Mycotoxins: presence and stability during processing of cereal based food

Arnau Vidal Corominas

Universitat de Lleida

TESI DOCTORAL

Mycotoxins: presence and stability during processing of cereal based food

Arnau Vidal Corominas

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia Agroalimentària

Director/a
Sonia Marín Sillué
Vicent Sanchis Almenar

Tutor/a
Vicent Sanchis Almenar

2017

Acknowledgements/Agraïments/Agradecimientos

- A l'Antonio, a la Sònia i al Vicent que m'ho han ensenyat tot sobre la recerca i m'han ajudat a crèixer com a persona. Gràcies per a tots els consells que m'heu donat.

- To Sarah de Saeger and Marthe de Boevre to permit me to be in your lab and for make me feel like at home during my stay in Ghent. To all the people that I met during my Belgium experience.

- A tots els membres del TECAL que hem compartit bons moments durant aquesta anys i m'han ajudat en tot.

- A la Sílvia per l'enorme paciencia que ha tingut, per les estones compartides i per les aventures que queden per viure.

- Als pares per ser qui sóc. En Roger per transmetre calma i a la tia per fer de segona mare.

Abbreviation list

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFB2a	Aflatoxin B2a
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
Afs	Aflatoxins
ALOP	Appropriate Level of Protection
CAC	Codex Alimentarius Commission
CCPs	Critical control points
CE	Collision energy
CXP	Cell exit potential.
DOM-1	Deepoxy-deoxynivalenol
DON	Deoxynivalenol
15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
DON-15-glucuronide	Deoxynivalenol-15-glucuronide
DON-3-glucoside	Deoxinivalenol-3-glucoside
DON-3-glucuronide	Deoxinivalneol-3-glucuronide
DON-7-glucuronide	Deoxynivalenol-7-glucuronide
DP	Declustering potential
EC	European Commission
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organisation of the United Nations
Fbs	Fumonisins
FLD	Fluorescence
FSO	Food Safety Objective
GAP	Good Agricultural Practices
HACCP	Hazard Analysis and Critical Control Points
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity chromatography
IARC	International Agency for Research on Cancer
ICMSF	International Commission on Microbiological Specifications for Foods
isoDON	Isodeoxynivalenol
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LOD	Limit of Detection

LOQ	Limit of Quantification
MS	Mass spectrometry
MS/MS	Double mass spectrometry
NIV	Nivalenol
norDON A	nor-deoxynivalenol A
norDON B	nor-deoxynivalenol B
norDON C	nor-deoxynivalenol C
norDON D	nor-deoxynivalenol D
norDON E	nor-deoxynivalenol E
norDON F	nor-deoxynivalenol F
norDON-3-glucoside A	nor-deoxynivalenol-3-glucoside A
norDON-3-glucoside B	nor-deoxynivalenol-3-glucoside B
norDON-3-glucoside C	nor-deoxynivalenol-3-glucoside C
norDON-3-glucoside D	nor-deoxynivalenol-3-glucoside D
norDON-3-glucoside lactone	nor-deoxynivalenol-3-glucoside lactone
OTA	Ochratoxin A
OTα	Ochratoxin alpha
PBS	Phosphate Buffered Saline
PDI	Probable Daily Intake
POs	Performance Objectives
RSDr	Relative Standard Deviation
SCF	Scientific Committee on Food
TDI	Tolerable Daily Intake
TFMSA	Trifluoromethanesulfonic acid
TWI	Tolerable Weekly Intake
UV	Ultraviolet
WHO	World Health Organisation
ZAN	Zearalanone
ZEN	Zearalenone
ZEN-14-glucoside	Zearalenone-14-glucoside
α-ZAL	alpha-zearalanol
α-ZEL	alpha-zearalenol
β-ZAL	beta-zearalanol
β-ZEL	beta-zearalenol

Index

1. Summary/Resum/Resumen

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. The most important producing genera are *Aspergillus*, *Fusarium* and *Penicillium*. Mycotoxins can be present in a wide range of products and their intake is of concern because they can produce a wide range of harmful effects to human and animal. Cereal products represent one of the main sources of exposure to mycotoxins. Several mycotoxins have been identified up to the present, but those of special interest in food and feed safety are: aflatoxins (B1, B2, G1 and G2), fumonisins (Fbs), ochratoxin A (OTA), patulin, trichothecenes (deoxynivalenol and nivalenol), and zearalenone (ZEN). However, unaltered mycotoxins might not be the only threat for health consumers, because they can be present in conjugated forms which cannot be detected in the routinary mycotoxins analysis, but they can become as dangerous as parent mycotoxins.

The objectives of this thesis were determine the presence of some mycotoxins in cereal products, study the stability of them during the food process and assess the mycotoxin exposure. To reach these aims different studies have been done. Firstly, cereals and cereal based food commercial samples have been analysed to study the presence of aflatoxins (Afs), deoxynivalenol (DON), nivalenol (NIV), ZEN, OTA and some of them conjugated mycotoxins. Bread making and pasta making processes have been deeply studied and some factors have been evaluated to observe the stability of mycotoxins. Mycotoxins exposure has been assessed through two different methods: 1) the combination of contamination data with consumption data and 2) using biomarkers.

All mycotoxin analyses have been conducted using liquid chromatography, by ultraviolet, fluorescence and mass spectrometer detectors were used in different studies. Mass spectrometer detectors were handled when conjugated mycotoxins were searched in the different matrixes. Moreover, some new analytical methods have been developed successfully to improve the detection of mycotoxins.

Analysis of cereals and cereal based foods showed that mycotoxins are very prevalent. Trichothecenes have showed the highest presence and also the largest concentrations. Thus, some bran analysed samples had DON levels over the limit set by the European legislation. Although DON and NIV were the most common analysed mycotoxins, the rest of the mycotoxins also showed a worrying high presence. More controls on conjugated mycotoxins should be done, because results showed their common presence in the analysed products and in some cases even the concentration of conjugated mycotoxins was higher than parent mycotoxins.

Although mycotoxins are considered extremely stable compounds, food processes can cause changes in mycotoxin concentration. Bread fermentation is a complex step where many factors play a role. OTA concentration does not change in concentration during fermentation. Unlike OTA, DON is highly affected by this step (it may either decrease or increase). Actually its fate depends on several factors: temperature, time, enzyme and sourdough presence. Similar to DON, DON-3-glucoside also depends on the same factors as DON during fermentation.

Although OTA is more stable than DON and its conjugates during baking, all of them can be reduced in function of some factors as: time, temperatures, enzyme presence and heat transmission. The DON reduction observed in boiled pasta was caused by DON transfer from pasta to broth due to his high solubility. Kinetics to anticipate the mycotoxins fate at the end of food processes have been built as a useful tool for food industry with a good fit ($r > 0.75$).

Because of the important levels of DON found in cereal bran analysed, exposure assessment of DON through bran consumption was calculated. Therefore, fibre supplements intake can be an important source of DON. Biomarkers of DON and OTA were studied through plasma and urine analysis and the results revealed that the exposure to DON and OTA could be of concern for the Catalonia population. Even, a high exposure to DON could be held throughout the time by the same person, exceeding the tolerable daily intake systematically instead of eventually.

1.2. Resum (Catalan)

Les micotoxines són metabòlits secundaris produïts per fongs que poden contaminar diferents productes agrícoles abans o després de la collita. Els gèneres més importants de fongs productors de micotoxines són *Aspergillus*, *Fusarium* i *Penicillium*. Les micotoxines poden estar presents en una gran varietat de productes i la seva ingesta pot ser de gran perillositat perquè produeixen una gran varietat d'afectes perjudicials per la salut dels humans i animals. Els productes a base de cereals representen una de les principals fonts d'exposició a les micotoxines. Varies micotoxines s'han identificat fins a dia d'avui, però aquelles micotoxines amb major interès per la seguretat dels aliments i el pinso són: les aflatoxines (B1, B2, G1 i G2), les fumonisines (Fbs), la ocratoxina A (OTA), la patulina, els tricotecens (deoxinivalenol i nivalenol) i la zearalenona (ZEN). Però, les micotoxines no alterades poden no ser l'únic problema per la salut dels consumidors, perquè també poden estar presents les micotoxines conjugades. Aquestes poden no ser detectades durant els anàlisis rutinaris encara que poden arribar a tenir la mateixa perillositat que les micotoxines lliures.

Els objectius d'aquesta tesis eren determinar la presència d'algunes micotoxines en els productes fets a base de cereals, estudiar l'estabilitat de les micotoxines durant el processat dels aliments i avaluar l'exposició a aquest tipus de compostos. Per aconseguir aquests objectius s'han elaborat diferents estudis. En primer lloc, s'han realitzat alguns anàlisis de cereals i aliments a base de cereals procedents del mercat per estudiar la presència de les aflatoxines (Afs), el deoxinivalenol (DON), el nivalenol (NIV), la ZEN, la OTA i alguns dels seus conjugats. A continuació s'ha estudiat en profunditat el procés d'elaboració del pa i de la pasta i alguns factors que afecten la seva estabilitat han estat avaluats. L'exposició a les micotoxines s'ha realitzat a través de dos mètodes diferents: 1) combinació de les dades de contaminació amb dades de consum i 2) utilitzant biomarcadors.

Tots els anàlisis de les micotoxines es van dur a terme mitjançant la cromatografia líquida, encara que detectors d'ultraviolat, de fluorescència i espectròmetre de masses van ser utilitzats pels diferents estudis. L'espectròmetre de masses fou utilitzat principalment per l'anàlisi de les micotoxines conjugades a les diferents matrius. A més, alguns nous mètodes d'anàlisi foren desenvolupats satisfactòriament per millorar la detecció de les micotoxines.

L'anàlisi de cereals i de productes a base de cereals ha mostrat que la presència de micotoxines és molt usual. Els tricotecens foren les micotoxines amb una major presència i també amb les més grans concentracions. Així, algunes mostres de fibra analitzades tenien concentracions de DON superiors els límits establerts per la legislació Europea. Encara que el DON i el NIV van ser les micotoxines amb major presència, la resta de micotoxines analitzades també van mostrar nivells preocupants de contaminació. Les micotoxines conjugades haurien d'estar més controlades, perquè els resultats obtinguts mostren l'elevada presència d'aquest tipus de micotoxines en els productes estudiats, inclús en alguns casos la concentració de les micotoxines conjugades és més elevada que la concentració de les micotoxines lliures.

Encara que les micotoxines són considerades compostos amb una extrema estabilitat, el processat dels aliments pot arribar a provocar canvis en les seves concentracions. La fermentació del pa és una etapa molt complexa del processat on una gran quantitat de factors influeixen. La concentració de la OTA no es veu afectada durant la fermentació. A diferència de la OTA, el DON és enormement afectat per aquesta etapa (increments o reduccions del DON s'han observat el final de la fermentació). De fet la seva estabilitat depèn de molts factors: temperatura, temps i presència d'enzims o de massa mare. Com el DON, el DON-3-glucosid també depèn dels mateixos factors que el DON durant la fermentació. Encara que la OTA sigui més estable que el DON i el seus conjugats durant el fornejat, totes aquestes micotoxines poden ser reduïdes en aquesta etapa en funció de varis factors: temps, temperatures, presència d'enzims i transmissió de la calor. La reducció del DON observada durant l'ebullició de la pasta va ser causada per la transferència del DON de la pasta al brou a causa de la seva elevada solubilitat. Cinètiques de reducció de les micotoxines pels diferents processos de transformació dels aliments han estat construïdes per poder anticipar el contingut d'aquestes al final del processat i poden ser d'una gran utilitat per a la indústria alimentaria perquè han presentat un bon ajust ($r > 0,75$).

A causa dels alts nivells de DON trobats en les mostres de segó analitzades, es van realitzar càlculs de l'exposició a DON a través del consum d'aquest producte. Els resultats indiquen que la ingesta de fibra pot suposar una important via d'exposició al DON. Biomarcadors del DON i la OTA han estat estudiats al plasma i orina i els resultats han revelat que l'elevada exposició al DON i a la OTA poden ser un problema per la població catalana. Inclús, l'elevada exposició el DON podria ser mantinguda al llarg del temps per la mateixa persona, superant la ingesta diària tolerable sistemàticament en lloc d'eventualment.

1.3. Resumen (Spanish)

Las micotoxinas son metabolitos secundarios producidos por hongos que pueden contaminar a varios productos agrícolas antes o después de la cosecha. Los géneros más importantes de hongos productores de micotoxinas son *Aspergillus*, *Fusarium* y *Penicillium*. Las micotoxinas pueden estar presentes en una gran variedad de productos y su ingesta puede ser peligrosa porque producen una gran variedad de efectos dañinos para la salud de los humanos y los animales. Los productos a base de cereales representan una de las principales fuentes de exposición a las micotoxinas. Varias micotoxinas se han identificado hasta la fecha, pero las micotoxinas con interés especial para la seguridad de los alimentos y los piensos son: las aflatoxinas (B1, B2, G1 y G2), las fumonisinas (Fbs), la ocratoxina A (OTA), la patulina, los tricotecenos (deoxinivalenol y nivalenol) y la zearalenona (ZEN). Sin embargo, las micotoxinas no alteradas pueden no ser el único problema para la salud de los consumidores, debido a que también pueden estar presentes las micotoxinas conjugadas. Este tipo de compuestos pueden no ser detectados en los análisis rutinarios aunque pueden llegar a ser igual de peligrosos que las micotoxinas libres.

Los objetivos de esta tesis fueron determinar la presencia de algunas micotoxinas en los productos a base de cereales, estudiar su estabilidad durante el procesado de los alimentos y evaluar la exposición a este tipo de compuestos. Para lograr estos objetivos se han elaborado distintos estudios. En primer lugar, se han realizado varios análisis de cereales y alimentos a base de cereales procedentes del mercado para estudiar la presencia de aflatoxinas (Afs), deoxinivalenol (DON), nivalenol (NIV), ZEN, OTA y algunos de sus conjugados. A continuación, se ha estudiado en profundidad el efecto del proceso de elaboración del pan y de la pasta en la concentración de las micotoxinas y también se han evaluado algunos de los factores que podrían afectar al contenido final de las micotoxinas. Para la evaluación de la exposición a los metabolitos secundarios producidos por hongos se han utilizado dos métodos distintos: 1) combinación de los datos de contaminación con los datos de consumo y 2) utilización de biomarcadores.

Todos los análisis de micotoxinas se han realizado con cromatografía liquida, utilizando detectores de ultravioleta, de fluorescencia y espectrómetro de masas para los distintos estudios. El espectrómetro de masas fue principalmente empleado para el análisis de las micotoxinas conjugadas en las distintas matrices. Además, algunos nuevos métodos de análisis fueron desarrollados satisfactoriamente para mejorar la detección de estas toxinas producidas por hongos.

El análisis de cereales y de productos a base de cereales ha demostrado que la presencia de micotoxinas es muy común en este tipo de productos. Los tricotecenos fueron las micotoxinas con una mayor presencia y también con las más altas concentraciones encontradas. Así, algunas muestras de fibra analizadas tenían concentraciones de DON por encima de los límites establecidos por la legislación Europea. Aunque el DON y el NIV fueros las micotoxinas con mayor presencia, el resto de las micotoxinas también mostraron niveles

preocupantes de contaminación. Las micotoxinas conjugadas deberían de estar más controladas, porque los resultados obtenidos muestran la elevada presencia de este tipo de micotoxinas en los productos estudiados, incluso en algunos casos su concentración es más elevada que la concentración de las micotoxinas libres.

Aunque las micotoxinas son consideradas compuestos con una extrema estabilidad, el procesado de los alimentos puede llegar a causar cambios en sus concentraciones. La fermentación del pan es una etapa muy compleja del procesado donde influyen una gran cantidad de factores. La concentración de OTA no se ve afectada durante la fermentación. A diferencia de la OTA, el DON está enormemente afectado por esta etapa (incrementos o reducciones del DON se han observado al final de la fermentación). De hecho su estabilidad depende de muchos factores: temperatura, tiempo y presencia de enzimas o de masa madre. Similar al DON, el DON-3-glucósido también depende de los mismos factores que el DON durante la fermentación. Aunque la OTA sea más estable que el DON y sus conjugados durante el horneado, todas estas micotoxinas pueden ser reducidas en esta etapa en función de varios factores: tiempo, temperaturas, presencia de enzimas y transmisión del calor. Por otra parte, la reducción de DON observada durante la ebullición de la pasta fue causada por la transferencia del DON de la pasta al caldo debido a su elevada solubilidad. Cinéticas de reducción de las micotoxinas para los distintos procesos de transformación de los alimentos se han elaborado para anticipar el contenido de estas al final del procesado y pueden ser de gran utilidad para la industria alimentaria porque han tenido un buen ajuste ($r > 0,75$).

Debido a los altos niveles de DON encontrados en las muestras de salvado analizadas, y teniendo en cuenta el alto consumo de estos productos, se realizó un cálculo de la exposición de los consumidores a esta micotoxina. Los resultados indican que la ingesta de fibra puede suponer una importante vía de exposición al DON. Los biomarcadores del DON y la OTA han sido estudiados en plasma y orina y los resultados han revelado que la elevada exposición al DON y a la OTA pueden ser un problema para la población catalana. Incluso, la elevada exposición a DON podría ser mantenida a lo largo del tiempo por la misma persona, superando la ingesta diaria tolerable sistemáticamente en lugar de eventualmente.

2. Introduction

2.1. Mycotoxins

Mycotoxins are toxic secondary metabolites produced by fungi and contaminate various agricultural commodities either before harvest or under post-harvest conditions. It is believed that their purpose is thought to be for fungal defence or competition (Magan & Aldred, 2007). A high number of filamentous moulds have the capability to produce mycotoxins; however the most important producing genera are *Aspergillus, Fusarium* and *Penicillium* (Pit & Hocking, 2009). These moulds have worldwide distribution and they are found in a wide range of environments. In general, mycotoxigenic moulds are not aggressive pathogens, but some species can invade and colonize plant tissues synthesizing mycotoxins during cultivation, harvesting, drying, transport, processing and storage (Moss, 1992). About 400 different mycotoxins have been identified, but only around 20, synthesized by different fungal species, are important as natural contaminants in feed and food. Mycotoxins can be present in a wide range of products and have been detected in a huge amount of vegetable products (rice, wheat, rye, barley, corn, soybeans, sorghum, nuts, spices), in foods (baking products, pasta, breakfast cereals), beverages (fruits, juices and puree, beer and wine), feed and animal products (dairy products, meat, eggs, etc.). The intake of mycotoxins can be dangerous because they can produce autoimmune illnesses, metabolic and biochemical deficiencies; they have allergenic properties, reduce reproductive efficiency, and some of them are teratogenic, carcinogenic, mutagenic, and can cause dead. Due to their high presence, maximum tolerable levels of dangerous mycotoxins have been set, and an effective management of food safety must achieve a safe food supply. European legislation aims to harmonize the maximum tolerance levels of mycotoxins in food and feed for all European Union members, and the requirements of the sampling and analytical techniques, committing all countries to follow the common rules.

Although there are many species of toxigenic moulds, only a few mycotoxins 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 (B1, B2, G1 and G2), fumonisins (Fbs), ochratoxin A (OTA), patulin, trichothecenes (deoxynivalenol, T-2 toxin, HT-2 toxin), and zearalenone (ZEN).

2.1.1. Aflatoxins B and G group

Aflatoxins (Afs) are the best known and most widely studied mycotoxins. They were first isolated in the early 1960s when 100,000 turkey poults died after consuming aflatoxin contaminated peanut meal in the United Kingdom. Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) (Figure 2.1) are the most important Afs and 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 (EFSA, 2007).

AFB1 is the most important compound with respect to its occurrence and toxicity for human and animals. It has been reported to be the most powerful natural carcinogen in mammals (Creppy, 2002) and there is evidence from human studies that AFB1 is a major risk factor for hepatocellular carcinoma, therefore it is classified in group 1 by International Agency for Research on Cancer (IARC, 2012). Moreover, Afs can cause a myriad of other effects: immunosuppresion, reduced growth rate, lowered milk and egg production, reduced reproductivity, reduced feed utilization and efficiency and anemia (Do et al., 2007).

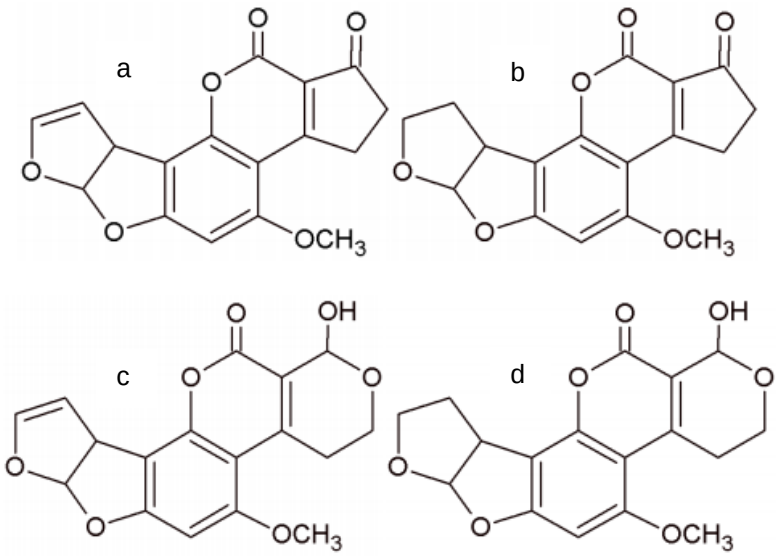


Figure 2.1. Molecular structure of Aflatoxins B1 (a), B2 (b), G1 (c) and G2 (d).

Afs have been found in a variety of agricultural commodities as cereals and their derivatives as flour, breakfast cereals or beer (Aydin et al., 2008; Burdaspal & Legarda, 2013; Hassan & Kassaify, 2014). They are also frequently detected in nuts (Milhome et al., 2014; Ezekiel et al., 2012), cocoa (Turcotte et al., 2013), spices (Hammami et al., 2014) and dried figs (Heperkan et al., 2012). Since cereals are the base of feeds, Afs or their metabolites have been detected in meat (Aziz & Youssef, 1991), milk and dairy products (Gul & Dervisoglu, 2014).

Then European Commission has established maximum levels for AFB1 and Afs (AFB1+AFB2+AFG1+AFG2) in cereal products (Table 2.1), nuts and dried fruit prior to sorting or other physical treatment, and lower values for foods for direct consumption likewise contamination in spices (EC, 2010, 2012).

2.1.2. Trichothecenes

Trichothecenes are a very large family of chemically related mycotoxins produced by various species of *Fusarium*. The most important structural features causing the biological activities of trichothecenes are: 12,13-epoxy ring, the presence of hydroxyl or acetyl groups at

appropriate positions on trichothecene nucleus and structure and position of the side-chain. They are produced on many different grains like wheat, oats or maize.

2.1.2.1. Deoxynivalenol

Deoxynivalenol (DON) (Figure 2.2) is a trichothecene mycotoxin produced by fungi of the *Fusarium* genus, for example *F. culmorum* and *F. graminearum*, which has been widely detected in cereal crops (wheat, maize, oats, barley and rye). Environmental conditions that favour DON production in the field are low temperature and high humidity. Cold and wet weather, which tends to delay harvest, permits continued growth of mould on the crop, thereby increasing the chance of higher concentrations of DON being produced (Scientific Committee on Food, SCF, 1999).

In general, acute exposure of animals to DON results in decreased feed consumption (anorexia), vomiting (emesis), abdominal pain, dizziness, headache, throat irritation, diarrhoea, and blood in stool (Rotter et al., 1996). While longer exposure causes reduced growth, and adverse effects on the thymus, spleen, heart, and liver (Sobrova et al., 2010). Thus, on cells of 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 rumiants, appear to have higher tolerance (Rocha et al., 2005). Concerning human safety, Scientific Committee on Food (SCF) established a temporary Tolerable Daily Intake (TDI) of 1 µg/kg body weight (b.w.) per day.

DON occurs predominantly in grains such as wheat, barley, oats, rye and maize, and less often in rice, sorghum and triticale. Thus, DON is found in cereal-based foods (Cano-Sancho et al., 2011) and feedstuffs (Döll et al., 2011). Due to the high presence of DON in cereals, European Commision (EC) set up maximum limits for DON in cereals (EC, 2006). However, different levels of DON presence exist for unprocessed cereals (≥ 1250 µg/kg) and processed cereals (≤ 750 µg/kg).

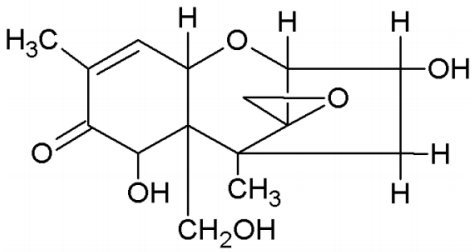


Figure 2.2. Molecular structure of deoxynivalenol (DON).

2.1.2.2. Nivalenol

Nivalenol (NIV) (Figure 2.3) is a mycotoxin produced mainly by *Fusarium* genera, mainly *F. cerealis*, *F. poae*, but also *F. graminearum* and *F. culmorum*. It is a field mycotoxin produced mainly in wheat. It is found in moist cool conditions (Eriksen, 2003).

Toxicity of NIV has been studied using *in vitro* and *in vivo* experiments demonstrating immunotoxicity, haematotoxicity/myelotoxicity and developmental and reproductive toxicity. IARC (1993) concluded on the available data at time that there is inadequate evidence of carcinogenicity of NIV in experimental animals and that its carcinogenicity for humans was not classifiable (Group 3). There have been reports of human toxicoses possibly linked to intake of trichothecene contaminated food, but this cannot be conclusively linked to any single toxin. More recently, exposure to dietary NIV has been associated with an increased incidence of oesophageal and gastric cancers in certain regions of China (Hsia et al., 2004).

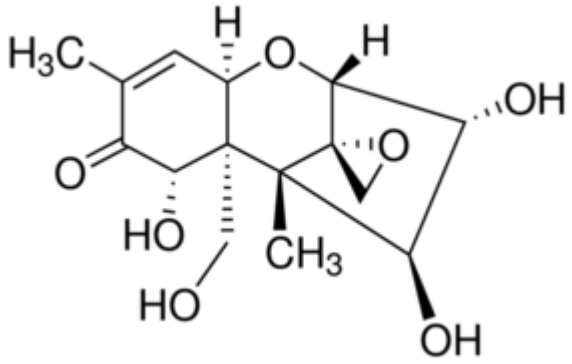


Figure 2.3. Molecular structure of nivalenol (NIV).

NIV has a considerable presence in cereals: wheat, maize, barley, oat and rye. Thus, Broekaert et al. (2015) found 32 % of the analysed samples (3062) with presence of NIV. However, legislation about NIV maximum permitted levels does not exist nowadays.

2.1.3. Zeralenone

Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* species, including *F. culmorum*, *F. roseum* and *F. graminearum* (Caldwell et al., 1970; Hestbjerg et al., 2002). These fungi are present on almost all continents, and they are known to infect both pre- and post-harvested wheat, barley, rice, maize, and other crops (Zinedine et al., 2007). ZEN is a macrocyclic β-resorcyclic acid lactone containing an unsaturated bond at C1'-C2' and a ketone function at position C6' (Figure 2.4). Either the double bond or the ketone, or both, can be reduced yielding a series of congeners and stereoisomers. Reductions are important because they affect the biological activity. Also because it represents a mechanism whereby organisms can biotransform ZEN (Jelinek et al., 1989; Kuiper-Goodman et al., 1987).

Absorption of ZEN 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). Natural exposure to ZEN and some of its metabolites through contaminated food has been pointed out as a cause of female reproductive changes as a result of their powerful estrogenic activity: their hormonal action exceeds that of most other naturally occurring non-steroidal estrogens (Bennett & Klich, 2003). Fertility problems have been observed in laboratory animals (mice, rats, guinea pigs, hamsters, and rabbits) and also in swine and sheep (Krska et al., 2003; Kuiper-Goodman et al., 1987). ZEN may be an important etiologic agent of intoxication in infants or foetuses exposed to this mycotoxin, which results in premature thelarche, pubarche, and breast enlargement (EFSA, 2011). In 2000, the SCF established a temporary TDI of 0.2 µg/kg b.w.

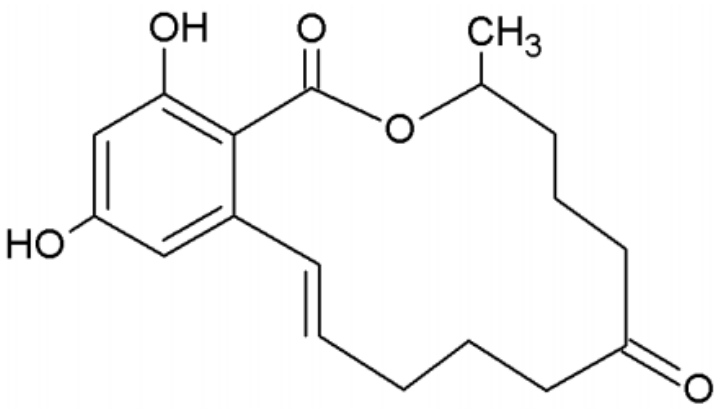


Figure 2.4. Molecular structure of zearalenone (ZEN).

ZEN is common in cereals, according to EFSA (2011), among grains for human consumption the frequency of occurrence of ZEN in maize is 33 %. Due to his high presence in cereals, EC set up maximum limits for ZEN presence in cereals and there is also a difference between unprocessed cereals (≥ 100 µg/kg) and processed cereals (≤ 75 µg/kg).

2.1.4. Ochratoxin A

Ochratoxin A (OTA) is recognized as a secondary metabolite of several *Aspergillus* and *Penicillium* which are characterized by widespread occurrence and different behaviour which depends on the ecological niches, the products affected and environment (Duarte et al., 2010). Both are considered to be storage fungi, rather than field contaminants or plant pathogens, and toxin production occurs mainly when susceptible commodities are stored under inappropriate conditions, particularly at high moisture levels. Its chemical structure consists of a dihydroisocoumarin moiety coupled to L-β-phenylalanine by a peptide bond (Figure 2.5).

Although there are other ochratoxin forms, OTA is the most important ochratoxin due to its incidence and toxicity.

OTA is a potent nephrotoxin and causes both acute and chronic effects in kidneys of all mammalian species tested (El Khoury & Atoui, 2010). Studies on animals have characterized this mycotoxin as nephrotoxic, hepatotoxic, neurotoxic, immunotoxic, teratogenic and carcinogenic, causing renal carcinoma (JECFA, 2008). Chronic human exposure to OTA has been related to development of urinary tract tumors and Balkan Endemic Nephropathy. The International Agency for Research on Cancer (IARC) classified OTA as possibly carcinogenic to humans (group 2B) (IARC 1993).

Occurrence of OTA has been found in a very wide range of raw and processed food commodities all over the world. It was first reported in cereals but has since been found in other products, including coffee, dried fruits, wine, beer, cocoa, nuts, beans, peas, bread and rice (Coronel et al., 2012). It has also been detected in meat, especially pork and poutry, following transfer from contaminated feed (Curtui et al., 2001). Major contributors to OTA in the diet in Europe are cereals, wine and coffee. OTA has been detected in human blood, urine and breast milk, demonstrating dietary exposure. Daily intakes have been estimated at between 0.2 and 4.7 ng/kg b.w. In 2006, European Food Safety Authority (EFSA) derived a TDI of 17 ng/kg bodyweight for OTA in the diet.

In order to prevent human toxicological damage, legal restrictions are applied to mycotoxins in food. The EC established maximum levels for OTA in cereals, dried fruits, coffee and wine in 2006 (EC, 2006). In the case of cereals, legislation discriminated between unprocessed (5 μg/kg) and intended for direct human consumption products (3 μg/kg).

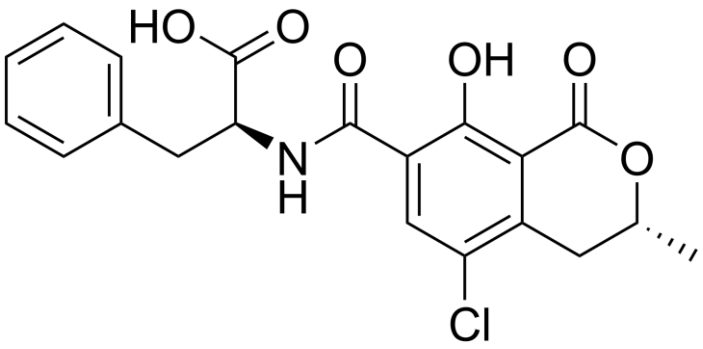


Figure 2.5. Molecular structure of OTA.

Table 2.1. Toxicity, tolerable daily intake (TDI), maximum values in European legislation (1881/2006), moulds producers for Afs, DON, ZEN and OTA.

Mycotoxin	Toxicity	TDI	European legislation in cereals (1881/2006)	Main food	Producer moulds
Aflatoxins	IARC: Group 1 Carcinogenic	0.14 ng/kg bw/day	2 µg/kg AFB1 4 µg/kg Afs	Maize, peanuts and pistachios.	*A. flavus* *A. parasiticus* *A. nomius* *A. tamarii*
Deoxynivalenol	IARC: Group 3 Immunological alterations Nutritional alterations	1 µg/kg bw/day	1250 µg/kg cereals 1750 µg/kg durum wheat and oat 1750 µg/kg maize	Bread, baking products and breakfast cereals.	*F. graminearum* *F. culmorum*
Nivalenol	IARC: Group 3 Immunotoxicity Haematotoxicity Myelotoxicity	1.2 µg/kg bw/day	-	Bread, baking products and breakfast cereals.	*F. cerealis* *F. poae*
Zearalenone	IARC: Group 3 Oestrogen	250 ng/kg bw/day	100 µg/kg cereals 200 µg/kg maize	Bread, baking products and breakfast cereals.	*F. graminearum* *F. culmorum* *F. cerealis* *F. semitectum.*
Ochratoxin A	IARC: Group 2B Nephrotoxic Hepatotoxic Neurotoxic Teratogenic Carcinogenic	17 ng/kg bw/day	5 µg/kg cereals	Beer, breakfast cereals, bread, coffee and nuts.	*A. ochraceus* *A. niger* *A. carbonarius* *A. terreus* *P. verrucosum* *P. Nordicum.*

2.2. Cereals

A cereal is any grass cultivated for the edible components of its grain (a type of fruit called caryopsis) composed of the endosperm, germ and bran. The word cereal derives from *Ceres*, name of the Roman goddess of harvest and agriculture. Cereal grains are grown in high quantities and provide more food energy worldwide than any other type of crop and are therefore staple crops. Due to their high energy content, in some developing countries, grain in form of rice, wheat, millet, or maize constitutes the main daily sustenance. In developed countries, cereal consumption is moderate and varied but still substantial. Depending on geographical area, different types of cereals have had more importance creating an entire culture around them: among European countries dominates consumption of wheat, among American countries, corn, rice in Asian countries and sorghum and millet in African communities. The form of consumption of cereals differs and examples of this variety are: bread, pastries, breakfast cereals, and also serve as raw material for industries of alcoholic beverages such as beer or whiskey. In addition, preparation and cooking of most of these foods are simple and versatile, from bread to pizza. Their consumption is appropriate for any age and condition.

The importance of cereals is estimated to grow in the next years, because surface of cereals cultivated around the world increases every year (FAO, 2016) (Table 2.2) (Figure 2.6). Wheat, as one of the most important cereals, follows the same trend (Table 2.2).

