0.0092	0.0388
Gamma	Lognormal	0.0003	0.0001	0.00149	0.0020	0.0029
Gamma	Gamma	0.0004	0.0001	0.0017	0.0022	0.0031
Gamma	Weibull	0.0003	0.0001	0.0015	0.0021	0.00281
Gamma	Chi-Square	0.0003	0.0001	0.0012	0.0019	0.0024
Weibull	Lognormal	0.0145	0.0048	0.0598	0.0869	0.1352
Weibull	Gamma	0.0173	0.0054	0.0727	0.1035	0.1596
Weibull	Weibull	0.0161	0.0045	0.0656	0.0992	0.1428
Weibull	Chi-Square	0.0128	0.0031	0.0546	0.0843	0.1200
Chi-Square	Lognormal	0.0146	0.0098	0.0435	0.0561	0.0840
Chi-Square	Gamma	0.0170	0.0113	0.0521	0.0698	0.0862
Chi-Square	Weibull	0.0149	0.0100	0.0439	0.0573	0.0693
Chi-Square	Chi-Square	0.0120	0.0139	0.0070	0.0392	0.0486

Values of exposure using the probabilistic approach are shown in Table 16.5 for all combinations of distributions (Log-normal, Gamma, Weibull and Chi-square) of apple juice intake and patulin contamination values obtained by Monte Carlo simulation using real data from this study. This methodology allowed us to simulate all kinds of scenarios from the existing data. Thus, the 99th percentile revealed that some individuals might be exposed to up to 0.16 μg kg^{-1} b.w. day^{-1}. Overall, mean values generated

through this method were also far from the PMTDI of 0.4 μg kg^{-1} b.w. day^{-1}.

16.5 Conclusion

In conclusion, it is confirmed that babies are the main population group exposed to patulin. However, the daily intake of baby consumers is below the PMTDI, including high consumers. Dietary intake does not represent a risk for adult and children consumers as their consumption is far below safe levels.

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Chapter 17. Global discussion

17.1 Global design. Going to the Total Diet Studies framework

The present study was designed on the basis of the global structure developed by the first and the second "Catalonian total diet study for chemical contaminants" carried out between 2000-2002 and 2005-2007, respectively. In these pioneering studies, samples were also purchased in 12 main cities from Catalonia, representative of 72 % of Catalonian population. Considering the same geographical regions and main cities: Barcelona, Hospitalet de Llobregat, Vilanova i la Geltrú, Mataró, Sabadell, Terrassa, Girona, Tarragona, Reus, Tortosa, Manresa and Lleida. Sampling was based on a pooling of a variable number of individual samples in order to obtain "composites" or analytic samples. Because the food contaminants selected in these studies were mainly environmental pollutans (such as arsenic, cadmium, mercury, lead, dioxins and furans, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, hexachlorobenzene, polybrominated diphenyl esthers, polychlorintated diphenyl esthers and polychlorinated naphtalenes), the food categories were extended to other susceptible food groups like meats, fish, vegetables, fruits, eggs or oils. In that case, food dietary intake data was provided from the national survey ENCAT 2002-2003, and combined with contamination datasets through a deterministic and probabilistic method (ACSA, 2005; Bocio et al., 2004; Llobet et al., 2007; Martí-Cid et al., 2008).

Despite of a worldwide standardization of exposure assessment methodologies is not available yet, big efforts are held by governmental organizations in order to build a harmonized framework in food risk analysis. EFSA, responsible to reach this objective in the European Union, has issued Scientific Opinions intended to draw a set of standard gold references to apply in exposure assessment studies for chemicals (EFSA, 2006, 2007, 2008, 2010, 2011a,b,c). EFSA supported the total diet studies (TDS) to provide the most accurate estimates of mean exposure of populations or collectives to food chemicals, but accuracy and reliability will always depend on the design (EFSA, 2011b). A TDS consists of selecting, collecting and analysing commonly consumed food purchased at retail level, processing the food as for consumption, pooling the prepared food items into representative food groups, homogenising the pooled samples and analysing them for harmful and/or beneficial chemical substances. Essential principles

of a TDS draw the study design 1) to be representative of the whole diet, 2) to analyse composite samples pooled from a variable number of individual samples and 3) to analyse food samples as commonly consumed (EFSA, 2011b).

Considering that most food categories included in the present work are mainly consumed as bought without cooking and/or additional transformation steps, we can assume the present work following a TDS approach. We only identified one food category susceptible of cooking step, pasta, which effect will vary depending on boiling procedure and mycotoxin. Accordingly, we assessed the impact of this process on the final exposure estimates to check the weight of the bias. In any case, the presented study design permits to be compared with other TDS reporting mycotoxin exposure levels in countries following TSD harmonisation towards…

Previous TDS to assess exposure to mycotoxins have been conducted in France. The First French Total Diet Study was published in 2005 considering 456 composites pooled from 2280 individual samples to analyse a wide range of mycotoxins. Recently, the Second French Total Diet Study was published including an improved sampling design based on the selection of two hundred twelve core foods, purchasing a huge set of 20280 individual samples to be pooled in 1352 composite samples (Sirot et al., 2009). One of the most important advantages, highlighted by the authors, about the core food sampling method is the fact that it is able to cover about 140 fewer products, in turn the high number of sub-samples (around 15 by composite) assures a high representativeness. From an analytical point of view, it seems complex and very expensive to develop a chemical analytical method to cover the wide range of complex matrices for all mycotoxins, in order to assure accuracy, validity and reproducibility. Moreover, pooling a large set of individual samples can really dilute mycotoxin levels, mainly important for those mycotoxins with severe acute effects as DON.

The high seasonal variability of the occurrence of mycotoxins indicates the high complexity to draw a realistic scenario of exposure to any mycotoxins across the time. Therefore, each exposure estimations should be considered as punctual, from a defined period and without correlation with future estimates predictions. Only continuous

sampling plans along time can guarantee reliable tendencies on exposures to a target mycotoxin.

17.2 Occurrence of mycotoxins in foods intended for human consumption

Considering the results from our studies, after performing more than 4500 chemical analysis, we found global occurrence of all mycotoxins to be almost of 32 % of total samples. The highest levels of positive samples (above of LOQ) were reached for DON and FBs with percentage levels of 47 and 40 %, respectively. Lowest levels of positive samples were found for A-type trichothecenes, T-2 and H-T2 (1 and 8 %, respectively), followed by aflatoxins (12 %). Corn snacks were one of the food matrices with highest percentages of *Fusarium* mycotoxins. Aflatoxins were most widely found in dried red pepper, followed by nuts. Very few samples were found to exceed the EU limits.

Most of the methods used in the present study were based on solid-liquid extraction (or liquid-liquid in case of beverages), commonly with a polar solvent solutions followed by a clean-up step with immunoaffinity columns. Mycotoxins have been mainly determined and quantified from the extract by HPLC coupled to fluorescence or absorbance detector. Trichothecenes were determined in breakfast cereals, snacks and pasta samples following an additional derivatisation and finally analysis by gas chromatography with electrochemical detection (GC-ECD). Exceptionally, AFM_1 content was analyzed using an Enzyme-Linked ImmunoSorbent Assay (ELISA) commercial kit. Advances on methods for analysis and sampling of mycotoxins have been subsequently reviewed by Shephard et al. (2010, 2011, 2012) from 2007 to date. Several of the most interesting improved methods are going to the multi-mycotoxin detection methods by UHPLC or HPLC-MS/MS covering a wide range of toxins also considering a wide range of secondary mycotoxins as case of DAS, MAS, nivalenol, DOM-1, 3-Ac-DON, 15-Ac-DON, Fus-Z or metabolites as α-ZAL, β-ZAL, α-ZOL or β-ZOL.

Fusarium species are also responsible for the production of another group of

bioactive compounds called emerging or "minor" mycotoxins. This group including enniatins (ENA, ENA1, ENB and ENB1), fusaproliferin and beauvericin is not well investigated by scientists. A few worldwide data are published about the occurrence of these toxins in commodities from the Nordic countries (Norway, Finland) and Italy (Jestoi, 2008), and little information is available in the literature about their presence in foods especially in the South of Europe and the rest of the world. The presence of emerging mycotoxins (ENs, BEA and FUS) was determined in samples of cereals commercialized in Spain. The percentage of contamination of total samples with ENs, BEA and FUS were 73.4%, 32.8% and 7.8%, respectively (Meca et al., 2010), therefore and considering the current knowledge on the toxicity of these contaminants, they should be considered in future studies.

"Hidden (masked) fumonisins" are of current interest because they are not detected by the usual analytical methods and could represent an additional potential risk for animals and humans. The occurrence studies revealed that various conjugated forms of DON, ZEA, and T-2 and HT-2 toxins (T-2 and HT-2) were often found, albeit usually at lower concentrations than their parent compounds. In the various studies found in the literature, LC-MS/MS methodology was the analytical method of choice. In addition to chromatographic methods, there are in principle other contemporary approaches to detect masked mycotoxins such as immunochemical methods, and the review by Goryacheva and De Saeger (2012) provides insights into the possibilities, problems and prospects of these tests. Although there is some preliminary evidence on possible release of DON from its conjugated form by action of hydrolytic enzymes catalyzing the cleavage of β-O-glucosidic bonds, the bioavailability of D3G is not fully understood yet. On this account, its content in a human diet has not been considered in TDI calculation. Risk assessment of mycotoxins may need to be reconsidered, taking into account the new data and knowledge on masked mycotoxins (De Saeger and van Egmond, 2012).

17.3 Left-censored data management

Contamination datasets of mycotoxins in foods are frequently characterised by a large range of samples under the limit of detection or quantification. In appearance, this fact could be positive from a food safety point of view, but the ignorance of the real level of non detects become a concern for the risk analyst specially in case of non-threshold substances, genotoxic cancerigens like aflatoxins. In this case, the levels of these pollutants are highly heterogeneously distributed, finding a moderate set of positive samples with several items highly contaminated. Accordingly, exposure assessment computation will become awkward and inaccurate with large ranges of uncertainty.

The most widely applied method to manage the uncertainties related with left-censored data is based on the substitution of those non-detects or non-quantified values by LOD or LOQ x 0.5 and in other occasions two scenarios are assumed, building a range from the upper bound (worst case), by substitution of non detects by LOD/LOQ, to the lower bound (best case), by substitution of non detects by 0.