Table 2.2. World production of cereals, wheat (millions of tonnes) and relative production of wheat compared to total cereal production (%).

	Year				
	2012	2013	2014	2015	2016
Cereal (millions of tonnes)	2292.8	2519	2563.5	2531.7	2570.8
Wheat (millions of tonnes)	655.1	711.5	730.5	734.2	742.4
Relative production of wheat (%)	28.57	28.25	28.50	29.00	28.88

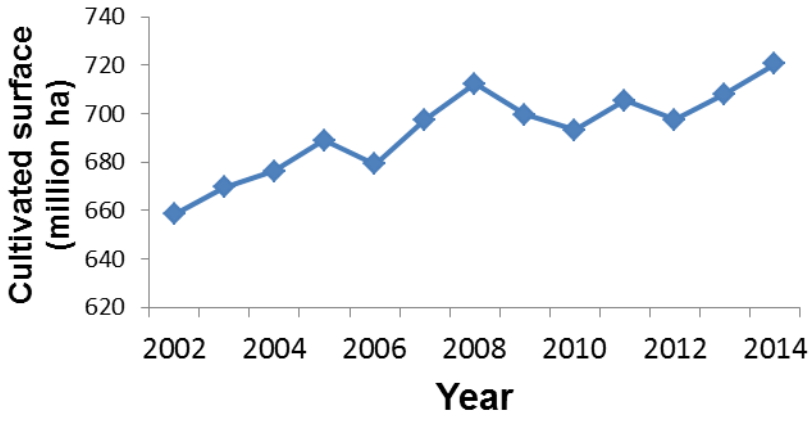


Figure 2.6. World cultivated surface of cereals from 2002 to 2014 (millions of hectares).

Whole cereal grains are the entire seed of a plant. This is made up of three key edible parts:

- **Bran:** it is the multi-layered outer skin of the edible kernel. It contains important antioxidants, B vitamins and fiber.

- **Germ**: it is the embryo which has the potential to sprout into a new plant. It contains many B vitamins, some protein, minerals, and healthy fats.

- **Endosperm:** it is the germ's food supply. Endosperm is by far the largest portion of the kernel. It contains starchy carbohydrates, proteins and small amounts of vitamins and minerals.

In their natural form (as in *whole grain*), they are a rich source of vitamins, minerals, carbohydrates, fats, oils and protein. But, cereals are usually submitted to a refining process to get flour. Refining normally removes the bran and the germ, leaving only the endosperm. Without bran and germ, about 25 % of a grain's protein is lost, and at least seventeen key nutrients are greatly reduced in. The remaining endosperm is mostly fast absorption carbohydrates (after ingestion pass to blood in a short time). Protein content is very variable, between 6 and 16 % by weight, depending on the type of cereal and industrial processing. Amino acid composition of proteins in grain depends on the species and variety; and is generally poor in essential amino acids.

Although flour, endosperm part, is the most consumed part of the grain and it is the most used in food industry to produce a wide range of products like bread or bakery products, and other parts are also consumed for the benefits of dietary fiber contained in the bran.

2.3. Presence of mycotoxins in cereals and cereal based food

Cereals, such as majority of crops, are susceptible to be contaminated with fungi, either in the field or during storage, as well as during processing, because moulds are common contaminants in soil, air, water and vegetation. Fungal contamination can cause damage in cereal grains, including low germination, low baking quality, discoloration, off-flavours, softening and rotting, and formation of pathogenic or allergenic propagules. Moreover, it may also produce mycotoxins, because cereals can be contaminated by the three main mycotoxin producing genera (*Penicillium, Aspegillus* and *Fusarium*). During the past decades a huge number of scientific papers have demonstrated that the list of raw cereals actually contaminated by mycotoxins is continuously increasing. Thus, despite efforts to control fungal contamination,

extensive mycotoxin contamination has been reported. Indeed, Devegowda et al. (1998) reported that 25 % of cereals approximately consumed in the world were contaminated by mycotoxins. Considerable differences regarding type and prevalence of mycotoxin contamination in different regions of the world have been reported (Binder et al., 2007; Marín et al., 2013; Schatzmayr et al., 2013). Overall results confirm that Afs, trichothecenes, Fbs, OTA and ZEN are the main contaminating mycotoxins in cereals. Moreover, their concentrations are sometimes over legislation European limit (EC, 1881/2006) as notifications received in the last years by Rapid Alert System for Food and Feed (RASFF) showed. Afs, OTA and DON caused most of the mycotoxins notifications from cereals (Figure 2.7). For this reason, within the European Union, European Commission in 2006 laid down maximum levels for the presence of DON, ZEN, fumonisin (Fbs), OTA and Afs in unprocessed cereals as well as in cereal-derived products and cereals intended for direct human consumption, with particular limits for baby food. Good agricultural practices are needed to limit presence of fungi and mycotoxins in cereal grains during cultivation, followed by good manufacturing practices, particularly related to storage, during the further cereal production chain (Edwards, 2004). Infection of cereal grains in field with fungi and subsequent mycotoxin production is known to be affected by agronomic and climatic conditions. The three fungi mentioned (*Penicillium, Aspegillus* and *Fusarium*) have their own optimal temperature and moisture conditions for infection of cereals, defining their spread and occurrence over regions and years, and subsequent mycotoxin production (Magan et al., 2011).

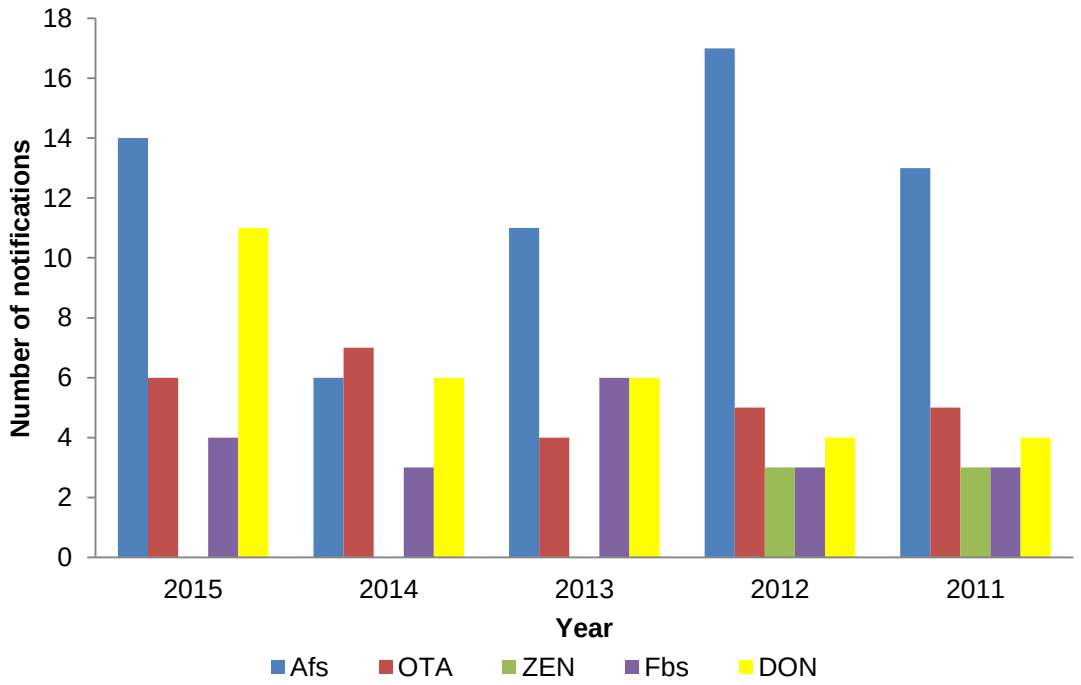


Figure 2.7. Number of notifications received by RASFF on mycotoxins (Afs, OTA, ZEN, Fbs and DON) in cereals from 2011-2015.

Mycotoxins can diffuse into the grain and can be found in all ground fractions and, due to their thermo-resistance properties, also in products subject to thermal processing. Such products make a serious threat to consumers' health due to the presence of mycotoxins. Several studies showed high presence of them in all types of cereal based food and sometimes levels were again over legislation limit. Cano-Sancho (2013) and Coronel et al. (2012) showed the high occurrence of Afs, DON, ZEN and OTA in cereal based food from Catalonia (Table 2.3). Moreover, cereal products represent one of the main sources of exposure to mycotoxins (Marin et al., 2013).

Table 2.3. Presence of mycotoxins in cereal based food from Catalonia (Cano-Sancho, 2013 and Coronel et al., 2012).

	Food	n	Positive samples (%)	Mean ± sd (µg/kg)	Max. (µg/kg)
Afs	Sweet corn	11	2.8	0.9±0.1	1.0
	Breakfast cereals	72	1	0.5	0.5
	Corn snacks	72	1	0.8	0.8
DON	Wheat flakes	27	74.1	190 ± 117	437
	Corn flakes	65	75.4	109 ± 78	580
	Beer	71	1.4	12	12
	Sweet corn	72	7.4	114 ± 36	139
	Corn snacks	71	78.9	153 ± 58	304
	Pasta	70	74.3	226 ± 177	946
	Sliced bread	72	16.7	68 ± 18	98
	Bread	31	100.0	246 ± 158	739
ZEN	Wheat flakes	29	13.8	6.3 ± 5.4	12.1
	Corn flakes	71	0	-	-
	Beer	71	11.3	3.1 ± 1.4	5.1
	Sweet corn	72	18.1	4.9 ± 0.7	5.9
	Corn snacks	72	23.6	5.9 ± 6.8	22.8
	Pasta	70	14.1	3.8 ± 1.8	5.9
	Sliced bread	71	43.7	3.7 ± 4.5	20.9
OTA	Beer	71	88.7	0.022 ± 0.023	0.126
	Corn-based breakfast cereals	71	2.8	0.728 ±0.764	1.268
	Wheat/rice-based	28	25.0	0.293±0.141	0.570
	Loaf bread	70	12.9	0.283±0.181	0.658

2.4. Control of mycotoxins

As commented in the last section, there is a frequent presence of mycotoxins in food, so, there is a need to control in the food chain because food safety must be guaranteed. As result, food industry in developed world demands raw ingredients of the best quality and that conform statutory limits where these have been set for contaminants such as mycotoxins. Ideally, formation of any mycotoxins should be prevented, but this is not always possible so it is important to know how concentrations of mycotoxins present in raw materials change through the food chain.

2.4.1. Hazard analysis and critical control points (HACCP)

Hazard analysis and critical control points systems or (HACCP)-like assessment of food chain is now often applied to key commodities to determine critical points at which mycotoxins can be controlled or eliminated. This approach enables strategies for minimizing consumer exposure to be developed through appropriate management of products, including safe disposal of any contaminated waste material. In some instances, different maximum allowed limits may be set for raw material and finished food products. When this is done, relationship between different limits must be realistic and based on sound data. Food industries identify critical control points (CCPs) in their processes that may result in mycotoxin contamination, such as mouldy grain or nut products, or storage conditions that may lead to the development of mould. Identification of appropriate CCPs along process chain will enable food industry to develop and apply proper HACCP and good agricultural practices (GAP) systems which will ensure that there are no unforeseen sources of mycotoxin contamination in their products; they have clearly supplied a great improvement in the Food Safety Management.

2.4.2. Food safety objective (FSO)

On the other hand, in the last years, food safety management approach for microbiological risks has been completed and developed through the inclusion of other metrics like the Food Safety Objective (FSO) (International Commission on Microbiological Specifications for Foods, ICMSF, 1998). FSO specifies a goal which can be incorporated into the design of control measurements in food chain corresponding with maximum permissible level of a hazard in a food at moment of consumption which leads to an Appropriate Level of Protection (ALOP).

Acceptable level of risk is the level adopted following consideration of public health impact, technological feasibility, economic implications, and that which a society regards as

reasonable in the context of and in comparison with other risks in everyday life (Schothorst, 1998).

In this context, the agro-food industry would use FSOs as means to co-ordinate risk management in the production process throughout farm-to fork production chain (De Swarte & Donker, 2005). Maximum hazard levels at other steps along food chain are called Performance Objectives (POs) (Codex Alimentarius commission, CAC, 2007).

Concept of FSO has mostly been applied to understanding the effects of handling and processing on levels of bacterial pathogens in foods, but it is also applicable to the formation and removal of mycotoxins (Pitt et al., 2013). FSO is calculated from the following equation:

$$H_0 - \sum I + \sum R \leq \text{FSO}$$

Increase in mycotoxin levels may occur before or after harvest, during drying, or during storage ($\sum I$) and reduction during processing ($\sum R$) (ICMSF, 2002). Initial mycotoxin level (H_0) for food industries could be designed as the time of sale from farm to distributors or processors. In the case of mycotoxins, FSO, H_0, R and I are expressed in µg/kg.

2.5. Food Processing

Processing of raw food commodities can be considered as the application of any combination of chemical, biological or physical methods used to produce final consumer food or animal feed. Processing of cereals or other commodities from harvest to the point where food is eaten by human or animal consumer involves a complex chain of actions. This may be as simple as hand grinding maize and boiling, while it can be large-scale semi-automated mills, bakeries, extrusion plants and breweries that involve many stages to produce retail product as purchased by the consumer.

At each stage in these processes concentration of a mycotoxin may decrease, increase or remain unaffected. There are many factors that influence changes in the amounts of mycotoxins present during processing, and understanding these factors will assist in minimizing mycotoxins formation, maximizing their elimination and ensuring that, as far as possible, no toxic reaction products are produced. Most mycotoxins are quite stable at room temperature and under neutral conditions. However, many factors must be considered during processing because temperature, pH, presence of other constituents or enzymes presence may affect stability of mycotoxins. Moreover, food industry should be sure that there are no moulds present in the commodity which may result in further mycotoxin formation during processing under specific conditions.

2.5.1. Cleaning and sorting

The first stage is usually to clean whole grain to remove dust, broken grains and other foreign material. This action of food processing can significantly reduce mycotoxin concentrations (Bullerman et al., 2002). Physical procedures generate little heat other than that caused by the operation of the machinery for cleaning, dehusking and abrasion so that not significant thermal breakdown of mycotoxin happens at this stage. However, mould and mycotoxins are generally concentrated in dust and broken grains that are more susceptible to fungal infection and toxin contamination, or in outer seed coat of the grains. The removal of this waste material can thus result in a considerable lowering of mean mycotoxin concentration, although reports of extent to which this occurs show very variable results. This may depend on quality of grain on receipt or on how mycotoxin is distributed within individual grains. For instance, only a 2-3 % of OTA reduction was achieved after cleaning barley (Scudamore et al., 2003), 19 % of DON reduction was got after cleaning wheat (Abbas et al., 1985; Zhang & Hang, 2014) or until 80 % of total Afs were removed after cleaning different kernels (Park et al., 2002).

2.5.2. Milling

Cleaned cereal is usually milled into component fractions, detailed specification of which depends on cereal and process. However, mycotoxins are not eliminated during milling but they are only redistributed and concentrated in certain milling fractions (Cheli et al., 2013). Cereal fractions intended for human consumption (flour or semolina) are usually less contaminated, while higher concentration after milling is detected in external fractions (bran, shorts and middlings) (Table 2.3). Significantly lower mycotoxin levels in finished flour may be attributed to potential of bran layer to behave as a physical barrier preventing the mycelia from penetrating further into kernel structure. The major mycotoxin concentration in external parts is of concern because although these parts are almost always used for animal feed, sometimes they are used for direct human consumption due to their benefits (improved large bowel function to slowed digestion and absorption of carbohydrate and fat and reduced risk for certain diseases). Level of mycotoxin reduction in the inner parts will depend on several factors. Some of these factors have been described: initial mycotoxin concentration, milling method, mould penetration in grain and moisture of grain (Cheli et al., 2013). So, there is a great variability of mycotoxin reduction in final flour, from a large reduction (> 80%) (Zhang et al., 2014) to a slight reduction (4 %) (Zheng et al., 2014).

2.5.3. Fermentation

Fermentation has been known since very early time and it is a food process with help of microorganisms. Fermentation occurs when yeast and bacteria convert carbohydrates transforming the food. A lot of products undergo fermentation (bread, beer, wine, dairy products, etc.). But fermentation can affect stability of mycotoxins mainly due to the presence of microorganisms. Moreover, hundreds of additional enzymatic activities are present in their cells, actively secreted into food matrix or released from disintegrated cells after autolysis. Literature shows a large variability for stability of mycotoxins in fermentation process and even some increases of mycotoxin have been found after fermentation. The loss of mycotoxins observed after fermentation can be caused by adsorption, as shown for some lactic acid bacteria (Haskard et al., 2001; Pierides et al., 2000), or irreversible degradation transformations as occurred in detected degradation products of transformation of ZEN by *Saccharomyces cerevisiae* (Böswald et al., 1995; Matsuura & Yoshizawa, 1985).

Different results with a large variability exist about stability of mycotoxins during different food and beverage fermentations. Firstly, a wide range of mycotoxins are widely reduced during beverage fermentation. Thus, an almost complete loss of OTA (> 98%) was detected and also an important reduction of ZEN (95 %) and patulin (98 %) (Inoue et al., 2013) after wort fermentation to beer. However, DON (Kostelanska et al., 2011) and Afs (Inoue et al., 2013) resisted widely this process. Different authors studied the OTA fate during wine fermentation; all results showed a large OTA reduction or even a total reduction of it (Piotrowska et al., 2013).

Manufacturing of many dairy products involves fermentation with lactic acid bacteria. The major mycotoxin of concern in milk is aflatoxin M1 (AFM1); important reductions of AFM1 (from 18 to 30 %) (Arab et al., 2012) have been detected after fermentation due to action of lactic acid bacteria.

Fermentation is an important step in commercial bread production. Proper fermentation provides a resilient crumb, which is soft and smooth to the touch. Although bread fermentation is shorter than other fermentations (beer, wine …), some changes in the mycotoxin concentrations have been detected. Thus, fermentation reduced concentration of Afs and reduction percentages in a dry matter basis got at low and high concentrations were: 28.0 % (AFG1), 33.6 % (AFB2), 30.6 % (AFG1) and 33.7 % (AFG2) (Gumus et al., 2009).The few existing studies about OTA in bread fermentation present contradictory results. For instance, Scudamore et al. (2003) did not find any reduction at 43 ºC for 35 min. On the other hand, Valle-Algarra et al. (2009) found a reduction from 29.8 to 33.5 % (30 ºC, 1 h), but they used spiked OTA contamination unlike Scudamore et al. (2003), which is likely to be more easily reduced.

There is poor information about ZEN in the bread making process shows that ZEN concentration can be reduced during this step. But the level of reduction is very variable. Cano-

Sancho et al. (2013) showed a total reduction of ZEN concentration after fermentation (25 ºC for 95 minutes) while Heidari et al. (2014) only had a 20 % of ZEN reduction after bread fermentation (30 ºC for 90 minutes).

The effect of bread fermentation in DON is controversial. While important reductions by more than 50 % were observed in some studies (Voss & Snook, 2010), no changes (Lancova et al., 2008) or even increases of DON concentrations were reported by others (Bergamini et al., 2010; Simsek et al. 2012; Suman et al. 2012). Samar et al. (2001) concluded that the reduction of DON in dough fermentation occurs with increasing fermentation temperature and time. They did not find any reduction working at 30 ºC (temperature generally used for the fermentation in bread process) until a fermentation of 95 minutes (25 %). While important reductions were found when they worked at 40 ºC (23 min-15 % and 45 min-40 %) and 50 ºC (20 min-29 % and 40 min-41 %), these reductions were more important when the time increased. However the spiked mycotoxin contamination used by Samar et al. (2001) could be the reason for DON reduction. So, some studies about DON stability during bread fermentation have been made. But the results are not conclusive because different factors can determine the variability of results.

The high variability of mycotoxin stability during fermentation can be caused by a huge amount of factors.Some of them are:

- **Microorganisms:** different yeasts and bacteria have a different effect in the reduction of mycotoxins. Although all of them can produce a correct fermentation, they can have a different impact on mycotoxins. For instance, a different reduction of Afs was obtained after fermentation of dairy products depending on the *Lactobacillus* strain used for the fermentation, from 5 to 80 % of AFB1 reduction (Peltonen et al., 2000).

- **Mycotoxin:** every mycotoxin has a different stability during fermentation, and it is common to obtain divergent results with different mycotoxins. Valle-Algarra et al. (2009) found more reduction of OTA (> 30 %) than DON (< 10 %) after bread fermentation.

- **Time:** the length of fermentation could be an essential factor for the mycotoxin stability. Longer fermentations will result in higher reductions, as it was observed in OTA during wine fermentation (Petruzzi et al., 2014).

Table 2.3. Effect of wheat milling in DON concentration in recent studies.

Reference	Type of product	Milling procedure	Moisture (%)	Wheat grain (µg/kg)	Clean wheat grain (µg/kg)	Type of cont.	Shorts			Bran		Flour			Comments
							Conc. (µg/kg)	Distribution factor (%)	Relative distribution (%)	Conc. (µg/kg)	Distribution factor (%)	Conc. (µg/kg)	Distribution factor (%)	Reduction (%)	
Brera et al., 2013	Durum wheat	Industrial milling	-	502	382	Natural	745	148	-	1051	209	207*	41	46	Cleaning process is not specified. They did two more bran separations before the final bran (pelled wheat and flour middlings not shown in the table).
				431	393		505	117	-	923	214	137*	32	65	
				369	252		507	137	-	802	217	112 *	30	56	
				739	328		865	117	-	1100	149	348*	47	-6	
Edwards et al.(2011)	Wheat	Pilot-scale mill (MLU-202, Bühler AG)	16	-	-	-	-	-	-	-	122	-	93	-	Cleaning method: Carter-Day dockage tester
Kostelanska et al., 2011	Wheat	Bühler laboratory mill	14	-	176	Natural			-	265	151	104	59	41	
					1446	Spiked			-	2561	177	778	54	46	
Savi et al., 2016	Wheat	Pilot-scale mill Quadrumat Senior® (Brabender)	14	-	1895	Natural	-	-	-	2278	120	1305	69	31	
Schwake-Anduschus et al., 2015	Wheat	Bühler laboratory scale mill (MLU-202) duster (Bühler MLU-302)	16.5	-	12119	Natural	40598	335	-	30297	250	10301	85	15	Moreover, they contributed the results with breaking flours and 2 more reduction flours not shown in this table.
					5804		13639	235	-	10157	175	4353	75	25	
					1702		3897	229	-	2553	150	1106	65	35	
Simsek et al. (2012)	Wheat	MLU 202 Bühler laboratory mill.	16.5	-	5230	Natural			-	14300	273	2000	38	62	Cleaning method: Carter - Day dockage tester
Stuper-Szablewska et al., 2016	Wheat	Industrial mill	-	8	1	Natural			-	1	100	<lod	0	100	They submitted the grain a pre cleaned grain to remove impurities. Semolina and different types of grain fractions obtained during milling not shown in the table.
Thammawong et al., 2011	Wheat	Bühler laboratory mill Model MLU-202	14.5-16	-	5270	Natural	9510	180	-	7230	137	4680	88	11	They obtained a low-grad flour with a DON concentration of 0.8 and 4.48 mg/kg respectively.
					896		1560	174	-	725	81	860	96	4	
Tibola et al., 2015	Wheat	Laboratory Mill 3100® (Perten)	14	2100	1050	Natural			-	1606	153	504	48	52	Air screen was used to clean (Kepler Webber, model: LC 160-1, Panambi/RS)
				1452	1300				-	1274	98	1118	86	14	
Zhang et al., 2014	Wheat	MLU 202 Bühler Laboratory Mill	16.5	-	4680	Natural	10240	219	43	10320	220	940	20	80	
					8990		14130	157	32	16110	179	1610	18	82	
					17100		26110	153	25	28550	167	1630	9	90	
					28401		32190	113	22	35590	125	5970	21	79	
					36720		48090	131	21	50200	137	5890	16	84	
Zheng et al., 2014	Wheat	Bühler laboratory mill (MLU-202)	14.5	-	1890	Natural	3.49	184	-	3070	162	1120	59	41	This process also gave a low-grad flour with a DON concentration of 0.91 mg/kg
					900		1570	174	-	730	81	860	95	4	This process also gave low-grad flour with a DON concentration of 0.80 mg/kg

- Data not provided

* Semolina of durum wheat.

- **Temperature:** some studies pointed out that warmer environments will help to reduce mycotoxin content. El-Nezami et al. (1998) detected more AFB1 reduction when fermentation of milk was at 37 ºC than 25 ºC.

- **pH:** alkaline or acid environment can affect the stability of the mycotoxins (Megalla & Hafez, 1982; Petruzzi et al., 2013). Moreover, pH also will be important for the development of the fermentation microorganisms.

- **Presence of other compounds:** enzymes or additives could produce changes in the mycotoxin stability. Some studies showed the possible importance of additives during fermentation (Lahtinen et al., 2004).

Although importance of some factors is intuited they have not been studied deeply as well as the possible interaction among factors. There is a lack of information about mycotoxins stability during fermentation.

2.5.4. Thermal processing

Application of heat to cooking and preservation of products is the basis of all thermal processes. These processes include mainly frying, boiling, baking and roasting. Studies of the stability of mycotoxins through thermal processing have been published. First of all, it is worthy to mention that mycotoxins are considered to be heat-stable molecules under 120 ºC, thus they are rarely eliminated by thermal treatment (Kabak, 2009).

Besides, effect of thermal tretments on mycotoxin stability varies depending upon the process itself (some processes could be more destructive than others). Dry heating (mainly baking and roasting) and wet heating (mostly boiling and frying) must be differenciated. Moreover, food matrix, especially once cooked, can bind mycotoxins, making recovery and analyses difficult and leading to erroneous results and conclusions in determining losses. All of these factors also need to be studied and taken into account when evaluating the ability of a certain process to reduce mycotoxin concentrations. Some factors which may affect the impact of heat treatments on mycotoxins:

- **Type of mycotoxin:** some mycotoxins can be more thermoresistant than others. For instance, OTA seems to be stable up to 180 °C; however aflatoxin B1 was almost completely degraded at heating temperatures of 160 °C and above (Raters et al., 2008).

- **Initial concentration:** it seems initial mycotoxin concentration affects level of mycotoxin reduction, when the initial mycotoxin concentration is higher more reduction is got (Bergamini et al., 2010).

- **Type of contamination:** spiked contamination could result in a weak link of mycotoxin to the matrix. So reducing mycotoxin by heat may be easier in spiked experiments.

- **Temperature:** a high temperature could cause a major reduction of mycotoxin (Bergamini et al., 2010; Suman et al., 2012).

- **Time:** a longer time of exposition to heat will cause larger mycotoxin degradation (Lancova et al., 2008).

- **Heat transmission:** smaller piece of food, faster temperature inside product will increase. Numanoglu et al. (2013) showed the importance of heat transmission for ZEN reduction in a maize product.

- **Heating method:** methods which use higher temperatures will cause larger mycotoxin degradation. Thus, roasting of coffee produced more OTA reduction (93%) (Pérez de Obanos et al., 2005) than boiling of pasta (35 %) (Sakuma et al., 2013).

2.5.4.1. Baking

Baking is a dry heating process which uses prolonged dry heat acting by convection, and not by thermal radiation. Some studies have focused on the effect of baking process on mycotoxin content in bakery products (bread, cakes, biscuits, etc.) and most of them investigated also the fate of mycotoxins during whole bread-making process due to its importance:

Little information about Afs stability during baking exits but all information found a wide Afs reduction during baking. Thus, almost a total reduction was got in corn muffins (87 %) (Stoloff & Trucksess, 1981) and a minimum reduction of 55 % was achieved in wheat bread (Gumus et al., 2009). These results indicate baking is effective to reduce Afs concentration.

Several investigations on the effects of baking on OTA reduction show conflicting results, however studies are scarce. Some authors reported no changes in OTA content during baking (Scudamore et al., 2003) whereas others (Valle-Algarra et al., 2009) observed a significant effect on the mycotoxin (33 % average) in wheat bread combining different time/temperature values (190 ºC/50 min, 207 ºC/40 min, 223 ºC/35 min and 240 ºC/30 min).

Subirade et al. (1996) also found reduction of OTA in baked wheat products (66 %), but they worked with biscuits and heat penetration is likely higher than in bread. Also ZEN seems to be extremly highly stable during baking. Thus, Cano-Sancho et al. (2013) did not observe any ZEN reduction during baking, while Heidari et al. (2014) only got a 16 %. Although Numanoglu et al. (2013) achieved some reduction of ZEN (28 % at 250 ºC for 15 minutes), they used an exceptionally thin matrix of maize (1 mm).

Some studies suggested that DON is highly stable in baking process (El-Banna et al., 1983; Scott et al., 1984). Thus, El-Banna et al. (1983) showed no reduction at high temperature with low time suggesting that the temperature effect is not so clear, and time may have more influence. Other authors; however, do not agree (Kostelanska et al., 2011; Pacin et al., 2010; Valle-Algarra et al., 2009) and they observed DON reductions. The stronger DON reduction was reported by Abbas (1985), who observed a reduction of 69% in wheat bread during the baking process. But other factors can affect which were not studied, like the presence of additives, the size of the baked product…

In summary, studies on mycotoxin reduction during baking reported high variability in the level of degradation, and similar time/temperature combinations do not seem to result in same reduction rate. Several variables such as presence of other ingredients or enzymes which are not taken into account may have to do with the variability reported.

2.5.4.2. Boiling

Boiling implies a heat treatment in water at the boiling point, so it is a wet heat treatment. It may be an intermediate step in cereal products production, or can be involved in the final cooking of cereals or pasta for direct consumption. Few works have been done about the behaviour of mycotoxins during boiling process. The existing studies show it may be a useful method to make safer food. In this case, mycotoxin reduction is not caused by degradation, but by leaching to the water used in the process.Thus, mycotoxin reduction during boiling will be more influenced by the hidrosolubility of compound than by their thermal stability. Afs are reduced during boiling and reduction seems to be around 30 % of total initial Afs concentration. For instance, ordinary cooking of rice contaminated with AFB1 showed an average reduction of 34% (Park et al., 2005). In another study boiling corn grits gave an average reduction of aflatoxins of 28% (Stoloff, 1981). Concerning OTA, the few existing assays report a significant reduction of OTA concentration. Some authors have also studied the soaking step previous to boiling in some cases, showing this step is also very important for the OTA reduction. For instance, Milanez et al. (1996) and Iha et al. (2009), observed an important reduction of OTA (up to 64% and 45 % respectively) by soaking and boiling beans. Iha et al. (2009) analysed the water of soaking and cooking, and they showed the reduction of OTA in

beans was produced by the transfer of OTA to the cooking water. According to Cano-Sancho et al. (2011), presence of DON in analysed uncooked pasta was 75 % of the analyzed samples. Different studies worked with DON stability during boiling and all of them got reduction of DON in cooked pasta (Brera et al., 2013; Cano-Sancho et al., 2013; Nowicki et al. 1988; Sugita-Konishi et al., 2006; Visconti et al., 2004). Percentages of reduction ranged from 20 % to 75 %. Most authors affirm that DON reduction is caused by its high water-solubility, analysis of boiling water resulting in high DON concentration (Nowicki et al., 1988; Sugita-Konishi et al., 2006; Visconti et al., 2004). DON reduction in pasta increases with increasing percentage of water used (Visconti et al., 2004).

2.5.5. Kinetics of toxin degradation

Chemical kinetics deals with the rates of chemical processes. Also known as reaction kinetics, includes investigations of how different experimental conditions can influence speed of a chemical reaction and yield information about the reaction's mechanism and transition states, as well as the construction of mathematical models that can describe characteristics of a chemical reaction. A study into the kinetics of a chemical reaction is usually carried out to determine absolute rate of reaction. Mathematical models that describe chemical reaction kinetics provide with tools to better understand and describe chemical or biological processes such as contaminants decomposition, microorganism growth and complex chemistry of biological systems. Reaction rate or speed of reaction for a reactant or product in a particular reaction is intuitively defined as how quickly or slowly a reaction takes place.

Reaction rate has the form:

$r = k\,[A]^m\,[B]^n$

Here k is the reaction rate constant. [A] and [B] are the molar concentrations of substances A and B in moles per unit volume of solution. Rate constant is constant for a given reaction only if only concentration of reactants changes. If temperature, for example changes, rate constant will change. Exponents m and n are called partial orders of reaction. They depend on the reaction mechanism and can be determined experimentally. The order of reaction with respect to a given substance is defined as the index, or exponent, to which its concentration term in the rate equation is raised.

Orders of reaction for each reactant are often positive integers, but they may also be zero, fractional, or negative. However, the most common orders are zero, first and second orders.

- Zero order reactions

Zero-order reactions (order = 0) have a constant rate. This rate is independent of the concentration of the reactants. The rate law is:

rate = *k*, with *k* having units of mols/second.

- First order reactions

A first order reaction (order =1) has a rate proportional to the concentration of one of the reactants. The rate law is:

rate = *k* [A], with k having the units of 1/second.

- Second order reactions

A second order reaction (order = 2) has a rate proportional to the concentration of the square of a single reactant or the product of the concentration of two reactants:

rate = $k[A]^2$, with the units of the rate constant 1/(mols second).

- Half life

Half-life (abbreviated $t_{1/2}$) is the time required for a reactant concentration to reduce to half its initial value. The half-life is calculated from rate law equation by allowing [A] to equal 0.5 [A_0].

Some studies developed kinetics for mycotoxin degradation in food processes. All of them used first order kinetic models (Castells et al., 2006; Jackson et al., 1996; Numanoglu et al., 2012). Knowing degradation kinetics can allow an approximate estimation of their concentration in final products, through prediction of expected final levels once the mycotoxin concentrations in raw materials are known.

2.6. Modified mycotoxins

Most investigations refer to the presence and reduction of mycotoxins in their unaltered form. However, unaltered mycotoxins might not be the only source of health hazard for consumers, because a group of metabolites called modified mycotoxins which cannot be detected in the routinary mycotoxin analysis exists. However they can become as dangerous as parent mycotoxins when we ingest them, because, some investigations have demonstrated the transformation of some conjugated mycotoxins to parent mycotoxins by human gastro intestinal microbiota (Berthiller et al., 2011). Conversion to parent mycotoxins cause the need to consider the admitted maximum levels in food of conjugated mycotoxins, confirming their importance and inclusion in risk assessment studies because the presence of modified mycotoxins leads to

underestimation of total mycotoxin concentration in samples. It is very important the toxicity assessment of all conjugated mycotoxins that occur in food because it will be possible to make an estimation of health risk posed by the sum of the different forms of a given mycotoxin. Moreover, most conjugated forms are not legislated. Recognition of toxicological relevance of masked mycotoxins in food commodities will enable overall toxicity estimates to be used by regulatory bodies, food manufacturers and monitoring authorities to protect consumers' health.

2.6.1. Origin of conjugated mycotoxins

Mycotoxin conjugates can be classified according to their origin, in fungal, plant, animal and food processing conjugates.

2.6.1.1. Fungal conjugates

Some fungi can directly produce conjugated mycotoxins. Bolliger and Tamm (1972) were the first to observe a fungal conjugation; they found four ZEN -related fungal metabolites in *F. graminearum*: 13-formyl-zearalenone, 5,6-dehydro-zearalenone, and the two stereoisomers of 5-hydroxy-zearalenone. After that, more fungal ZEN conjugates have been observed. The most important fungal ZEN conjugates, for their oestrogenic activity are α-zearalenol (α-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), β-zearalenol (β-ZEL) and zearalanone (McMullen, 1977; Mirocha et al., 1979; Richardson et al., 1985). Transformation to these products could be regarded as an intoxification pathway. For example, α-ZEL was able to bind to oestrogen receptors 10-20 times stronger than ZEN (Fitzpatrick et al., 1989). Pfeiffer et al. (2010) reported that fungal conjugate production in their culture was approximately 10 % of the total amount of ZEN. However it is known that pattern of mycotoxins and their amounts may vary considerably, depending on response of particular strain of the fungus to temperature, moisture, soil, host plant, and other factors (Pfeiffer et al., 2010).

Other two conjugated mycotoxins produced by fungi are two precursors of DON: 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON). *F. graminearum* is the producer of 3-ADON and 15-ADON, and some genetic differentiations have been identified between the 3-ADON and 15-ADON producer populations (Gale et al., 2007). Moreover, some geographical predominance in one conjugated genotype has been observed. In southern of Europe and Chinathe 15-ADON genotype is more predominant (Boutigny et al., 2014; Prodi et al., 2009; Somma et al., 2014; Yli-Mattila et al., 2009); while 3-ADON phenotypic *Fusarium* species are predominant in South America and the north of Europe (Scandinavia, Finland and North-western Russia) (Castañares et al., 2014; Yli-Matilla et al., 2010). These compounds are common in cereals (Ogiso et al., 2013; Yoshinari et al., 2012). For example, Ogiso et al. (2013) found 15-ADON in the 100 % of corn gluten feed samples analyzed and a maximum level

founded was 580 µg/kg. The study of scientific cooperation (SCOOP) task 3.2.10 found only presence of 3-ADON in 8% of analysed samples, but the 27% of positive samples presented a concentration over 520 µg/kg. Due to their high presence, their toxicity has been studied. 15-ADON has the highest level of toxicity compared to DON, and 3-ADON the lowest toxic level. 15-ADON impairs the barrier function, whereas DON and 3-ADON do not have a significant effect. Moreover 15-ADON caused more histological effects than DON and 3-ADON (Kadota et al., 2013; Pinton et al., 2012). Rapid transformation of 3-ADON and 15-ADON to DON has been observed in *in vivo* experiments (Eriksen et al., 2002), for this reason, acetylated deoxynivalenol has been determined to exhibit equivalent toxicity to deoxynivalenol; so the joint FAO/WHO expert committee on Food Additives has updated the provisional maximum tolerable daily intake to 1 µg/kg body weight per day for both DON and its acetyl derivatives.

Other fungal conjugated mycotoxins may be produced, however, their presence is not detected in feed or food. In contrast, the commented fungal conjugates may be very common in food and it is important to enlarge the knowledge about them.

2.6.1.2. Plant conjugates

Plants transform mycotoxins in conjugated forms to protect themselves. They convert them to more polar metabolites which are easier to store in the cell. Plant use different compounds to attach to mycotoxins.

The first assay in plant conjugates from ZEN was by Engelhardt et al. (1988); they found enzymes in maize are able to transform ZEN to zearalenone-14-glucoside (ZEN-14-glucoside) (Engelhardt et al., 1988). Then other studies have found more ZEN plant conjugates, for example, Berthiller et al. (2006) showed that *Arabidopsis thaliana* can rapidly transform ZEN into an array of 17 different compounds. However, the most important is ZEN -14-glucoside due to his high presence in cereals as for instance in wheat and oat (De Boevre et al., 2013). Schneweis et al. (2002) found that 42 % of the wheat analyzed samples contained also ZEN-14-glucoside, and the concentration of ZEN -14-glucoside represented between 10 to 20 % of the total ZEN concentration. It is important to control ZEN -14-glucoside because it is completely cleaved to ZEN during digestion in swine (Gareis et al., 1990).

DON-3-glucoside is the plant conjugated which comes from DON; the hydroxyl group in position C13 of DON is replaced by a glucose unit. DON is considered as a plant pathogen inhibiting protein synthesis (Lemmens et al., 2005; Poppenberger et al., 2003). Hence, glucosylation of DON could represent a detoxification process in plants. Glucosylated mycotoxins can be stored in plant vacuoles, where they are protected against cleavage by β-glucosidases. DON-3-glucoside in cereal grains has been known since some years ago; Sewald et al. (1992) were the first to detect this DON metabolite in maize plants. More recently, it has

been also identified in wheat (Berthiller et al., 2005), barley grains (Lancova et al., 2008; Malachova et al., 2010), triticale (Rasmussen et al., 2012) and oat (Rasumssen et al., 2012).

The assays show high percentage of DON-3-glucoside presence in some crops, its presence may reach the 100 % of analysed samples in wheat, maize, oat and triticale (Berthiller et al., 2009; Dall'asta et al. 2013; Desmarchelier et al., 2010; Rasmussen et al., 2010).The ratio DON-3-glucoside/DON concentration is similar among cereal assays, from 10 to 30 % (Berthiller et al., 2009; Dall'asta et al., 2013; Desmarchelier et al., 2010; Rasmussen et al., 2010) and it could vary in relation to years, genotypes and type of plant. On the other hand, it is important to control DON-3-glucoside concentration in food because human intestinal microbiota hydrolise DON-3-glucoside to DON (Berthiller et al., 2011; Gratz et al., 2013).

Other trichothecene have their glucoside derivates, however their knowledge is scarcer. Yoshinari et al. (2014) noted the percentage of nivalenol-3-glucoside to nivalenol ranged from 12 to 27 % in wheat samples. Also T-2 and HT-2 glucoside toxin conjugates have been identified (Busman et al., 2011); these conjugated mycotoxins have been found in wheat, oat and barley (Lattanzio et al., 2012; Veprikova et al., 2012). Fusarenon-X-glucoside was identified for the first time by Nakagawa et al. (2011) in wheat grains, moreover it has been quantified that approximately more than 15 % of total fusarenon is converted to glucoside form.

OTA has also been shown to be transformed in wheat and maize cell suspension cultures to ochratoxin α (OTα), ochratoxin A methyl ester, two isomers of hydroxyochratoxin A and the glucosides and methyl esters of both hydroxyochratoxin A isomers (Ruhland et al., 1994, 1996). The OTα has a lower toxicity than OTA, otherwise hydroxyl-ochratoxin A is an immunosuppressant as effective as OTA itself (Bruinink et al., 1998). The other OTA plant conjugates toxicities have not been studied.

Plant mycotoxin conjugates are common in cereals their ratio to parent mycotoxins is between 10 to 30 %. So, they are important enough to quantify them in food and feed.

2.6.1.3. Food-processing conjugates

Degradation products are conjugated mycotoxins which are formed during foodprocessing. Food processing, especially heating steps can potentially alter mycotoxins. Although mycotoxins are generally very stable, degradation products can occur to some extent. Their level of toxicity and condition in which they appear for each mycotoxin will suppose an important step to establish correctly the PO for all food processes.

2.6.1.3.1. Degradation products from fermentation process

The biodegradation has been studied in some assays, and it is also another method to reduce the concentration of mycotoxins in the food. The main disadvantage of fermentation is that is only useful for some products and conditions of the medium need to be very strict for fermentation microorganisms.

Fermentations in wine and in beer converted AFB1 to aflatoxin B2a (AFB2a). AFB2a is a hydrolyte of AFB1 and it was firstly synthesized chemically by Takahashi et al. (1977). During wine fermentation 60 % of AFB1 was reduced and converted to AFB2a due to *Saccharomyces cerevisae* action (Inoue et al., 2013). On the other hand, in beer fermentation although *S. cerevisiae* is also used, a low reduction of AFB1 was detected and this caused low detection of AFB2a in final beer (Inoue et al., 2013b). Although both winemaking and brewing are based on a fermentation carried out by yeast, AFB1 appears to be preferentially hydrated during wine-making process. It may be explained by differences with respect to yeast, fermentation conditions, or raw materials used. Specifically, although *S. cerevisiae* was used in both processes, different strains were present in wine versus beer fermentation. Furthermore, fermentation conditions for wine (25 °C and pH 3) were distinct from those for beer (20 °C and a change in pH from 5 to 4). In addition, raw materials used for wine making (grapes) are different to those used to brew beer (wort made from barley, corn, and hops). In yogurt fermentation AFB2a was also detected (Megalla et al., 1982). AFB2a is a less toxic compound than AFB1 as showed Lillehoj et al. (1969) and Inoue et al. (2013b).

Regarding DON, DON-3-glucoside could be a degradation product of DON in fermentation. It has been observed in beer production; Lancova et al. (2008) found a spectacular increase of DON-3-glucoside during malting and brewing experiments, between 880 and 630 % for malt and beer, respectively. Zachariasova et al. (2012) found three more products after fermentation of wort: deoxynivalenol-3-diglucoside, deoxynivalenol-3-triglucoside and deoxynivalenol-3-tetraglucoside. However, DON-3-glucoside had the most important increase. Fermentation of breadmaking process also caused an increase of DON-3-glucoside (30 %) (Zachariasova et al., 2012).

ZEN can be affected in fermentations process and it can be reduced in the food causing formation of two ZEN conjugates: β-ZEL and α-ZEL. Mizutani et al. (2011) showed a clear ZEN reduction in beer fermentation because of *S. cerevisae*. *S. cerevisae* converted 85.9 % of ZEN to β-ZEL and α-ZEL in a low concentration (4 % of initial ZEN concentration). The higher production of β-ZEL agrees with Zöllner et al. (2000) who detected only the presence of ZEN and β-ZEL in beer samples.

A wide range of microorganisms are able to reduce OTA when they are in contact. These organisms produce intra and extracellular enzymes which have the ability of degrading OTA. This results in OTA degradation to OTα (Abrunhosa et al., 2014; Stander et al., 2000). For instance, *Pediococcus parvulus* isolated from wines achieved a 90 % of OTA degradation with a

transformation of OTα (Abrunhosa et al., 2014) and Stander et al. (2000) got an almost total transformation of OTA to OTα after 120 minutes of treatment with lipase from *Aspergillus niger.*

The use of microorganisms in food processing may be an useful way to reduce mycotoxin concentration, but they transform mycotoxin to degradation products which may still have toxicological effects, although usually lessthan the original mycotoxin. However more knowledge about this is necessary to use with major benefit the microorganisms.

2.6.1.3.2. Degradation products from heating processes

Heating can cause reduction of mycotoxins in food but it causes apparition of degradation products. They usually have a lower toxcitiy level than parent mycotoxin, however, it is important to know which degradation products are formed when parent mycotoxins are destroyed.

Because of its high presence in cereals, the more common studied degradation products are the degradation products from DON. In general it is considered to be a relatively stable compound. However, some studies have shown that DON is especially unstable under alkaline conditions and at high temperatures. Most of studies on their stability observed their degradation depends on time, temperature, or pH in aqueous solutions. Greenhalgh et al. (1984) were the first to found isodeoxynivalenol (isoDON) due to a baking process. After, other degradation products found were named by Young et al. (1986) as nor-deoxynivalenol A (norDON A), nor-deoxynivalenol B (norDON B), nor-deoxynivalenol C (norDON C), and DON lactone. Bretz et al. (2006) found some new DON degradation products which are 9-hydroxymethyl DON lactone, nor-deoxynivalenol D (norDON D), nor-deoxynivalenol F (norDON F) and nor-deoxynivalenol E (nor DON E). Moreover, degradation products from DON-3-glucoside have been identified in bakery simulation wheat products; Kostelanska et al. (2011) identified nor-deoxynivalenol-3-glucoside A (norDON-3-glucoside A), nor-deoxynivalenol-3-glucoside B (norDON-3-glucoside B), nor-deoxynivalenol-3-glucoside C (norDON-3-glucoside C), nor-deoxynivalenol-3-glucoside D (norDON-3-glucoside D) and nor-deoxynivalenol-3-glucoside lactone (norDON-3-glucoside lactone). Moreover, origination of breakdown products, namely isoDON, norDON A-C (norDON A more abundant than norDON B and norDON C) has not been only found in model experiments, but in some commercial samples (Bretz et al., 2006; Malachova et al., 2012). Degradation food processing products toxicity is lower than DON. The immortalized human kidney epithelial cells (IHKE cells) incubated for 24 h with norDON A-C were 100 % viable (Bretz et al., 2006).

OTA degradation was studied by Cramer et al. (2008) in roasted coffee; the main degradation product was 14-(R)-ochratoxin A and which was formed in amounts of up to 25.6% relative to OTA. The toxicity is lower and it has levels of IC50 = 163 µM. Cramer et al. (2008)

found another degradation product in coffee only in traces, 14-decarboxy-ochratoxin A, which is less toxic than OTA.

Food processing may reduce mycotoxin levels, however degradation products are formed. It is necessary to improve the knowledge about degradation products formed and their toxicity.

2.6.1.4. Animal conjugates

Animal conjugates are mycotoxins that arise from metabolism. These bioconversions generally take place in liver or gastrointestinal tract and are a consequence of the action of tissue enzymes or microflora (Galtier, 1999) and they are further excreted. Although these conjugates are extremely unlikely to play any role in food or feedstuffs, they are very important as biomarkers to evaluate mycotoxin exposure.

More common mycotoxin conjugation products in mammals are glucuronides, as found for *Fusarium* mycotoxin DON and ZEN. Besides unchanged mycotoxins, both deoxynivalenol glucuronide and zearalenone glucuronide were detected in urine of animals (Thanner et al., 2016). Also sulfates of DON and ZEN are formed and excreted by various animals. In recent years, a great number of studies improved the analysis of glucuronide metabolites. In DON, deoxynivalenol-15-glucuronide, which is the most common DON glucuronide form, deoxynivalenol-3-glucuronide (Warth et al., 2012) and deoxynivalenol-7-glucuronide (Šarkanj et al., 2013) were discovered and glucuronide forms represented more than 75 % of total DON forms in urine (Warth et al., 2012).

Other glucuronided mycotoxins detected in urine, plasma or milk are T-2 glucuronide, HT-2 3-glucuronide and HT-2 toxin 4-glucuronide (Welsch & Humpf, 2012) and all of them would produce an increase of parent mycotoxin if samples were submitted to a β-glucuronidase incubation. Glucuronidation occurs mainly in liver and the responsible enzyme for its catalysis is UDP-glucuronyltransferase.

OTA metabolites detected in animals or *in vitro* studies indicate several routes of biotransformation (Ringot et al., 2006): only a small percentage of OTA is hydroxylated, mainly by hepatic cytochrome P540 enzymes of several species, to 4(R/S)-OH-Ochratoxin A (Ringot et al., 2006). Phase I reactions could also yield OTB, the less toxic dechlorination product of OTA (Ringot et al., 2006). Another reaction involves opening of isocoumarin ring and results in a ring-opened lactone form of OTA, a product of similar toxicity detected so far only in rats by special analytical procedures (Li et al., 2000). A major pathway is apparently hydrolysis of amide bond resulting in phenylalanine and ochratoxin alpha (OTα) as cleavage products. OTα is the main metabolite detected in rats, ruminants and human urine (Li et al., 2000; Muñoz et al., 2010). Contrary to DON, OTA gluruconide form in urine is not very clear. On the other hand, urine,

plasma and milk samples presented evidence of OTα glucuronide forms (Muñoz et al., 2010a; Muñoz, et al., 2010b; Pena, et al., 2006; Solfrizzo et al., 2011).

2.6.2. Analysis of conjugates

The importance of modified mycotoxins causes the need to analyse them in food and feed. However, they escape routine analysis for several reasons; these substances are usually more polar than precursor toxins, so they are hard to extract with the usual solvents and/or get lost in cleanup process. Furthermore, the major constraint for determination of conjugated mycotoxins is limited availability of measurement standards (either pure substances or calibrants). To avoid this problem, indirect measures are made and conjugated mycotoxins are transformed to parent mycotoxins through enzymatic (Beloglazova et al., 2013) or alkaline treatment (Dall'Asta et al., 2009). Nowadays, LC-MS is often used for quantification of modified mycotoxins. A major prerequisite for several of these methods is adequate clean-up prior to detection. Commercial cleanup devices for mycotoxins (multifunctional columns or immunoaffinity columns (IACs)) as well as tailor-made clean-up procedures (solid-phase extraction, liquid/liquid partition, etc.) might not necessarily be suitable for conjugated mycotoxins. Extraction and clean-up have to be adapted or at least verified for their applicability. Antibodies against mycotoxins, immobilized in IACs, might not recognize conjugated derivatives, so a previous study of cross reactivity with IAC columns is necessary (Veršilovskis et al., 2011; Zachariasova et al., 2014). During the last few years, the importance of clean-up techniques has slightly decreased, as numerous multianalyte LC-MS/MS methods do not use any clean-up at all. With highly sensitive LC-MS equipment, it has become feasible to simply dilute the sample extracts to minimize possible matrix effects (dilute and shoot approaches).

2.7. Risk assessment

Risk analysis has three main components: risk assessment (scientific advice and information analysis), risk management (regulation and control) and risk communication. Risk assessment provides scientific knowledge for the establishment of guidelines and other recommendations. Risk assessment is performed in a four-step process: hazard identification, hazard characterisation, exposure assessment, and risk characterisation (European Commission, 2000). So, dietary exposure assessment is considered as an essential component of risk assessment.

2.7.1. Exposure assessment

Exposure assessment is defined as 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. Although several scientific reports have been published in order to propose the best methodologies for the exposure assessment framework, to date harmonization is far from being achieved. A common approach to estimate exposure is the combination of contamination data with consumption data. Several methods can be used to estimate intake of a food chemical, and choice will depend on what information is available and how accurate and detailed the estimate needs to be.

2.7.1.1. Biomarkers

Concerning the limitation of combination of contamination data with consumption data, novel methods like biological markers (biomarkers) have been proposed as successful methods to assess the exposure of individuals to chemicals through estimation of food contaminant or their metabolites in biological fluids or molecules induced specifically in response to toxic effect. 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.

One of the first applications of biomarkers to human exposure to food chemicals is illustrated in studies on AFB1 in the late 1980s. The use of AFB1-albumin adducts as biomarkers of Afs exposure has been validated in experimental and human sample analyses (Wild et al., 1990, 1992). The use of urinary aflatoxin B1-N7-guanine adducts validated in the laboratory with human samples, provides a measure of acute exposure to 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 OTA. Due to physical features of OTA, it has a long half-life in human blood. So, OTA blood levels are useful to know the exposure over longer periods, whilst biomarkers levels in urine could be better for short-term variations (Castegnaro et al., 1991; EFSA 2006; Gilbert et al., 2001). While OTA is the predominant form in blood, OTα is the most common compound in urine (Coronel et al., 2011a, b; Klapec et al., 2012; Muñoz et al., 2010a). While other mycotoxins have a higher percentage of glucuronide conjugates in urine, as trichotecens (> 70 %), OTA glucuronides in human urine samples are scarce and even they were not detected in large number of urine analysis (Pena et al., 2006; Vatinno et al., 2007). In a study on OTA-exposure of pregnant women, urine samples were analysed in parallel, with and without enzymatic hydrolysis, an evidence for glucuronides conjugates of OTA was weak or absent (Klapec et al., 2012). So, analysis of OTA and OTα in blood and in urine is a correct way to evaluate OTA exposure. In the case of DON and considering its short excretion half-life, only urinary levels of DON or its glucuronide forms have been proposed as reliable biomarkers. A preliminary approach was developed based on an indirect method: samples were submitted to an enzymatic glucuronide treatment for the subsequent determination of “total DON” (sum of free DON and DON released by hydrolysis) (Turner et al., 2010). DON glucuronides are the main DON forms in urine (> 70 % of total DON). Therefore, it is essential to analyse them. Recent advances in analytical methods permitted a direct method for quantification of DON metabolites such as deoxynivalenol-3-glucuronide (DON-3-glucuronide) (Warth et al., 2011).

2.8. References

Abbas, H.K., Mirocha, C.J., Pawlosky, R.J., & Pusch, D.J. 1985. Effect of cleaning, milling, and baking on deoxynivalenol in wheat. *Applied and Environmental Microbiology, 50*, 482-486.

Abrunhosa, L., Inês, A., Rodrigues, A.I., Guimarães, A., Pereira, V.L., Parpot, P., Mendes-Faia, A., & Venâncio, A. 2014. Biodegradation of ochratoxin A by *Pediococcus parvulus* isolated from Douro wines. *International Journal of Food Microbiology, 188*, 45-52.

Arab, M., Sohrabvandi, S., Mortazavian, A.M., Mohammadi, R., & Tavirani, M.R. 2012. Reduction of aflatoxin in fermented milks during production and storage. *Toxin Reviews, 31*, 44-53.

Aydin, A., Gunsen, U., & Demirel, S. 2008. Total aflatoxin, aflatoxin B1 and ochratoxin a levels in Turkish wheat flour. *Journal of Food and Drug Analysis, 16*, 48-53.

Aziz, N.H., & Youssef, Y.A. 1991. Occurrence of aflatoxins and aflatoxin-producing moulds in fresh and processed meat in Egypt. *Food Additives and Contaminants, 8*, 321-331.

Beloglazova, N.V., De Boevre, M., Goryacheva, I.Y., Werbrouck, S., Guo, Y., & De Saeger, S. 2013. Immunochemical approach for zearalenone-4-glucoside determination. *Talanta, 106*, 422-430.

Bennett, J.W., & Klich, M. 2003. Mycotoxins. *Clinical Microbiology Reviews, 16*, 497-516.

Bergamini, E., Catellani, D., Dall'asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. 2010. Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. 2009. Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Dall'Asta, C., Schuhmacher, R., Lemmens, M., Adam, G., & Krska, A.R. 2005. Masked mycotoxins: Determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 53*, 3421-3425.

Berthiller, F., Werner, U., Sulyok, M., Krska, R., Hauser, M.-T., & Schuhmacher, R. 2006. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant *Arabidopsis thaliana*. *Food Additives and Contaminants, 23*, 1194-1200.

Berthiller, F., Krska, R., Domig, K. J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. 2011. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264–267.

Biehl, M.L., Prelusky, D.B., Koritz, G.D., Hartin, K.E., Buck, W.B., & Trenholm, H.L. 1996. Biliary excretion and enterohepatic cycling of zearalenone in immature pigs. *Toxicology and Applied Pharmacology, 121*, 152-159.

Binder E.M., Tan L.M., Chin L.J., Handl J., & Richard J. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal Feed Science and Technology, 137*, 265–282

Bolliger, G., & Tamm, C. 1972. Vier neue metabolite von *Giberella zeae*: 5-formyl-zearalenon, 7′-dehydrozearalenon, 8′-hydroxy- und 8′-epi-hydroxy-zearalenon. *Helvetica Chimica Acta, 55*, 3030–3048.

Böswald, C., Engelhardt, G., Vogel, H., & Wallnöfer, P.R. 1995. Metabolism of the *Fusarium* mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. N*atural Toxins, 3*, 138-144.

Boutigny, A.-L., Ward, T.J., Ballois, N., Iancu, G., & Ioos, R. 2014. Diversity of the *Fusarium graminearum* species complex on French cereals. *European Journal of Plant Pathology, 138*, 133-148.

Brera, C., Peduto, A., Debegnach, F., Pannunzi, E., Prantera, E., Gregori, E., De Giacomo, M., & De Santis, B. 2013. Study of the influence of the milling process on the distribution of deoxynivalenol content from the caryopsis to cooked pasta. *Food Control, 31*, 309–312.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.-U. 2006. Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445-6451.

Bruinink, A., Rasonyi, T., & Sidler, C. 1998. Differences in neurotoxic effects of ochratoxin A, ochracin and ochratoxin-α *in vitro*. *Natural Toxins, 6*, 173-177.

Bullerman, L.B. 2002. Processing effects on mycotoxins: Introduction. *Advances in Experimental Medicine and Biology, 504*, 155-156.

Burdaspal, P.A., & Legarda, T.M. 2013. Survey on aflatoxin in beer sold in Spain and other European countries. *World Mycotoxin Journal, 6*, 93-101.

Busman, M., Poling, S.M., & Maragos, C.M. 2011. Observation of T-2 toxin and HT-2 toxin glucosides from *Fusarium sporotrichioides* by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). *Toxins, 3*, 1554-1568.

CAC. 2007. Principles and guidelines for the conduct of microbiological risk management (MRM). CACGL, 63-2007.

Caldwell, R.W., Tuite, J., Stob, M., & Baldwin, R. 1970. Zearalenone production by *Fusarium* species. *Applied Microbiology, 20*, 31-34.

Cano-Sancho, G., Gauchi, J.-P., Sanchis, V., Marín, S., & Ramos, A.J. 2011. Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1098-1109.

Cano-Sancho, G. 2013. Exposure assessment of Catalonian population to mycotoxins. Thesis University of Lleida.

Cano-Sancho, G., Sanchis, V., Ramos, A.J., & Marín, S. 2013. Effect of food processing on exposure assessment studies with mycotoxins. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 867-875.

Castañares, E., Albuquerque, D.R., Dinolfo, M.I., Pinto, V.F., Patriarca, A., & Stenglein, S.A. 2014. Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina. *International Journal of Food Microbiology, 179*, 57-63.

Castegnaro, M., Maru, V., Petkova-Bocharova, T., Nikolov, I., & Bartsch, H. 1991. Concentrations of ochratoxin A in the urine of endemic nephropathy patients and controls in Bulgaria: lack of detection of 4-hydroxyochratoxin A. *IARC scientific publications, 115*, 165-169.

Castells, M., Pardo, E., Ramos, A. J., Sanchis, V., & Marín, S. 2006. Reduction of ochratoxin A in extruded barley meal. *Journal of Food Protection, 69*, 1139–1143.

Cheli, F., Pinotti, L., Rossi, L., & Dell'Orto, V. 2013. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. *LWT-Food Science and Technology, 54*, 307-314.

Coronel, M.B., Sanchis, V., Ramos, A.J., & Marin, S. 2011a. Ochratoxin A in adult population of Lleida, Spain: Presence in blood plasma and consumption in different regions and seasons. *Food and Chemical Toxicology, 48*, 2697-2705.

Coronel, M.B., Marin, S., Tarragó, M., Cano-Sancho, G., Ramos, A.J., & Sanchis, V. 2011b. Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain. *Food and Chemical Toxicology, 49*, 1436-1442.

Coronel, M.B., Marín, S., Cano-Sancho, G., Ramos, A.J., & Sanchis, V. 2012. Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 979-993.

Cramer, B., Königs, M., & Humpf, H.-U. 2008. Identification and *in vitro* cytotoxicity of ochratoxin A degradation products formed during coffee roasting. *Journal of Agricultural and Food Chemistry, 56*, 5673-5681.

Creppy, E.E. 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127*, 19-28.

Curtui, V.G., Gareis, M., Usleber, E., & Märtlbauer, E. 2001. Survey of Romanian slaughtered pigs for the occurence of mycotoxins ochratoxins A and B, and zearalenone. *Food Additives and Contaminants, 18*, 730-738.

Dall'Asta, C., Mangia, M., Berthiller, F., Molinelli, A., Sulyok, M., Schuhmacher, R., Krska, R., Galaverna, G., Dossena, A., & Marchelli, R. 2009. Difficulties in fumonisin determination: The issue of hidden fumonisins. *Analytical and Bioanalytical Chemistry, 395*, 1335-1345.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. 2013. Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal, 6*, 83–91.

De Boevre, M., Di Mavungu, J.D., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut, A., Berthiller, F., Van Peteghem, C., & De Saeger, S. 2012. Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 819-835.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J.D., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B., & De Saeger, S. 2013. Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281-292.

De Swarte, C., & Donker, R. A. 2005. Towards an FSO/ALOP based food safety policy. *Food Control, 16*, 825-830.

Desmarchelier, A., & Seefelder, W. 2010. Survey of deoxynivalenol and deoxynivalenol-3-glucoside in cereal-based products by liquid chromatography electrospray ionization tandem mass spectrometry. *World Mycotoxin Journal, 4*, 29–35.

Devegowda, G., Raju, M.V.L.N., Afzali, N., & Swami, H.V.L.N. 1998. Mycotoxin picture worldwide: novel solutions for their counteraction. *Biotechnology in the Feed Industry, Proceedings of the 14th Annual Symposium, Nottingham University Press*, 241-255.

Do, J.H., & Choi, D.-K. 2007. Aflatoxins: Detection, toxicity, and biosynthesis. *Biotechnology and Bioprocess Engineering, 12*, 585-593.

Döll, S., & Dänicke, S. 2011. The *Fusarium* toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Preventive Veterinary Medicine, 102*, 132-145.

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

Edwards, S.G. 2004. Influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. *Toxicology Letters, 153*, 29-35.

Edwards, S.G., Dickin, E.T., MacDonald, S., Buttler, D., Hazel, C.M., Patel, S., & Scudamore, K.A. 2011. Distribution of *Fusarium* mycotoxins in UK wheat mill fractions. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1694-1704.

EFSA (European Food Safety Authority). 2011. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal, 9*, 2197.

EFSA, 2006. European Food Safety Authority, EFSA. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA Journal, 365*, 1-56.

EFSA, 2007. European Food Safety Authority, EFSA. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *EFSA Journal, 446*, 1-127.

El Banna, A.A., Lau, P.Y., & Scott, P.M. 1983. Fate of mycotoxins during processing of foodstuffs II. Deoxynivalenol (vomitoxin) during making of Egyptian bread. *Journal of Food Protection, 46*, 484-486.

El Khoury, A.E., & Atoui, A. 2010. Ochratoxin A: General overview and actual molecular status. *Toxins, 2*, 461-493.

El-Nezami, H., Kankaanpaa, P., Salminen, S., & Ahokas, J. 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B1. *Food and Chemical Toxicology, 36*, 321-326.

Engelhardt, G., Zill, G., Wohner, B., & Wallnöfer, P.R. 1988. Transformation of the *Fusarium* mycotoxin zearalenone in maize cell suspension cultures. *Naturwissenschaften, 75*, 309-310.

Eriksen, G.S., Pettersson, H., Johnsen, K., & Lindberg, J.E. 2002. Transformation of trichothecenes in ileal digesta and faeces from pigs. *Archiv fur Tierernahrung, 56*, 263-274.

Eriksen, G.S. 2003. Metabolism and toxicity of trichothecenes. PhD thesis. Acta Universitatis Agriculturae Sueciae – Agraria 400. *Swedish Agricultural University, Uppsala*, Sweden.

European Commission (EC). 2000. White Paper on Food Safety. Brussels, 12 January 2000.

European Commission (EC). 2006. European Commission N° 1881/2006 of December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L 364 (2006)*, 5-24.

European Commission (EC). 2010. European Commission, N° 165/2010 of 26 February 2010 amending Regulation (EC) N° 1881/2006 setting maximum levels for certain contaminants in foodstuff as regard aflatoxins. *Official Journal of the European Union, L 50*, 8-12.

European Commission (EC). 2012. European Commission, N° 1058/2012 of 12 November 2012 amending Regulation (EC) N° 1881/2006 as regards maximum levels for aflatoxins in dried figs. *Official Journal of the European Union, L 50*, 313-314.

Ezekiel, C.N., Sulyok, M., Warth, B., Odebode, A.C., & Krska, R. 2012. Natural occurrence of mycotoxins in peanutcake from Nigeria. *Food Control, 27*, 338-342.

FAO. 2016. Crop Prospects and Food Situation. N°3, September 2016.

Fitzpatrick, D.W., Picken, C.A., Murphy, L.C., & Buhr, M.M. 1989. Measurement of the relative binding affinity of zearalenone, α-zearalenol and β-zearalenol for uterine and oviduct estrogen receptors in swine, rats and chickens: An indicator of estrogenic potencies. *Comparative Biochemistry and Physiology. Part C, Comparative, 94*, 691-694.

Gale, L.R., Ward, T.J., Balmas, V., & Kistler, H.C. 2007. Population subdivision of *Fusarium graminearum* sensu stricto in the upper midwestern United States. *Phytopathology, 97*, 1434-1439.

Galtier, P. 1999. Biotransformation and fate of mycotoxins. *Journal of Toxicology – Toxin Reviews, 18*, 295-312.

Gareis, M., Bauer, J., Thiem, J., Plank, G., Grabley, S., & Gedek, B. 1990. Cleavage of Zearalenone-Glycoside, a “Masked” Mycotoxin, during Digestion in Swine. *Journal of Veterinary Medicine, Series B, 37*, 236-240.

Gilbert, J., Brereton, P., & MacDonald, S. 2001. Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples. *Food Additives and Contaminants, 18*, 1088-1093.

Gratz, S.W., Duncan, G., & Richardson, A.J. 2013. The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. *Applied and Environmental Microbiology, 79*, 1821-1825

Greenhalgh, R., Gilbert, J., King, R.R., Blackwell, B.A., Startin, J.R., & Shepherd, M.J. 1984. Synthesis, characterization, and occurrence in bread and cereal products of an isomer of 4-deoxynivalenol (vomitoxin). *Journal of Agricultural and Food Chemistry, 32*, 1416-1420.

Groopman, J.D., Hasler, J.A., Trudel, L.J., Pikul, A., Donahue, P.R., & Wogah, G.N. 1992a. Molecular dosimetry in rat urine of aflatoxin-N7-guanine and other aflatoxin metabolites by multiplemonoclonal antibody affinity chromatography and immunoaffinity/high performance liquid chromatography. *Cancer Research, 52*, 267-274.

Groopman, J.D., Jiaqui, Z., Donahue, P.R., Pikul, A., Lisheng, Z., Jun-shi, C., & Wogan, G.N. 1992b. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guanxi Autonomous Region, People’s Republic of China. *Cancer Research, 52*, 45-52.

Groopman, J.D., Wild, C.P., Hasler, J., Junshi, C., Wogan, G.N., & Kensler, T. W. 1993. Molecular epidemiology of aflatoxin exposures: validation of aflatoxin-N7-guanine levels in urine as a biomarker in experimental rat models an humans. *Environmental Health Perspectives, 99*, 107-113.

Gul, O., & Dervisoglu, M. 2014. Occurrence of aflatoxin M1 in vacuum packed kashar cheeses in Turkey. *International Journal of Food Properties, 17*, 273-282.

Gumus, T., Arid, M., Daglioglu, O., & Velioglu, M. 2009. Fate of aflatoxins during bread making. *Journal of Food Science and Technology, 46*, 384-387.

Hammami, W., Fiori, S., Al Thani, R., Ali Kali, N., Balmas, V., MIgheli, Q., & Jaoua, S. 2014. Fungal and aflatoxin contamination of marketed spices. *Food Control, 37*, 177-181.

Harwig, J., Chen, Y.K., & Collins-Thompson, D.L. 1974. Stability of ochratoxin A in beans during canning. *Canadian Institute of Food Science and Technology Journal, 7*, 288-289.

Haskard, C.A., El-Nezami, H.S., Kankaanpää, P.E., Salminen, S., & Ahokas, J.T. 2001. Surface Binding of Aflatoxin B1 by Lactic Acid Bacteria. *Applied and Environmental Microbiology, 67*, 3086-3091.

Hassan, H.F., & Kassaify, Z. 2014. The risks associated with aflatoxin M1 occurrence in Lebanese dairy products. *Food Control, 37*, 68-72.

Heidari, S., Milani, J., & Nazari, S.S.S.J. 2014. Effect of the bread-making process on zearalenone levels. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 2047-2054.

Heperkan, D., Güler, F.K., & Oktay, H.I. 2012. Mycoflora and natural occurrence of aflatoxin, cyclopiazonic acid, fumonisin and ochratoxin A in dried figs. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 277-286.

Hestbjerg, H., Nielsen, K.F., Thrane, U., & Elmholt, S. 2002. Production of trichothecenes and other secondary metabolites by *Fusarium culmorum* and *Fusarium equiseti* on common laboratory media and a soil organic matter agar: An ecological interpretation. *Journal of Agricultural and Food Chemistry, 50*, 7593-7599.