Despite of scientific reports recommend other methods than substitution, these sophisticated methods have not been extended to mycotoxin risk analysis studies yet. EFSA, recommended by the Scientific Committee has recently produced a deep report were thoroughly, most of the important methods (substitution, log-probit regression, maximum likelihood estimation, Kaplan-Meier estimation), were assessed to characterise their robustness. This study concluded that in the majority of previous studies, the substitution methods resulted in bad estimates. From the simulation study good estimations derived from MLE methods when data could be accurately fitted with the implemented distributions. KM method also resulted to be a good approach, either for the mean and percentile 95. Hewet and Ganser (2007) and Helsel (2005) recommended MLE particularly when the percentage of censored data is between 50 and 80 %, while KM method was recommended by Helsel (2005) when fraction of censored data is 50 % or lower. Under the assumption of random feature of censorship, Tressou et al. (2006) applied KM estimator to draw the distribution of exposure to OTA by French

populations and to quantify the probability to exceed the related PTWI. The authors also compared KM method with substitution methods and a parametric approach, showing lower estimates than for the parametric distributions.

17.4 Food dietary intake assessment

Several dietary intake assessment methods can be used in exposure assessment studies, from gross estimates given by national food balance sheets to accurate individual dietary surveys. The selection of the method will depend on a wide range of factors like the food chemical nature, the required accuracy, availability of previous data or in most of cases or the assigned budget.

Reliable food consumption information has been collected by EFSA over the last five years at an increasing level of detail. In 2008, following recommendations issued by the EFSA Scientific Committee (2005), EFSA created the "Concise European Food Consumption Database" (EFSA, 2008) and subsequently improved by the Comprehensive European Food Consumption Database to be used for detailed exposure calculations. Currently this last database includes food consumption data concerning infants, toddlers, children, adolescents, adults, elderly and very elderly from a total of 32 different dietary surveys carried out in 22 different Member States (EFSA, 2011a). Dietary patterns in Catalonia have been assessed subsequently from 1992 to 2003 in two individual studies, ENCAT-92 (n=2641) and ENCAT-02 (n=2060). These studies were carried out using two 24-h recall and a food frequency questionnaire administered by trained interviewers. Previously, in 1986 it was carried out the first nutritional questionnaire in Catalonia and no more research has been done to date (Jiménez et al., 1988). In the rest of Spain, there are few studies with the main goal focused to assess the nutritional status. EnKid Study 1998-2000 was carried out to young population and children between 1 and 14 years old by means of 24-hours recall (Serra-Majem et al., 2001). AESAN (Agencia Española de Seguridad Alimentaria y Nutrición) also conducted a couple of studies, one of them using a 24-hours recall and the other a food records with similar purposes (Requejo et al., 2002; Ortega et al., 2010).

To date, most of previous exposure assessment studies for mycotoxins have been performed using national surveys, food balance data sheets or consumption assumptions. Considering datasets available in the Comprehensive Database, 43 % of studies were carried out by means of food records and 53 % by 24-h recall (only one study through a 48-h recall). Most of these studies (73 %) were applied at individual level and 27 % at household level.

The most important drawbacks when applying these studies in chemical exposure assessment are, in one hand, that those studies were designed to assess the nutritional status, therefore most of the food categories are formulated to assess energy, nutrients, minerals or vitamins intake, not specifically for target pollutants. On the other hand, those methods do not reflect long term dietary habits and also, they could be carried out during a period far from the chemical analysis or in other regions of the country, when or where dietary habits may be different.

Thus, a specific design should be considered in order to assess the intake of those foods susceptible to mycotoxin contamination. The food frequency questionnaire (FFQ) is currently the most widely used tool in epidemiological research. They have great advantages over other methods in the sense that they are cost-effective and can be used in large populations with relative ease and they facilitate repeated assessments. Main disadvantages of food records, diet history or 24-h record are the time consuming for volunteers and researchers, then too expensive to large epidemiological studies. Moreover, these methods are not suitable to assess chronic exposure because they mostly cover short periods in the life of individuals, in contrast of the common dietary habits derived from the FFQ. Considering chronic toxicity to be the main endpoint in mycotoxin risk assessment, FFQ seems to be the best method. However to obtain a useful FFQ in terms of reliability and accuracy, it should be statistically assessed to check the validity and reproducibility. Despite of several approaches can be used to assess the performance of FFQs, the most common proceeds involve the comparison with an independent standard to check the validity and describe its reproducibility between measures. Measurement errors in FFQs may lead to underestimation of causative or protective risks, thus reducing these errors should be a priority (Willet, 1998). Few

studies have studied the exposure to mycotoxins through other methods than the available national databases described above. One of them is 24-h duplicate diet performed in Netherlands in 1994 for AFB_1, AFM_1 and OTA (Sizoo and Egmond, 2005).

Concerning selection of the population group selection, non-average individuals should always be taken into account, focusing mainly in high consumers drawn from the high percentiles of the exposure. Infants and children are considered the most exposed groups because they present the highest consumption levels per kilogram body weight. The most vulnerable collectives are also considered infants, young children, pregnant and lactating women. Elderly (older than 75 years) are most vulnerable than the rest of adults due to their deficiencies on the immune system but they are not a priority in exposure to chemical substances. Other collectives have been identified to be vulnerable to have special dietary requirements derived from health problems (celiac, diabetics…) or personal choices (vegetarians). In Catalonia during 2009, the immigrant population represented 15.9 % of the total population. The most important origins of immigrants were Morocco, Rumania, Ecuador, Bolivia and Colombia, who represented 19, 8, 7, 5 and 4 % of total immigrant population, respectively (Migracat, 2010). Health Department from Catalonian Government published a report about dietary habits in origin, and also, the new dietary patterns adopted by the immigrants in Catalonia. The report highlighted the maintaining of origin patterns, with use of cuscus, spices, bread and pastries among immigrants from Maghreb; in case of citizens from Colombia and Ecuador, the most commonly consumed cereal is corn, its flour is the basis to cook the homemade "tortas" or "tortillas"; Romanians use also the corn flour in traditional cooking to make some dishes as "mamaliga" or soups (Vidal-Ibáñez and De la Cruz, 2007). On the other hand, according to the Catalonian Celiac-sufferers Association, the population who suffers this anomaly could be around 1 %, only the 10 % of patients being diagnosed. This collective needs to change their dietary habits in order to substitute cereal-based foods by gluten-free foods, commonly manufactured with rice and corn flour. Considering corn based food the most susceptible to be contaminated by a wide range of *Fusarium* mycotoxins, immigrants and celiac sufferers should be

highlighted to be high consumers of this cereal, can be more exposed to these toxins.

17.5 Integration of contamination and consumption raw datasets

In the present work we have applied the two essential techniques proposed to combine contamination with consumption datasets: deterministic and probabilistic approach. In each study we have first estimated the exposure by means of the deterministic method and subsequently we have refined the estimation through simulation methods. In the context of exposure assessments, the term 'point estimates' refers to a method whereby a fixed value for food consumption (such as the average or high level consumption value) is multiplied by a fixed value for the residue/concentration (often the average residue level or upper tolerance or permitted level according to legislation) and the intakes from all sources are then summed. Probabilistic analysis involves describing variables in terms of distributions to characterise their variability and/or uncertainty. It then takes account of all the possible values that each variable could take and weights each possible model outcome by the probability of its occurrence. In the presented works, different probabilistic methods have been used depending on the situation. The first step was always to check carefully each histogram, evaluating the number of quantified samples, the number of extreme values, and distribution of the mycotoxin levels. In case of consumption datasets also we needed to check individually outliers or biased responses in the FFQ. Subsequently, each dataset was fitted to the best distribution, commonly gamma or lognormal, checking in each case their goodness of fit. Then, it was the time to build the model, in non-parametric approaches there is an iterating resample from each raw dataset performing random combination from observed values. In case of parametric approaches, re-sampling is carried out from the fitted distributions. 10.000 iterations were considered to be enough to get stable estimations of percentiles. One of the main drawbacks of the deterministic approach is that it does not enable to calculate complicated statistics such as high quantiles, but the definition of high-level consumers is crucial to the outcome of the risk assessment because, in practice, it determines the proportion of the population

that would exceed a health based limit. The reliability of high percentiles is related to the number of subjects used to calculate them, being not statistically robust when there are a limited number of subjects. Therefore, simulation methods are proposed to be the best approach when refinements are required, especially for high quantiles. Additionally, simulation methods permit us to know the validity and accuracy of the high quantiles estimated for the simulation method through the confidence interval. Pseudo-parametric confidence intervals with a sample size of 100,000 were computed to the boundaries of the 95% confidence interval taking the 0.025th and 0.975th empirical quantiles of the final bootstrap distribution. In several cases, such as aflatoxins, raw contamination data was only computed through the mean level because it is the best choice in chronic exposure models as the case of the mechanism of action of these carcinogens. But in case of DON or T2-HT2 extreme values from true observations have been considered because acute toxicity is one of the most important health concern derived from these toxins. The most commonly occurring mycotoxins in Catalonian market were the fumonisins and deoxynivalenol, which were widely found in cereal-based food. Despite the low levels of T2 and HT2 toxins in foods, high exposure values have been computed through modelling because their high toxicity. We concluded that A-type trichothecenes and DON were the main health concern of the mycotoxin assessed in the line of other studies carried out in France (Leblanc et al., 2005, 2012). Studies reporting the toxic effect of simultaneous mycotoxins and co-occurrence with bioactive dietary substances have been increasing during the last years (Guerra et al., 2005; Ranaldi et al., 2007). Therefore, a novel food safety challenge seems to take into account the high complexity of the real dietary mixtures on exposure assessment models.

17.6 Use of mycotoxin biomarkers

Biomarkers measures are objective and may, therefore, have a superficial aura of impartiality, especially to the epidemiologist trained to be suspicious of the answers provided by forgetful and potentially biased humans and their interviewers. Before a biochemical indicator can be used as a measure of nutritional intake, it must be evaluated

with respect to its sensitivity to intake that nutrient or non-nutrient. Most common validation approach in individuals is to estimate their dietary exposure and determine its relation with biochemical levels (Willet, 1998).