Hidy, P.H., Baldwin, R.S., Greasham, R.L., Keith, C.L., & Mcmullen, J.R. 1977. Zearalenone and some derivatives: production and biological activity. *Advances in Applied Microbiology, 22*, 59-82.

Hsia, C.C., Wu, Z.Y., Li, Y.S., Zhang, F. & Sun, Z.T., 2004. Nivalenol a main *Fusarium* toxin in dietary foods from high-risk areas of cancer of esophagus and gastric cardia in China, induced benign and malignant tumors in mice. *Oncology Reports, 12*, 449-456.

IARC (International Agency for Research on Cancer). 1993. Ochratoxin A. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Working Group on the Evaluation of Carcinogen Risks to Humans Meeting; June 9-16, 1992; Lyon, France. 1993. 1992. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol 56. Lyon, France: World Health Organization (WHO), International Agency for Research on Cancer, 489-521. Available at: http://monographs.iarc.fr/ENG/Monographs/vol56/index.php.

IARC (International Agency for Research on Cancer). 2012. A Review of Human Carcinogens. vol 100F: Chemical Agents and Related Occupations. Lyon, France: IARC Working Group on the Evaluation of Carcinogenic Risks to Humans; 2012. Available at: http://monographs.iarc.fr/ENG/Monographs/vol100F/mono100F.pdf

ICFMSF. 1998. International Commission on Microbiological Specifications for Foods. Microorganisms in Food 6: Microbial ecology of food commodities. London: Blackie Academic & Professional. ISBN: 041247350X.

ICMSF. 2002. International Commission on Microbiological Specifications for Foods. Microorganisms in Food 7: Microbiological testing in food safety management. Kluwer Academic/Plenum Publishers. ISBN: 0306472627.

Iha, M.H., Trucksess, M.W., & Tournas, V.H. 2009. Effect of processing on ochratoxin A content in dried beans. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 1389-1395.

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. 2013a. Fate of mycotoxins during beer brewing and fermentation. *Bioscience, Biotechnology and Biochemistry, 77*, 1410-1415.

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. 2013b. Degradation of aflatoxin B1 during the fermentation of alcoholic beverages. *Toxins, 5*, 1219-1229.

Jackson, L. S., Hlywka, J. J., Senthil, K. R., Bullerman, L. B., & Musser, S. M. 1996. Effects of time, temperature, and pH on the stability of fumonisin B1 in an aqueous model system. *Journal of Agricultural and Food Chemistry, 44*, 906–912.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) Ochratoxin A (addendum). 2008. Safety Evaluation of Certain Food Additives and Contaminants. Prepared by the Sixty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives; June 19-28, 2007; Geneva, Switz. 2008. WHO Food Additive Series, vol 59. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), 357-429, 454. Available at: http://whqlibdoc.who.int/publications/2008/9789241660594_eng.pdf.

Jelinek, C.F., Pohland, A.E., & Wood, G.E. 1989. Worldwide occurrence of mycotoxins in foods and feeds--an update. *Journal of the Association of Official Analytical Chemists, 72*, 223-230.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2011. Seventy-second report. Evaluation of certain contaminants in food. Available at: http://tinyurl.com/zk7ow4k.

Kabak, B. 2009. The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture, 89*, 549-554.

Kadota, T., Furusawa, H., Hirano, S., Tajima, O., Kamata, Y., & Sugita-Konishi, Y. 2013. Comparative study of deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol on intestinal transport and IL-8 secretion in the human cell line Caco-2. *Toxicology in Vitro, 27*, 1888-1895.

Klapec, T., Šarkanj, B., Banjari, I., & Strelec, I. 2012. Urinary ochratoxin A and ochratoxin alpha in pregnant women. *Food and Chemical Toxicology, 50*, 4487-4492.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. 2011.Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Kostelanska, M., Zachariasova, M., Lacina, O., Fenclova, M., Kollos, A.-L., & Hajslova, J. 2011. The study of deoxynivalenol and its masked metabolites fate during the brewing process realised by UPLC-TOFMS method. *Food Chemistry, 126*, 1870-1876.

Krska, R., Pettersson, H., Josephs, R.D., Lemmens, M., Mac Donald, S., & Welzig, E. 2003. Zearalenone in maize: Stability testing and matrix characterisation of a certified reference material. *Food Additives and Contaminants, 20*, 1141-1152.

Kuiper-Goodman, T., Scott, P.M., & Watanabe, H. 1987. Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology, 7*, 253-306.

Lahtinen, S.J., Haskard, C.A., Ouwenhand, A.C., Salminen, S.J., & Ahokas, J.T. 2004. Binding of aflatoxin B1 to cell wall components of *Lactobacillus rhamnosus* strain GG. *Food Additives and Contaminants, 21*, 158-164.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H., & Vanova, M. 2008. Fate of trichothecene mycotoxins during the processing: Milling and baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25*, 650-659.

Lattanzio, V.M.T., Visconti, A., Haidukowski, M., & Pascale, M. 2012. Identification and characterization of new *Fusarium* masked mycotoxins, T2 and HT2 glycosyl derivatives, in naturally contaminated wheat and oats by liquid chromatography-high-resolution mass spectrometry. *Journal of Mass Spectrometry, 47*, 466-475.

Lemmens, M., Scholz, U., Berthiller, F., Dall'Asta, C., Koutnik, A., Schuhmacher, R., Adam, G., Buerstmayr, H., Mesterházy, Á., Krska, R., & Ruckenbauer, P. 2005. The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for *Fusarium* head blight resistance in wheat. *Molecular Plant-Microbe Interactions, 18*, 1318-1324.

Li, S., Marquardt, R.R., & Frohlich, A.A. 2000. Identification of ochratoxins and some of their metabolites in bile and urine of rats. *Food and Chemical Toxicology, 38*, 141-152.

Lillehoj, E.B., & Ciegler, A. 1969. Biological activity of aflatoxin B2a. *Applied microbiology, 17*, 516-519.

Magan, N., & Aldred, D. 2007. Why to fungi produce mycotoxins? In Food Mycology: A Multifaceted Approach to Fungi and Food; Dijksterhuis, J., Samson, R.A., Eds.; Taylor & Francis: Boca Raton, FL, USA, 121–133.

Magan, N., Medina, A., & Aldred, D. 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathology, 60*, 150-163.

Malachova, A., Cerkal, R., Ehrenbergerova, J., Dzuman, Z., Vaculova, K., & Hajslova, J. 2010. *Fusarium* mycotoxins in various barley cultivars and their transfer into malt. *Journal of the Science of Food and Agriculture, 90*, 2495-2505.

Malachova, A., Varga, E., Schwartz, H., Krska, R., & Berthiller, F. 2012. Development, validation and application of an LC-MS/MS based method for the determination of deoxynivalenol and its conjugates in different types of beer. *World Mycotoxin Journal, 5*, 261-270.

Matsuura, Y., & Yoshizawa, T. 1985. Conversion of zearalenone, an estrogenic mycotoxin, by brewing microorganisms. *Journal of the Food Hygienic Society of Japan, 26*, 24-28.

Megalla, S.E., & Hafez, A.H. 1982. Detoxification of aflatoxin B1 by acidogenous yoghurt. *Mycopathologia, 77*, 89-91.

Milanez, T.V., & Leitão, M.F. 1996. The effect of cooking on ochratoxin A content of beans variety ‘Carioca’. *Food Additives and Contaminants, 13*, 89–93.

Milhome, M.A.L., Lima, C.G., de Lima, L.K., Lima, F.A.F., Sousa, D.O.B., & Nascimiento, R.F. 2014. Occurrence of aflatoxins in cashew nuts produced in northeastern Brazil. *Food Control, 42*, 34-37.

Mirocha, C.J. 1979. Biology and action of Zearalenone. *Toxicon, 17*, 127.

Mizutani, K., Nagatomi, Y., & Mochizuki, N. 2011. Metabolism of zearalenone in the course of beer fermentation. *Toxins, 3*, 134-141.

Moss, M.O. 1992. Secondary metabolism and food intoxication – Moulds. *Journal of Applied Bacteriology Symposium Supplement, 21*, 80-88.

Muñoz, K., Blaszkewicz, M., & Degen, G.H. 2010a. Simultaneous analysis of ochratoxin A and its major metabolite ochratoxin alpha in plasma and urine for an advanced biomonitoring of the mycotoxin. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 878*, 2623-2629.

Muñoz, K., Campos, V., Blaszkewicz, M., Vega, M., Alvarez, A., Neira, J., & Degen, G.H. 2010b. Exposure of neonates to ochratoxin A: First biomonitoring results in human milk (colostrum) from Chile. *Mycotoxin Research, 26*, 59-67.

Nakagawa, H., Ohmichi, K., Sakamoto, S., Sago, Y., Kushiro, M., Nagashima, H., Yoshida, M., & Nakajima, T. 2011. Detection of a new *Fusarium* masked mycotoxin in wheat grain by high-resolution LC-Orbitrap™ MS. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1447-1456.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. 1997. The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37*, 21-25.

Nowicki, T. W., Gaba, D. G., Dexter, J. E., Matsuo, R. R., & Clear, R. M. 1988. Retention of the *Fusarium* mycotoxin deoxynivalenol in wheat during processing and cooking of spaghetti and noodles. *Journal of Cereal Chemistry, 8*, 189–202.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. 2012. Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants –Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 423–430.

Ogiso, M., Ito, S., Kimura, A., Saito, M., Sasaki, A., Kibune, N., & Watai, M. 2013. Survey of 7 trichothecenes in corn-derived feed and feed ingredients. *Journal of the Food Hygienic Society of Japan, 54*, 213-218.

Pacin, A., Ciancio Bovier, E., Cano, G., Taglieri, D., & Hernandez Pezzani, C. 2010. Effect of the bread making process on wheat flour contaminated by deoxynivalenol and exposure estimate. *Food Control, 21*, 492-495.

Park, D.L. 2002. Effect of processing on aflatoxin. *Advances in Experimental Medicine and Biology, 504*, 173-179.

Park, J.W., Lee, C., & Kim, Y.B., 2005. Fate of aflatoxin B1 during the cooking of Korean polished rice. *Journal of Food Protection, 68*, 1431–1434.

Peltonen, K.D., El-Nezami, H.S., Salminen, S.J., & Ahokas, J.T. 2000. Binding of aflatoxin B1 by probiotic bacteria. *Journal of the Science of Food and Agriculture, 80*, 1942-1945.

Pena, A., Seifrtová, M., Lino, C., Silveira, I., & Solich, P. 2006. Estimation of ochratoxin A in portuguese population: New data on the occurrence in human urine by high performance liquid chromatography with fluorescence detection. *Food and Chemical Toxicology, 44*, 1449-1454.

Pérez de Obanos, A., González-Peñas, E., & López de Cerain, A. 2005. Influence of roasting and brew preparation on the ochratoxin A content in coffee infusion. *Food Additives and Contaminants, 22*, 463-471.

Petruzzi, L., Sinigaglia, M., Corbo, M.R., Campaniello, D., Speranza, B., & Bevilacqua, A. 2014. Decontamination of ochratoxin A by yeasts: Possible approaches and factors leading to toxin removal in wine. *Applied Microbiology and Biotechnology, 98*, 6555-6567.

Pfeiffer, E., Hildebrand, A.A., Becker, C., Schnattinger, C., Baumann, S., Rapp, A., Goesmann, H., Syldatk, C., & Metzler, M. 2010. Identification of an aliphatic epoxide and the corresponding dihydrodiol as novel congeners of zearalenone in cultures of *Fusarium graminearum*. *Journal of Agricultural and Food Chemistry, 58*, 12055-12062.

Pierides, M., El-Nezami, H., Peltonen, K., Salminen, S., & Ahokas, J. 2000. Ability of dairy strains of lactic acid bacteria to bind aflatoxin M1 in a food model. *Journal of Food Protection, 63*, 645–50.

Pinton, P., Tsybulskyy, D., Lucioli, J., Laffitte, J., Callu, P., Lyazhri, F., Grosjean, F., Bracarense, A.P., Kolf-clauw, M., & Oswald, I.P. 2012. Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: Differential effects on morphology, barrier function, tight junction proteins, and mitogen-activated protein kinases. *Toxicological Sciences, 130*, 180-190.

Piotrowska, M., Nowak, A., & Czyzowska, A. 2013. Removal of ochratoxin A by wine *Saccharomyces cerevisiae* strains. *European Food Research and Technology, 236*, 441-447.

Pitt, J.I., & Hocking, A.D. 2009. Fungi and Food Spoilage. 3rd Edtion. London; New York: Blackie Academic & Professional.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. 2003. Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Prodi, A., Tonti, S., Nipoti, P., Pancaldi, D., & Pisi, A. 2009. Identification of deoxynivalenol and nivalenol producing chemotypes of *Fusarium graminearum* isolates from durum wheat in a restricted area of northern Italy. *Journal of Plant pathology, 91*, 727-731.

Ramos, A.J., Hernández, E., Plá-Delfina, J.M., & Merino, M. 1996. Intestinal absorption of zearalenone and in vitro study of non-nutritive sorbent materials. *International Journal of Pharmaceutics, 128*, 129-137.

RASFF, 2013. Rapid Alert System for Food and Feed http://ec.europa.eu/food/food/rapidalert/index_en.htm

Rasmussen, R. R., Storm, I. M. L. D., Rasmussen, P. H., Smedsgaard, J., & Nielsen, K. F. 2010. Multi-mycotoxin analysis of maize silage by LC-MS/MS. *Analytical and Bioanalytical Chemistry, 397*, 765–776.

Rasmussen, P.H., Nielsen, K.F., Ghorbani, F., Spliid, N.H., Nielsen, G.C., & Joørgensen, L.N. 2012. Occurrence of different trichothecenes and deoxynivalenol- 3-β-D-glucoside in naturally and artificially contaminated Danish cereal grains and whole maize plants. *Mycotoxin Research, 28*, 181-190.

Raters, M., & Matissek, R. 2008. Thermal stability of aflatoxin B1 and ochratoxin A. *Mycotoxin Research, 24*, 130-134.

Richardson, K.E., Hagler Jr., W.M., & Mirocha, C.J. 1985. Production of zearalenone, α- and β-zearalenol, and α- and β-zearalanol by *Fusarium* spp. in rice culture. *Journal of Agricultural and Food Chemistry, 33*, 862-866.

Ringot, D., Chango, A., Schneider, Y.-J., & Larondelle, Y. 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions, 159*, 18-46.

Rocha, O., Ansari, K., & Doohan, F.M. 2005. Effects of trichothecene mycotoxins on eukaryotic cells: A review. *Food Additives and Contaminants, 22*, 369-378.

Rotter, B.A., Prelusky, D.B., & Pestka, J.J. 1996. Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health - Part A, 48*, 1-34.

Ruhland, M., Engelhardt, G., Schafer, W., & Wallnofer, P.R. 1996. Transformation of the mycotoxin ochratoxin A in plants: 1. Isolation and identification of metabolites formed in cell suspension cultures of wheat and maize. *Natural toxins, 4*, 254-260.

Ruhland, M., Engelhardt, G., Wallnöfer, P.R., & Schäfer, W. 1994. Transformation of the mycotoxin ochratoxin A in wheat and maize cell suspension cultures. *Naturwissenschaften, 81*, 453-454.

Sakuma, H., Watanabe, Y., Furusawa, H., Yoshinari, T., Akashi, H., Kawakami, H., Saito, S., & Sugita-Konishi, Y. 2013. Estimated dietary exposure to mycotoxins after taking into account the cooking of staple foods in Japan. *Toxins, 5,* 1032-1042.

Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. 2001. Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants, 18*, 1004-1010.

Šarkanj, B., Warth, B., Uhlig, S., Abia, W.A., Sulyok, M., Klapec, T., Krska, R., & Banjari, I. 2013. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. *Food and Chemical Toxicology, 62*, 231-237.

Savi, G.D., Piacentini, K.C., Tibola, C.S., Santos, K., Sousa, M. G., & Scussel, V.M. 2016. Deoxynivalenol in the wheat milling process and wheat-based products and daily intake estimates for the Southern Brazilian population. *Food Control, 62*, 231-236.

SCF (Scientific Committee of Food). 1999. Opinion of the Scientific Committee on Food on *Fusarium*-toxins Part 1: Deoxynivalenol (DON), (expressed on 2 December 1999) http://ec.europa.eu/food/fs/sc/scf/out44_en.pdf

SCF (Scientific Committee of Food. 2000. Opinion of the Scientific Committee on Food on *Fusarium*-toxins Part 2: Zearalenone (ZEA), (expressed on 22 June 2000) http://ec.europa.eu/food/fs/sc/scf/out65_en.pdf.

Schatzmayr G., & Streit E. 2013. Global occurrence of mycotoxins in the food and feed chain: Facts and figures. *World Mycotoxin Journal, 6*, 213–222.

Schneweis, I., Meyer, K., Engelhardt, G., & Bauer, J. 2002. Occurrence of zearalenone-4-β-D-glucopyranoside in wheat. *Journal of Agricultural and Food Chemistry, 50*, 1736-1738.

Schothorst, M.V. 1998. Principles for the establishment of microbiological food safety objectives and related control measures. *Food Control, 9*, 379-384.

Schwake-Anduschus, C., Proske, M., Sciurba, E., Muenzing, K., Koch, M., & Maul, R. 2015. Distribution of deoxynivalenol, zearalenone, and their respective modified analogues in milling fractions of naturally contaminated wheat grains. *World Mycotoxin Journal, 8*, 433-443.

SCOOP. 2003. Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU member states Report on Tasks for Scientific Cooperation Task 3.2.10. Accessed 03.06.01 http://europa.eu.int/comm/food/fs/scoop/task3210.pdf

Scott, P.M., Kanhere, S.R., Dexter, J.E., Brennan, P.W., & Trenholm, H.L. 1984. Distribution of the trichothecene mycotoxin deoxynivalenol (vomitoxin) during the milling of naturally contaminated hard red spring wheat and its fate in baked products. *Food Additives and Contaminants, 1*, 313-323.

Scudamore, K.A., Banks, J., & MacDonald, S.J. 2003. Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. *Food Additives and Contaminants, 20*, 1153-1163.

Sewald, N., Von Gleissenthall, J.L., Schuster, M., Müller, G., & Aplin, R.T. 1992. Structure elucidation of a plant metabolite of 4-deoxynivalenol. *Tetrahedron-Asymmetry, 3*, 953-960.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. 2012. Analysis of Deoxynivalenol and Deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. 2010. Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology, 3*, 94-99.

Solfrizzo, M., Gambacorta, L., Lattanzio, V.M.T., Powers, S., & Visconti, A. 2011. Simultaneous LC-MS/MS determination of aflatoxin M1, ochratoxin A, deoxynivalenol, de-epoxydeoxynivalenol, α and β-zearalenols and fumonisin B1 in urine as a multi-biomarker method to assess exposure to mycotoxins. *Analytical and Bioanalytical Chemistry, 401*, 2831-2841.

Somma, S., Petruzzella, A.L., Logrieco, A.F., Meca, G., Cacciola, O.S., & Moretti, A. 2014. Phylogenetic analyses of *Fusarium graminearum* strains from cereals in Italy, and characterisation of their molecular and chemical chemotypes. *Crop and Pasture Science, 64*,52-60.

Stander, M.A., Bornscheuer, U.T., Henke, E., & Steyn, P.S. 2000. Screening of commercial hydrolases for the degradation of Ochratoxin A. *Journal of Agricultural and Food Chemistry, 48*, 5736-5739.

Stoloff, L., & Trucksess, M.W. 1981. Effect of boiling, frying, and baking on recovery of aflatoxin from naturally contaminated corn grits or cornmeal. *Journal Association of Official Analytical Chemists, 64*, 678-80.

Streit, E., Schwab, C., Sulyok, M., Naehrer, K., Krska, R., & Schatzmayr, G. 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins, 5*, 504-523.

Stuper-Szablewskaa, K., Szablewskib, T., Buśkoa, M., & Perkowskia, J. 2016. Changes in contents of trichothecenes during commercial grain milling. *LWT-Food Science Technology, 69*, 55-58.

Subirade, I. 1996. Fate of ochratoxin A during breadmaking. *Food Additives and Contaminants, 13*, 25–26.

Sugita-Konishi, Y., Park, B.J., Kobayashi-Hattori, K., Tanaka, T., Chonan, T., Yoshikawa, K., & Kumagai, S. 2006. Effect of cooking process on the deoxynivalenol content and its subsequent cytotoxicity in wheat products. *Bioscience, Biotechnology and Biochemistry, 70*, 1764-1768.

Suman, M., Manzitti, A., & Catellani, D. 2012. A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of wholegrain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Sundstøl Eriksen, G., Pettersson, H., & Lundh, T. 2004. Comparative cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. *Food and Chemical Toxicology, 42*, 619-624.

Takahashi, D.M. 1977. High pressure liquid chromatographic determination of aflatoxins in wines and other liquid products. *Journal of the Association of Official Analytical Chemists, 60*, 799-804.

Thammawong, M., Okadome, H., Shiina, T., Nakagawa, H., Nagashima,H., Nakajima, T., & Kushiro, M. 2010. Distinct distribution of deoxynivalenol, nivalenol, and ergosterol in *Fusarium* infected Japanese soft red winter wheat milling fractions. *Mycopathologia, 172*, 323-330.

Thanner, S., Czeglédi, L., Schwartz-Zimmermann, H.E., Berthiller, F., & Gutzwiller, A. 2016. Urinary deoxynivalenol (DON) and zearalenone (ZEA) as biomarkers of DON and ZEA exposure of pigs. *Mycotoxin Research, 32*, 69-75.

Tibola, C.S., Fernandes, J.M.C., & Guarienti, E.M. 2015. Effect of cleaning, sorting and milling processes in wheat mycotoxin content. *Food Control, 60*, 174-179.

Turcotte, A. M., Scott, P.M., & Tague, B. 2013. Analysis of cocoa products for ochratoxin A and aflatoxins. *Mycotoxin Research, 29*, 193-201.

Turner, P.C., White, K.L.M., Burley, V.J., Hopton, R.P., Rajendram, A., Fisher, J., Cade, J.E., & Wild, C.P. 2010. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK adults. *Biomarkers, 15*, 553-562.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. 2009. Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 896-906.

Vatinno, R., Aresta, A., Zambonin, C.G., & Palmisano, F. 2007. Determination of ochratoxin A in human urine by solid-phase microextraction coupled with liquid chromatography-fluorescence detection. *Journal of Pharmaceutical and Biomedical Analysis, 44*, 1014-1018.

Veprikova, Z., Vaclavikova, M., Lacina, O., Dzuman, Z., Zachariasova, M., & Hajslova, J. 2012. Occurrence of mono- and di-glycosylated conjugates of T-2 and HT-2 toxins in naturally contaminated cereals. *World Mycotoxin Journal, 5*, 231-240.

Veršilovskis, A., Huybrecht, B., Tangni, E.K., Pussemier, L., de Saeger, S., & Callebaut, A. 2011. Cross-reactivity of some commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns to DON- and ZEN-conjugated forms and metabolites. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1687-1693.

Visconti, A., Haidukowski, E. M., Pascale, M., & Silvestri, M. 2004. Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters, 153*, 181–189.

Voss, K.A., & Snook, M.E. 2010. Stability of the mycotoxin deoxynivalenol (DON) during the production of flour-based foods and wheat flake cereal. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 1694-1700.

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Fröhlich, J., & Krska, R. 2012. Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins. *Rapid Communications in Mass Spectrometry, 26*, 1533-1540.

Welsch, T., & Humpf, H.-U. 2012. HT-2 toxin 4-glucuronide as new T-2 toxin metabolite: Enzymatic synthesis, analysis, and species specific formation of T-2 and HT-2 toxin glucuronides by rat, mouse, pig, and human liver microsomes. *Journal of Agricultural and Food Chemistry, 60*, 10170-10178.

Wild, C.P., Chapot, B., Hall, A.J., Montesano, R., Hudson, G.J., Sabbioni, G., Wogan, G.N., Whittle, H., & Groopman, J.D. 1992. Dietary Intake of Aflatoxins and the Level of Albumin-bound Aflatoxin in Peripheral Blood in The Gambia, West Africa. *Cancer Epidemiology Biomarkers and Prevention, 1*, 229-234.

Wild, C.P., Jiang, Y.Z., Allen, S.J., Jansen, L.A.M., Hall, A.J., & Montesano, R. 1990. Aflatoxin-albumin adducts in human sera from different regions of the world. *Carcinogenesis, 11*, 2271-2274.

Yang, D., Geng, Z.M., Yao, J.B., Zhang, X., Zhang, P.P., & Ma, H.X. 2013. Simultaneous determination of deoxynivalenol, and 15- and 3-acetyldeoxynivalenol in cereals by HPLC-UV detection. *World Mycotoxin Journal, 6*, 117-125.

Yli-Mattila, T., & Gagkaeva, T. 2010. Molecular chemotyping of *Fusarium graminearum*, *F. culmorum*, and *F. cerealis* isolates from Finland and Russia. *Molecular Identification of Fungi,* 159-177.

Yli-Mattila, T., Gagkaeva, T., Ward, T.J., Aoki, T., Kistler, H.C., & O'Donnell, K. 2009. A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. *Mycologia, 101*, 841-852.

Yoshinari, T., Ohnishi, T., Kadota, T., & Sugita-Konishi, Y. 2012. Development of a purification method for simultaneous determination of deoxynivalenol and its acetylated and glycosylated derivatives in corn grits and corn flour by liquid chromatography-tandem mass spectrometry. *Journal of Food Protection, 75*, 1355-1358.

Yoshinari, T., Sakuda, S., Furihata, K., Furusawa, H., Ohnishi, T., Sugita-Konishi, Y., Ishizaki, N., & Terajima, J. 2014. Structural determination of a nivalenol glucoside and development of an analytical method for the simultaneous determination of nivalenol and deoxynivalenol, and their glucosides, in wheat. *Journal of Agricultural and Food Chemistry, 62*, 1174-1180.

Young, J.C., Blackwell, B.A., & ApSimon, J.W. 1986. Alkaline degradation of the mycotoxin 4-deoxynivalenol. *Tetrahedron Letters, 27*, 1019-1022.

Zachariasova, M., Cuhra, P., & Hajslova, J. 2014. Cross-reactivity of rapid immunochemical methods for mycotoxins detection towards metabolites and masked mycotoxins: The current state of knowledge. *World Mycotoxin Journal, 7*, 449-464.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. 2012. Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

Zhang, H., & Wang, B. 2014. Fate of deoxynivalenol and deoxynivalenol-3-glucoside during wheat milling and Chinese steamed bread processing. *Food Control, 44,* 86-91.

Zheng, Y., Hossen, S.M., Sago, Y., Yoshida, M., Nakagawa, H., Nagashima, H., Okadome, H., Nakajima, T., & Kushiro, M. 2014. Effect of milling on the content of deoxynivalenol, nivalenol, and zearalenone in Japanese wheat. *Food Control, 40*, 193-197.

Zinedine, A., Soriano, J.M., Moltó, J.C., & Mañes, J. 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology, 45*, 1-18.

Zöllner, P., Berner, D., Jodlbauer, J., & Lindner, W. 2000. Determination of zearalenone and its metabolites α- and β-zearalenol in beer samples by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications, 738*, 233-241.

3. Objectives

General objectives of the present thesis were to determine the stability of mycotoxins during food processing, as well as to evaluate the mycotoxin presence in cereal based foods and human exposure to them.

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

- To determine mycotoxin levels in some cereal based food.

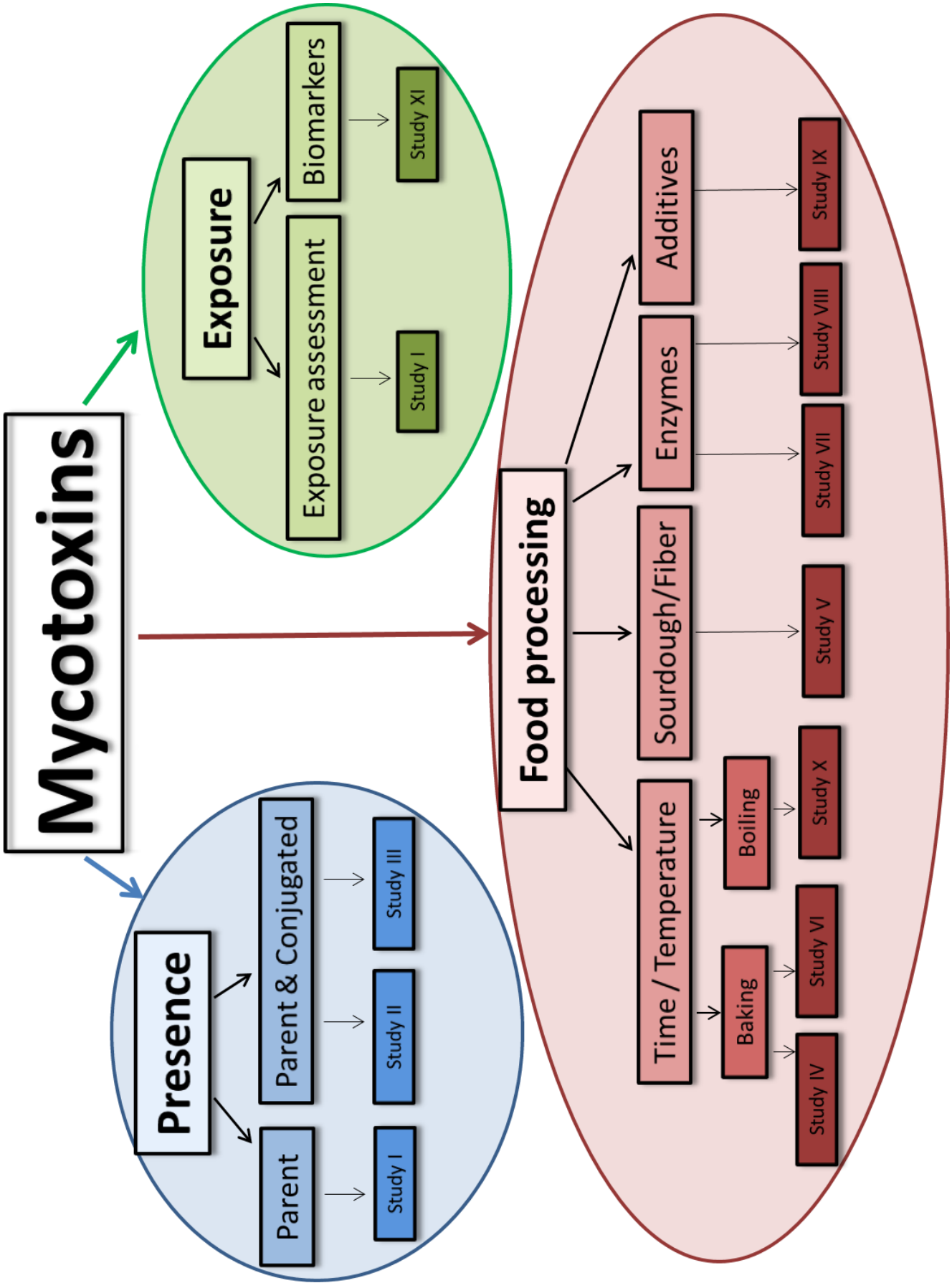
- To develop analytical methods for the correct determination of conjugated mycotoxins.

- To evaluate stability of OTA, DON and their conjugates in food processing, to identify Performance Objectives (POs).

- To deeply study the different factors which affect stability of OTA, DON and their conjugates.

- To evaluate exposure levels of the population to mycotoxins through intake studies and biomarkers.

4. Work Plan


Mycotoxins
Exposure
Biomarkers
Study XI
Exposure assessment
Study I
Presence
Parent & Conjugated
Study III
Study II
Parent
Study I
Food processing
Additives
Study IX
Enzymes
Study VIII
Study VII
Sourdough/Fiber
Study V
Time / Temperature
Boiling
Study X
Baking
Study VI
Study IV

5. Methodology

Although the methodology is described with more detail in each study, information about general metholologies followed in this thesis is described in this section.

5.1. Mycotoxins presence

These studies (I, II and III) deal with the presence of mycotoxins in cereals and cereal based food. It is really important to publish studies about mycotoxins presence because they allow to know the real situation under the current risk management. Moreover results permit exposure assessment. However, choice of samples is an essential step to get representative results.

Study I. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market.

Sixty-seven bran samples were collected from different Spanish cities and analysed for Afs, DON, ZEN and OTA. Wheat and oat were the only cereals bran analysed because the bran from these cereals is the mostly consumed. Maximum possible information was collected for each sample:

- **Shoping city:** samples were purchased from three different Spanish cities (Lleida, Valencia and Olot).
- **Country of origin:** although the samples were bought in Spain, the cereal could have been harvested in different countries.
- **Distribution company.**
- **Submitted to thermal treatment:** some brands are submitted to heat treatment as extrusion or toasting. This heat process could affect mycotoxin stability.
- **Organic/no organic production of cereal.**
- **Presence of other ingredients:** some brand samples could bring other ingredients to improve the flavour, but they may produce changes in mycotoxins concentration.

Later, results were analysed based on these characteristics. Moreover, data were used for an exposure assessment included in the same study.

Study II. Investigation on the incidence of free and modified mycotoxins in cereal-based processed foods.

This study was made during the stay in Ghent University; specifically, in the Food analysis laboratory of Dr. Sarah De Saeger. Bread samples (56) were bought in all types of shops. All types of bread were collected (bran bread, multicereal bread, etc.) and they were

analysed for parent mycotoxin (NIV, DON and ZEN) and conjugated mycotoxin (DON-3-glucoside, 3-ADON, 15-ADON, ZEN-14-glucoside, α-ZEL and β-ZEL). The analysed mycotoxins are the most usual mycotoxins in cereal products. As bread is one of the most consumed products around the world, it is very important to control mycotoxin presence, especially conjugated mycotoxins which have been rarely analysed. Raw materials are more commonly analysed than final products but information about mycotoxins in final products is required to be compared to raw material, and for risk assessment.

Study III. Looking for modified aflatoxins

This study was also made during the stay in Ghent University. African samples of maize (18) and sorghum (1) were analysed for Afs (AFB1, AFB2, AFG1 and AFG2). Samples were collected from different fields from Nigeria and were analysed in a previous study in which Afs content was studied. So, we selected Afs positive samples with large content of mycotoxin, as they were likely to contain conjugated aflatoxins.

5.2. Preparation of samples for food processing studies

One main objective of this thesis was to study the stability of mycotoxins during food processing. We studied fermentation, baking and boiling in cereal processing. Samples were lab-made with the aim to have more control of the factors. So, in all cases raw ingredients were bought and food processes were simulated trying to follow industrial process. OTA, DON and their conjugates were the analysed mycotoxins because they are some of the most common mycotoxins in wheat products. So, all food processes studied involved wheat as cereal. Before to start all studies, mycotoxin content of flour or semolina was analysed and if mycotoxin concentration was too low, mould producers of OTA (*Aspergillus ochraceus*, TA 3.201) and DON (*Fusarium graminearum*, TA 3.234) were inoculated to obtain desired mycotoxin concentration. These moulds are kept in the Food Technology Department collection, University of Lleida. They were previously proved to be OTA and DON producers when cultured on wheat. However, conjugated mycotoxin concentration was difficult to change. Prior to mould inoculation, flour was sterilised and water was added in flour or semolina to get better water activity for mould growth. Later, a spore suspension of each strain was made in water and Tween 80 (0.005 % v/v) and five millilitres of either *F. graminearum* or *A. ochraceus* suspension were inoculated in glass flasks containing flour or semolina with the optimum moisture. After inoculation, flasks were stored at 25 °C and periodic mycotoxin analysis was made, thus it could be know when mycotoxins concentration was enough. Then, this contaminated material was mixed with ordinary one, and food processes started.

5.2.1. Bread making process

Some studies required the elaboration of breads or baking analogues to study the stability of mycotoxins during the bread making process.

Study IV. Stability of DON and OTA during the breadmaking process and determination of process and performance criteria.

Study V. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.

Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products

Study VII. Enzyme bread improvers affect stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Study VIII. Effect of xylanase and α-amylase in DON and his conjugates during breadmaking process.

Study IX. Stability of DON and DON-3-glucoside with food additives presence.

252 loaves of bread were made to study the stability of OTA, DON and their conjugates (DON-3-glucoside, 3-ADON and 15-ADON): 180 loaves for study IV and V, 42 for study VII and 30 for study VIII. All loaves were made in pilot plant of the Food Technology Department from University of Lleida. Standard recipe was used, however, some ingredients changed among studies as well as temperatures and times of fermentation and baking. Different times (from 45 to 130 minutes) and temperatures (from 160 to 220 °C) of baking were used in the study IV (Figure 5.1). Brand and sourdough were added only in Study V (Figure 5.2). Bread improvers were not added in Studies VII and VIII because different enzymes (xylanase, α-amylase, cellulase, protease, lipase and glucose-oxidase) and fermentation temperatures (30 and 45 °C) were used in Study VII (Figure 5.3) and xylanase and α-amylase were added in Study VIII (Figure 5.4). Moreover, different initial parent mycotoxins concentrations were tested. Round small thermoprobes were located in the middle of the dough to control evolution of temperature in bread during all breadmaking process. In all cases, a piece of sample was taken after fermentation and baking for analysis.

Figure 5.1. Scheme of bread making process and ingredients used for the Study IV. Stability of DON and OTA during breadmaking process and determination of process and performance criteria.

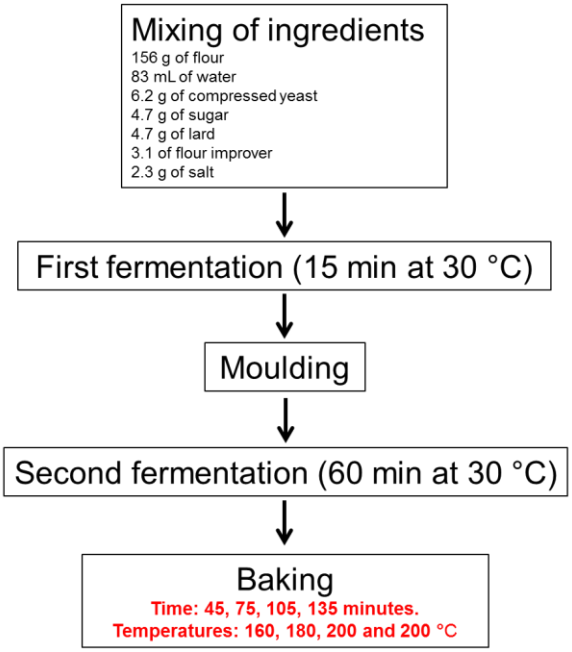


Figure 5.2. Scheme of bread making process and ingredients used for the Study V. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.

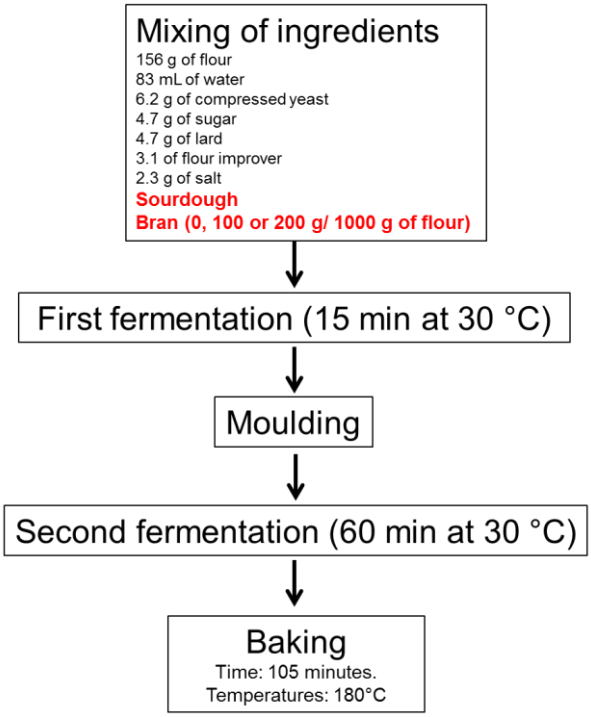


Figure 5.3. Scheme of the bread making process and ingredients used for the Study VII. Study VII. Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

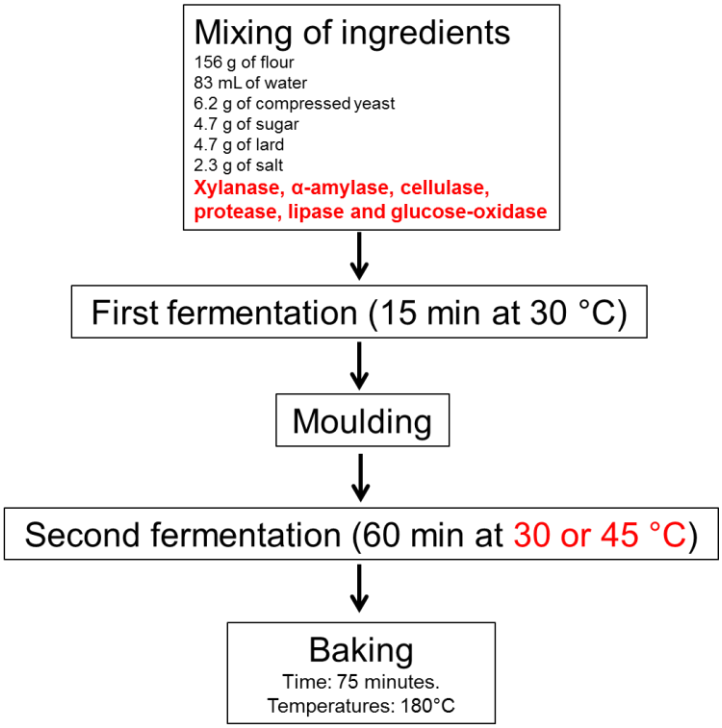
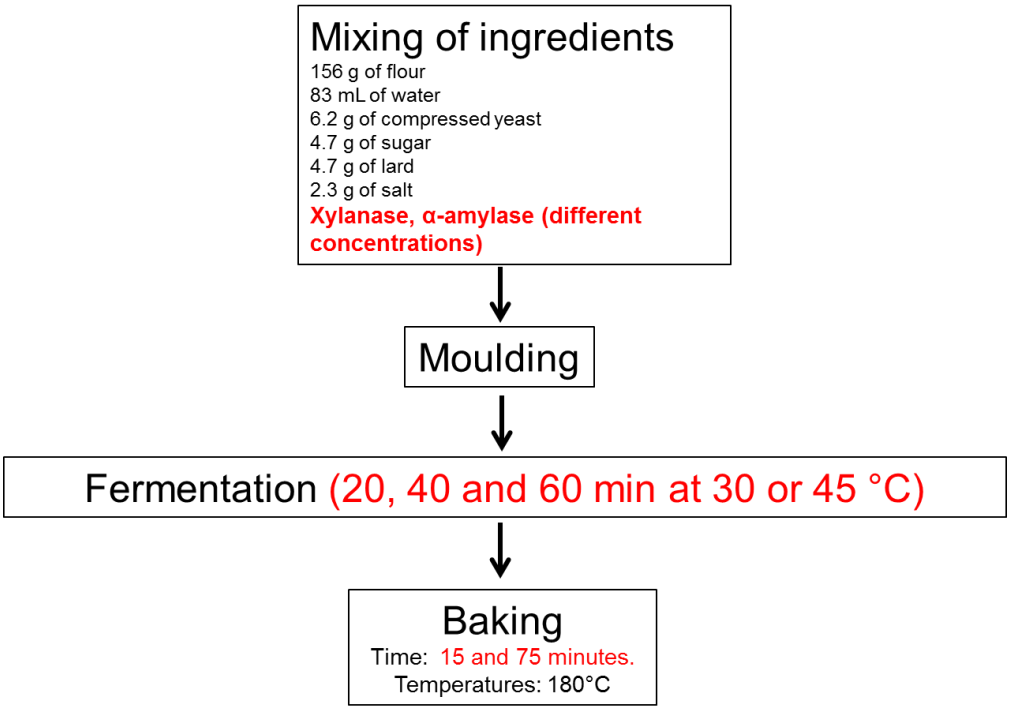


Figure 5.4. Scheme of the bread making process and ingredients used for the Study VIII. Study VIII. Effect of xylanase and α-amylase in DON and its conjugates during the breadmaking process.



Fate of OTA, DON and their conjugates during baking step was studied deeply. For this reason, more than 500 small round wheat flour doughs (3 g) were made in the pilot plant of the Food Technology of University of Lleida. These tiny matrixes permitted a fast heat transmission, so, a high temperature was achieved easily inside the studied item. Small thermoprobes were located inside the doughs to check the temperature evolution during baking. Ingredients used were the common for a bakery product. However ingredients formula changed in different studies. Thus, a in-depth study of the effect of temperature and time of baking in OTA, DON and its conjugates (DON-3-glucoside, 3-ADON and 15-ADON) was made in the Study VI, so different temperatures (from 140 to 200 °C) and times (from 0 to 40 minutes) were tested (Figure 5.5). On the other hand, a large list of common food industry additives was added to the standard dough to check their possible effect on the stability of DON and DON-3-glucoside in Study IX (Figure 5.6).

Figure 5.5. Scheme of small flour wheat matrixes making process and ingredients used for the Study VI. Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.

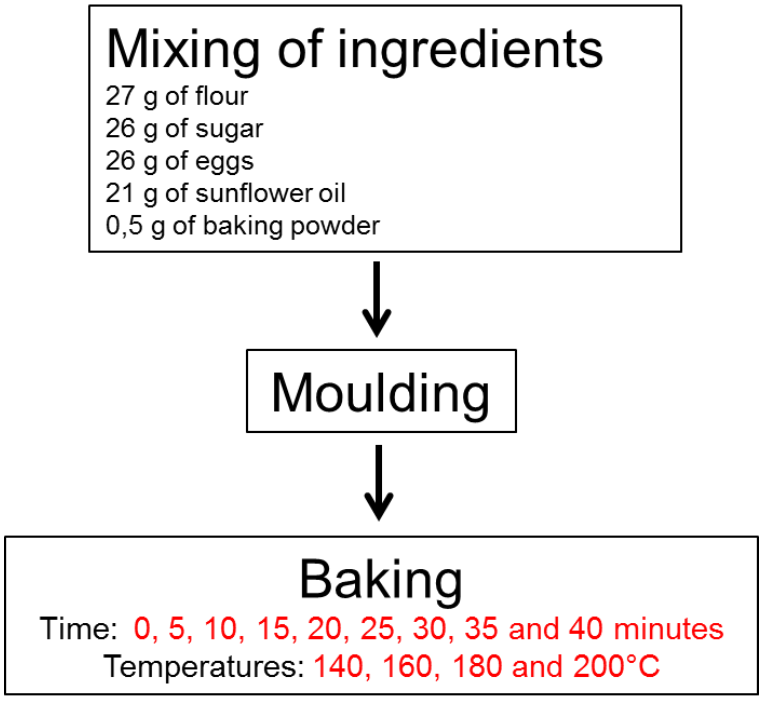
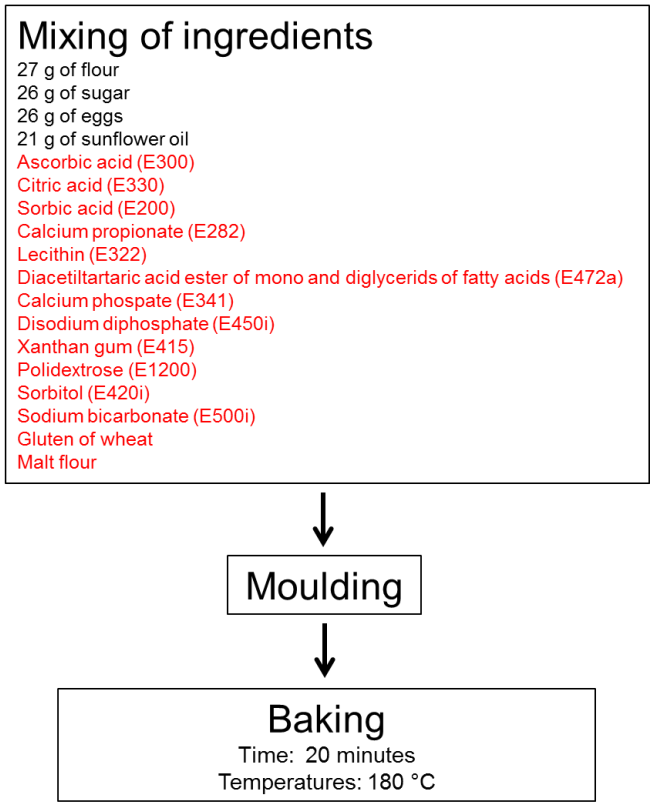


Figure 5.6. Scheme of bread making process and ingredients used for the Study IX. Study IX. Stability of DON and DON-3-glucoside with food additives presence.



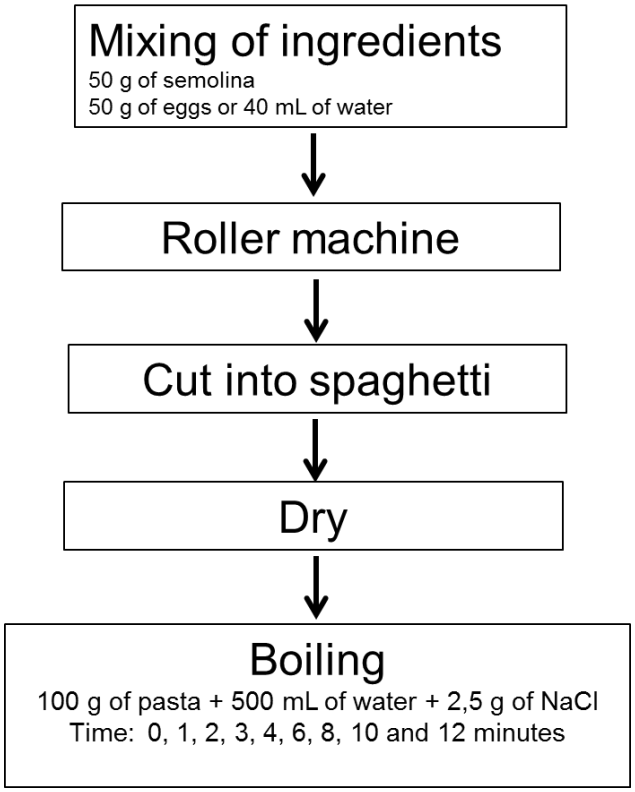
5.2.2. Boiling

Spaghettis were made and stability of mycotoxins during breadmaking pasta and boiling was assessed.

Study X. Stability and kinetics of leaching of deoxynivalenol, deoxynivalenol-3-glucoside and ochratoxin A during boiling of wheat spaghettis.

Durum wheat semolina was used to produce pasta samples which were boiled to study stability of OTA, DON and DON-3-glucoside. Initial wheat semolina was previously inoculated with producing moulds to get the required mycotoxin concentration. As for bread making studies process followed was as similar as possible to industrial one (Figure 5.7). 6 kg of pasta was necessary to produce all necessary samples (3 replicates x 2 different initial concentrations x 9 sampling times). Pasta was prepared mixing durum wheat semolina with water or egg. Presence of egg was studied as a possible factor which affects mycotoxin stability. Then the mix was manually blended and dough was transferred to a roller machine to get a thin dough sheet with was later cut into spaghetti. Later, resulting spaghettis were dried before cooking. 9 different times (from 0 to 12 min) were considered for determination of mycotoxins concentration over time. Moreover, broths were also analysed to see the possible transfer of mycotoxins to boiling water.

Figure 5.7. Scheme of the pasta making process followed and ingredients used for the Study X. Stability and kinetics of leaching of deoxynivalenol, deoxynivalenol-3-glucoside and ochratoxin A during boiling of wheat spaghettis



5.3. Exposure assessment studies

Mycotoxin exposure has been studied and some studies of this thesis dealt with this topic.

Study I. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market.

Study XI. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.

Firstly, from contamination data got in Study I an exposure assessment to DON due to bran consumption was made. DON was chosen for exposure assessment because its concentration in analysed bran samples was large. DON concentrations obtained for each contamination data set were fitted with the method of maximum likelihood using the CAPABILITY procedure of SAS software. Anderson-Darling statistic was used in the goodness-of-fit test, and a significance level greater than 95 % was necessary to accept distribution as a suitable candidate. Nine different simulation scenarios of consumption were used from 1 to 30 g of fibre per day and normalised to an average individual body weight of 70 kg. Random

contamination, drawn from corresponding adjusted gamma probability density function, was combined with the normalised consumption vector over simulation set. The mean exposure and other statistics where computed on a histogram built with simulation of the set (n = 10,000).

Secondly, a biomarkers study for DON and OTA was made. Twenty three volunteers from Lleida participated in the study and gave 6 plasma and 6 urine samples for analysis. DON, OTA and their conjugates (DON-3-glucuronide, DON-3-glucoside, 3-ADON, 15-ADON, DOM-1 and OTα) were analysed in urine, while OTA and OTα were analysed in plasma. Moreover, volunteers were submitted to a restriction diet for five days. Diet restricted those products susceptible of DON and/or OTA contamination (Table 5.1). The subsequent 7 days, volunteers returned to their common diet, but they had to register their food intake (Table 5.2). So, levels of DON, OTA and their metabolites were monitored in urine and OTA and OTα in plasma over the whole period (Table 5.3).

Table 5.1. Restricted and permitted food during th restriction period.

Restricted food	Permitted food
Cereals and derivatives	
• Pasta made from wheat. • Bread • Baking products (cookies, muffins ….). • Cereal breakfast. • Snacks (extrusions maize, popcorn…).	• Rice • Cereal breakfast from rice or oat.
Beverages	
• Wine, beer and coffe	• The rest
Species	
• Cayenne	• All except cayenne.
Nuts	
• Peanuts, pistachios, raisins.	• Hazelnuts, walnuts and almonds.
Chocolate	**All meat and fish**
	Fruit and vegetables
	Milk and derivatives

Table 5.2. Example of questionnaire given to volunteers for food consumption recording during the study.

	FOOD REGISTER	
	Day X	**ATTENTION!**
Breakfast		You should write down with the maximum precision, the type and quantity of food you eat during all day. You should put the weight of the intake food as long as you can do it. You must increase the precision in the following food: • **Coffee** • **Beer** • **Wine** • **Pasta** • **Bread** • **Baking products** • **Breakfast cereals** • **Snacks** • **Nuts** • **Chocolate**
At half morning		
Lunch		
At half afternoon		
Dinner		
Others		

Table 5.3. Scheme of the experimental setup and sampling strategy.

Day	-5	-4	-3	-2	-1	0	1	2	3	4	5	6
Diet	R*	R	R	R	R	N*	N	N		N	N	N
Blood analysis			X		X	X		X		X		X
Urine analysis			X		X	X	X			X		X

*R = Restricted diet
*N = Normal diet

5.4. Chemical analysis

Different analyses were made to obtain results in the different studies. Although mycotoxin analyses were the most common, enzyme activity and creatinine levels were also analysed.

5.4.1. Enzyme assays

Enzymes are considered as a possible factor which affects mycotoxins stability during food process. Two studies involved deep work with enzymes and their relation with mycotoxin stability.

Study VII. Enzyme bread improvers affect stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Study VIII. Effect of xylanase and α-amylase in DON and its conjugates during breadmaking process.

Enzyme activity was measured for initial mix of ingredients (12 samples), fermented dough (78 samples) and final bread (78 samples) and we determined six different enzyme activities in the Study VII (xylanase, α-amylase, cellulase, protease, lipase and glucose-oxidase) and two enzyme activities in Study VIII (xylanase and α-amylase).

Enzyme activity is defined as the moles of substrate converted per unit time. So, enzyme activity is a measurement of the quantity of active enzyme present. Moreover, enzyme assays are quiet sensitive to some conditions which should be defined in an enzyme assay: salt concentration, temperature, pH and substrate saturation.

All enzyme assays techniques measure either the consumption of substrate or production of product over time. A large number of different methods of measuring concentrations of substrates and products exist and many enzymes can be assayed in several different ways. However, a first classification in enzyme assays can be made according to their sampling method: continuous assays, where the assay gives a continuous reading of activity, and discontinuous assays, where samples are taken, reaction stopped and then concentration of substrates/products is determined. The most common enzyme assays are: spectrophotometric, fluorometric, calorimetric, chemiluminescent, light scattering, microscale thermophoresis, radiometric and chromatographic. Each method has some odds and drawbacks and all of them are a good choice in function of the required study.

Spectrophotometry was the method followed in enzyme determinations made in this thesis, besides this method provides a good precision, it is widely used in food industry and it can be used for all the enzymes tested in the thesis.

Spectrophotometric assays measure a change in light that assay solution absorbs. If light is in the visible region as in the methods followed in this thesis, you can actually see a change in colour of the assay, and these are called colorimetric assays.

To quantify enzymatic activity with a spectrophotometer is necessary to make essential steps before enzyme activity determination:

1. Blank reagent: it is used to set the spectrophotometer to zero absorbance. It is prepared with neither substrate nor enzyme, only reagents.
2. Calibration curve: it has to be made with different concentrations. It is prepared using substrates with known added enzyme concentration.

5.4.2. Creatinine assays

To obtain correct mycotoxin results in urine analysis it is necessary to quantify the creatinine values.

Study XI. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.

Creatinine is a breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). It is an important indicator of renal health. Furthermore, creatinine concentration is also checked during standard urine drug tests so normal creatinine levels indicate the test sample is undiluted. Creatinine levels have been determined in Study XI to correct mycotoxin concentration in urine (138 samples). Creatinine levels were analysed according to Jaffé reaction. It is widely employed as method of choice for creatinine testing due to its speed, precision and cost-effectiveness. It is based on the properties of creatinine and picric acid in alkaline solution. Colour change that occurs is directly proportional to concentration of creatinine.

5.4.3. Mycotoxin analysis

Mycotoxins have been analysed in all studies of this thesis and chromatography has been the only method used to quantify them. Analysis in the thesis have been made through liquid chromatography because it was the easier method available in the laboratory. Nonetheless different liquid chromatography techniques have been handled. It is essential to appraise which is the best method to analyse mycotoxins before to start the study.

5.4.3.1. HPLC with fluorescence or ultraviolet detector

In the following studies the HPLC with fluorescence or ultraviolet detector were used:

Study I. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market.

Study IV. Stability of DON and OTA during the breadmaking process and determination of process and performance criteria.

Study V. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.

Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.

Study VII. Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Study IX. Stability of DON and DON-3-glucoside with food additives presence.

Study X. Stability and kinetics of leaching of deoxynivalenol, deoxynivalenol-3-glucoside and ochratoxin A during boiling of wheat spaghettis.

Study XI. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.

HPLC from Food Technology Department of University of Lleida has been used for most studies in this thesis. This analytical technique permits to analyse easily all studied parent mycotoxins. But, some drawbacks appear with the use of HPLC because it is not able to detect a wide range of conjugated mycotoxins or the limits of detection are too high. Moreover, purification is customarily needed. Thus, mycotoxins analysed with this equipment were parent mycotoxins Afs, DON, ZEN and OTA and conjugated mycotoxin DON-3-glucoside. Fluorescence (FLD) or ultraviolet (UV) detector was changed in function of the analysed mycotoxins. Ultraviolet was demanded with DON and DON-3-glucoside and FLD was needed for Afs, ZEN and OTA. Moreover, a post-column photochemical derivatization device was added for the derivatization of Afs. All analysis with HPLC needed a purification step which was made with IAC columns specific for each mycotoxin. DON-3-glucoside showed an acceptable good cross reactivity with IAC DON column and the limit of detection was good enough. Validation studies for all mycotoxins and matrixes were made to be sure used analytical methods were acceptable for analysing mycotoxins (Table 5.4).

5.4.3.2. LC with mass spectrometer

In the following studies the LC with mass spectrometer detector were used:

Study II. Investigation on the incidence of free and modified mycotoxins in cereal-based processed foods.

Study III. Looking for modified aflatoxins.

Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.

Study VIII. Effect of xylanase and α-amylase in DON and his conjugates during breadmaking process.

Study XI. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.

HPLC had some limitations to analyse conjugated mycotoxins or scant concentrations. For this reason, when studies required analysing conjugated or lower concentrations LC with a tandem mass spectrometry (LC-MS/MS) was used. An important advantage of LC-MS/MS is that a purification of samples is not compulsory, so IAC columns are not necessary if sample is clean enough. The lack of purification helps to analyse conjugates because they are not lost during this previous step. Thus, LC-MS/MS has been employed in some studies; also this type of equipment was handled during the stay in Ghent University. Many mycotoxins in different matrixes have been analysed with this equipment (Table 5.4). Moreover two new analytical methods were developed in two studies (Study VI and XI). One for bakery products (Study VI) and another one for urine samples (Study XI) both permit to detect DON conjugates with an extremely low level of detection. The two new methods worked correctly as the results showed.

Table 5.4. Most important analytical information for all mycotoxin analysis performed in this thesis work.

Study	Lab.	Matrix	Equipment	Mycotoxin	Validation	New method	Other
I	Lleida	Bran	HPLC-FLD HPLC-UV	Afs ZEN OTA DON	Yes	No	
II	Ghent	Bread	LC-MS/MS	NIV DON DON-3-glucoside 3-ADON 15-ADON ZEN ZEN -14-glucoside α-ZEL β-ZEL	Yes	No	
III	Ghent	Cereals	LC-MS/MS	Afs	No	No	
IV and V	Lleida	Flour, fermented dough and bread	HPLC-UV HPLC-FLD	DON DON-3-glucoside OTA	Yes	No	
VI	Lleida	Baked products	HPLC-UV HPLC-FLD	DON OTA	Yes	No	
			LC-MS/MS	DON DON-3-glucoside 3-ADON 15-ADON DOM-1	Yes	Yes	
VII	Lleida	Flour, fermented dough and bread	HPLC-UV	DON DON-3-glucoside	No	No	Enzymes
VIII	Lleida	Flour, fermented dough and bread	LC-MS/MS	DON DON-3-glucoside	No	No	Enzymes
IX	Lleida	Baked products	HPLC-UV	DON DON-3-glucoside	No	No	
X	Lleida	Semolina, Pasta and broth	HPLC-UV HPLC-FLD	DON DON-3-glucoside OTA	Yes	No	
XI	Lleida	Plasma	HPLC-FLD	OTA OTα	Yes	No	
		Urine	LC-MS/MS	DON DON-3-glucoside DON-3-glucuronide 3-ADON 15-ADON DOM-1 OTA OTα	Yes	Yes	Creatinine

6. Study I. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in Spain's markets.

Food and Chemical Toxicology (2013) 53, 133-138.

Authors: Vidal, A., Marín, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V.

6. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market.

Arnau Vidal, Sonia Marín, Antonio J. Ramos, German Cano-Sancho and Vicente Sanchis.

Applied Mycology Unit, Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio, Av. Rovira Roure 191, 25198 Lleida, Spain

*Corresponding author. Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: vsanchis@tecal.udl.es. Postal address: Avda. Rovira Roure, 191. 25198 Lleida (Spain)

6.1. Abstract

The aflatoxins (Afs), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN) are mycotoxins produced by fungal species which can contaminate, alone or simultaneously, cereal-based raw materials. Usually, the higher mycotoxins concentrations in cereals are found in the external layers of the grain (bran). Nowadays bran is increasingly consumed for its high fiber concentration. The objectives of this study were determining the concentration of these mycotoxins in bran samples intended for direct human consumption and to study the influence of some characteristics of the samples that may affect the mycotoxins content, there aren't studies about fiber for direct human consumption. 67 bran samples from shops and supermarkets from two different Spanish cities were analyzed, being 37 samples of wheat bran and the remaining of oat bran. The results showed a major presence of DON in the analyzed samples, with levels above the EU legislation in some samples. Presence of DON was more frequent in wheat samples, compared to oats ones ($p<0.05$). Extruded or toasted samples, subjected to a heat treatment during processing, presented a significantly lower concentration of OTA, and differences between the organically and conventionally produced samples were also detected in OTA, which showed higher levels in the organic samples. Co-occurrence was frequently found between the Fusarium mycotoxins (ZEN and DON). Due to the high levels of DON in the analyzed samples, a calculation of DON intake has been made and it has been demonstrated that bran can account for an important percentage of DON exposure in the total diet.

Keywords: aflatoxins, deoxynivalenol, ochratoxin A, zearalenone, dietary fiber.

6.2. Introduction

To date, over 300 mycotoxins have been identified, but the most important groups in foods are produced by these three genera: aflatoxins (AFs), produced by some *Aspergillus* species, ochratoxin A (OTA) produced by both *Aspergillus* and *Penicillium*, and *Fusarium* toxins, mainly trichothecenes (type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)), zearalenone (ZEN) and fumonisins B1 and B2. Moreover, Fusarium mycotoxins can be found alone or simultaneously, as well as co-occurring with other mycotoxins such as AFs, in cereals and in cereal-based foods (Jestoi, 2008). The co-occurrence is a usual situation in cereals, especially in mycotoxins potentially produced by the same fungal specie.

AFs are the most potent mutagenic and carcinogenic natural substances known. There are 6 types of AFs that frequently contaminate foods: B1, B2, G1, G2, M1 and M2. although the latter two are only found in the milk and derivatives. Aflatoxin B1 (AFB1) is consistently carcinogenic and genotoxic *in vitro* and *in vivo* (EFSA, 2007), and therefore it was classified in the group 1 by the International Agency for Research on Cancer (IARC, 2002). OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (SCF, 1998). This mycotoxin has been classified as a possible human carcinogen, in the group 2B, by IARC (IARC, 2002). DON is one of the most regular contaminants in cereals (Jelinek et al., 1989), also known as vomitoxin. Although no indication of carcinogenic or mutagenic effects has been reported, DON has been linked with human gastroenteritis (Pestka, 2010a; Pestka 2010b). At the molecular level, DON disrupts normal cell function by inhibiting protein synthesis, affecting cell signalling, differentiation, and proliferation. An acute and high dose of DON can induce vomiting, whereas chronic dietary exposure to DON causes reduced feed intake, decreased nutritional efficiency, reduced weight gain, and immune dysregulation. It is the predominant mycotoxin with highest levels detected in the bran fraction (Edwards et al., 2011). ZEN is a non-steroidal estrogenic toxin, which the International Agency for Research on Cancer (IARC) has categorized as a class 3 carcinogen (IARC, 1993). ZEN produces estrogenic effects in humans and animals leading to hyperestrogenism. ZEN can act as an estrogen analog and in humans has been recently considered as a triggering factor for central precocious puberty at least in prepubertal girls (Massart and Saggese, 2010).

Due to their toxicological effects, the European Commission has set maximum permitted levels in processed cereal products for direct human consumption: 2 µg/kg for AFB1 and 4 µg/kg for the sum of AFB1, AFG1, AFB2 and AFG2; 3 µg/kg for OTA, 750 µg/kg for DON and 75 µg/kg for ZEN (European Commission, 2006a, 2010).

Dietary fiber refers to plant cell wall components, mainly polysaccharides and is not digestible by human or other mammalian digestive enzymes. The benefits of dietary fiber intake are numerous, ranging from improved large bowel function to slowed digestion and absorption of carbohydrate and fat and reduced risk for certain diseases. Based on the effects on bowel function, the EFSA Panel considered fiber intake of 25 g/day to be adequate for normal laxation

in adults (EFSA, 2010). Despite of the widely reported benefits of fiber intake, a study showed that Spanish population only eats 16.5 g/day (Ruiz-Roso and Pérez, 2010). Because of the low levels of fiber intake, some fiber supplements can be found in the market. Most of the fiber supplements are based on cereal bran (the bran is the external layer of the grain and it is obtained as the result of abrasion), because it is cheap and contain a high level of fiber. However, cereal bran is the part of the grain with the highest concentration of mycotoxins (Thammawong et al., 2010). Wheat bran is the most common source of dietary fiber. In recent years, due to the wide spreading of several slimming diets, like Dukan diet, the consumption of bran has increased. For example, a rule of Dukan diet is that high quantities of oat bran must be eaten everyday (Hansel et al., 2011), usually up to 3 spoonfuls daily, which in some cases could represent more than 60 grams per day.

There are few researches on the presence of mycotoxins in oat and wheat bran and, to our knowledge, none dealt with dietary bran for human consumption. This is the first research with fiber intake. The objectives of this paper were, in one hand, to determine the AFs, OTA, DON and ZEN levels of oat and wheat bran commercially available in the Spanish market and, on the other hand, to assess the safety of this type of product for usual consumers.

6.3. Materials and methods

6.3.1. Cereal bran samples

Bran samples were purchased in 2012 from different shops from two different Spanish cities, Lleida and Valencia. The total number of samples was 67. They were packaged in plastic bags and pack weight ranged from 0.2 to 1 kg. There were 37 samples of wheat bran and 30 samples of oat bran. The different samples were taken from hypermarkets or supermarkets and health food stores. The samples were transported and stored under cool conditions until analysis (mean aw of the samples was 0.42). Full sample details including the type of cereal, the processing treatments, and the type of production, if available, were recorded for later statistical analysis.

6.3.2. Chemicals and reagents

Mycotoxin standards, including ZEN , DON, OTA, AFB1, AFB2, AFG1 and AFG2 were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were

purchased from J.T. Baker (Deventer, The Netherlands). Benzene and n-hexane were purchased from Merck (Damstadt, Germany). All solvents were LC grade. Filter papers (Whatman number 1) and glass microfiber filters (Whatman GF/A) where purchased from Whatman (Maidstone, UK). Immunoaffinity columns (IAC) for AFs (Easi-extract® Aflatoxin), DON (DONPREP®) and ZEN (Easi-extract Zearalenone®) extracts clean-up were purchased from R-Biopharm (Rhône LTD Glasgow, UK). 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) and sodium chloride (8.0 g) (J.T Baker) in 1 L of pure water; the pH was brought to 7.4.

6.3.3. Preparation of standard solutions

The standards of ZEN, OTA, AFB1, AFB2, AFG1 and AFG2 were dissolved in methanol at a concentration of 5.0 mg/mL and stored at 4 ºC in a sealed vial until use. The concentration in the stock solutions was checked by UV spectroscopy according to AOAC Official methods of analysis chapter 49 (Horwitz, 2006). Working standard solutions (2.0, 1.0, 0.5, 0.1, 0.05, 0.01 and 0.005 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system. The standard of DON was dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. Working standards (10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriate chromatographic system.