One of the first applications of biomarkers to human exposure to food chemicals is illustrated in the studies on the mycotoxin aflatoxin B_1 in the late 1980s (Zhu et al., 1987; Gan et al., 1988). In these studies statistically significant correlations were observed between the dietary intake of aflatoxin B_1 and urinary excretion of the metabolite aflatoxin M_1. Subsequent investigations have tended to utilise aflatoxin B_1 bound to serum/plasma albumin (Wild et al., 1992; Turner et al., 1998) and urinary aflatoxin B_1–DNA adducts (Groopman et al., 1992), where a correlation coefficient of 0.80 with dietary exposure was obtained. Subsequently, developing of biomarkers in exposure assessment studies for mycotoxins have been increasing during the last decades mainly focused in aflatoxin B_1, ochratoxin A, fumonisins and more recently deoxynivalenol. Further information about the current situation of *Fusarium* mycotoxin biomarkers is summarized in the Annex 2 concluding that successful methods have been developed to biomonitor exposure to ochratoxin A and aflatoxins, however few studies and unsuccessful results have been obtained with biomarkers of *Fusarium* toxins exposure. Urinary levels of fumonisins have been reported as an effective method to assess short-term intake of this toxin. Despite the ratio Sa:So has been validated as biomarker of fumonisin exposure in animal species, unsuccessful results have been reported among human populations. Further studies are required to understand accurately basal levels of these contaminants, interactions with other contaminants or variability sources. Regarding DON, several studies have reported data on absorption, toxicokinetics, toxicodynamics and metabolism in animals, but few studies have been conducted in human populations. Urinary level of DON has been used as biomarker to assess the exposure of human populations showing successful results, with positive correlations between estimated dietary intake of the toxin and urinary levels. In spite of the interest of researchers in zearalenone contamination of food and its toxicity in animal species, very few studies have been conducted to assess the real impact on human population. Finally, no studies have been conducted to assess possible biomarkers to

assess the exposure to T-2 toxin or HT-2 toxin.

Recent analytical advances focus to the detection of multi-mycotoxin by LC-MS methods. For example liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI□MS/MS) method for the quantitative measurement of 15 mycotoxins and key metabolites in human urine using polarity switching. DON, D3G, $D_{15}G$), de-epoxy DON, NIV, T-2 toxin, HT-2 toxin, zearalenone, zearalenone-14-O-glucuronide, α- and β-zearalenol, fumonisins B_1 and B_2 (FB_1, FB_2), ochratoxin A (OTA) and aflatoxin M_1 (AFM_1) were determined without the need for any cleanup using a rapid and simple dilute and shoot approach. The co-occurrence of several mycotoxins in the investigated samples clearly emphasizes the great potential and importance of this method to assess exposure of humans and animals to naturally occurring mycotoxins (Warth et al., 2012). To sum up, considering the recent advances in chemical analysis of biological fluids, it seems that biomarkers or mycotoxin exposure can have a promising role on mycotoxin risk assessment. However, further research is necessary from nutritional epidemiologist in order to fit the correlation of mycotoxins biological signals with dietary intakes.

17.7 Effect of food processing

In the present work we have dedicated a full chapter to assess the food processing on final exposure assessment estimates, focusing on DON to be responsible of highest health risk estimates. Fate of DON was assessed through two very common processes: traditional bread-making and boiling of pasta, both elaborated from naturally contaminated flour. Considering our results, high stability of DON resulted during bread-making process, from dough kneading to final baking, including fermentation steps.

The effect of baking on DON was hardly reported with controversial effects between previous studies. Head treatment at 190°C and 200°C temperature resulted in minor reduction of DON level, between 1.7 and 4.1%, and major effects at 230°C (7.6 - 9.9%) (Israel-Roming and Avram, 2010). Young et al. (1984) observed that the

production of yeast containing products resulted in an increase in DON, which they attributed to the enzymatic conversion of DON precursors. Neira et al. (1997) found reductions in stages of fermentation and oven baking, with mean reduction rates of 21.6 and 28.9 %, respectively, and final reductions ranged between 16.8 and 96.6 %. A significant decrease occurred of approximately 38–46% of the original content during fermentation, but these rates were reverted after baking without any final reduction (Lancova et al., 2008). Authors suggested transformation of DON to conjugated forms as a possible mechanism of reversible mycotoxin depletion. Boyacioglu et al. (1993) examined the effects of bread additives on DON level post-bake and has shown that potassium bromate and l-ascorbic acid had no effect, but sodium bisulphite, l-cysteine and ammonium phosphate resulted in drops of up to 40%. Greenhalgh et al. (1984) identified a DON isomer in baked bread at levels of 3–13% of DON originally present. Further information has recently been produced by Kostelanska et al. (2011) about fate of DON and it masked form D3G during baking. The authors found a moderate decrease on the final content of both DON and D3G (87 and 90 %, respectively), finding significant increase of D3G (145 %) when enzymatic bakery improvers were used in the process.

The high solubility of DON in water provides the suitable conditions to its depletion during wet food processing. Despite this chemical property, reduction of DON through common cooking processes such us boiling of pasta, has been poorly studied to date. We found high reduction rates, almost 75 %, where DON is mainly transferred to the boiling water without other eventual losses. Previous studies found moderate depletion rates of 50-60% (Sugita-Koonishi et al., 2006; Nowicki et al., 1988). On the basis of these findings, and in order to assess the bias, we subsequently weighted the impact of this reduction on simulation exposure assessment models. Accordingly we computed the exposure models from Catalonian population raw datasets, introducing the new parameter, DON reduction from boiling of pasta, and compared with the previous estimations. Despite the statistically significant differences were found in most of parameters and collectives, the impact on risk characterization was not so pronounced to avoid health concern estimation in the most exposed groups. In this

model, reduction effect was counteracted by low contribution of pasta to the global DON intake.

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Chapter 18. Concluding remarks and future research

(English, Spanish, Catalan)

Concluding remarks

- **Aflatoxins**. The highest percentages of positive samples were found in red pepper, pistachios and peanuts (57, 20 and 11 %, respectively), but not found or found in a few samples from the other categories. The most exposed group should be the celiac sufferer collective, followed by the adolescents. The MoEs built in the present study for the worst case scenario ranged between 662 and 8,208. The cancer incidence attributable to AFs ranged from 0.000 to 0.004 cancers/year per 100,000 people for children and celiac sufferers, respectively.

- **Aflatoxin M_1**. The occurrence of AFM_1 in cheese and yoghourt from Catalonian market is very low. Although high occurrence in milk was observed, the contamination levels were very low, far from the safe limit established by EU. Moreover, results from all studied scenarios suggest that it should not be expected AFM_1 exposure to contribute to the risk of liver cancer among Catalonian population, including high consumers.

- **Deoxynivalenol.** High incidence of DON was found in cereal-based foodstuffs from Catalonian market, especially in wheat flakes, corn flakes, corn snacks, pasta and bread, with a percentage of positive samples ranging from 73.4 to 100.0%. Despite the high percentage of positive samples in foodstuffs from Catalonian market, only five samples were above the limit established by European Commission. Catalonian population should be expected to be exposed to moderated levels of deoxynivalenol, being the infants and individuals with ethnic dietary patterns the most exposed population groups. Although the majority of the population do not exceed the TDI of 1 $\mu g\ kg^{-1}$ bw day^{-1}, there is still a large population exceeding this safety value.

- **Fumonisins.** Despite fumonisins can be found regularly in Catalonian market, especially in ethnic food, beer and corn snacks, all levels remain far from EU maximum levels. Infants should be expected to be the main risk group due to

fumonisin intake; however these results suggest that no human risk derived from FBs should be expected in any Catalonian population group.

- **Patulin.** Mean patulin levels for positive samples in apple juice, solid apple-based food and apple-based baby food were 8.05, 13.54 and 7.12 μg kg^{-1}, respectively. No samples exceeded the maximum permitted levels established by European Commission. Infants were the main group exposed to patulin, however no risk was detected at these levels of contamination. Adults and children were far from risk levels.

- **T-2 and HT-2 toxins.** T-2 toxin was only quantified in 5 out of total samples of cereal-based food. HT-2 was present in a low percentage of the samples (from 6.2% in corn flakes to 15.3% in sliced bread), and it was not detected in samples of bread and beer. The most exposed group population to the toxins T-2 and HT-2 should be expected to be the children. Despite of the drawbacks to set the exposure outputs derived from the large censored data, we have found that a group of child population could exceed the safety limits of 0.06 μg^{-1} kg bw^{-1} day.

- **Zearalenone.** The results showed that the occurrence of ZEA in food marketed in Catalonia was low. Sliced bread, corn snacks and sweet corn were the commodities where it was most often present. Considering our results, either from *NP-NP* and *P-P* method, the most exposed group should be expected to be the infants, especially for the high quantiles, however, in all cases the estimates were far from the TDI of 250 ng kg^{-1} body weight, established by the EFSA.

- **Co-occurrence of mycotoxins in special commodities**. The results showed that FBs were widely found in corn-based foods purchased from special shops specialized on imported foods. DON was mainly found in cuscus and several corn-based ethnic foods. Several gluten-free food samples were contaminated by FBs (6 out of 18) and DON (2 out 18), while AFs and ZEA were only found in 6 ethnic food samples. Considering these results, these special commodities should not be a health concern for these collectives; however, special attention should be focused on bulk imported foods distributed in special retail shops.

- **Food processing effect.** DON was stable during bread-making process, considering fermentation and baking. ZEA showed depletion during the first fermentation but without enough statistical differences. Major decreases were found for DON during boiling of pasta, with almost 75 % of transference from pasta to water. When we applied the reduction factor in the simulation exposure models, the final reduction was statistically significant but not enough to consider the process as protective because the contribution of pasta to the global DON intake was commonly low.

- **Biomarker of exposure to FBs.** The results showed higher Sa and So levels in plasma than in urine, and significant differences were shown when males were compared to females. Concerning Sa/So ratios from maize-food consumers and non consumers, significant differences were found in urine and plasma samples but evidences of mechanism of action of fumonisins were not apparent. Through time-course study, we have narrowed down the day in which the maximum alteration of Sa/So ratio should be expected in humans. More studies are required to better understand the use of this biomarker with human population, mainly, to improve the accuracy at low levels of exposure.