6.2.4. Analytical methods

6.3.4.1. AFs and OTA

Five g of ground bran sample was mixed with 30 mL of extractant solution (60% acetonitrile, 40% water) for 10 min and filtered with filter Whatman number 1. 2 mL of filtered solution was diluted with 18 mL of PBS solution and drained through the IAC corresponding column. After this, the columns were washed with 20 mL of PBS and AFs and OTA were eluted by applying 1 mL of methanol grade HPLC and 1 mL of milli-Q water, consecutively. The equipment used for the HPLC simultaneous detection of AFs and OTA was a separation Module Alliance 2695 Waters®, an analytical column Water Spherisorb® 5mm ODS2, 4.6 x 250 mm and a Multi λ Fluorescence Detector Waters 2475®. Excitation and emission wavelengths

were set, respectively, at 365 nm and 455 nm for AFs (0 to 16 minutes), and 333 nm and 463 nm for OTA (16 to 25 minutes). Derivatization of aflatoxins was obtained using a post-column photochemical derivatization device (UVETM Derivatizer LC Tech, Germany). Mobile phase consisted of methanol, acetonitrile and acetic acid 0.1 %, using the following proportions: 27 % of methanol, 14 % of acetonitrile and 59 % of acetic acid 0.1 %, until minute 16, and then 50 % of methanol and 50 % of acetonitrile until the end of the run. The mobile phase flow rate was 0.8 mL/min. The injection volume was 100 µL.

6.3.4.2. DON

Five g of ground bran sample was mixed with 40 mL of distilled water for 10 min. Then the sample was centrifuged for 8 min at 1780 *g*. Supernatant was filtered through a glass microfiber filter. 5 mL of filtered sample was drained through the IAC column and washed with 10 mL of distilled water. DON was eluted by applying 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water, consecutively. The purified samples were dried under nitrogen stream. Each dried sample was resuspended with the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON was determined by HPLC coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The mobile phase flow rate was 1.2 mL/min. The injection volume was 100 µL.

6.3.4.3. ZEN

Five g of ground bran sample was mixed with 25 mL of extractant solution (acetonitrile:milli-Q water, 75:25) for 10 min and filtered with Whatman number 1. 10 mL of filtered solution was diluted with 40 mL of PBS and drained through the IAC column. Columns were washed with 20 mL of PBS and ZEN eluted by applying 1.5 mL of HPLC grade acetonitrile and 1.5 ml of milli-Q water, consecutively. Finally, ZEN was determined using the HPLC system described at 2.4.1 and a mobile phase of acetonitrile-water (60:40), adjusted at pH 3.2 with acetic acid. Excitation and emission wavelengths were set at 274 nm and 455 nm, respectively. The mobile phase flow rate was 1 mL/min. The injection volume was 100 µL.

6.3.5. 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 was established by

determining AFB1, AFB2, AFG1, AFG2, OTA, DON and ZEN levels, in wheat bran food samples at least by triplicate, in those samples (blank samples) fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for AFs, DON, OTA and ZEN are summarized in Table 6.1. These values are in accordance to performance criteria established by Commission Regulation (EC) Nº 401/2006 (European Commission 2006a).

Table 6.1. Method performance for aflatoxins (AFs), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN) determination in cereal bran.

	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery (%)[c]	RSDr[d](%)
Aflatoxins						
B_1	0.30	0.90	5	2.5	103 ± 20	20
			5	5.0	89 ± 4	4
			5	7.5	102 ± 29	28
B_2	0.05	0.15	5	2.5	110 ± 15	14
			5	5.0	115 ± 8	7
			5	7.5	109 ± 9	15
G_1	0.10	0.30	5	2.5	110 ± 14	12
			5	5.0	98 ± 4	4
			5	7.5	108 ± 14	13
G_2	0.10	0.30	5	2.5	72 ± 3	4
			5	5.0	70 ± 6	8
			5	7.5	70 ± 12	17
OTA	0.2	0.6	5	2.5	74 ± 16	21
			5	5.0	103 ± 13	13
			5	7.5	95 ±12	13
DON	100	300	5	100	124 ± 8	6
			5	500	86 ± 10	12
			5	1000	99 ± 3	3
ZEN	2	6	5	25	95 ± 5	5
			5	50	98 ± 6	6
			5	100	102 ± 5	5

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value±standard deviation.
[d] RSDr = relative standard deviation.

6.3.6. Statistical analysis

ANOVA was applied to the whole data matrix in order to detect significant interactions among factors. Kruskal-Wallis test was applied to assess the significance of sample traits in the observed mycotoxin concentration levels. Moreover, Spearman rank correlation test allowed to establish correlations among the observed levels of the different mycotoxins analysed. For data analysis left-censored data were substituted by LOD/2.

6.3.7. Exposure assessment simulation model

For each contamination dataset, the gamma pdf was fitted with the method of maximum likelihood using the CAPABILITY procedure of SAS software (SAS, 2010). The Anderson-Darling statistic was used in the goodness-of-fit test, considering a significance level greater than 95%, to accept the distribution as a suitable candidate. Nine simulation consumption scenarios were taken from 1 to 30 g of fiber per day, normalised to an average individual body weight corresponding to adults of 70 kg.

The stochastic exposure model was adapted from the parametric model reported by Cano-Sancho et al. (2011). Random contamination, drawn from the corresponding adjusted gamma probability density function, was combined with the normalised consumption vector over the simulation set. The mean exposure and other statistics where computed on the histogram built with the simulation of the set (n= 10000).

6.4. Results and discussion

Three from the four mycotoxins (ZEN, DON, AFs and OTA) studied were frequently detected in many samples. AFs were not detected in any sample (LOD 0.55 µg/kg). Only 10 out of 67 samples were free of mycotoxins. The analysed mycotoxin with a major incidence in the samples was DON. It was present in 28 samples (42 %), the range of concentration being between < LOD and 6178 µg/kg. 13 samples (19%) showed levels of DON above the legislative limit in the EU for this kind of products, 750 µg/kg (EC Regulation 1881/2006). ZEN was present in 10 samples of 67 (15%); concentration ranged from < LOD to 25 µg/kg. OTA was present in 17 of 67 samples (25%) and the range of concentration was between < LOD and 2.3 µg/kg.

The predominance of DON in cereals and cereal food products has been reported by other authors (Trigo-Stockeli et al., 1996; Cano-Sancho et al., 2012). DON was the only analyzed mycotoxin that was found in samples at concentration above the EU legal limits for fiber samples. As fungal development in intact grains always starts from the outside of the grain, bran samples usually contain higher concentrations than the whole grains, as it only contains the outer layers of the seed. Mean mycotoxin concentrations in milled cereal samples found in other studies were 4.8 µg/kg bran of ZEN (maize), and 8.8, 3.4 and 0.5 mg/kg bran of DON (maize, wheat and wheat respectively) (Lauren & Ringrose, 1997, Trigo-Stockli et al., 1996 and Thammawong et al., 2010). However, those studies did not mention the destination of the milling fractions analysed, either animal or human consumption, while in our case samples were final products intended only for direct human consumption.

6.4.1. Differences in mycotoxin levels due to type of cereal

From the 67 samples analyzed, 37 belonged to wheat bran and 30 to oat bran categories (Table 6.2). Kruskal-Wallis test revealed significant differences in the levels of DON ($p < 0.05$); no significant interactions were observed with other factors. Sixty-two percent of wheat samples were contaminated by DON (1308 µg/kg of mean concentration for positive samples), while 17 % of oat samples contained DON (mean concentration for positive samples was 230 µg/kg). OTA was detected in 30 and 20 % of wheat and oat bran samples, respectively, with a mean level of 1.1 (wheat) and 0.3 (oat) µg/kg. ZEN was present in 13 and 17 % of wheat and oat samples, respectively, with mean levels between 8 (wheat) and 8 (oat) µg/kg.

In summary, oat samples showed less concentration of DON than wheat samples. Higher concentration of DON and ZEN in wheat samples has been reported by Martos et al. (2010). As a result, exposure to DON through dietary fiber would be lower when bran oat is the source of such fiber.

Table 6.2. Occurrence of zearalenone (ZEN), deoxynivalenol (DON) and ochratoxin A (OTA) in fiber samples, based on the type of cereal (µg/kg).

	n [a]	ZEN					DON					OTA				
		%[b]	M[c]	SD[d]	Max[e]	m[f]	%	M	SD	Max	m	%	M	SD	Max	m
Wheat	37	13	8	8	21	4	62	1308	1463	6178	825	30	1.1	0.8	2.3	0.9
Oat	30	17	8	9	25	4	17	230	34	276	230	20	0.3	0.1	0.4	0.3

[a] n, total number of analysed samples (% of samples >LOD)
[b] Percentage of samples >LOD
[c] Mean of positive samples (>LOD)
[d] Standard deviation of positive samples (>LOD)
[e] Maximum concentration.
[f] Median of positive samples (>LOD).

6.4.2. Differences in mycotoxin levels due to thermal processing treatment

Some of the fiber samples are sold not as raw fiber but as processed, which primarily involves the application of heat or thermo-mechanical treatment. Thus, there were samples which had been subjected to toasting or extrusion. The results have shown some differences in the levels of OTA in the samples ($p < 0.05$). No significant interactions were observed with the remaining factors. Although the percentage of contaminated samples was higher in the heat treated group, the mean levels of contamination were significantly lower in the treated group compared to the unheated (0.4 µg/kg vs 0.9 µg/kg, for the OTA positive samples) (Table 6.3).

Despite the relative thermostability of mycotoxins, some studies have shown that ZEN , DON, AFs and OTA could be affected to some extent by heat treatment of cereals and investigation on the effect of heat treatment on called mycotoxins have been done (Scudamore et al., 2008; Neira et al., 1997; Valle-Algarra et al., 2009; Saalia and Philips, 2011; Castells et al., 2006; Boudra et al., 1995).

ZEN was also more often detected in treated samples (18 vs 14%) but, although not significant, the ZEN level was higher in the untreated samples. Some studies show low decreases in ZEN concentration in the samples which are subjected to heat treatments (Ryu et al. 2002; Ryu et al, 1999 and Scudamore et al., 2008), and they conclude that higher temperatures are required for reduction of ZEN concentration. ZEN concentration in treated and untreated samples were not statistically different ($p > 0.05$).

Finally, samples which were not subjected to a heat treatment were less often contaminated by DON and with lower levels, although the difference was not significant ($p>0.05$) (Table 6.3). The temperature for DON degradation has been widely studied linked to extrusion cooking. However, contradictory conclusions are reached ranging from no changes in DON concentration to complete loss (Cazzaniga et al., 2001; Scudamore et al., 2008).

In summary, in this study only OTA have demonstrated lower levels in the samples which suffered a heat treatment.

Table 6.3. Occurrence of zearalenone (ZEN), deoxynivalenol (DON) and ochratoxin A (OTA) in fiber samples, based on the presence of a heat treatment (µg/kg).

	n [a]	ZEN					DON					OTA				
		%[b]	M[c]	SD[d]	Max[e]	m[f]	%	M	SD	Max	m	%	M	SD	Max	m
No heat treatment	56	14	9	9	25	4	37	981	991	3079	789	20	0.9	0.8	2.3	0.4
Heat treatment	11	18	3	1	4	3	64	1520	2260	6178	448	45	0.4	0.0	0.4	0.4

[a] n, total number of analysed samples
[b] Percentage of samples >LOD
[c] Mean of positive samples (>LOD)
[d] Standard deviation of positive samples (>LOD)
[e] Maximum concentration.
[f] Median of positive samples (>LOD).

6.4.3. Differences in mycotoxin levels due to type of production (organic vs conventional)

There were 26 samples from organic production and 41 samples from conventional production. Kruskal-Wallis test revealed that there was significant differences in the levels of

OTA in wheat bran samples ($p < 0.05$), but not in oat bran ones. No significant differences were found for DON and ZEN in any of the cereal brans. Table 6.4 shows the results for wheat bran samples. 45 % of organic wheat samples contained OTA while only 15 % of the conventional samples were positive; moreover, the mean OTA level of positive samples was 1.4 µg/kg in the organic samples compared to 0.6 µg/kg in the conventional ones. Our investigation shows higher OTA levels in the organic than in the conventional production, as in other previous reports (Leifert and Cooper, 2008; Anselme et al. 2006).

For ZEN, a major percentage of contaminated samples were observed in the conventional group, although mean concentration levels were higher in the organic group, while for DON it was just the opposite situation (although differences were not significant in any of the cases). Some reports show differences between conventional/organic productions in the Fusarium mycotoxins, with lower incidence of Fusarium contamination and Fusarium mycotoxins in the organic cereals than in the conventional ones (Bernhoft et al., 2010; Klinglamyr et al., 2010).

Table 6.4. Occurrence of zearalenone (ZEN), deoxynivalenol (DON), ochratoxin A (OTA) in fiber samples based on the type of production in the wheat fiber samples (µg/kg).

	n [a]	ZEN					DON					OTA				
		%[b]	M[c]	SD[d]	Max[e]	m[f]	%	M	SD	Max	m	%	M	SD	Max	m
Organic	11	9	21	0	21	21	72	655	565	1662	426	45	1.4	1.0	2.3	1.6
Conventional	26	15	4	3	9	3	58	1657	1681	6178	923	15	0.6	0.3	1.0	0.4

[a] n, total number of analysed samples.
[b] Percentage of samples >LOD.
[c] Mean of positive samples (>LOD).
[d] Standard deviation of positive samples (>LOD).
[e] Maximum concentration.
[f] Median of positive samples (>LOD).

6.4.4. Differences in mycotoxin levels due to other factors

Other factors have been analyzed (city of sample purchase, type of presentation, presence of other ingredients…), but none of them showed any effect in the analyzed mycotoxins.

The samples were purchased in two different cities (Lleida and Valencia), thus consumers from both cities are expected to be equally exposed to ZEN , DON and OTA through

wheat and oat bran consumption. Globalization of the market tends to diminish the impact of geographical differences in food consumption.

The presence of other ingredients (sugar, maize, honey, salt, egg …) in the samples did not either increase or reduce the mycotoxin presence in the analyzed samples, as they were present in small percentages.

6.4.5. Co-occurrence

Co-occurrence of 2 or 3 different mycotoxins was observed in 18 % of samples (Table 6.5), although no samples with the four studied mycotoxins were detected. A positive correlation between ZEN and DON content in the samples ($p < 0.05$) was observed. In 90 % of the samples where ZEN was detected, DON was also present. The two mycotoxins are produced by Fusarium and their co-occurrence in cereals has been reported in other studies (Stanković et al., 2012; Lee et al., 2011).

No significant correlation was observed among the remaining mycotoxins.

Table 6.5. Number and percentage of samples with mycotoxin co-occurrence.

	Two mycotoxins						Three mycotoxins			
	ZEN x DON	ZEN x AFs	ZEN x OTA	DON x AFs	DON x OTA	AFs x OTA	ZEN x DON x AFs	ZEN x DON x OTA	DON x OTA x AFs	ZEN x AFs x OTA
Number samples (%)	7 (10.5)	0 (0.0)	3 (4.5)	0 (0.0)	7 (10.5)	0 (0.0)	0 (0.0)	3 (4.5)	0 (0.0)	0 (0.0)

6.4.6. Exposure assessment

The low presence of ZEN and OTA in bran did not permit us to carry out an accurate exposure assessment simulation model; therefore we excluded these mycotoxins from the statistics. The DON contamination dataset was used in the calculations with previous substitution of left-censored data by LOD/2.

Results of exposure to DON considering the different consumption scenarios are shown in the Table 6.6. The range considered in the model was between 1 and 30 g/day, and the mean DON intake from bran was expected between 0.006 and 0.208 μg/kg bw/day. Highest estimations were found for the percentile 95 with extreme values, reaching estimations of 0.800 μg/kg bw/day.

In order to assess the expected contribution of DON exposure through dietary bran consumption to the global exposure of Catalonian population, our estimations were added to the estimations for adults given in Cano-Sancho et al. (2011). The median exposure values of 0.22 and 0.32 µg/kg bw/day were considered in the statistics, for adult females and males, respectively (results shown in Table 6.7).

Table 6.6. Estimated deoxynivalenol (DON) exposure from different fiber intake scenarios (from 1 to 30 g/day) for one adult (70 kg) computed with the raw contamination datasets (10000 simulations).

Fiber intake	Fiber intake (70 kg)	*Mean*	*sd*	*p50*	*p75*	*p90*	*p95*
g/day	*g/kg bw/day*	*µg DON kg bw day*					
1	0.014	0.006	0.009	0.003	0.009	0.018	0.026
2.5	0.036	0.017	0.024	0.008	0.023	0.048	0.067
5	0.071	0.034	0.047	0.016	0.046	0.094	0.132
7.5	0.107	0.052	0.072	0.024	0.069	0.142	0.199
10	0.143	0.069	0.096	0.032	0.092	0.190	0.266
15	0.214	0.104	0.144	0.048	0.138	0.285	0.399
20	0.286	0.139	0.193	0.064	0.185	0.381	0.533
25	0.357	0.173	0.240	0.080	0.231	0.475	0.666
30	0.429	0.208	0.289	0.097	0.278	0.571	0.800

Our results showed a great contribution of fiber supplements to the global exposure of Catalonian adults to DON. Most of fibre supplements are offered in supermarkets and health food stores to reach the dietary recommendations of fiber (between 25 and 30 g/day) because low fibre intake is commonly provided by the current diet in Spain (16.5 g/day). Based on a consumption of 5 g fiber/day (common recommendation in several diets), we estimated a contribution between 4.8 and 6.8 % for the percentile 50, this range being 22.8-30.1 % in case of percentile 90, depending of gender (female/male). Therefore, fiber supplements intake can be an important source of DON, thus it is important to include this category in global exposure assessment studies of this mycotoxin.

Table 6.7. Relative contribution of fiber intake to the global exposure of Catalonian population to deoxynivalenol (DON). Median exposure values of 0.22 and 0.32 µg/kg bw/day for males and females, were considered, respectively (from Cano-Sancho et al. 2011).

Fiber intake scenarios	*Percentile 50*					*Percentile 90*				
		Males		Females			Males		Females	
	DIF	DGE	*C*	DGE	*C*	DIF	DGE	*C*	DGE	*C*
g/day	µg/kg bw/day	µg/kg bw/day	%	µg/kg bw/day	%	µg/kg bw/day	µg/kg bw/day	%	µg/kg bw/day	%
1	0.003	0.223	1.4	0.323	1.0	0.019	0.239	7.8	0.339	5.5
2.5	0.008	0.228	3.6	0.328	2.5	0.048	0.268	17.9	0.368	13.0
5	0.016	0.236	6.8	0.336	4.8	0.095	0.315	30.1	0.415	22.8
7.5	0.024	0.244	9.9	0.344	7.1	0.143	0.363	39.3	0.463	30.8
10	0.032	0.252	12.8	0.352	9.2	0.191	0.411	46.4	0.511	37.3
15	0.049	0.269	18.1	0.369	13.2	0.285	0.505	56.5	0.605	47.1
20	0.065	0.285	22.8	0.385	16.9	0.381	0.601	63.4	0.701	54.4
25	0.081	0.301	26.9	0.401	20.2	0.476	0.696	68.4	0.796	59.8
30	0.097	0.317	30.7	0.417	23.3	0.572	0.792	72.2	0.892	64.1

DIF, Deoxynivalenol exposure from fiber supplement intake (units in µg/kg bw/day).
DGE, Deoxynivalenol global exposure considering other sources of exposure (median values) from Cano-Sancho et al. (2011) (units in µg/kg bw/day).
C, Relative contribution of fiber supplement intake to the global exposure to DON (%)

6.5. References

Anselme, M., Tangni, E.K., Pussemier, L., Molte, J., Van Hove, F., Schenieder, Y., Van Peteghem, C., Larondelle, Y. 2006. Comparison of ochratoxin A and deoxynivalenol in organically and conventionally produced beers sold on the Belgian market. Food Additives and Contaminants. Part A. Chemical Analytical Control Exposition Risk Assessment, 23, 910-918.

Bernhoft, A., Clasen, P.E., Kristoffersen, A.B., Torp, M. 2010. Less Fusarium infestation and mycotoxin contamination in organic than in conventional cereals. Food Additives and Contaminants. Part A. Chemical Analytical Control Exposition Risk Assessment, 27, 842-852.

Boudra, H., Le Bars, P., Le Bars, J. 1995. Thermostability of ochratoxin A in wheat under two moisture conditions. Applied. Environmental Microbiology, 61, 1156-1158.

Cano-Sancho, G., Gauchi, J.P., Sanchis, V., Marin, S., Ramos, A.J. 2011. Quantitative dietary exposure assessment of the Catalonian population (Spain) to the mycotoxin deoxynivalenol. Food Additives and Contaminants. Part A. Chemical Analytical Control Exposition Risk Assessment, 28, 1098-1109.

Cano-Sancho, G., Ramos, A.J., Marín, S., Sanchis, V. 2012. Presence and co-occurrence of aflatoxins, deoxynivalenol, fumonisins and zearalenone in gluten-free and ethnic foods. Food Control, 26, 282-286.

Castells, M., Marin, S., Sanchis, V., Ramos, A.J. 2006. Reduction of aflatoxins by extrusion-cooking of rice meal. Food Chemistry and Toxicology, 71, 369-377.

Cazzaniga, D., Basilico, J.C., Gonzalez, R.J., Torres, R.L., de Greef, D. 2001. Mycotoxins inactivation by extrusion cooking of corn flour. Letters in Applied Microbiology, 33, 144-147.

Edwards, S.G., Dickin, E.T., MacDonald, S., Buttler, D., Hazel, C.M., Patel, S., Scudamore, K.A. 2011. Distribution of Fusarium mycotoxins in UK wheat mill fractions. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28, 1694-1704.

European Commission. (2006a). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L364, 5-24.

European Comission (2010). Comission Regulation (EC) No 165/2010 of 26 February 2010 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L446, 1-127.

European Food Safety Authority (EFSA), 2007. Definition and description of ‘emerging risks’ within EFSA’s mandate. ESAF/SC/415 Final. Available at: http://www.efsa.europa.eu/. Accessed: 19/4/2012

European Food Safety Authority (EFSA), 2010. Scientific Opinion on Dietary Reference Values for carbohydrates and dietary fibre .EFSA Jounral, 8 (3), 1462. Available at: http://www.efsa.europa.eu/. Accessed: 19/4/2012

Hansel, B., Giral, P., Coppola-Xaillé, A., Monfort, G., Regnault, M., Bruckert, E. 2011. L’enquête Internet nationale « Dukan, et après?» : premiers résultats. Obesite, 6, 146-153.

Horwitz, W., Latimer, G.W. 2006. Official methods of analysis of AOAC International (18th ed). AOAC Gaithersburg, Maryland.

IARC (International agency for research on cancer). 1993. Monographs on the evaluation of the carcinogenic risk of chemicals to humans: Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. Vol 56, 397-444. Lyon, France.

IARC (International Agency for Research on Cancer). 2002. Monograph on the evaluation of carcinogenic risk to humans: Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Vol. 82, 1-556. Lyon, France.

Jelinek, C.F., Pohland, A.E., Wood, G.E. 1989. Worldwide occurrence of mycotoxins in foods and feeds an update. Journal of the Association of Oficial Analytical Chemists, 72, 223-230.

Jestoi, M. 2008. Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin - A review. Critical Reviews in Food Science and Nutrition, 48, 21-49.

Klinglamyr, C., Nöbauer, K., Razzazi-Fazeli, E., Cichna-Markl, M. 2010. Determination of deoxynivalenol in organic and conventional food and feed by sol-gel immunoaffinity chromatography and HPLC-UV detection. Journal of Chromatography. Analytical Technologies in the Biomedical and Life Sciences, 878, 187-193.

Lauren, D.R., Ringrose, M.A. 1997. Determination of the fate of three Fusarium mycotoxins through wet-milling of maize using an improved HPLC analytical technique. Food Additives and Contaminants, 14, 435-443.

Lee, T., Lee, S.-H., Lee, S.-H., Shin, J.Y., Yun, J.-C., Lee, Y.-W., Ryu, J.-G. 2011. Occurrence of Fusarium mycotoxins in rice and its milling by-products in Korea. Journal of Food Protection, 74, 1169-1174.

Leifert, C., Cooper, J. 2008. Safety issues in the organic food supply chain- Mycotoxins and enteric pathogens. Food Science and Technology, 22, 34-36.

Martos, P.A., Thompson, W., Diaz, G.J. 2010. Multiresidue mycotoxin analysis in wheat, barley oats, rye and maize rain by high-performance liquid chromatography-tandem mass spectrometry. World Mycotoxin Journal, 3, 205-223.

Massart, F., Saggese, G. 2010. Oestrogenic mycotoxin exposures and precocious pubertal development. International Journal of Andrology, 33. 369-376.

National Academy of Science (NAS). 1989. Recommended dietary allowances. National Academy Press.Washington, D.C. U.S.A.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., Resnik, S.L. 1997. The effects of bakery processing on natural deoxynivalenol contamination. International Journal of Food Microbiology, 37, 21-25.

Pestka, J.J. 2010. Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. World Mycotoxin Journal, 3, 323-347.

Pestka, J.J. 2010. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology, 84, 663-679.

Ruiz-Roso, B., Pérez, L. 2010. Avance de resultados sobre consumo de fibra en España y beneficios asociados a la ingesta de fibra insoluble. Revista Española de Nutrición Comunitaria, 16, 147-153.

Ryu, D., Hanna, M.A., Bullerman, L.B. 1999. Stability of zearalenone during extrusion of corn grits. Journal of Food Protection, 62, 1482-1484.

Ryu, D., Jackson, L.S., Bullerman, L.B. 2002. Effects of processing on zearalenone. Advances in Experimental Medicine and Biology, 504, 206-216.

Saalia, F., Phillips, R. 2011. Degradation of aflatoxins by extrusion cooking: Effects on nutritional quality of extrudates. Food Science and Technology, 44, 1496-1501.

Scientific Committee on Food (SCF) (1998). Commission of the European Communities, Directorate General XXIV, Outcome of discussions 14, Expressed on 17.09.88.

Scudamore, K., Guy, R.C.E., Kelleher, B., MacDonald, S. 2008. Fate of the Fusarium mycotoxins, deoxynivalenol, nivalenol and zearalenone, during extrusion of wholemeal wheat grain. Food Additives and Contaminants, 25, 331-337.

Stanković, S., Lević, J., Ivanović, D., Krnjaja, V., Stanković, G., Tančić, S. 2012. Fumonisin B1 and its co-occurrence with other fusariotoxins in naturally-contaminated wheat grain. Food Control, 23, 384-388.

Thammawong, M., Okadome, H., Shiina, T., Nakagawa, H., Nagashima,H., Nakajima, T., Kushiro, M. 2010. Distinct distribution of deoxynivalenol, nivalenol, and ergosterol in Fusarium infected Japanese soft red winter wheat milling fractions. Mycopathologia, 172, 323-330.

Trigo-Stockli, D.M., Deyoe, C.W., Satumbaga, R.F., Pederesen, J.R. 1996. Distribution of deoxynivalenol and zearalenone in milled fractions of wheat. Cereal Chemistry, 73, 388-391.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., Jiménez, M. 2009. Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26, 896-906.

7. Study II. Investigation on the incidence of free and modified mycotoxins in cereal-based processed foods.

Food Control, send.

Authors: Vidal, A., Marín, S., Sanchis, V., De Boevre M., & De Saeger, S.

7. Investigation on the incidence of free and modified mycotoxins in cereal-based processed foods.

Arnau Vidal[1], Sonia Marín[1] ,Vicente Sanchis[1], Marthe De Boevre[2*] and Sarah De Saeger[2*].

[1] Food Technology Department, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

[2] Laboratory of Food Analysis, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Belgium.

* equal last author

Corresponding author: Tel. +34-973-702535. Fax: +34-973-702596. email: arnau.vidal@tecal.udl.cat. Postal address: Avda. Rovira Roure, 191. 25198 Lleida (Spain).

7.1. Abstract

The present study describes the analysis of 56 commercial bread samples from Catalonia (Spain) for nine different mycotoxins, namely nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, zearalenone, α-zearalenol, β-zearalenol and zearalenol-14-glucoside. More than 80% of the analysed breads contained one or more mycotoxins, however no samples exceeded the maximum levels described in the EU legislation for free mycotoxins. Nivalenol was the most common mycotoxin (41%), while 21% of the analysed breads contained deoxynivalenol and 13% zearalenone. Regarding modified mycotoxins, high concentrations of DON-3-glucoside were found. Also, a high ratio deoxynivalenol-3-glucoside/deoxynivalenol was observed (0.75). The incidence of the acetylated deoxynivalenol forms was low (<8%), and a ratio of 0.44 for the sum of acetylated deoxynivalenol forms/deoxynivalenol was defined. A high incidence of α-zearalenol and β-zearalenol was observed (>60 % of presence) with concentrations larger than zearalenone. The calculated ratio for the sum of α-zearalenol and β-zearalenol/zearalenone was 5.09. ZEN-14-glucoside, however was detected in one sample with a concentration of 15.75 µg/kg. Fifty-seven % of the samples had more than one mycotoxin, which highlight the importance of mycotoxin co-occurrence in breads. This study proves the necessity to monitor free and modified mycotoxins in commercial cereal-based products.

Keywords: Nivalenol, deoxynivalenol, deoxynivalenol-3-glucoside, zearalenone, modified mycotoxins, bread.

7.2. Introduction

Cereal-based foods such as bread provide more nutrients to the world population than any other single food source (Peña, 2002). Bread, however, is made from cereals, and these are prone to mould infestations, and consequent mycotoxin contamination. *Fusarium* species produce a heterogeneous blend of mycotoxins such as trichothecenes and myco-oestrogens. Different studies show the high incidence of mycotoxins, especially fusariotoxins, in cereals and cereal-based foods (Chrpová et al., 2016; De Boevre et al., 2013).

The most abundant trichothecene, deoxynivalenol (DON), is mainly produced by *F. graminearum* and *F. culmorum* (Richard et al., 2007). The toxin is not classified as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC) (1993), however DON has been linked to human gastroenteritis (Pestka, 2010). In addition, cereal and cereal-based products are considered to be a major DON-source for human intake (Cano-Sancho, Gauchi, Sanchis, Marín, & Ramos, 2011a). Nivalenol (NIV), another trichothecene is also a common mycotoxin in cereals (Trombete et al., 2016). NIV showed higher acute toxicity than DON in animal studies (IARC, 1993). Zearalenone (ZEN) is a prevalent mycotoxin in cereals like DON and NIV (De Boevre et al., 2014; Vidal, Marín, Ramos, Cano-Sancho, & Sanchis, 2013). This myco-oestrogen has been categorized by IARC as a class 3 carcinogen (IARC, 1993).

Free mycotoxins, like DON and ZEN, might not be the only hazard for consumers' health, because the so-called modified mycotoxins are not detected in routine mycotoxins analysis. Modified mycotoxins are toxins attached to more polar functional groups, such as glycosyl residues or sulfates, or to polymeric carbohydrates or protein matrices (Berthiller, Schuhmacher, Adam, & Krska, 2009a; Rychlik et al., 2014). Modified mycotoxins may have plant, fungal, mammalian and food processing origins. A major concern and potential risk for consumers is the possible hydrolysis of modified mycotoxins into their toxic free forms during mammalian digestion (Broekaert, 2015a; Grabley, Gareis, Bockers, & Thiem, 1992; Nagl et al., 2014). Contrary to the wealth of information on the free mycotoxins, only limited data are available for mycotoxin derivatives in foods. The co-occurrence of free and modified DON forms has been documented in raw wheat, especially with focus on deoxynivalenol-3-glucoside (DON-3-glucoside), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). Reported levels of DON-3-glucoside are variable, however, the ratio of DON-3-glucoside/DON concentrations in unprocessed cereals is similar among assays, ranging from 0.1 to 0.3 (Berthiller et al. 2009b; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013; De Boevre et al., 2012a). 3-ADON and 15-ADON have also been detected in raw cereals with a lower incidence than DON-3-glucoside (Amarasinghe, Simsek, Brûlé-Babel, & Fernando, 2016; De Boevre et al., 2012a; Tibola, Fernandes, & Guarienti, 2015). Berthiller et al. (2011) demonstrated that several lactic acid bacteria hydrolyse DON-3-glucoside *in vitro*, which has been a first step to prove the toxicological relevance of DON-3-glucoside. On the other hand, 3-

ADON and 15-ADON are rapidly converted to DON during digestion (Broekaert, 2015a; Veršilovskis et al., 2012).

α-Zearalenol (α-ZEL) and β-zearalenol (β-ZEL) have been frequently detected in cereal-based food. De Boevre et al. (2013) observed that more than 30% of the analyzed bread samples were contaminated with α-ZEL and β-ZEL with an average contamination level of more than 15 µg/kg. Ayed, Ayed-Boussema, Ouanes, & Bacha (2011) argued that ZEN and α-ZEL exhibited the same range of cytotoxicity and genotoxicity, and both were more cyto- and genotoxic than β-ZEL. Finally, zearalenone-14-glucoside (ZEN-14-glucoside) has not been widely investigated in terms of occurrence-studies due to the lack of reference standards, but the toxin has been found in cereal samples (De Boevre et al., 2012a; Nathanail et al., 2015). The monitoring of ZEN-14-glucoside is important, because Dall'Erta et al. (2013) proved that ZEN-14-glucoside is easily and rapidly deconjugated to ZEN during digestion in rats.

To avoid consumer health risks, the European Commission has set maximum levels for DON (500 µg/kg) and ZEN (50 µg/kg) in bread. Despite the threat of other free and modified mycotoxins there is no specific legislation due to the lack of occurrence and toxicity data. However, the Joint European Commission FAO/WHO Expert Committee (JECFA) considered DON-3-glucoside and the acetylated forms, 3-ADON and 15-ADON, as an additional contributing factor of the total dietary exposure to DON (JECFA, 2011). It is important to take modified forms into account because their concentrations in food cannot be neglected. In some cases, DON-3-glucoside concentrations proved to be higher than its free toxin DON as stated in bread (De Boevre et al. 2013) and beer (Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012; Varga, Malachova, Schwartz, Krska, & Berthiller, 2013).

The present paper describes the simultaneous determination of the mycotoxins NIV, DON and ZEN and their respective metabolites or conjugates including DON-3-glucoside, 3-ADON, 15-ADON, α-ZEL, β-ZEL and ZEN-14-glucoside in a total of 56 Spanish commercial bread samples.

7.3. Material and methods

7.3.1. Reagents and chemicals

The individual mycotoxin solid calibration standards (1 mg) of DON, 3-ADON, 15-ADON, deepoxy-deoxynivalenol (DOM) (internal standard), NIV, zearalanone (ZAN) (internal standard), ZEN-14-glucoside, α-ZEL and β-ZEL were obtained from Sigma Aldrich (Bornem, Belgium). ZEN (5 mg) was supplied by Fermentek (Jerusalem, Israel). DON-3-glucoside (50.2 ng/µl, in acetonitrile) was purchased from Biopure Referenzsubstanzen (Tulln, Austria). All mycotoxin solid standards were dissolved in methanol (1 mg/mL) and were storable for a minimum of 1

year at -18 °C (Spanjer et al., 2008); DON-3-glucoside solution was kept at 4 °C. ZEN-14-glucoside, α-ZEL and β-ZEL were prepared based on the procedures of Grabley et al. (1992). The working solutions of DON, 3-ADON, 15-ADON, DOM, NIV, ZEN, ZEN-14-glucoside, α-ZEL, β-ZEL and ZAN (10 ng/µl) were prepared in methanol, and stored at -18 °C, while DON-3-glucoside was dissolved in acetonitrile, stored at 4 °C, and renewed monthly. Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Disinfectol® (denaturated ethanol with 5% ether) was supplied by Chem-Lab (Zedelgem, Belgium). Methanol (LCMS grade) was purchased from BioSolve (Valkenswaard, the Netherlands), while acetonitrile (Analar Normapur), n-hexane (Hipersolv Chromanorm) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany).