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Conclusiones finales

- **Aflatoxinas.** Los mayores porcentajes de muestras positivas se encontraron en pimentón, pistachos y cacahuetes (57, 20 y 11%, respectivamente), pero no se encontraron o se encontraron en pocas muestras de las otras categorías. El grupo más expuesto se estimó que eran los celíacos, seguidos de los adolescentes. Los márgenes de exposición (MoE) construidos en el presente estudio para el peor de los casos oscilaron entre 662 y 8,208. La incidencia de cáncer atribuible a las aflatoxinas varió desde 0.000 hasta 0.004 cánceres/año por cada 100,000 habitantes para los niños y los celíacos, respectivamente.

- **Aflatoxina M_1.** La presencia de AFM_1 en queso y yogur procedentes del mercado catalán es muy baja. A pesar de la alta presencia en la leche, los niveles de contaminación eran muy bajos, alejados del límite de seguridad establecido por la UE. Además, los resultados obtenidos en todos los escenarios estudiados sugieren que no se espera que la exposición a AFM_1 contribuya al riesgo de cáncer de hígado entre población Catalana, incluyendo los grandes consumidores.

- **Deoxinivalenol.** El DON se encontró de forma ampliamente extendida en los alimentos a base de cereales del mercado catalán, especialmente en los copos de trigo, copos de maíz, aperitivos de maíz, pasta y pan, con un porcentaje de muestras positivas que van desde 73.4 hasta 100.0%. A pesar del alto porcentaje de muestras positivas en estos productos alimenticios, sólo cinco muestras estaban por encima del límite establecido por la Comisión Europea. Se debe esperar que la población catalana esté expuesta a niveles moderados de deoxinivalenol, siendo los niños y las personas con patrones dietéticos étnicos, los grupos de población más expuestos. Aunque la mayor parte de la población no supera el TDI de 1 $\mu g\ kg^{-1}$ de peso corporal por $día^{-1}$, todavía hay una parte de población que puede superar este valor de seguridad.

- F**umonisinas**. A pesar de que las fumonisinas se encontraron regularmente en los

alimentos analizados, especialmente en la comida étnica, la cerveza y los aperitivos de maíz, todos estaban lejos de los niveles máximos establecidos por la UE. Se espera que los bebés sean el principal grupo de riesgo derivado de la exposición a FBs; sin embargo, estos resultados sugieren que no existe riesgo humano derivado de las FBs en cualquier grupo de la población catalana.

- **Patulina**. La media de concentración de patulina en muestras positivas de zumo de manzana, comida sólida y comida para bebés a base de manzana fueron 8.05, 7.12 y 13.54 $\mu g\ kg^{-1}$, respectivamente. Ninguna muestra superó los niveles máximos establecidos por la UE. Los recién nacidos eran el principal grupo expuesto a patulina, sin embargo no se detectó riesgo a esos niveles de contaminación, ya que tanto los adultos como los niños estaban muy alejados de los niveles de seguridad.

- **Toxinas T-2 y HT2**. La toxina T-2 se cuantificó en sólo 5 de las muestras totales analizadas. La toxina HT-2 estaba presente en un bajo porcentaje de las muestras (6.2 % en copos de maíz a 15.3 % en el pan de molde), y no se detectó en muestras de pan y cerveza. El grupo de población más expuesto a las toxinas T-2 y HT-2 se debe espera que sean los niños. A pesar de los inconvenientes para establecer la exposición derivada de datos con una gran parte de valores censurados, hemos encontrado que un grupo de población infantil puede exceder los límites de seguridad de 0.06 $\mu g\ kg^{-1}$ de peso corporal $día^{-1}$.

- **Zearalenona**. Los resultados mostraron que la presencia de ZEA en los alimentos comercializados en Catalunya fue baja. El pan de molde, los aperitivos de maíz y el maíz dulce fueron los productos en los cuales se encontraron más frecuentemente. Teniendo en cuenta nuestros resultados de exposición, ya sean del método NP-NP como del método P-P, el grupo más expuesto se debe esperar que sean los niños, especialmente para los percentiles más altos, sin embargo, en todos los casos las estimaciones estaban lejos de la TDI de 250 $ng\ kg^{-1}$ peso corporal y día, establecido por la EFSA.

- **Presencia simultánea de micotoxinas en productos especiales.** Los resultados mostraron que las FBs se encontraron ampliamente distribuidas en los alimentos a

base de maíz comprados en tiendas especializadas en alimentos de importación. El DON se encontró principalmente en cuscús y en varios alimentos étnicos a base de maíz. Algunas pocas muestras de alimentos sin gluten estaban contaminadas por FBs (6 de 18) y DON (2 de 18), mientras que las aflatoxinas y la ZEA sólo se encontraron en 6 muestras de alimentos étnicos. Teniendo en cuenta estos resultados, los productos especiales analizados no deben suponer un problema para la salud de estos colectivos, sin embargo, se debe prestar especial atención en los alimentos especiales importados i distribuidos a granel en tiendas de barrio.

- **Efecto del procesamiento de alimentos.** El DON se mantuvo estable durante el proceso de panificación, teniendo en cuenta la fermentación y horneado. La ZEA mostró una ligera disminución durante la primera fermentación pero sin suficientes diferencias estadísticas. Las principales disminuciones se encontraron en DON durante el hervido de la pasta, con casi el 75% de transferencia de la pasta al agua. Cuando aplicamos el factor de reducción en los modelos de exposición por simulación, la reducción final fue estadísticamente significativa, pero no lo suficiente como para considerar el proceso como protector debido a la contribución de la pasta a la ingesta global de DON era generalmente baja.

- **Biomarcadores de exposición a FBs.** Los resultados mostraron unos mayores niveles de Sa y So en plasma que en orina, y diferencias significativas cuando se compararon los hombres con las mujeres. En cuanto al ratio Sa/So procedente de consumidores de alimentos de maíz y no consumidores, se encontraron diferencias significativas en las muestras de orina y plasma, pero no se encontraron evidencias del mecanismo de acción de las fumonisinas. A través del estudio de la evolución temporal de los niveles, se determinó la jornada en la cual debe esperarse la máxima alteración del ratio Sa/So en humanos. Se requieren más estudios para optimizar la utilización de este biomarcador en población humana, principalmente, para mejorar la precisión en los niveles bajos de exposición.

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Conclusions finals

- **Les aflatoxines.** Els percentatges més elevats de mostres positives es van trobar en el pebre vermell, els festucs i els cacauets (57, 20 i 11%, respectivament), però no es van trobar o es van trobar en poques mostres d'altres categories. El grup més exposat es va estimar que són els celíacs, seguits dels adolescents. Els marges d'exposició (MoE) construïts per al pitjor dels casos va oscil·lar entre 662 i 8,208. La incidència de càncer atribuïble a les aflatoxines va variar entre 0.000 i 0.004 càncers/any per cada 100,000 habitants, per als nens i pels celíacs, respectivament.

- **L'aflatoxina M_1.** La presència d'AFM_1 en formatge i iogurt del mercat català és molt baixa. Malgrat l'alta incidència en la llet, es van determinar nivells molt baixos de contaminació, molt allunyats del límit establert per la UE. A més, els resultats d'exposició en tots els escenaris estudiats suggereixen que no s'ha d'esperar que l'AFM_1 contribueixi al risc de càncer de fetge entre la població Catalana, incloent-hi els grans consumidors.

- **Deoxinivalenol.** Es va trobar una elevada presència de DON en aliments a base de cereals procedents del mercat català, especialment en els flocs de blat, flocs de panís, aperitius de panís, pasta i pa, amb percentatges de mostres positives que van de 73 a 100 %. Malgrat l'alt percentatge de mostres positives, només cinc mostres estaven per sobre del límit establert per la UE. Es va estimar que la població catalana pot estar exposada a nivells moderats de deoxinivalenol, essent els nens i les persones amb patrons dietètics ètnics, els grups de població més exposats. Encara que la major part de la població no superava el TDI de 1 $\mu g\ kg^{-1}$ de pes corporal per dia^{-1}, encara hi ha una part de població que pot excedir aquest valor de seguretat.

- **Les fumonisines.** Malgrat les fumonisines es troben regularment en el mercat català, especialment en el menjar ètnic, la cervesa i els aperitius de panís, tots els nivells de concentració estaven lluny dels nivells màxims de la UE. El principal grup de risc

derivat de l'exposició a fumonisines es va estimar que eren els nadons, però els resultats suggereixen que no hi ha risc per a la salut derivat de FB en qualsevol grup de població.

- **La patulina.** La mitjana dels nivells de patulina en les mostres positives de suc de poma, menjar sòlid a base de poma i menjar per a nadons a base de poma van ser 8.05, 7.12 i 13.54 $\mu g\ kg^{-1}$, respectivament. Cap mostra va superar els nivells màxims establerts per la UE. Els nadons eren el grup més exposat a la patulina, però no es va detectar risc en aquests nivells de contaminació. Els adults i els nens estaven molt lluny dels nivells de seguretat.

- **Toxines T-2 i HT-2 toxines.** La toxina T-2 es va quantificar només en 5 de les mostres analitzades. La toxina HT-2 estava present en un baix percentatge de les mostres (6.2% en flocs de panís a 15.3% el pa de motlle), i no es va detectar en mostres de pa i cervesa. El grup de població més exposats a les toxines T-2 i HT-2 van ser els nens. Malgrat els inconvenients per establir l'exposició derivada les dades amb una gran proporció de valors censurats, hem trobat que un grup de població infantil pot excedir els límits de seguretat de 0.06 μg^{-1} kg de pes corporal dia^{-1}.

- **La zearalenona.** Els resultats van mostrar que la presència de ZEA en els aliments comercialitzats a Catalunya va ser baixa. El pa de motlle, els aperitius de panís i el panís dolç eren els aliments més contaminants. Tenint en compte els nostres resultats d'exposició, ja siguin del mètode NP-NP o del mètode P-P, el grup més exposat s'ha d'esperar que siguin els nens, especialment per als percentils alts, però en tots els casos les estimacions estaven lluny de la TDI de 250 ng kg^{-1} pes corpora i dia, establert per l'EFSA.