7.3.2. Collection of bread samples

A total of 56 bread samples including fibre-enriched bread, oat bread and multi-cereal bread from different manufacturers were randomly collected at several supermarkets in February 2016 from Lleida (Spain). Bread samples were kept in freezing conditions (-20 °C) until analysis to prevent moulding of the matrix.

7.3.3. Sample preparation and extraction

20 g of each bread sample was lyophilized and ground (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany). After each milling step, cleaning and decontamination of the equipment was performed using water and bleach. The ground material was vigorously homogenised with a spatula before weighing. Sample extraction was performed according to De Boevre et al. (2012b). Concisely, 2.5 g of the ground material was extracted with 10 mL acetonitrile:water:acetic acid (79:20:1, v/v/v) combined with hexane defatting (5 mL) using the agitator decanter overhead shaker (Agitelec; J. Toulemonde & Cie., Paris, France) for 60 min. After centrifugation (3,000 *g*, 15 min) the supernatant (hexane layer) was removed; the aqueous layer was filtered and evaporated to dryness (N_2, 40 °C). Finally, the residue was redissolved in 100 µL injection solvent, consisting of methanol:water (50:50, v/v) and 10 mM ammonium acetate with 0.3 % glacial acetic acid.

7.3.4. LC-MS/MS methodology

A Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) was used to analyse the samples, equipped with MassLynx™ version 4.1 and QuanLynx® version 4.1 software (Waters, Manchester, UK) for data acquisition and

processing. A ZORBAX Eclipse XDB C18-column (1.8 µm, 100×2.1 mm) was applied (Agilent Technologies, Diegem, Belgium). The mobile phase consisted of water:methanol (95:5, v/v (A)) and methanol:water (95:5, v/v (B)), both buffered with 10 mM ammonium acetate and adjusted with 0.3% of glacial acetic acid, at a flow rate of 0.2 mL/min. The gradient elution programme started at 70 % mobile phase A for 4.25 min. Then, the mobile phase B increased with a linear increase to 99 % in the 8 min. The mobile phase B was kept at 99 % for 2 min. The mobile phase linearly decreased till 30 % for 0.5 min. The 70 % of mobile phase A and 30 % of mobile phase B was running till 12 min. Duration of each HPLC run was 12 min, including reequilibration. Mass spectrometer was operated in the positive electrospray ionisation mode (ESI+). The capillary voltage was 3.2 kV, and nitrogen was applied as spray gas. Source and desolvation temperatures were set at 120 °C and 300 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 20 L/h and the desolvation gas flow 500 L/h. Two selected reaction monitoring (SRM) transitions with a specific dwell time were chosen for each analyte, in order to increase sensitivity and selectivity of the mass spectrometric conditions. The SRM-transitions for every analyte are described in Table 7.1. Developed LC-MS/MS method was successfully validated based on 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 (EC, 2006b) (Table 7.2). Matrix-matched calibration plots were constructed for the determination of the analytes. DOM and ZAN, structural analogues of type B trichothecenes and ZEN, respectively, were used as internal standards in the multi-mycotoxin analysis. Evaluating linearity, homogeneity of variance was checked before fitting the linear model. Linearity was tested graphically using a scatter plot. The obtained values were in conformity with ranges set in legislation (EC, 2006b). The precision was calculated in terms of relative standard deviation (RSD). Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by slope of standard curve; limit of quantification (LOQ) was similar, differing by six times the standard error. Calculated LOD and LOQ were verified by the signal-to-noise ratio (s/n), which should be more than 3 and 10, respectively according to the IUPAC guidelines. Results of the performance characteristics of LC-MS/MS method were in good agreement with the criteria mentioned in Commission Regulation (EC) No 401/2006 (EC, 2006b) and were described in detail in De Boevre et al. (2012b).

7.3.5. Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2007 (Redmond, WA, USA) and IBM SPSS 19 (Armonk, NY, USA).

Table 7.1. The optimized ESI-MS/MS parameters for the confirmation and quantification of nivalenol (NIV), deoxynivalenol-3-glucoside (DON-3-glucoside), deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), zearalenol-14-glucoside (ZEN-14-glucoside), zearalenone (ZEN), β-zearalenol (β-ZEL), zearalanone (ZAN) and α-zearalenol (α-ZEL).

Mycotoxin	Precursor ion (m/z)	Product ions [a](m/z)	CE[a,b] (eV)	CV[c] (v)	Retention time (min)
NIV	313.1	125.0/205.0	13/12	26	0.84
DON-3-glucoside	476.1	248.6/296.9	18/12	15	0.97
DON	297.4	249.2/231.2	15/10	26	1.00
DOM-1	281.1	109.1/137.0	19/15	26	1.55
3-ADON	356.1	203.1/339.2	16/15	25	2.52
15-ADON	356.1	339.2/137.4	25/8	18	2.52
ZEN-14-glucoside	498.1	283.2/319.2	23/15	19	6.79
ZEN	319.2	283.3/301.3	15/10	37	6.81
β-ZEL	321.0	303.3/285.1	10/8	25	7.58
ZAN	321.2	189.1/303.3	22/14	35	7.59
α-ZEL	321.3	285.4/303.3	12/8	30	7.60

[a] Values are given in the order: quantifier ion/ qualifier ion
[b] CE: Collision energy
[c] CV: Cone Voltage
NIV = nivalenol, DON-3-glucoside = deoxynivalenol-3-glucoside, DON = deoxynivalenol, DOM-1 = deepoxy-deoxynivalenol, 3-ADON = 3-acetyldeoxynivalenol, 15-ADON = 15-acetyldeoxynivalenol, ZEN-14-glucoside = zearalenol-14-glucoside, ZEN = zearalenone, β-ZEL = β-zearalenol, ZAN = zearalanone and α-ZEL = α-zearalenol.

Table 7.2. Validation parameters for the analyzed mycotoxins in bread.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	Calibration Range (µg/kg)	R [c](mean)	SE[d]	Apparent recovery (%)	RSD_r[e] (%)	RSD_R[f] (%)	U[g] (%)
NIV	10	20	25-400	0.97	0.005	95	9	18	35
DON-3-glucoside	13	26	25-400	0.97	0.011	97	8	11	23
DON	10	20	25-400	0.97	0.005	95	12	20	41
3-ADON	10	20	25-400	0.97	0.008	98	10	16	33
15-ADON	9	18	25-400	0.98	0.008	99	9	14	29
ZEN-14-glucoside	8	16	2.5-80	0.97	0.009	89	24	22	47
ZEN	6	12	2.5-80	0.99	0.004	74	15	10	21
β-ZEL	7	14	2.5-80	0.98	0.019	79	10	11	23
α-ZEL	5	10	2.5-80	0.97	0.012	77	18	22	45

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] R = Pearson's correlation coefficient.
[d] SE = Standard error of mean.
[e] RSD_r = relative standard deviation intra-day precision.
[f] RSD_R = relative standard deviation inter-day precision.
[g] U = measurement uncertainty.
NIV = nivalenol, DON-3-glucoside = deoxynivalenol-3-glucoside, DON = deoxynivalenol, 3-ADON = 3-acetyldeoxynivalenol, 15-ADON = 15-acetyldeoxynivalenol, ZEN-14-glucoside = zearalenol-14-glucoside, ZEN = zearalenone, β-ZEL = β-zearalenol and α-ZEL = α-zearalenol.

7.4. Results and discussion

7.4.1. Nivalenol

NIV was the most common mycotoxin in the analysed breads (Table 7.3), 41% of the analysed breads contained NIV with an average concentration of 292 ± 452 µg/kg with a maximum concentration of 1786.9 µg/kg. Although NIV is extensively studied in cereal samples, the presence of NIV in breads (= processed foods) has not been widely investigated. Our NIV results agreed with other studies of the NIV incidence in cereals; a recent review (Broekaert et al. (2015b) found 32% of cereals samples with NIV in wheat, maize, barley, oat and rye. Nonetheless, recent studies showed a large variability of NIV presence in cereal samples: 40% in wheat flour (China, Li et al., 2016); 40% in corn (China, Zhao et al., 2014); 30% in wheat grains and whole-wheat flour (Portugal, Trombete et al., 2016), 73% in wheat grains (Brazil, Calori-Dominguez et al., 2016); 74 % in barley, 71% in oats, 43% in wheat (Finland, Nathanail et al., 2015) and 6% in barley (Germany, Habler, & Rychlik, 2016). Also the maximum contamination level of NIV is different among published studies such as 1859 µg/kg in wheat grains (Tjechia, Chrpová et al., 2016), 1329 µg/kg in wheat grains (Spain, Calori-Dominguez et al., 2016) and 530 µg/kg in maize (Hove et al., 2016). The high variability of NIV levels is caused by the presence of different factors for mould growth like environmental factors, agricultural practices and storage (Wegulo, 2012). The large variance of NIV concentrations highlights the importance of a regular control in cereals. All commented results originate from unprocessed cereals, and there is little information about NIV presence in food samples. NIV in foods may be different, because the food process could affect the stability of mycotoxins. Valle-Algarra et al. (2009) showed that the mycotoxin was widely reduced after baking (>80% of reduction), but NIV-spiked breads were used and a probably larger reduction could be obtained with naturally contaminated samples. Others trichothecenes, for instance DON, which is also a trichothecene, showed a high stability during bread-making processes when naturally DON-contaminated flour was used (Vidal, Morales, Sanchis, Ramos, & Marín, 2014). Besides, the high presence of NIV detected in our breads means that NIV might not be widely removed during the bread-making process. More studies about the fate of NIV during food processing should be explored to anticipate on the concentration of NIV in food.

Detected high concentration of NIV is of concern due to its toxicity. *In vitro* and *in vivo* toxicity of NIV has been studied, demonstrating immunotoxicity, haematotoxicity/myelotoxicity and reproductive toxicity (EFSA-CONTAM 2013). Other studies showed that exposure to a high NIV-diet is associated with an increased incidence of oesophageal carcinoma in China (Hsia et al. 2004) however this toxin is unlikely genotoxic. Due to its toxigenic threat EFSA (EFSA-CONTAM 2013) established a tolerable daily intake (TDI) of 1.2 µg/kg body weight per day, but no EU maximum limits were established.

So, a high variability of NIV contamination levels was observed in breads, and the large incidence in the present study confirms that consumers are exposed to NIV. Regular controls of NIV levels in cereals and cereal-based foods should be performed on regular basis.

Table 7.3. Positive samples (%), mean concentration (µg/kg) and maximum concentration (µg/kg) of free mycotoxins and modified ones in the 56 analysed bread samples.

	% of positive samples	Mean ± standard deviation (µg/kg)	Median (µg/kg)	Maximum concentration (µg/kg)
NIV	41	292.4 ± 452.2	144.3	1786.9
DON	21	43.5 ± 36.2	31.7	144.0
3-ADON	3.6	10.8 ± 0.4	10.8	11.1
15-ADON	7.1	10.5 ± 0.1	10.5	10.6
DON-3-glucoside	11	29.4 ± 14.4	30.5	47.3
Total DON*	25	54.5 ± 46.1	42.4	178.5
ZEN	13	13.4 ± 2.8	12.6	19.1
α-ZEL	63	21.5 ± 21.0	13.9	104.9
β-ZEL	32	20.8 ± 14.6	15.0	56.7
ZEN-14-glucoside	1.8	15.7	15.7	15.7

* Total DON = sum of DON, 3-ADON, 15-ADON and DON-3-glucoside.
NIV = nivalenol, DON = deoxynivalenol, 3-ADON = 3-acetyldeoxynivalenol, 15-ADON = 15-acetyldeoxynivalenol, DON-3-glucoside = deoxynivalenol-3-glucoside, ZEN = zearalenone, α-ZEL = α-zearalenol, β-ZEL = β-zearalenol and ZEN-14-glucoside = zearalenol-14-glucoside.

7.4.2. DON, acetylated DONs and DON-3-glucoside

Twenty-one % of the analysed samples contained DON (Table 7.3) with an average concentration of 44 ± 36 µg/kg. All DON levels found were below the maximum levels set by the European Union (European Commission 1881/2006) for bread samples (500 µg/kg). The maximum concentration found was 144 µg/kg.

DON is one of the most prevalent contaminants in cereal and cereal-based products (Cano-Sancho et al., 2011b). Due to its common presence, studies describe DON presence in bread and baking products. Rodriguez-Carrasco et al. (2014) found DON was the most common mycotoxin in analysed bread sticks from Spain with a similar average DON concentration of 32 ± 8 µg/kg (present study, 43.5 ± 36.2 µg/kg). In the same way, González-Osnaya, Cortés, Soriano, Moltó, & Mañes (2011) analysed Spanish breads with a similar percentage of DON presence (28%), their average content of DON in bread was 42.5 µg/kg and a maximum concentration of 146.6 µg/kg of DON. On the other hand, Cano-Sancho et al. (2011b) and Beltran et al. (2013) found DON in all the analysed breads with maximum DON concentrations of 739 and 203 µg/kg respectively. Moreover, Cano-Sancho et al. (2011b) got an average level of 246 ± 158 µg/kg.

The discrepancies of contamination levels of DON in bread can be caused by different factors as commented for NIV: environmental factors, agricultural practices and storage. Moreover, bread-making process can also affect DON concentration. Fermentation temperature (Generotti et al., 2015; Vidal, Ambrosio, Sanchis, Ramos, & Marín, 2016), time, baking temperature (Generotti et al., 2015; Vidal et al., 2014 and Vidal, Sanchis, Ramos, & Marín, 2015) and presence of enzymes or flour improvers (Generotti et al., 2015; Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal et al., 2016) are factors which affect its stability during the bread-making process. However, DON cannot be totally reduced at the end of bread-making process, and even a toxin increase at the end of process can be detected in function of food processing factors such as temperature and the presence of enzymes (Vidal et al., 2014).

DON-3-glucoside was found in the bread samples (11%) with an average concentration of 29.4 ± 14.4 µg/kg. It has been solely detected when also DON was present; this relationship is expected as DON-3-glucoside is a plant metabolite originating from DON (Berthiller et al., 2009a). Fifty % of the analysed samples with DON contained DON-3-glucoside, and this relationship is also observed in other studies. Zachariasova et al. (2012) detected DON-3-glucoside in all bread samples when DON was present and De Boevre et al. (2013) detected DON-3-glucoside in 65% of the samples with DON. Presumably, when DON is detected in bread there is a high probability to observe DON-3-glucoside in the same sample.

The ratio of DON-3-glucoside/DON among assays of unprocessed cereals ranges from 0.1 to 0.3 (Berthiller et al., 2009b; Dall'Asta et al., 2013). However, average ratio of DON-3-glucoside/DON observed in the present study was 0.75 (Table 7.4). Other studies found a similar high ratio in bread samples: De Boevre et al. (2013) observed ratios from 1.00 to 0.84 in fibre and bran-enriched bread. These ratios in final processed products have also been observed in beer, as detected by Zachariasova et al. (2012) with 1.78, and Varga et al. (2013) with 0.56. The high DON-3-glucoside concentration detected in the final product can be linked with the increase of DON-3-glucoside during the bread-making process (Vidal et al., 2014; Zachariasova et al., 2012). Increase of DON-3-glucoside concentration may be caused by the presence of DON-3-glucoside embedded in the matrix, which is released during the process due to enzymatic activity (Simsek et al., 2012; Vidal et al., 2016). So, DON-3-glucoside embedded in the original raw flour is missed in routine analysis.

Table 7.4. Co-occurrence of mycotoxins (%), mean ratio of concentration between (modified) mycotoxins and their free mycotoxins and maximum ratio of concentration.

	% of co-occurrence	Mean ratio of concentration	Maximum ratio of concentration
DON-3-glucoside / DON	11	0.75	1.73
(3-ADON + 15-ADON) / DON	5.4	0.44	0.82
(α-ZEL + β-ZEL) / ZEN	11	5.09	8.47

DON-3-glucoside = deoxynivalenol-3-glucoside, DON = deoxynivalenol, 3-ADON = 3-acetyldeoxynivalenol, 15-ADON = 15-acetyldeoxynivalenol, α-ZEL = α-zearalenol, β-ZEL = β-zearalenol and ZEN = zearalenone.

The high concentration of DON-3-glucoside in breads is of concern. Although DON-3-glucoside is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003), Berthiller et al. (2011) have shown that this modified form can be hydrolysed to DON by several intestinal lactic acid bacteria. Thus, the FAO/WHO Expert Committee (JECFA) considered DON-3-glucoside to be an additional contributing factor to total dietary exposure to DON (JECFA, 2011).

3-ADON and 15-ADON had a low level of incidence, 3.6% and 7.1% respectively. Also, the detected concentrations were close to the limit of detection (LOD), 10.8 ± 0.4 µg/kg for 3-ADON and 10.5 ± 0.1 µg/kg for 15-ADON. Acetylated DON forms are mycotoxins produced by moulds, and the presence of 3-ADON versus 15-ADON is a phenotypic difference within *Fusarium* species. In the South of Europe the 15-ADON genotype is more predominant (Somma et al., 2014), and this agrees with our results where more 15-ADON was observed than 3-ADON. However, the two mycotoxins are not very common in the analysed breads. Obtained levels agree with other results as De Boevre et al. (2013) found an average concentration of 16 µg/kg for 3-ADON and 7 µg/kg for 15-ADON in bran-enriched bread. On the contrary higher concentrations of acetylated forms have been detected in unprocessed cereals (Tamura et al., 2015; De Boevre et al., 2012a). Lower level of acetylated DON forms detected in bread is caused by the rapid transformation of 3-ADON and 15-ADON to DON during the bread-making process (Wu, & Wang, 2015).

Although the levels of acetylated DON were low in bread, it is important to control their levels because their toxicity is equivalent or stronger than DON (Eriksen & Pettersson, 2004). Due to their level of toxicity, the FAO/WHO Expert Committee (JECFA) considered acetylated forms, as DON-3-glucoside, to be an additional contributing factor to the total dietary exposure to DON (JECFA, 2011).

7.4.3. ZEN, α-ZEL, β-ZEL, and ZEN-14-glucoside

ZEN and their derivatives were investigated in breads (Table 7.3). ZEN was detected in 13% of the analysed samples with an average concentration of 13.4 ± 2.8 µg/kg, and the maximum ZEN concentration detected was 19.1 µg/kg which complies with the maximum limit for bread (50 µg/kg) set by the European Commission (European Commission, 2006).

Only few studies about ZEN presence in cereal-based products exist, but all of them proved that ZEN is a common mycotoxin in food. Cano-Sancho, Marin, Ramos, & Sanchis (2012) observed a 44% of ZEN presence in Spanish breads, and Quiles, Saladino, Mañes, Fernández-Franzón, & Meca (2016) found ZEN in refrigerated pizza dough (100%). De Boevre et al. (2013) found more than 39% of ZEN presence in fibre-enriched bread and bran enriched bread from Belgium, and Iqbal et al. (2014) found 43% of ZEN presence in breads from Pakistan.

Although ZEN is very common in cereal-based foods, average concentrations of ZEN detected in other studies were usually lower: Cano-Sancho et al. (2012) found 3.7 ± 4.5 µg/kg in breads (Cano-Sancho et al., 2012), 29 ± 54 and 38 ± 52 µg/kg in fibre and bran-enriched bread, respectively (De Boevre et al., 2013) and 9.5 ± 2.8 µg/kg in breads (Iqbal et al., 2014). The average ZEN content is normally below the maximum legal limits, but some samples from different studies contained more ZEN. Iqbal et al. (2014) observed that 14% exceeded 50 µg/kg, De Boevre et al. (2013) detected 230 µg/kg and 157 µg/kg as maximum levels in fibre- and bran-enriched bread, respectively, and 12% of samples exceeded the EU maximum limits with a maximum detected concentration of 176 µg/kg in refrigerated pizza doughs from Spanish market (Quiles et al., 2016). The high levels of ZEN in processed products mean that cereals could contain even more ZEN because some studies showed ZEN is widely reduced during baking: Bol, Araujo, Veras, & Welke (2016) got an 89% reduction in the final bread, and Heidari, Milani, & Nazari, (2014) observed an 46% of ZEN reduction in the final bread.

α-ZEL and β-ZEL were observed in 63% and 32% of the analysed breads, respectively (Table 7.3). Also, the two mycotoxins have been found in higher concentrations than ZEN (21.5 ± 21.0 µg/kg (α-ZEL) and 20.8 ± 14.6 µg/kg (β-ZEL)) (Table 7.4).

Presence of these compounds is barely studied. The few information about them showed similar results: ≤ 7 ug/kg for α-ZEL and β-ZEL (De Boevre et al., 2013), besides, maximum concentration of α-ZEL was also similar (110 µg/kg) in fibre-enriched bread and bran-enriched bread. Other investigations also detected low concentrations of these conjugates: Nathanail et al. (2015) observed concentrations of α-ZEL and β-ZEL below 7 µg/kg in the analyzed cereals (wheat, oat and barley). Juan, Raiola, Mañes, & Ritieni (2014) detected only a 20% incidence of β-ZEL and a mean concentration of 2.5 µg/kg in cereal-based baby foods. Other authors did not detect these compounds in cereal samples (Juan, Ritieni, & Mañes, 2013). Although α-ZEL and β-ZEL are not regulated, the high levels detected are of concern, especially for α-ZEL. β-ZEL has a 2.5 times lower affinity for the estrogen receptor than ZEN, while α-ZEL has a 92 times higher affinity in comparison to ZEN. So, more control of these mycotoxins should be considered, especially for α-ZEL.

ZEN-14-glucoside was detected in one sample with a concentration of 15.7 µg/kg (Table 7.3). ZEN-14-glucoside has not been widely analysed due to the lack of a commercial reference standard, and nowadays few information is available. The existing information shows ZEN-14-glucoside is not a common mycotoxin, and its presence is scarce in cereals and cereal-based products. Nathanail et al. (2015) observed a higher presence in barley (18%) than in oats (3.2%) and wheat (6.7%). Iincidence of ZEN-14-glucoside in breads has only been analysed in one study: 15% incidence in fibre-enriched bread and 2% in bran enriched bread with an average concentration below 20 µg/kg (De Boevre et al., 2013). Although high concentrations of ZEN-14-glucoside were detected in some cereals, for instance 9750 µg/kg in maize (De Boevre et al., 2014), much lower concentrations were detected in bread samples (max = 155 µg/kg). The cleaning and milling of cereals cause an extensive reduction of ZEN-14-glucoside

(Schwake-Anduschus et al., 2015). For this reason, ZEN-14-glucoside has not been found in large concentrations in foods. No studies exist on the stability of ZEN-14-glucoside during baking. Nonetheless more information about the stability of ZEN-14-glucoside is necessary, because other modified mycotoxins showed an increase of concentration during baking such as DON-3-glucoside (Vidal et al., 2014). Although the toxicity of ZEN-14-glucoside is not known, the transformation from ZEN-14-glucoside to ZEN during digestion is probable.

7.4.4. Co-occurrence of mycotoxins

Co-occurrence of different mycotoxins was observed in analysed samples (57%). Whereas 36% of analysed breads contained three or more mycotoxins, 21% contained 2 mycotoxins, 21% 3 mycotoxins, 9% 4 mycotoxins and 5% 5 mycotoxins. Combination NIV/α-ZEL was the most common co-occurrence of mycotoxins (16 %). The high co-occurrence of mycotoxins in cereal-based products has been reported many times. Juan et al. (2014) found that 92% of cereal-based baby foods were co-contaminated with different mycotoxins, and Juan, Covarelli, Beccari, Colasante, & Mañes (2016) found that 91% of the positive samples showed co-contamination in durum wheat, where different enniatin combinations were the most prevalent mycotoxins. Rodríguez-Carrasco, Font, Moltó, & Berrada (2013) reported that 65% of cereal-based foods from Spain showed mycotoxin co-occurrence, especially among the *Fusarium* mycotoxins DON, HT-2 toxin and NIV.

Co-occurrence of mycotoxins in breads is an important research subject because the effects of co-exposure on human and animal health remain unclear. Most of the mycotoxin mixtures lead to additive or synergistic effects, which can cause even more health-related issues for humans and animals upon consumption of mycotoxin-contaminated food or feed (Smith, Madec, Coton, & Hymery, 2016). The obtained results clearly demonstrate the co-occurrence of NIV, DON, ZEN and their derivatives as a real threat in cereal-based products.

7.5. Conclusions

Our results showed a high incidence of *Fusarium* mycotoxins in Spanish breads, whereas more than 80% of the analyzed breads contained one or more *Fusarium* mycotoxins. Mycotoxin levels, however, were below the maximum limits set by the European Commission for bread. NIV was the most common free mycotoxin (41%) followed by DON (21%) and ZEN (13%). Consumers are likely to be exposed to a high level of NIV because of its high prevalence (maximum concentration up to 1785.9 µg/kg). DON and ZEN were not so common, however a high variability within concentration levels was observed. A consistent monitoring program for

DON-3-glucoside is proposed, because high concentrations, similar to DON, were found in bread. Also, the DON-3-glucoside/DON ratio showed to be as high as 0.75, which is only observed in processed foods and not in raw foods. The authors hypothesize that DON-3-glucoside is embedded to the flour, and released during the bread-making process. 3-ADON and 15-ADON were scarcely detected (<10%) in bread samples with rather low concentration levels (maximum <12 µg/kg). α-ZEL and β-ZEL were very common (63% and 32%, respectively). Their concentrations were higher in some samples (maximum of 104.9 µg/kg), and they appeared in much higher concentrations than ZEN (ratio sum α-ZEL and β-ZEL/ZEN, 5). Interestingly, the presence and concentration of α-ZEL is worrying due to its proven toxicity. ZEN-14-glucoside was detected in only one sample (15.7 µg/kg). Although analysed samples were below the maximum legal limits, research on both free and modified mycotoxin reduction in the food chain is imperative to protect consumers.

7.6. Acknowledgments

A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant (FPUAP2012-3636) and for the mobility grant (EST15/00023). The authors are grateful to the Spanish Government (project AGL2014-55379-P) for the financial support.

7.7 References

Amarasinghe, C.C., Simsek, S., Brûlé-Babel, A., & Fernando, W.G.D. (2016). Analysis of deoxynivalenol and deoxynivalenol-3-glucosides content in Canadian spring wheat cultivars inoculated with *Fusarium graminearum. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 33*, 1254-1264.

Ayed, Y., Ayed-Boussema, I., Ouanes, Z., & Bacha, H. (2011). *In vitro* and *in vivo* induction of chromosome aberrations by alpha- and beta-zearalenols: Comparison with zearalenone. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis, 726*, 42-46.

Beltrán, E., Ibáñez, M., Portolés, T., Ripollés, C., Sancho, J.V., Yusà, V., Marín, S., & Hernández, F. (2013). Development of sensitive and rapid analytical methodology for food analysis of 18 mycotoxins included in a total diet study. *Analytica Chimica Acta, 783*, 39-48.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009b). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. F*ood Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller. F., Schuhmacher, R., Adam, G., & Krska, R. (2009a). Formation, determination and significance of masked and other conjugated mycotoxins. *Analytical and Bioanalytical Chemistry, 395*, 1243-1252.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Bol, E.K., Araujo, L., Veras, F.F., & Welke, J.E. (2016). Estimated exposure to zearalenone, ochratoxin A and aflatoxin B1 through the consume of bakery products and pasta considering effects of food processing. *Food and Chemical Toxicology, 89*, 85-91.

Broekaert, N. (2015a). Disposition of 3- and 15-Acetyl-Deoxynivalenol and Deoxynivalenol-3-β-D-Glucoside in broiler chickens and pigs. Dissertation of thesis, University of Ghent.

Broekaert, N., Devreese, M., De Baere, S., De Backer, P., & Croubels, S. (2015b). Modified *Fusarium* mycotoxins unmasked: From occurrence in cereals to animal and human excretion. *Food and Chemical Toxicology, 80*, 17-31.

Calori-Domingues, M.A., Bernardi, C.M.G., Nardin, M.S., de Souza, G.V., Dos Santos, F.G.R., Stein, M.A., Gloria, E.M., Dias, C.T.S., & De Camargo, A.C. (2016). Co-occurrence and distribution of deoxynivalenol, nivalenol and zearalenone in wheat from Brazil. *Food Additives and Contaminants: Part B Surveillance, 9*, 142-151.

Cano-Sancho, G., Gauchi, J.P., Sanchis, V., Marín, S., & Ramos, A.J. (2011a). Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. F*ood Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1098-1109.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., & Marín, S. (2011b). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22*, 490-495.

Cano-Sancho, G., Marin, S., Ramos, A.J., & Sanchis, V. (2012). Occurrence of zearalenone, an oestrogenic mycotoxin, in Catalonia (Spain) and exposure assessment. *Food and Chemical Toxicology, 50*, 835-839.

Chrpová, J., Šíp, V., Sumíková, T., Salava, J., Palicová, J., Štočková, L., Džuman, Z., & Hajšlová, J. (2016). Occurrence of *Fusarium* species and mycotoxins in wheat grain collected in the Czech Republic. *World Mycotoxin Journal, 9*, 317-327.

Dall'Asta, C., Dall'Erta A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal, 6*, 83-91.

De Boevre, M., Di Mavungu, J.D., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., De Saeger, S. (2012a). Natural occurrence of mycotoxins and their masked forms in food and feed products. *World Mycotoxin Journal, 5*, 207-219.

De Boevre, M., Di Mavungu, J.D., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut, A., Berthiller, F., Van Peteghem, C., De Saeger, S. (2012b). Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 819-835.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J.D., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B., & De Saeger, S. (2013). Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281-292.

De Boevre, M., Landschoot, S., Audenaert, K., Maene, P., Di Mavungu, D., Eeckhout, M., Haesaert, G., & De Saeger, S. (2014). Occurrence and within field variability of *Fusarium* mycotoxins and their masked forms in maize crops in Belgium. *World Mycotoxin Journal, 7*, 91-102.

Scientific opinion on risks for animal and public health related to the presence of nivalenol in food and feed. (2013). EFSA Journal, 11, 3262.

Eriksen, G.S., & Pettersson, H. (2004). Toxicological evaluation of trichothecenes in animal feed. *Animal Feed Science and Technology, 114*, 205-239.

European Commission (EC), 2006. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal European Union L364*, 5-24.

Generotti, S., Cirlini, M., Malachova, A., Sulyok, M., Berthiller, F., Dall'Asta, C., & Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins, 7*, 2773–2790.

González-Osnaya, L., Cortés, C., Soriano, J.M., Moltó, J.C., & Mañes, J. (2011). Occurrence of deoxynivalenol and T-2 toxin in bread and pasta commercialised in Spain. *Food Chemistry, 124*, 156-161.

Grabley, S., Gareis, M., Bockers, W., & Thiem, J. (1992). Glycosylation of mycotoxins. *Synthesis, 11*, 1078-1080.

Habler, K., & Rychlik, M. (2016). Multi-mycotoxin stable isotope dilution LC-MS/MS method for *Fusarium* toxins in cereals. *Analytical and Bioanalytical Chemistry, 408*, 307-317.

Heidari, S., Milani, J., & Nazari, S.S.S.J. (2014). Effect of the bread-making process on zearalenone levels. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 2047-2054.

IARC (International Agency for Research on Cancer). (1993). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans. 56. Lyon, France.

Iqbal, S.Z., Rabbani, T., Asi, M.R., & Jinap, S. (2014). Assessment of aflatoxins, ochratoxin A and zearalenone in breakfast cereals. Food Chemistry, 257-262.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2011). Seventy-second report. Evaluation of certain contaminants in food. Accessed: 03.08.16.

Juan, C., Ritieni, A., & Mañes, J. (2013). Occurrence of *Fusarium* mycotoxins in Italian cereal and cereal products from organic farming. *Food Chemistry, 141*, 1747-1755.

Juan, C., Raiola, A., Mañes, J., & Ritieni, A. (2014). Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control, 39*, 227-236.

Juan, C., Covarelli, L., Beccari, G., Colasante, V., & Mañes, J. (2016). Simultaneous analysis of twenty-six mycotoxins in durum wheat grain from Italy. *Food Control, 62*, 322-329.

Li, F., Jiang, D., Zhou, J., Chen, J., Li, W., & Zheng, F. (2016). Mycotoxins in wheat flour and intake assessment in Shandong province of China. *Food Additives and Contaminants: Part B Surveillance, 9*, 170-175.

Nagl, V., Woechtl, B., Schwartz-Zimmermann, H.E., Hennig-Pauka, I., Moll, W.-D., Adam, G., & Berthiller, F. (2014). Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. *Toxicology Letters, 229*, 190-197.

Nathanail, A.V., Syvähuoko, J., Malachová, A., Jestoi, M., Varga, E., Michlmayr, H., Adam, G., Sieviläinen, E., Berthiller, F., & Peltonen, K. (2015). Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. *Analytical and Bioanalytical Chemistry, 407*, 4745-4755.

Pestka, J. J. (2010). Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal, 3*, 323–347.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Pralatnet, S., Poapolathep, S., Giorgi, M., Imsilp, K., Kumagai, S., & Poapolathep, A. (2016). Survey of deoxynivalenol and aflatoxin B1 in instant noodles and bread consumed in Thailand by using liquid chromatography-tandem mass spectrometry. *Journal of Food Protection, 79*, 1269-1272.

Richard, E., Heutte, N., Sage, L., Pottier, D., Bouchart, V., Lebailly, P., & Garon, D. (2007). Toxigenic fungi and mycotoxins in mature corn silage. *Food and Chemical Toxicology, 45*, 2420-2425.

Rychlik, M, Humpf, H.-U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including “masked” mycotoxins. *Mycotoxin Research, 30*, 197-205.

Rodríguez-Carrasco, Y., Ruiz, M.J., Font, G., & Berrada, H. (2013). Exposure estimates to *Fusarium* mycotoxins through cereals intake. *Chemosphere, 93*, 2297-2303.

Rodríguez-Carrasco, Y., Font, G., Moltó, J.C., & Berrada, H. (2014). Quantitative determination of trichothecenes in breadsticks by gas chromatography-triple quadrupole tandem mass spectrometry. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 1422-1430.

Schwake-Anduschus, C., Proske, M., Sciurba, E., Muenzing, K., Koch, M., & Maul, R. (2015). Distribution of deoxynivalenol, zearalenone, and their respective modified analogues in milling fractions of naturally contaminated wheat grains. *World Mycotoxin Journal, 8*, 433-443.

Simsek, S., Burgess, K., Whitney, K. L., Gu, Y., & Qian, S. Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287–292.

Smith, M.-C., Madec, S., Coton, E., & Hymery, N. (2016). Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins, 8*, 94.

Somma, S., Petruzzella, A. L., Logrieco, A. F., Meca, G., Cacciola, O. S., & Moretti, A. (2014). Phylogenetic analyses of *Fusarium* graminearum strains from cereals in Italy, and characterization of their molecular and chemical chemotypes. *Crop and Pasture Science, 65*, 52–60.

Quiles, J.M., Saladino, F., Mañes, J., Fernández-Franzón, M., & Meca, G. (2016). Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population. *Food and Chemical Toxicology, 94*, 19-24.

Tamura, M., Mochizuki, N., Nagatomi, Y., Harayama, K., Toriba, A., & Hayakawa, K. (2015). A method for simultaneous determination of 20 *Fusarium* toxins in cereals by high-resolution liquid chromatography-orbitrap mass spectrometry with a pentafluorophenyl column. *Toxins, 7*, 1664-1682.