- **Presència simultània de micotoxines en els productes especials.** Els resultats van mostrar que les FBs es trobaven àmpliament distribuïdes en els aliments a base de panís comprats en botigues especialitzades en aliments d'importació. El DON es va trobar principalment en cuscús i alguns aliments ètnics a base de panís. Algunes mostres d'aliments sense gluten estaven contaminades per FBs (6 de 18) i DON (2 de 18), mentre que les aflatoxines i la ZEA només es van trobar en 6 mostres d'aliments ètnics. Tenint en compte aquests resultats, els aliments especials no haurien de suposar

un problema per la salut d'aquests col·lectius, però s'ha de centrar especial atenció en els aliments especials importats i distribuïts al detall en botigues de barri.

- **Efecte dels aliments de processament.** El DON es va mantenir estable durant el procés de panificació, tenint en compte la fermentació i l'enfornat. La ZEA va mostrar una lleugera disminució durant la primera fermentació però sense prou diferències estadístiques. Les principals disminucions es van trobar per al DON durant l'ebullició de la pasta, amb gairebé el 75% de la transferència de la pasta a l'aigua. Quan apliquem el factor de reducció en els models d'exposició, la reducció final va ser estadísticament significativa, però no suficient com per considerar el procés com a protector a causa de la baixa contribució de la pasta a la ingesta global del DON.

- **Biomarcadors d'exposició a FBs.** Els resultats van mostrar un major nivell de Sa i So en plasma que en orina, i es van trobar diferències significatives quan es van comparar els homes amb les dones. Quant a la relació Sa/So dels consumidors d'aliments de panís i dels no consumidors, es van trobar diferències significatives en les mostres d'orina i plasma en van comparar ambdós grups, però no es van trobar evidències del mecanisme d'acció de les fumonisines. A través de l'estudi temporal, s'ha determinat la jornada en quan es dóna l'alteració màxima de la relació Sa/So en humans. Tanmateix, es requereixen més estudis per comprendre millor la utilització d'aquest biomarcador amb la població humana, principalment, per millorar la precisió en els nivells baixos d'exposició.

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Future research

- Because occurrence of mycotoxins in raw foods is highly variable depending of multiple ambiental factors, subsequent exposure assessment studies should be performed in order to overseeing the safety and health protection of Catalonian citizens.
- Special attention should be focused on high risk mycotoxins as deoxynivalenol, T-2 and HT-2 toxins. Sampling, analytical and computations methodologies should be improved to get the best estimation of outputs.
- In the present study we have find the immigrants to be a risk group of exposure to DON, accordingly, future studies should take this collective into account to assess accurately their exposure.
- Sampling method could be improved including more highly consumed foodstuffs missed in the present work
- Analytical method should cover emergent mycotoxins and also, hidden forms of those mycotoxins highly susceptible to be conjugated.
- Biomonitoring of exposure to mycotoxins may largely contribute to estimate individual exposure scenarios, thus more studies are required to develop and apply biomonitoring methods in the exposure framework.

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Investigaciones futuras

- Debido a que la aparición de micotoxinas en los alimentos es muy variable dependiendo de múltiples factores ambientales, se deben realizar consecutivos estudios de evaluación de la exposición, con el fin de velar por la seguridad y protección de la salud de los ciudadanos catalanes.
- Se debería prestar especial atención a las micotoxinas de alto riesgo como son el deoxinivalenol, la toxina T-2 y la HT-2. El muestreo, análisis y metodologías de cálculos deben ser mejoradas para obtener una estimación precisa de los resultados.
- En el presente estudio se ha concluido que los inmigrantes son un grupo de riesgo de exposición al DON, en consecuencia, los estudios futuros deben tomar este colectivo en cuenta para evaluar con precisión su exposición.
- El método de muestreo se puede mejorar incluyendo algunos alimentos consumidos que no se consideraron en el presente trabajo.
- El método analítico debería de cubrir micotoxinas emergentes y también, formas ocultas de aquellas micotoxinas altamente susceptibles a conjugar.
- El biomonitoreo de la exposición a las micotoxinas puede contribuir en gran medida a estimar escenarios de exposición individual, con lo que más estudios son necesarios para desarrollar y aplicar métodos de biomonitoreo en el marco de la exposición.

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Investigacions futures

- Degut a que l'aparició de micotoxines en els aliments és molt variable depenent de múltiples factors ambientals, s'haurien de realitzar estudis consecutius d'avaluació de l'exposició per tal de vetllar per la seguretat i protecció de la salut dels ciutadans catalans.
- S'hauria de para especial atenció a les micotoxines d'alt risc com són el deoxinivalenol, la toxina T-2 i la HT-2. El mostreig, anàlisi i metodologies de càlculs han de ser millorades per obtenir una precisa estimació dels resultats.
- En el present estudi s'ha trobat que els immigrants són un grup de risc en l'exposició al DON, en conseqüència, els estudis futurs han de tenir en compte aquest col·lectiu per avaluar amb precisió la seva exposició.
- El mètode de mostreig es pot millorar incloent alguns aliments consumits que no es van considerar en aquest treball.
- El mètode analític hauria de cobrir les micotoxines emergents i també, formes emmascarades de micotoxines altament susceptibles a ser conjugades.
- El biomonitoreig de l'exposició a les micotoxines pot contribuir en gran mesura a estimar escenaris d'exposició individual, de manera que són necessaris més estudis per a desenvolupar i aplicar mètodes de biomonitoreig en el marc de l'exposició.

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Annex 1. Biomonitoring of *Fusarium* spp. mycotoxins: perspectives for an individual exposure assessment tool

Food Science and Technology International 2010 16, 266-76

Introduction

Fusarium species are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia (Creppy, 2002). Among the toxins formed are trichothecenes of the A-type, such as T-2 toxin (T-2), HT-2 toxin (HT-2), T-2 triol, T-2 tetraol, neosolaniol (NEO), di- and 15-monoacetoxyscirpenol (DAS, MAS), scirpentriol (SCIRP); trichothecenes of the B-type such as deoxynivalenol (DON), 3- and 15-acetylDON (3- and 15-ADON), nivalenol (NIV), fusarenon-X (FUS-X) as well as zearalenone (ZEA) and fumonisins (FB) (Gelderblom et al., 1988; De Nijs et al., 1996; Glenn, 2007).

Fumonisins

Fumonisins (FB) are a group of mycotoxins mainly produced by *F. verticillioides* and *F. proliferatum*, which usually contaminate corn. Among them, the most important are fumonisin B_1 (FB_1) and B_2 (FB_2) (Nelson et al., 1992). FB occur mainly in maize and maize-based foods, therefore populations with high maize consumption can be exposed to significant amounts of these mycotoxins through the ingestion of fumonisin contaminated maize (Marasas, 1996; Shephard et al., 1996; Visconti et al., 1996; WHO, 2001).

Toxicity of FB has been widely reviewed by Soriano et al. (2005), Voss et al. (2007), Stockmann-Juvala and Savolainen (2008) and Wan Norhasima et al. (2009). Human exposure to fumonisin contaminated commodities has been linked to esophageal and liver cancer in South Africa and China (Sydenham et al., 1991; Yoshizawa et al., 1994). Acute and chronic toxicity of FB has been largely demonstrated in several animal species, including carcinogenicity and cardiovascular toxic effects (Gelderblom et al., 1988, 1991). FB_1 is a cancer promoter but a poor cancer initiator. It is not genotoxic because FB_1 does not induce unscheduled DNA synthesis in primary rat hepatocytes (Norred et al., 1992). Based on toxicological evidence, the International Agency for

Research on Cancer (IARC) has classified FB_1 as possibly carcinogenic (group 2B) to humans (IARC, 1993). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated FB and allocated a provisional maximum tolerable daily intake (PMTDI) of 2 μg kg^{-1} body weight day^{-1} for FB on the basis of the non observed effect level (NOEL) of 0.2 mg kg^{-1} body weight day^{-1} and a safety factor of 100 (WHO, 2001). This PMTDI for FB has recently been confirmed by the Scientific Committee on Food (ECSCF; European Commission, 2003).

Zearalenone

Zearalenone (ZEA) is a nonsteroidal mycotoxin produced by *Fusarium* spp. that is found commonly in a number of cereal crops, such as maize, barley, oats, wheat, rice and sorghum, being most frequently encountered on corn (Kuiper-Goodman et al., 1987; Tanaka et al., 1988; Krska et al., 2003). ZEA and some of its metabolites have shown to competitively bind to oestrogen receptors. Thus, the toxicity is associated with reproductive problems in specific animals and possibly in humans (Wood, 1992). Fertility problems have been observed in animals such as swine and sheep (Krska et al., 2003). ZEA may be an important etiologic agent of intoxication in infants or fetuses exposed to this mycotoxin, with results in premature thelarche, pubarche and breast enlargement (CAST, 2003). Risk assessment of ZEA by the EC SCF concluded on a PTDI of 0.2 mg kg^{-1} body weight whereas the tolerable daily intake (TDI) established by JECFA was 0.5 mg kg^{-1} body weight (EFSA, 2004).

Type-A Trichothecenes: T-2 and HT-2 Toxins

T-2 toxin and HT-2 toxin are type-A trichothecene mycotoxins produced by different *Fusarium* species, that may contaminate a variety of cereal grains, especially in cold-climate regions or during wet storage conditions (Bottalico, 1998; WHO, 2001; SCOOP, 2003).

They have been widely studied in animals, but despite their toxic effects, the toxicology has never been assessed in humans. T-2 is a potent inhibitor of protein synthesis and mitochondrial function both *in vivo* and *in vitro* and shows immunosuppressive and cytotoxic effects. Moreover, it has been reported that the toxin has extremely toxic effects on skin and mucous membranes (Visconti et al., 1991; Visconti, 2001; Sudakin, 2003; Eriksen and Pettersson, 2004). In poultry, T-2 toxin has been reported to inhibit DNA, RNA and protein synthesis in eukaryotic cells, to affect the cell cycle and to induce apoptosis both in vivo and in vitro (Sokolovic et al., 2008). It has been shown that through deacetylation of T-2, HT-2 is obtained as major metabolite; however, little information is available regarding toxicity of HT-2 alone (Visconti, 2001; Sudakin, 2003). JECFA, after assessing the toxic effect of both mycotoxins, has concluded that the toxic effects of T-2 and HT-2 could not be differentiated. Thus, the PMTDI for these toxins, combined or separately, was set at 0.06 μg kg^{-1} body weight day^{-1} (FAO, 2001).

Type-B Trichothecenes: Deoxynivalenol (vomitoxin)

The mycotoxin deoxynivalenol (DON) is a type-B trichothecene, produced by molds of *Fusarium* genus, mainly *F. graminearum* or *F. culmorum* (Greenhalgh et al., 1986) when grown on various cereals crops (wheat, maize, barley, oat and rye). Although DON is not as toxic as other trichothecenes such as T-2 toxin, HT-2 toxin or fusarenon-X, this mycotoxin is one of the most common contaminants of cereals worldwide (Jelinek et al., 1989; Scott, 1989; IARC, 1993). Upon ingestion it can cause severe toxicosis in humans and farm animals. Acute effects of food poisoning in humans are abdominal pain, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea and blood in the stool (Rotter et al., 1996). The TDI of 1 μg kg^{-1} body weight based on a reduction of body weight gain (Iverson et al., 1995) was established by the EC SCF (SCF, 2002).

Biological markers as an exposure assessment tool for mycotoxins

Exposure assessment is the qualitative and/or quantitative evaluation of the likely intake of chemical agents via food as well as exposure from other sources if relevant (WHO, 1997). Thereby, through knowledge of the mycotoxin occurrence in the food and dietary habits of the population, we can quantify the mycotoxin dietary intake. To assess food consumption, four different types of data can be used: food supply data, data from householdconsumption surveys, data from dietary surveys among individuals and the collection of duplicate diets (Hulshof and Löwik, 1998). The current exposure assessment schemes are largely deterministic and uncertainty and/or variability issues are accounted for by means of cautionary measures which are implicitly embedded in calculation schemes and rules (Verdonck et al., 2005). More recently, probabilistic methods as Monte Carlo simulations have been developed to quantify the sources of uncertainty and variability of human exposure (Verdonck et al., 2006). Although these methods are suitable to assess the exposure of populations to contaminants and to identify risk groups, they are not recommended to evaluate the individual exposition due to a low accuracy and sensitivity. The use of biochemical indicators has been proposed as a suitable method to assess individual exposure to contaminants. The WHO defined in 1993, a biomarker as 'any parameter that can be used to measure an interaction between a biological system and an environmental agent, which may be chemical, physical or biological'. This method allows effective exposure considering variability among food contamination levels, cooking effect, individual consumption, variations in toxicokinetics or toxicodynamics (WHO, 1993; Paustenbach and Galbraith, 2006). Among the potential valuable application of biomarkers in epidemiologic studies and in clinical trials, there is the possibility of measuring them earlier than the observed true endpoint of interest, given their property of relating the effect of exposures or treatments on cellular and molecular changes to the true endpoint/outcome (Merlo et al., 2006). Interpretation of biomarkers of effect is hampered by lack of knowledge on the metabolism of most non-nutrients and their mechanisms of action in humans *in vivo*. Before a biochemical indicator can be used as a measure of dietary intake, it must be

evaluated with respect to its sensitivity to the intake of those contaminants. If these indicators are to be used as measures of dietary exposure, however, the epidemiologist is obviously responsible for ensuring that the exposure measure is a valid representation of long-term intake. Several strategies are available to define the relationships between longterm dietary intake and biological levels: (i) animal studies; (ii) geographic correlation of intake and biological marker; (iii) correlation with individual intake; (iv) dietary manipulation in humans and (v) repeated measures (Walter, 1998). Regarding biomonitoring of mycotoxin intake, successful results have been reported about biomarkers of ochratoxin A and aflatoxins. Higher levels of ochratoxin A have been found in blood samples from people with kidney or urinary disorders than in healthy people, showing good correlation among dietary intake and blood levels of this toxin (Scott, 2005). The use of serum aflatoxin B_1-albumin adducts as biomarkers of aflatoxin exposure has been validated in experimental and human sample analyses (Wild et al., 1990a, b, 1992). The use of urinary aflatoxin B_1-N7-guanine adduct validated in the laboratory with human samples, provides a measure of acute exposure to aflatoxin B_1 (AFB_1) and reflects a relatively short-term (24-48 h) exposure (Groopman et al., 1992a, b, 1993). Another problem to conduct an accurate exposure assessment is the presence of conjugated forms of mycotoxins, known as 'masked' mycotoxins. More important 'masked' mycotoxins have been reported to be produced by *Fusarium* species. For example, more common mycotoxin conjugation products in mammals are glucuronides, as found in ZEA-4-glucoside and DON-3-glucoside. These conjugated metabolites are usually stable under extraction conditions, maintaining the capability to produce toxic effects (Berthiller et al., 2009). Unfortunately they cannot be detected through routine analysis making necessary alternative methods.

Biomonitoring Exposure to Fumonisins

Methods to biomonitor the exposure to FB have been reviewed previously in several cases (Turner et al., 1999; Shephard et al., 2007). Mainly, two analytical procedures have been reported as fumonisin biomarker: fumonisin B_1 and sphingoid

bases ratios.

Fumonisin B_1 as Biomarker

Absorption, distribution and excretion of FB_1 have been widely studied in several animal species including rats, laying hens, vervet monkeys, swine or piglets (Prelusky et al., 1994; Shephard et al., 1994a, b; Fodor et al., 2006, 2008) and FB_2 toxicokinetics has been studied in rats and vervet monkeys (Shephard et al., 1995; Shephard and Snijman, 1999). These toxicokinetics studies have shown that FB_1 had low oral bioavailability, with values ranging from 3% to 6% (Prelusky et al., 1994; Fodor et al., 2008) and short half-life when dosedintraperitoneally or intravenously. Half-life in rats has been reported at 18 min and 40 min in vervet monkeys and estimated by regression analysis in 70 kg human as 128 min giving an animal's weight to fit the prediction (Shephard et al., 1992; Delongchamp and Young, 2001). As FB_1 is mainly excreted in faeces, HPLC with fluorescence detection method was initially developed as suitable tool to exposure assessment to FB. Determination of FB_1 in faeces was applied on exposure assessment of rural and urban populations from South Africa with mean fumonisin levels in maize for consumption of 2.2 and 0.3 $\mu g\ kg^{-1}$, respectively. Results showed significant differences among FB_1 concentrations in rural and urban feces ($p<0.014$). Considering faecal samples were taken 24 h after maize consumption, FB_1 could be expected to be a suitable short-term biomarker of this toxin exposure (Chelule et al., 2000). Moreover, FB have been detected in human hair using LC-MS-MS analytical method. Hair samples were obtained from South African population highly exposed to fumonisin with probable daily intake for 70 kg individuals of 13.8 $\mu g\ kg^{-1}$ body weight per day. Results showed that mean values of FB_1 ranged from 33.0 to 22.2 $\mu g\ kg^{-1}$ hair, with maximum values of 93.5 $\mu g\ kg^{-1}$ hair, concluding that human hair analysis could be an useful tool to measure the cumulative exposure to FB (Sewram et al., 2003). Urinary FB_1 have been reported recently as a sufficiently sensitive tool to assess the human exposition to FB. A liquid chromatograph-mass spectrometry method and extraction on Oasis MAZ

cartridges was performed to determine urinary FB_1. Urinary FB_1 was correlated with maize intake ($p<0.001$) and the correlation remained significant after adjusting for age, education and place of residence (Gong et al., 2008).

Sphingoid Base Levels and Ratios in Plasma

Due to rapid elimination and low bioavailability of FB, an indirect indicator of human exposition to these toxins has been required. FB have a remarkable structural similarity to sphingolipids (Merrill et al., 1996; Riley et al., 2001). This group of mycotoxins, especially FB_1 potently inhibits the enzyme ceramide (CER) synthase, which catalyzes the acylation of sphinganine and reacylation of sphingosine. The inhibition of CER synthase by FB increases the intracellular sphinganine concentration, process described as the main contributor to the toxicity and carcinogenicity of FB_1 (Wang et al., 1991; Merrill et al., 1993; Yoo et al., 1996; Riley et al., 2001). Based on this biological perturbation, particularly elevation of sphinganine (Sa) to sphingosine (So) or Sa 1-phosphate to So 1-phosphate ratios in tissues, urine and blood, have been proposed as potential biomarkers of fumonisin exposure in various animal species (Wang et al., 1992; Riley et al., 1993; Morgan et al., 1997; Wang et al., 1999; Van der Westhuizen et al., 2001; Kim et al., 2006; Tran et al., 2006; Cai et al., 2007). This biomarker was validated initially in Wistar rats by Solfrizzo et al. (1997) and recently in F344 rats by Cai et al. (2007), obtaining more sensitive results in urine than in serum for acute and sub-chronic exposure to FB_1. Furthermore, several studies have been conducted to assess the effectiveness of this biomarker on human population without successful results to obtain an accurate validation due to the low sensitivity when applied to individuals (Van der Westhuizen et al., 1999, 2008; Abnet et al., 2001; Qiu and Liu 2001; Solfrizzo et al., 2004; Missmer et al., 2006; Nikiema et al., 2008). Van der Westhuizen et al. (1999) initially conducted a study to assess Sa : So ratio in human plasma and urine from three different populations from Africa (Centane, n=154; Bomet, n=29 and KwaZulu-Natal, n=27) with mean fumonisin intake of 3.8, 0.06 $\mu g\ kg^{-1}$ body weight day^{-1} and nondetected levels, respectively. Despite these differences among exposures,

nonsignificant differences in Sa : So ratios were found, showing mean levels of serum ratios of 0.34, 0.43 and 0.28 in Centane, Bomet and KwaZulu-Natal population and urinary ratios of 0.41 and 0.38 in Centane and Bomet. More recently, they conducted a cross-sectional study in two areas from the same region of South Africa (Bizana, n=150 and Centane, n=152), concluding that although significant and contrasting differences in plasma and urinary sphingoid base levels in the areas were observed, there was no significant difference in the mean total fumonisin levels in the maize consumed, mean plasmatic ratio and urinary ratio from Bizana population (Van der Westhuizen et al., 2008). Croatia is a country located in the region affected by endemic nephropathy (EN), chronic renal disease geographically restricted to several European Eastern countries. Ribar et al. (2001) conducted a study to determine the possible modifications in the concentrations of urinary and serum Sa, So and Sa : So ratio of healthy subjects and EN patients from EN endemic area in Croatia. Eighty-nine serum samples and 30 urine samples were obtained from men and women affected (n=1), suspected (n=7) or at risk (n=12) to EN as well as healthy (n=27) and control from nonendemic area (n=20). Sphingolipids were extracted from serum and urine according to the method of Riley et al. (1994). Results showed nonstatistically significant difference in the serum Sa : So ratio in either men or women from the endemic area as compared with the control group of subjects. While urinary Sa : So ratio was found to differ significantly in the male group of healthy, suspected and affected people, among women it differs significantly in subjects at risk and suspected to EN. The authors did not report conclusions but suggested that study subjects could be presumed to have been exposed to FB and sphingolipid metabolism impairment could be postulated as an early indicator of EN (Ribar et al., 2001). Mexico is one of the most important countries regarding maize consumption. Human consumption is approximately 300 g day^{-1} providing 56% of the calories. Population of 38 Mexican volunteers (categorized with different maize based food consumption level: high, medium and low) participated in a trial to determine urinary Sa : So ratio. Urine samples were collected at three stages: A) at the beginning of the experiment with normal diet, B) after two weeks without consumption of any type of maize based food and C) one week after the re-assumption of normal maize based

food consumption. Urine samples were analyzed according to methodology described by Solfrizzo et al. (1997). Results showed that there were no significant differences among the groups in the estimated mean fumonisin intake and the Sa : So ratio. Sa : So ratio was significantly higher during exposed stage A and C, with respective mean fumonisin intake of 6 and 5.1 μg kg^{-1} bw day^{-1} than Sa : So ratio obtained during nonexposed period B (Landeros et al., 2005). Other study was performed in China with 15 females and 13 males exposed to FB_1 in corn diets over 1 month to analyze So and Sa in human urine and monitor the Sa : So ratio. The estimated daily FB_1 intake was ranged between 0.4 and 457 μg kg^{-1} body weight day^{-1} in females and between 0.5 and 740 μg kg^{-1} body weight day^{-1} in males. Urinary Sa : So ratio did not change over the month in females (0.2 initially and 0.18 at the end), while mean urinary ratio increased from 0.11 to 0.21 in males. However, it could be ascribed to a single participant with a high value, as they reported (Qiu and Liu, 2001). Solfrizzo et al. (2004) assessed urinary sphingoid bases of population from northern Argentina (n=74) and southern Brazil (n=116) as exposed population with mean fumonisin intake of 0.56 μg kg^{-1} body weight day^{-1} and urinary sphingoid bases of population from southern Italy (n=66) and central Argentina (n=20) with low or no fumonisin exposure (control group). Mean Sa : So ratio in regions with exposure to FB was 1.24, significantly higher than regions without exposure, where the Sa : So ratio was 0.36. However, mean Sa : So ratio from northern Argentina was 0.69, not significantly different from the control population and significantly lower than the value 1.57 showed in the southern Brazil population. Therefore, the highest value obtained in southern Brazil cannot be associated to fumonisin exposure, existing with other confounding factors. Moreover, the ratios have been assessed in simultaneous matrices as buccal cells, urine and serum in population from Burkina Faso, without showing any association between urinary Sa : So ratios and fumonisin intake, but suggesting a positive trend between fumonisin intake and Sa : So ratios in serum (Nikiema et al., 2008). Latest study was performed to assess Sa : So ratio and frequency of detection in urine samples from urban and rural population from Portugal. A total of 68 urine samples were collected from male and female adult healthy volunteers from

urban (n=38) and rural (n=30) zone. Optimized extraction method, based on the procedures described by Castegnaro et al. (1996, 1998) and Qiu and Liu (2001), followed by derivatization with naphtalene-2,3-dicarboxyaldehyde (NDA) and injection to HPLC-FD system, was carried out to detect and quantify urinary Sa and So. Sa : So ratio was between 0.11 and 0.95, with a mean value of 0.43±0.22. Significant differences were not found when the results of Sa, So and Sa : So ratio of males, females as well as combined (males and females together) were compared between rural and urban population (Silva et al., 2009). In our latest study (unpublished data), performed with two exposure groups from the same region (exposed and nonexposed group from Catalonia, (Spain), significant differences were observed among mean plasma Sa : So ratios. Results showed significant differences in sphingosine levels in groups considering both sexes combined or among males ($p<0.05$), while no significant differences were observed in females between groups ($p>0.05$). Thus these results suggest that the decrease of the ratios could be due to a decrease of sphingosine level, as should be expected according to the mechanism of action. However, wide ranges of Sa : So ratios and bad correlation coefficients were observed when linear regression was fitted, which suggests that this biomarker is low sensitive and imprecise to apply over individuals. Further studies are required to better understand all physiological factors that lead to Sa : So ratios variations as reported by Abnet et al. (2001) as well as biochemical processes that can modify sphingoid metabolism as extensive cell death, metabolization by other bioactive molecules or alteration by other components of the diet (Merrill et al., 2001). Other main problem is the lack of information about sphingoid bases basal levels in tissues, urine and blood of healthy human population.

Biomonitoring Exposure to DON

Absorption, distribution, accumulation, metabolism and elimination of DON have been reported in a wide range of animal species. Toxicodynamic studies have shown low absorption in poultry (<1%), in sheep ranged 61% and at least 29% was absorbed by dairy cows when DON toxin was administered; on the other hand, high

absorption has been estimated in swine (47-82%). Plasma elimination of DON tended to be slower in pigs, taking approximately 7 times longer than sheep, 2 times longer than cow and slightly longer than laying hens to clear the toxin after a single oral dose. Numerous studies have reported that swine is very sensitive to DON in contaminated feedstuffs. The distribution characteristics of DON in swine are also different than in other species. Only a small proportion of the dose can be found in the blood, although the toxin is extensively absorbed (Yoshizawa et al., 1981; Prelusky et al., 1985, 1986, 1988, 1994; Friend et al., 1986). DON and DON-glucuronide excretion in the urine represented 37% and 50% of the ingested DON in rats (Meky et al., 2003) and swine respectively (Goyarts and Danicke, 2006). Goyarts and Danicke (2006) have confirmed that not all animals are able to detoxify DON to the metabolite de-epoxy-DON and that this metabolism occurs principally in the large intestine, where unlikely absorption proceeds. Furthermore, it was shown that quantitative urinary recovery of DON can be considered as an indicator for its systemic absorption, as it approximates the bioavailability as estimated by the kinetic study. Assuming a high comparability of digestion and excretion in humans and swine, they concluded that although DON is poorly detoxified, it is rapidly excreted and so is not found in remarkable concentrations in serum after 24 h. Regarding human population, an earlier study was performed to develop, to validate and to measure urinary concentrations of DON and its metabolites in 15 females from Henan (Linxian) region, where the staple diet was based on corn and wheat (high-risk region of esophageal cancer, n=11), or rice (low-risk region of esophageal cancer, n=4). The mean levels of DON detected in the samples from high-risk and low-risk areas were 37 ng mL^{-1} and 12 ng mL^{-1}, respectively. Through these values and specific assumptions regarding excretion, urine production and recoveries, the authors estimated a daily exposure ranged from 1.9 to 13.0 and 0.6-2.5 mg kg^{-1} day^{-1} for high- and low-risk population respectively, in the line of previous studies that have been estimated this exposure through classic methods (Meky et al., 2003). Urinary DON was widely surveyed in a large-scale study conducted in UK and compared with cerealbased food intake (Turner et al., 2008a). Three population groups were selected according to low, medium or high cereal intake, estimated previously through 7-day weighted food

diary. From each group, 100 individuals were selected and urinary samples were collected during the period on the basis of available data in the 7-day diary that was provided. DON was detected in 296 of 300 (98.7%) urine sample, with geometric mean of 9.42 mg DON day^{-1} (nd-65.97 μg day^{-1}). Cereal intake was significantly associated with urinary DON ($p<0.0005$), showing mean levels of 6.55, 9.63 and 13.24 μg day^{-1} in low, medium and high cereal intake groups, respectively. The food groups associated with urinary DON were predominantly wheat based, particularly the three main bread groups (white, wholemeal and other bread). A crude estimation was made based on: (i) the amount of urinary DON, (ii) an assumption that 50% of the ingested DON was being excreted in the urine (Meky et al., 2003; Goyarts and Danicke, 2006) and (iii) the urinary DON originated from DON intake in the previous 24 h. For the 300 individuals the mean intake was estimated as 319 ng kg^{-1} body weight day^{-1}, below TDI for DON ingestion of 1 mg kg^{-1} body weight (SCF, 2002) and slightly higher than previous estimation that showed DON daily intakes of 176 and 142 ng kg^{-1} body weight day^{-1} for males and females respectively (SCOOP, 2003). Briefly, more detailed analysis of these data will be published. In this report, food diary information (n=255) for (a) the day of urine collection, (b) the previous 24-h period and (c) the day of urine collection plus the previous 24 h combined, were further examined to assess whether the recent intake of cereal correlated more strongly with urinary DON, compared with (d) the longer term assessment of usual cereal intake from 7-day food diaries. Results suggest that the inter-individual variation in urinary DON was somewhat better explained by recent cereal intake compared with usual cereal intake assessed over 7 day (Turner et al., 2009). An intervention study was conducted to assess the effect of wheat-restricted diet over DON urinary levels. The study was performed with 25 healthy adult volunteers and involved 2 days of normal diet and 4 days of a wheat-restricted diet. Food diaries were kept for normal diet days and for the two latest days of intervention diet. Initial morning urinary samples were collected the following day of each period. Samples were analyzed and adjusted using a creatinine concentration in mg mL^{-1} of urine and subsequent data were expressed as ng DON mg^{-1} creatinine. Results showed that during intervention diet

period there was a low percentage of detected samples (36%) while during normal diet all samples had detectable levels of DON. Mean levels and ranges were 1.0 (nd-8.4) and 10.8 (0.7-61.3) ng mL^{-1} for intervention and normal diet respectively, results in the line of previous study conducted in UK (Turner et al., 2008b). Recently, another intervention trial conducted with 22 urine samples from UK volunteers to correlate urinary DON level with one or more metabolite in the urine was published. A1H-Nuclear Magnetic Resonance-based chemometrics approach (metabolomics) was utilized to examine samples from individuals eating a normal diet. Urinary DON was determined using an in-house immunoaffinity-LC-MS assay (Turner et al., 2008b). Model was built on 16 individuals, eight with low urinary DON and eight with high urinary DON level; and validated with a further six urine samples, of which there were three in each category of DON level. Through statistical analysis two possible biomarkers were identified: hippurate and mannitol, the first one being the more interesting candidate (Hopton et al., 2010). Turner et al. (2008c) emphasized urinary DON as a good tool to assess exposure to this contaminant at the individual level. In contrast, they reported several uncertainties to resolve the full validating of this biomarker to apply in epidemiological studies. Their questions were: (a) what is the relationship at the individual level between DON intake and the urinary biomarker; (b) what are the pharmacokinetics of DON and DON-glucuronide excretion and what are the consequent temporal variations in this biomarker; (c) Does the ratio of DON to DON-glucuronide in urine vary by individual Therefore, they concluded that in humans, such studies require validated methods of exposure assessment to compare exposure to toxins both individually and in combination with health outcomes (Turner et al., 2008c).

Biomonitoring the Exposure to ZEA

Absorption of ZEA has been reported as extensive and quick in rats and rabbits (Kuiper-Goodman et al. 1987; Ramos et al. 1996), being estimated in the range of 80-85% in pigs (Biehl et al., 1993). In mammals, zearalenone is mainly metabolized into α-zearalenol (α-ZEA) and β-zearalenol (β-ZEA), while the first is the most predominant

in pigs, the second is the most predominant metabolite in cows (Jodlbauer et al., 2000; Kleinova et al., 2002; Zöllner et al., 2002). Earlier studies of Ueno et al. (1983) showed that there are two types of ZEA reductase differing in optimum pH. In humans as in pigs, ZEA probably can be absorbed after oral administration and can be metabolized in intestinal cells into α-ZEA and β-ZEA, and would be excreted significantly in bile and urine (Döll et al., 2003).

In a previous study conducted with one male volunteer, 100 mg of ZEA were administered and α-ZEA and β-ZEA concentrations were determined in the urine at 6, 12 and 24 h after the administration. The concentrations of ZEA, α-ZEA and β-ZEA were 3.7 and 3 μg mL^{-1} and not detected after 6 h; 6.9, 6 and 2.7 μg mL^{-1} after 12 h; and 2.7, 4 and 2 μg mL^{-1} after 24 h (Mirocha et al., 1981).

Furthermore, ZEA and its metabolites were studied in serum from 32 girls affected by central precocious puberty (CPP) and in 31 healthy female. Results showed increased serum levels of ZEA and α-ZEA in 6 girls with CPP. ZEA levels correlated with patient height and weight. The authors concluded that ZEA is suspected to be a triggering factor for CPP development in girls, and may also represent a growth promoter in exposed patients (Massart et al., 2008).

ZEA dietary intake was estimated by JECFA to European region reporting ranges of 0.004-0.029 and 0.006-0.055 μg kg^{-1} body weight per day for adults and infants respectively (CAST 2003). Despite the high consumption of cereals in European countries, few studies have been conducted to assess the exposure to this mycotoxin, neither through conventional method nor biomarkers, thus more studies are required to accurately characterise the risk of this endocrine disruptor and confirm it as a dangerous problem for human health (Minervini et al., 2005).

Biomonitoring the Exposure to T-2 and HT-2 Toxins

T-2 toxin is more rapidly absorbed than DON after its ingestion by most species,

its plasmatic half-life being less than 20 min. The fraction of T-2 toxin eliminated as parent compound in the urine was showed as negligible. In spite of administration of a lethal oral dose in swine (2.4 mg kg^{-1}) and toxic oral doses (up to 3.6 mg kg^{-1}) in calves, no parent T-2 toxin was detected in plasma or urine (Beasley et al., 1986; Larsen et al., 2004). T-2 toxin can be detected in pig blood before 30 min after their ingestion (Eriksen et al., 2004). The main reactions in trichothecene metabolism are hydrolysis, hydroxylation and deep oxidation. Typical metabolites of T-2 toxin in an organism are HT-2 toxin, T-2-triol, T-2-tetraol, 3-pm-hydroxy-T-2, and 3'-hydroxy-HT-2 toxin. There are significant differences in the metabolic pathways of T-2 toxin between ruminants and non-ruminants. Ruminants are more resistant to the adverse effects of T-2 toxin due to microbial degradation within rumen microorganisms (Dohnal et al., 2008). The patterns of distribution and excretion suggest that T-2 toxin and/or its metabolites are excreted into the intestine through the bile and that the liver is a major organ for excretion of the toxin (Chi et al., 1978). No studies have been conducted with humans until now to assess the presence of this toxin or its metabolites in biological fluids.

Conclusions

Conventional methods of exposure assessment are based on the combination of food analysis data with dietary intake data. That combination of data can be deterministic or probabilistic, however, in both cases have been proven to be limited, due to low sensitivity and accuracy. A useful method to assess effective exposition of human populations to contaminants could be through the study of the effect on biological molecules or monitoring these toxins directly on biological fluids. These biological markers, known as biomarkers, allow the assessment of exposure of human populations to mycotoxins considering the variability within dietary intake, cooking effect, intestinal absorption, metabolism or distribution over individuals. Thus, the understanding of mechanism of action, toxicokinetics and toxicodynamics of the mycotoxins, is required to develop useful biomarkers.

Successful methods have been developed to biomonitor exposure to ochratoxin A and

aflatoxins, however few studies and unsuccessful results have been obtained with biomarkers of *Fusarium* toxins exposure. Urinary levels of fumonisins have been reported as an effective method to assess short-term intake of this toxin. Despite the ratio Sa:So has been validated as biomarker of fumonisin exposure in animal species, unsuccessful results have been reported among human populations. Further studies are required to understand accurately basal levels of these contaminants, interactions with other contaminants or variability sources.

Regarding DON, several studies have reported data on absorption, toxicokinetics, toxicodynamics and metabolism in animals, but few studies have been conducted in human populations. Urinary level of DON has been used as biomarker to assess the exposure of human populations showing successful results, with positive correlations among estimated dietary intake of the toxin and urinary levels. In despite of the interest of researchers in zearalenone contamination of food and its toxicity in animal species, very few studies have been conducted to assess the real impact on human population. Finally, no studies have been conducted to assess possible biomarkers to assess the exposition to T-2 toxin or HT-2 toxin.

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Annex 2. Regulations of mycotoxins

Maximum levels in foods intended for human consumption

Aflatoxins

Commission Regulation (EC) Nº 165/2010 of 26 February 2010 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins.

Foods	Levels AFB_1 μg kg^{-1}	Levels sum AF ($B_1+B_2+G_1+G_2$) μg kg^{-1}
Almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in foodstuffs	8	10
Hazelnuts and Brazil nuts, intended for direct human consumption or use as an ingredient in foodstuffs	5	10
Tree nuts, other than the tree nuts listed above, and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2	4
Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2	4
All cereals and all products derived from cereals, including processed cereal products	2	4
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 fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) Mixtures of spices containing one or more of the abovementioned spices	5	10
Processed cereal-based foods and baby foods for infants and young children	0.10	-

Aflatoxin M_1

Commission Regulation (EC) Nº 165/2010 of 26 February 2010 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins.

Foods	Levels μg kg^{-1}
Raw milk, heat-treated milk and milk for the manufacture of milk-based products	0.050
Infant formulae and follow-on formulae, including infant milk and follow-on milk	0.025
Dietary foods for special medical purposes intended specifically for infants	0.025

Fumonisins

Commission Regulation Nº 1126/2007 of 28 September 2007 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products.

Foods	**Levels** $\mu g\ kg^{-1}$
Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exception of foodstuffs listed in ([a])	1000
[a] Maize-based breakfast cereals and maize-based snacks	800
[a] Processed maize-based foods and baby foods for infants and young children	200

Patulin

Commission Regulation (EC) Nº 401/2006 Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. OJEU. L70,12-34.

Foods	**Levels** $\mu g\ kg^{-1}$
Fruit juices, concentrated fruit juices as reconstituted and fruit nectars	50
Solid apple products, including apple compote, apple puree intended for direct consumption	25
Apple juice and solid apple products, including apple compote and apple puree, for infants and young children	10
Baby foods other than processed cereal-based foods for infants and young children	10

A-type trichothecenes. T2 and HT2 toxins

Despite of the European Commission recently asked to the EFSA for a scientific opinion on the risk to human and animal health related to the presence of T-2 and HT-2 toxin in food and feed, in order to assess if the combined t-TDI was still appropriate, the maximum levels in foodstuffs have not been set yet to date.

B- type trichothecenes. Deoxynivalenol.

Commission Regulation (EC) Nº 401/2006 Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. OJEU. L70,12-34.

Foods	**Levels** $\mu g\ kg^{-1}$
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of ([b])	750
Pasta (dry)	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
[b] Processed cereal-based foods and baby foods for infants and young children	200

Zearalenone

Commission Regulation (EC) Nº 401/2006 Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. OJEU. L70,12-34.

Foods	**Levels** $\mu g\ kg^{-1}$
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs ([c])	75
Refined maize oil	400
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
[c] Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
[c] Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
[c] Processed maize-based foods for infants and young children	20

Risk characterization

Mycotoxin	Nomenclature	Safety level (ng kg^{-1} bw day^{-1})	Reference
Dexynivalenol	TDI	1000	SCF, 1999
Fumonisins	TDI	2000	SCF, 2003
Patulin	PMTDI	400	WHO, 1995
T2 and HT2	TDI	60	SCF, 2001
	TDI	1000	EFSA, 2011b
Zearalenone	PMTDI	500	EFSA, 2004
	TDI	250	EFSA, 2011a

TDI: Tolerable Daily Intake; PTDI: Provisional Tolerable Daily Intake

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