Tibola, C.S., Fernandes, J.M.C., & Guarienti, E.M. (2015). Effect of cleaning, sorting and milling processes in wheat mycotoxin content. *Food Control, 60*, 174-179.

Trombete, F., Barros, A., Vieira, M., Saldanha, T., Venâncio, A., & Fraga, M. (2016). Simultaneous determination of deoxynivalenol, deoxynivalenol-3-glucoside and nivalenol in wheat grains by HPLC-PDA with immunoaffinity column cleanup. *Food Analytical Methods, Article in press*, 1-8.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 896-906.

Varga, E., Malachova, A., Schwartz, H., Krska, R., & Berthiller, F. (2013). Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 137-146.

Vanheule, A., Audenaert, K., De Boevre, M., Landschoot, S., Bekaert, B., Munaut, F., Eeckhout, M., Höfte, M., De Saeger, S., & Haesaert, G. (2014). The compositional mosaic of *Fusarium* species and their mycotoxins in unprocessed cereals, food and feed products in Belgium. *International Journal of Food Microbiology, 181*, 28-36.

Veršilovskis, A., Geys, J., Huybrechts, B., Goossens, E., De Saeger, S., & Callebaut, A. (2012). Simultaneous determination of masked forms of deoxynivalenol and zearalenone after oral dosing in rats by LC-MS/MS. *World Mycotoxin Journal, 5*, 303-318.

Vidal, A., Marín, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. *Food and Chemical Toxicology, 53*, 133-138.

Vidal, A., Morales, H., Sanchis, V., Ramos, A. J., & Marín, S. (2014). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control, 40*, 234–242.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry, 178*, 276-286.

Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, 208*, 288-296.

Wegulo, S.N. (2012). Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins, 4*, 1157-1180.

Wu, L., & Wang, B. (2015). Evaluation on levels and conversion profiles of DON, 3-ADON, and 15-ADON during bread making process. *Food Chemistry, 185*, 509-516.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New ‘‘masked” *Fusarium* toxins occurring in malt, beer, and breadstuff. Journal of Agricultural and Food Chemistry, 60, 9280–9291.

Zhao, Z., Rao, Q., Song, S., Liu, N., Han, Z., Hou, J., & Wu, A. (2014). Simultaneous determination of major type B trichothecenes and deoxynivalenol-3-glucoside in animal feed and raw materials using improved DSPE combined with LC-MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 963*, 75-82.

8. Study III. Looking for modified aflatoxins.

Food Chemistry (sent)

Authors: Vidal, A., Marín, S., Sanchis, V., De Boevre M., & De Saeger, S.

8. Looking for modified aflatoxins.

Arnau Vidal[1#], Sonia Marín[1] ,Vicente Sanchis[1], Marthe De Boevre[2*] and Sarah De Saeger[2*].

[1] Food Technology Department, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

[2] Laboratory of Food Analysis, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Belgium.

* equal last author

Corresponding author: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: email: arnau.vidal@tecal.udl.cat. Postal address: Avda. Rovira Roure, 191. 25198 Lleida (Spain)

8.1. Abstract

The main objective of this study was looking for conjugated aflatoxins in cereal samples. Parent mycotoxins are not the only form of mycotoxins in food, there are also modified mycotoxins. Recently, different hydrolysis methods to convert modified mycotoxins to parent mycotoxins have been developed successfully (alkaline conditions, acid environment and enzyme treatments). 19 different aflatoxin contaminated maize and sorghum samples were incubated with potassium hydroxide, trifluoromethanesulfonic acid and enzymes (β-glucuronidase, protease, α-amylase and cellulase). After incubation, samples were analysed to find variations in aflatoxins concentration. Potassium hydroxide incubation caused the total reduction of aflatoxins because they cannot resist in alkaline conditions. Trifluoromethanesulfonic acid incubation did not produce an increase of parent mycotoxins, neither β-glucuronidase nor protease incubation. On the other hand, α-amylase and cellulase incubation turned out slight increases of aflatoxins, 14.9 ± 8.3 and 12.5 ± 5.0 % respectively. The results showed that a small proportion of aflatoxins could be embedded in the carbohydrates from matrix.

Keywords: Aflatoxins, conjugated aflatoxin, aflatoxin derivatives, aflatoxin hydrolysis.

8.2. Introduction

Mycotoxins are toxic secondary metabolites produced by a variety of filamentous fungi. The most important mycotoxins are produced by species in the genera *Fusarium*, *Aspergillus* and *Penicillium*, which can grow on a variety of crops. Fungal infection and mycotoxin production may occur during growth, harvest, and storage of agricultural commodities. Aflatoxins (Afs) are the most potent genotoxic and carcinogenic mycotoxins and the four main aflatoxins are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). AFB1, AFB2, AFG1 and AFG2 are consistently carcinogenic and genotoxic *in vitro* and *in vivo* (EFSA, 2007) and therefore they were classified in the group 1 by the International Agency for Research on Cancer (IARC, 2002).

Afs can occur in a wide range of important raw food commodities including cereals, nuts, spices, figs and dried fruits (EFSA, 2007). Presence of Afs in cereals has been extensively inquired (Andrade & Caldas, 2015). Jager, Tedesco, Souto and Oliveira (2013) analysed corn flour and 75 % of analysed samples had Afs presence with an average concentration of 0.56 ± 0.70 µg/kg. Huong et al. (2016) obtained 26 % of positive analysed maize samples, furthermore the average concentration was considerably high: 24.51 µg/kg. Abia et al. (2013) found 30 % positive maize samples with Afs presence. Finally, Njumbe Ediage, Van Poucke and De Saeger (2015) found Afs presence in 10 % of the analysed sorghum samples with a maximum concentration of 50 µg/kg. The few existing studies about Afs exposure showed a high probably daily intake (PDI) around the world. Blankson and Mill-Robertson (2016) calculated PDI maximum levels of 1.054 µg/kg bw/day and 0.838 µg/kg bw/day for infants and young children, respectively, from Ghana. Jager et al. (2013) calculated a PDI of 1.58 µg/kg bw/day for Brazilian population. Regarding Europe, Reports by members of European Union (Scientific Cooperation on Questions Relating to Food Projects (SCOOP), 1996) showed estimated dietary exposure for Europeans to AFB1 of 0.03 to 1.28 ng/kg bw/day. Besides, most of the European studies got similar Afs exposure results: Cano-Sancho, Sanchis, Marín, and Ramos (2013) got a mean of 0.14 ng/kg bw/day in Spanish population and Leblanc, Tard, Volatier and Verger (2005) got 0.117 ng/kg bw/day in French population. However, a larger dietary exposure was estimated among Swedish population (0.8 to 2.1 ng/kg bw/day) (Thuvander et al., 2001).

Nonetheless, free mycotoxins, like Afs, might not be the only hazard for consumers' health. Mycotoxin derivatives can be present in food and they are not detected in routine mycotoxins analysis. Contrary to the wealth of information on the free mycotoxins, only limited data are available for mycotoxin derivatives. Moreover, a major concern and potential risk for consumers is the possible hydrolysis of no free mycotoxins into their toxic parent forms during mammalian digestion (Broekaert, 2015; Grabley, Gareis, Bockers, & Thiem, 1992; Nagl et al., 2014).

Some of the derivative forms are called matrix-associated mycotoxins because they are attached to more polar functional groups (Rychlik et al., 2014) and they may have plant, fungal,

mammalian and food processing origins. This type of compounds are potentially bound or strongly associated to glycosyl residues or sulfates, or to polymeric carbohydrates or protein matrices or other food components through supramolecular complexation. The rest of the derivative forms are modified mycotoxins and they have been biologically (plants, animals or fungi) or chemically modified (thermally or non-thermally) (Rychlik et al., 2014). Nowadays, some of the derivative forms can be determined only in an indirect way through the application of a hydrolysis step (Dall'Asta et al., 2009a) in which derivative forms are converted to parent mycotoxins. Basic, acid or enzymatic treatments have been used successfully as hydrolytic steps to determine derivative mycotoxins. For instance, hidden fumonisin in corn flour was detected after basic treatment with potassium hydroxide (KOH) 2 M (Dall'Asta et al., 2009a) due to the release of hidden fumonisins. Acid treatment, with trifluoromethanesulfonic acid (TFMSA) and enzymes (glucosidase, cellulase and cellobiase) hydrolysed the glycoside bond in zearalenone-4-glucoside (ZEN -4-glucoside) (Beloglazova et al., 2013) and it caused an increase of his parent mycotoxin, zearalenone (ZEN). Finally, common enzymes used in the food industry (xylanase, α-amylase, cellulase and protease) caused an increase of deoxynivalenol (DON) concentration during bread making process (Vidal, Sanchis, Ramos, & Marín, 2016a).

Given that mycotoxins can be present in modified forms which could be hydrolysed and transformed to parent mycotoxins, the objective of this study was looking for modified Afs through the application of a hydrolytic step. Acid, base and hydrolytic enzymes (β-glucuronidase, protease, α-amylase and cellulase) treatment were tested to release modified Afs.

8.3. Material and methods

8.3.1. Reagents and chemicals

The individual mycotoxin solid calibration standards (1 mg) of AFB1, AFB2, AFG1, AFG2 and zearalanone (ZAN) (internal standard) were obtained from Sigma Aldrich (Bornem, Belgium). All mycotoxin solid standards were dissolved in methanol (1 mg/mL) and were storable for a minimum of 1 year at - 18 °C (Spanjer, Rensen, & Scholten, 2008). The working solutions of AFB1, AFB2, AFG1, AFG2 and ZAN (10 ng/µl) were prepared in methanol, and stored at -18 °C and renewed monthly. Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Disinfectol® (denaturated ethanol with 5% ether) was supplied by Chem-Lab (Zedelgem, Belgium). Methanol (LCMS grade) was purchased from BioSolve (Valkenswaard, the Netherlands), while acetonitrile (Analar Normapur) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic

acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany). MultiSep ® 228 Aflapat were purchased by Romer Labs (Tulln, Austria). The four enzymes used in the study, namely, β-glucuronidase (*Escherichia coli*, 25 KU), protease (*Aspergillus oryzae*, 500 U/g), α-amylase (*Aspergillus oryzae,* 30 U/mg) and cellulase (*Aspergillus niger*, 0.3 U/mg) were purchased from Sigma Aldrich (Bornem, Belgium) as TFMSA (≥ 99 %) and KOH (≥ 99 %). Sodium acetate buffer (SAB) was prepared with sodium acetate (27.21 g, ≥ 99 % from Sigma Aldrich), glacial acetic acid (6 mL) and milli-Q water (994 mL), the pH was adjusted to 5.0 with NaOH (10 M).

8.3.2. Collection of the cereal samples

A total of 18 maize and 1 sorghum samples from different Nigerian fields with Afs presence were kept in freezing conditions (-20 °C) until analysis to prevent moulding of the matrix.

8.3.3. Sample preparation,treatment and extraction

Each sample was milled (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany). After each milling step, cleaning and decontamination of the equipment was performed using water and bleach. The ground material was vigorously homogenised with a spatula before weighing. Then, the treatments depended on the hydrolytic process (control, enzymatic, acid and basic):

8.3.4. Treatments

8.3.4.1. Acid treatment

Acid treatment was performed according to Beloglazova et al. (2013). Briefly, 2.5 g of the ground sample were dipped with 7 mL TFMSA (0.025 M) for 13 h. at 40 ºC in an incubation bath.

8.3.4.2. Basic treatment

Basic treatment was performed according to Dall'Asta et al. (2009a). Briefly, 2.5 g of the ground sample were blended in an Ultraturrax T25 high-speed blender (IKA, Stauffen, Germany) with 7 mL of 5 M KOH for 10 min at 6000 rpm.

8.3.4.3. Enzymatic treatment

Enzymatic treatment was performed according to Beloglazova et al. (2013). Briefly, 2.5 g of the ground sample were dipped with 7 mL of SAB and weight of each used enzyme (β-glucuronidase, α-amylase, cellulase and protease) (Table 8.1). The ground sample with buffer and enzyme were incubated overnight at optimum enzyme temperature (Table 8.1).

8.3.5. Extraction

For control, 2.5 g of the ground material was extracted with 35 mL acetonitrile:water:acetic acid (79:20:1, v/v/v). For treated samples, 28 mL of acetonitrile:acetid acid (99:1, v/v) were added, so all of them (control and treated) had 35 mL of acetonitrile:water:acetic acid (79:20:1, v/v/v). A shaking process was made using the agitator decanter overhead shaker (Agitelec; J. Toulemonde & Cie., Paris, France) for 60 min. After centrifugation (3,000 *g*, 15 min), 32 mL were placed in another tube and evaporated to dryness (N_2, 40 ºC). Then, the residue was redissolved in 30 mL of acetonitrile:formic acid (99:1, v/v) and the 30 mL were loaded on the Multisep column, and the column was washed with 2 mL of acetonitrile:formic acid (99:1, v/v). The purified extracts were dried under a stream of nitrogen (N_2, 40 ºC). Finally, the residue was redissolved in 100 µL injection solvent, consisting of water:methanol:acetic acid (57:42:1, v/v) and 5 mM ammonium acetate. Samples were prepared to determine the AFB1, AFB2, AFG1, AFG2 and internal standard (ZAN).

Table 8.1. Enzymes used for enzymatic hydrolysis and their optimal conditions.

	Mould producer	Concentration (U/g)	Optimum temperature (ºC)
β-glucuronidase	*Escherichia coli*	750 U/g	37
Protease	*Aspergillus oryzae*	2 U/g	37
α-amylase	*Aspergillus oryzae*	50 U/g	37
Cellulase	*Aspergillus niger*	3 U/g	37

8.3.6. LC-MS/MS methodology

A Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) was used to analyse the samples, equipped with MassLynx™ version 4.1 and QuanLynx® version 4.1 software (Waters, Manchester, UK) for data acquisition and processing. A ZORBAX Eclipse XDB C18-column (1.8 µm, 100×2.1 mm) was applied (Agilent Technologies, Diegem, Belgium). The mobile phase consisted of water:methanol:acetic acid (94:5:1, v/v (A)) and methanol:water:acetic acid (97:2:1, v/v (B)), both buffered with 5 mM ammonium acetate, at a flow rate of 0.3 mL/min. The gradient elution programme started at 70 % mobile phase A for 4.25 min. Then, the mobile phase B increased with a linear increase to 99 % in the 8 min. The mobile phase B was kept at 99 % for 2 min. The mobile phase linearly

decreased till 30 % for 0.5 min. The 70 % of mobile phase A and 30 % of mobile phase B was running till 12 min. The duration of each HPLC run was 12 min, including reequilibration. The mass spectrometer was operated in the positive electrospray ionisation mode (ESI+). The capillary voltage was 20 kV, and nitrogen was applied as spray gas. Source and desolvation temperatures were set at 120 °C and 400 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 L/h and the desolvation gas flow 800 L/h. Two selected reaction monitoring (SRM) transitions with a specific dwell time were chosen for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric conditions. The SRM-transitions for every analyte are described in Table 8.2. The developed LC-MS/MS method was successfully validated based on 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 (EC, 2006b) (Table 8.3). Matrix-matched calibration plots were constructed for the determination of the analytes. ZAN was used as internal standards in the multi-mycotoxin analysis. Evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. The linearity was tested graphically using a scatter plot. The obtained values were in conformity with the ranges set in legislation (EC, 2006b). The precision was calculated in terms of the relative standard deviation (RSD). Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; limit of quantification (LOQ) was similar, differing by six times the standard error. The calculated LOD and LOQ were verified by the signal-to-noise ratio (s/n), which should be more than 3 and 10, respectively according to the IUPAC guidelines. The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in Commission Regulation (EC) No 401/2006 (EC, 2006b) and were described in detail in Njumbe Ediage et al. (2015).

Table 8.2. The optimized ESI-MS/MS parameters for the confirmation and quantification of aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2) and zearalanone (ZAN).

Mycotoxin	Precursor ion (m/z)	Product ions [a](m/z)	CE[a,b] (eV)	CV[c] (v)	Retention time (min)
AFG1	329.0	243.0/311.2	25/20	40	7.09
AFG2	331.0	313.1/245.2	25/30	53	6.73
AFB1	313.0	285.1/241.2	24/36	51	7.70
AFB2	315.0	287.2/259.2	27/30	51	7.42
ZAN	321.2	189.1/303.3	22/14	12	7.59

[a] Values are given in the order: quantifier ion/ qualifier ion
[b] CE: Collision energy
[c] CV: Cone Voltage
AFG1 = aflatoxin G1, AFG2 = aflatoxin G2, AFB1 = aflatoxin B1, AFB2 = aflatoxin B2 and ZAN = zearalanone.

8.3.7. Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2007 (Redmond, WA, USA) and IBM SPSS 19 (Armonk, NY, USA).

Table 8.3. Validation parameters for the analyzed mycotoxins in maize.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	Calibration Range (µg/kg)	R [c](mean)	SE[d]	Apparent recovery (%)	RSD_r[e] (%)	RSD_R[f] (%)	U[g] (%)
AFB1	10	20	25-400	0.97	0.005	95	9	18	39
AFB2	13	26	25-400	0.97	0.011	97	8	11	39
AFG1	10	20	25-400	0.97	0.005	95	12	20	41
AFG2	10	20	25-400	0.97	0.008	98	10	16	56

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] R = Pearson's correlation coefficient.
[d] SE = Standard error of mean.
[e] RSD_r = relative standard deviation intra-day precision.
[f] RSD_R = relative standard deviation inter-day precision.
[g] U = measurement uncertainty.
AFB1 = aflatoxin B1, AFB2 = aflatoxin B2, AFG1 = aflatoxin G1 and AFG2 = aflatoxin G2.

8.4. Results and Discussion

8.4.1. Control

All selected samples (18 of maize and 1 of sorgum) were positive for AFB1. The concentrations of AFB1 in all of them were very different, from 73.7 to 1820.3 µg/kg and an average concentration of 615.2±563.1 µg/kg (Table 8.4). All samples with presence of AFB2 had also AFB1, however, AFB2 average concentrations (157±164.8 µg/kg, max = 602.3 µg/kg) were always lower than AFB1 concentrations, as other studies about Afs presence showed (Rodrigues & Chin, 2012; Hove et al., 2016). The larger AFB1 concentration than AFB2 was expected because AFB2 is the dihydro derivative of the AFB1. AFG1 and AFG2 were less common (< 40 %), notwithstanding, some huge concentrations were detected (max AFG1 = 3817.1 µg/kg). As for AFB2, the average concentration of AFG2 (48.9±56.8 µg/kg) was lower than AFG1 (1037.3±1556.7 µg/kg), because AFG2 is the dihydro derivative of the AFG1.

AFB1 was found at higher concentrations than AFG1 in 89 % of our samples, however, larger concentration of AFG1 than AFB1 could be found due to different storage conditions. For instance, AFG1 is produced at lower temperatures than AFB1 (Lin, Ayres, & Koehler, 1980).

All the analysed cereals contained Afs levels over the legal limit permitted by the European legislation (1881/2006, EU, 2006). The maximum permitted levels of Afs in cereals are 2 µg/kg of AFB1 and 4 µg/kg of total sum of Afs and in unprocessed maize are 5 µg/kg of AFB1 and 10 µg/kg of total sum of Afs.

Controls of Afs levels in cereals should be carried on next years, because although most of the studies found samples below 10 µg/kg, for example, Rodrigues, Handl and Binder (2011) found less than 20 % of the samples over 10 µg/kg when analysing 324 samples of 13 different African countries. For instance, Matumba et al. (2013) detected some maize samples from Malawi with more than 592 µg/kg of AFB1 and Rodrigues et al. (2011) detected also some huge levels of Afs in maize from different countries, with a maximum detected level of 556 µg/kg of total Afs. Control samples are important to avoid a lofty chronic exposure.

The great levels of Afs detected in the selected samples were useful as they were going to be analysed for conjugated Afs. Different studies showed a concentration correlation between parent mycotoxin and conjugates as it has been shown in DON with deoxynivalenol-3-glucoside (DON-3-glucoside). The ratio DON-3-glucoside/DON concentration is similar in raw cereals among the studies, from 10% to 30% (Berthiller et al., 2009).

Table 8.4. Afs content variation (mean% ± standard deviation) as affected by treatment (basic treatment, acid treatment, α-amylase, cellulase, protease and β-glucosidase).

Treatment	Control			Basic treatment (KOH)	Acid treatment (TFMSA)	β-glucosidase	Protease	α-amylase	Cellulase
Mycotoxin	n (%)	µ (µg/kg)	max. (µg/kg)	% increase	% increase	% increase	% increase	% increase	% increase
AFB1	19 (100.0)	615.2±563.1	1820.3	-100.0	-1.4±1.9	0.4±16.7	-8.9±12.5	12.9±9.5	14.8±1.9
AFB2	19 (100.0)	157.8±164.8	602.3	-100.0	4.6±8.4	-6.2±7.4	-4.8±5.3	17.1±7.3	16.6±9.2
AFG1	7 (36.8)	1037.3±1556.7	3817.1	-100.0	1.5±3.8	-3.3±3.9	6.4±7.8	18.5±9.6	10.9±5.4
AFG2	6 (31.6)	48.9±56.8	138.0	-100.0	2.7±5.6	3.1±5.0	7.5±10.9	11.4±6.9	7.6±3.5
Total	19 (100)	-	-	-100.0	1.9±4.9	-1.5±8.3	0.1±9.1	14.9±8.3	12.5±5.0

n = number of positive samples.
AFB1 = aflatoxin B1, AFB2 = aflatoxin B2, AFG1 = aflatoxin G1 and AFG2 = aflatoxin G2.

8.4.2. Basic treatment

The treatment with KOH caused a very high pH in the environment (pH = 12) and all analysed Afs concentrations were drastically reduced after treatment with KOH (Table 8.4).

Obviously the results were significantly statiscal different ($p < 0.05$) compared with control samples.

The total reduction of aflatoxin after basic treatment could be linked with pH. Afs are extremely sensitive to pH variations and alkaline environment reduces widely Afs concentrations (Lee, Her, & Lee, 2015, Saalia, & Dixon Phillips, 2010). Alkaline conditions are present in some food process, for instance during tortilla making process where during nixtamalization pH values over 10 are generated. Some studies showed the total reduction of Afs (100 %) during tortilla making process (Moreno-Pedraza et al., 2015; Anguiano-Ruvalcaba, Verver y Vargas-Cortina, & Guzmán-De Peña, 2005; Elias-Orozco, Castellanos-Nava, Gaytán-Martínez, Figueroa-Cárdenas, & Loarca-Piña, 2002; De Girolamo, Lattanzio, Schena, Visconti, & Pascale, 2016). Our results at pH = 12 agree with the total Afs remotion from nixtamalization ($pH \approx 10$).

The reduction of Afs after KOH treatment should result in Afs degradation products. Although we did not look for degradation products after the alkaline treatment, two degradation products from AFB1 have been detected after nixtamalización due to alkaline conditions (Moreno-Pedraza et al., 2015). Degradation Afs products should be submitted to toxicological studies to determine their toxicity.

Although basic treatment with KOH was not useful to detect conjugated aflatoxins because they do not resist alkaline conditions, other mycotoxins are not so sensitive to pH. Thus, treatment with KOH permitted to detect conjugated fumonisins in large amount of food samples. Dall'Asta et al. (2009a,2009b) found hidden fumonisins in all corn flour, snacks, bread, pasta and extruded products after alkaline treatment. Oliveira et al. (2015) analysed raw maize samples (72) and all of them contained covert fumonisins. Also, Bryla, Roszko, Szymczyk, Jedrzejczak and Obiedziński (2016) 100 % of hidden fumonisins in 88 of maize-based food products analysed (groats, starch concentrates, flour, noodles, corn flakes and maize snacks). Moreover, the total concentration of hidden fumonisins was always high and hidden fumonisins represented more than 25 % of total fumonisins. Furthermore, several results of fumonisin content, which were found to be acceptable according to the EU limits, were found contaminated above the limit when also the bound forms were considered (Dall'Asta et al., 2009a). Some authors pointed out that hidden fumonisins could be embedded in the starch or proteins of the matrix. Regarding DON, alkaline conditions cause the transformation of 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) to DON (Malachova et al., 2015; Wu & Wang, 2016). Thus, Malachova et al. (2015) showed that 32 % of the spiked 3-ADON and 47 % of the spiked 15-ADON in flour matrix were hydrolysed to DON when samples were submitted to KOH treatment. Besides, the molar sum of 3-ADON, 15-ADON and DON was higher than the initial sum of these toxins before hydrolysis. This may have been caused by the presence of additional (unknown) sources of DON in the sample. Some DON could be hidden in starch or proteins of the matrix as fumonisins. KOH treatment is a valid method to quantify

modified mycotoxins from certain parent mycotoxins, especially fumonisins and DON, but it is not useful for conjugated Afs because alkaline conditions cause Afs degradation.

8.4.3. Acid treatment

TFMSA is one of the strongest known Brønsted acids (pKa = 13), characterized with extreme thermal stability and a high resistance towards reductive and oxidative cleavage, and it has showed better efficiency in release parent mycotoxins than other similar acids as trichloroacetic acid (TCA) and TFA trifluoroacetic acid (TFA) (Tran & Smith, 2011)

Similar Afs concentrations in the analysed samples were obtained after TFMSA treatment (Table 8.4), so TFMSA did not cause any change in Afs content and no differences were detected when the samples were compared to control samples.

While alkaline environment caused Afs reduction, acid environment did not produce changes in Afs stability. It is known Afs are stable at low pH (Rastegar et al., 2017). However, acid conditions can produce some changes in other parent mycotoxin concentrations. For instance, an increase of ZEN was detected after acid hydrolysis with TFMSA (0.025 M). Acidic environment hydrolysed (60 %) the glycoside bond in ZEN -14-glucoside (Beloglazova et al., 2013) causing ZEN increase. DON stability after TFMSA treatment has been also studied (Malachova et al., 2015, Tran & Smith, 2011). In the same way, high increases of DON (70 %) were got after TFMSA treatment (Tran & Smith, 2011). Tran et al. (2011) observed the sum of DON acetates was lower than the DON increase after TFMSA treatment. So, the increase of DON could be caused by some hidden DON in the food matrix that could not be detected in routine analysis. As commented in the basic treatment section (see section 3.2), DON could be bound to starch or proteins of the matrix. On the other hand, the increase of DON could not be caused by a release of DON-3-glucoside, because, although Malachova et al. (2015) observed a total reduction ofdeoxynivalenol-3-glucoside after acid hydrolysis, it did not produce an increase of parent mycotoxin (DON) and authors pointed out that the reaction lead to the formation of other undetected products (Malachova et al., 2015).

On the contrary, some mycotoxins are sensitive to low pH and they are reduced in acidic environments. Some examples of reduced mycotoxins in acidic environment are: nivalenol (Humer et al., 2016), beauverecin (Luciano, Meca, Manyes, & Mañes, 2014) and enniatins (García-Moraleja, Font, Mañes, & Ferrer, 2015).

Although acid treatment can produce an increase of parent mycotoxins due to hydrolysis of modified mycotoxins, Afs concentration has not been modified due to TFMSA treatment.

8.4.4. Enzymatic treatment

There are many different enzymes which cleave the linkage between two molecules. The physiological functions of enzymes depend on the origin (plant, fungi, animals or bacteria) and the substrate specificity.

We checked Afs concentration after treatment of samples with different common enzymes (β-glucuronidase, protease, α-amylase and cellulase). The results depended on the enzyme used (Table 8.4).

β-glucuronidase is an enzyme that catalyzes hydrolysis of β-D-glucuronic acid residues and there were no changes in Afs concentration after treatment with it. Some studies agree with our results and β-glucuronidase treatment did not cause a change in the concentration of parent mycotoxin. For instance this enzyme was tested for ZEN in wheat and maize samples (Beloglazova et al., 2013) without obtaining variations in parent mycotoxin concentration. However, treatment with β-glucuronidase caused increases in parent mycotoxin concentrations when biological (urine plasma and milk) samples where analysed. Thus, for example high increases of ZEN (> 100 %) and DON (between 79 and 98 %) have been detected in human urine after treatment of urine with β-glucuronidase (Warth et al., 2012; Vidal et al., 2016; Heyndrickx et al., 2015). The increases of parent mycotoxins showes that a large percentage of mycotoxins are in glucuronidated form in urine. High presence of glucuronided forms in biological samples occurs as a result of animal metabolism. Glucuronidation occurs mainly in the liver and the responsible enzyme for its catalysis is UDP-glucuronyltransferase. So, the absence of change in Afs concentration after β-glucuronidase treatment of cereal samples is expected because the glucuronided forms in cereals may be really scarce.

Proteases (also called peptidases or proteinases) are enzymes that perform proteolysis, that is, begin protein catabolism through hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Afs concentration did not change after treatment with proteases (Table 8.4).Contrary to β-glucuronidase, protease is barely used in mycotoxin analysis. There are some studies about protease affect in DON. Unlike in our results with Afs, protease caused an increase of parent mycotoxin concentration in all of them (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal, Ambrosio, Sanchis, Ramos, & Marín, 2016c; Zhou, Schwarz, He, Gillespie, & Horsley, 2008) with a maximum DON increase of 35 % after treatment with protease (Vidal et al., 2016c). These results pointed out that some DON could be embedded in cereal wall proteins and it is of concern because an increase of DON could be detected after some food process where protease is added, as for example in bread making process (Vidal et al., 2016c). The absence of Afs increase after protease treatment showed there could not be Afs bound to proteins. Unfortunately, other parent mycotoxins (different to DON or Afs) stability after protease treatment has not been studied yet.

α-Amylase is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and hydrolyse them into polymers composed of glucose units.

It is found in saliva and it has a large amount of uses in food industry. Slight increases for all analysed Afs (AFB1, AFB2, AFG1 and AFG2) have been detected after α-amylase treatment (Table 8.4). The release of Afs after α-amylase treatment agrees with other studies where α-amylase activity caused increases of parent mycotoxin as ZEN and DON. Regarding ZEN , α-amylase produces total transformation of ZEN -14-glucoside to ZEN (Beloglazova et al., 2013). Therefore, the increase of ZEN will depend on the original ZEN -14-glucoside content. However Afs glucoside forms has not been detected yet, but, the slight increase of Afs could be caused by some glucoside conjugation. In the same way, DON concentration also increased due to α-amylase and although DON glucoside forms have been identified (Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012) DON variation may not be linked to them (Kostelanska et al., 2011). Thus, α-amylase caused increases in DON concentration and even a 99 % of DON increase was detected due to α-amylase presence during bread fermentation (Simsek et al., 2012). Other studies have also reported large increases of DON during the bread making process when α-amylase is present in the recipe; Vidal et al. (2016c) got a 20 % of DON increase at the end of bread making process when they added α-amylase in the mix of ingredients. Contrary to ZEN -14-glucoside, DON-3-glucoside is increased by the presence of α-amylase and larger increases than for DON (~ 500 %) occurred (Vidal et al., 2016c). The increase of parent DON during α-amylase treatment could be due to a possible splitting of glycosidic bonds between parent mycotoxin and cell polysaccharides, as some authors suggested (Kostelanska et al., 2011). This may also imply that parent mycotoxins with increased after α-amylase treatment could be more likely to be bound to starch and polysaccharides than other molecules. So, Afs could be also embedded in some polysaccharides from the matrix and they could be released during enzyme treatment. The embedded Afs could be released during food process or digestion, because α-amylase are used in some food industry and are also present in animal saliva, and it will produce an increase of Afs exposure.

Cellulase is an enzyme which produces decomposition of cellulose and some related polysaccharides. It is widely used in the food industry and when added to bread hydrolyses non-starch polysaccharides, leading to an improvement of the rheological properties of dough, bread loaf volume and crumb firmness. The cellulase treatment caused increases of Afs in small percentages (12.5 ± 5.0 µg/kg) (Table 8.4).

Relation between cellulase and parent mycotoxins has been observed in few studies and analogous results to α-amylase were obtained. Firstly, cellulase is able to cleave all ZEN -14-glucoside after 600 minutes of treatment (Beloglazova et al., 2013) and transform it to ZEN. Regarding DON, cellulase also released DON from wheat,and wheat flour , 27 and 26 % in Simsek et al. (2012) and Vidal et al. (2016c), respectively. As in α-amylase, increases of DON-3-glucoside also occurred during the bread making process, confirming that DON and DON-3-glucoside are not linked and that both mycotoxins are embedded in the carbohydrates of the cereal matrix (Vidal et al., 2016c). Similar to DON, Afs could be embedded in the cell wall

cellulose. . It is a concern because cellulase is a common enzyme used in the food industry and the presence of cellulase would produce an increase of Afs concentration at the end of food processing, leading to an increased Afs exposure.

The presence of Afs modified mycotoxins in raw cereals could be lower compared to other mycotoxins because the increases of parent Afs shown were not high. For instance, in raw cereals, parent fumonisins could be only the 37 % of the total presence of fumonisin (Dall'Asta et al., 2009) or parent DON could represent 50 % of the total DON (Berthiller et al., 2009; De Boevre et al., 2014). The lower level of modified Afs could be due to Afs formation during storage, contrary to other mycotoxins with high percentage of conjugated forms (DON, ZEN , fumonisins, etc.) which are formed in the field. Some of the most common conjugates found in food have a plant origin, mostly glucoside conjugates (DON-3-glucoside, ZEN -14-glucoside, T-2 and HT-2 glucoside …) and all of their parent mycotoxins are formed in the field. On the other hand, matrix associations with mycotoxins could occur in the field. These two reasons could explain the lower level of Afs conjugates detected.

The presence of modified mycotoxins in raw cereals or in food is of concern because they can be transformed to parent mycotoxins during food processing or during digestion (Hahn, et al., 2015, Nagl et al., 2014). Thus, official organisms are considering some modified mycotoxins may be an additional contribution factor of the total dietary exposure to parent mycotoxin, for example, the Joint European Commission FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside, 3-ADON and 15-ADON as additional contributing factors of the total dietary exposure to DON (Codex, 2011; JEFCA, 2010).

8.5. Conclusions

Afs are very sensitive to the alkaline conditions and KOH treatment is not a useful method to detect embedded Afs in the matrix. TFMSA treatment did not cause Afs increase. Afs could be embedded to matrix carbohydrates because α-amylase and cellulase caused a slight increase of AFs concentration. Conversely, β-glucosidase and protease did not produce any change in the Afs concentration showing that Afs glucuronide conjugates did not exist in the analysed cereals and Afs are not bound to proteins. Although the low level of modified Afs found in our samples research on them in the food chain is imperative to protect consumers.

8.6. Acknowledgements

The authors are grateful to the Spanish government (project AGL2014-55379-P) for financial support. A. Vidal thanks the Spanish government (Ministry of Education) for the pre-doctoral grant.

8.7. References

Abia, W.A., Warth, B., Sulyok, M., Krska, R., Tchana, A.N., Njobeh, P.B., Dutton, M.F., & Moundipa, P.F. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS*). Food Control, 31*, 438-453.

Andrade, P.D., & Caldas, E.D. (2015). Aflatoxins in cereals: Worldwide occurrence and dietary risk assessment. *World Mycotoxin Journal, 8*, 415-431.

Anguiano-Ruvalcaba, G.L., Verver, Y., Vargas-Cortina, A., & Guzmán-De Peña, D. (2005). Inactivation of aflatoxin B1 and aflatoxicol through traditional "nixtamalización" of corn and their regeneration by acidification of corn dough. *Salud Publica de Mexico, 47*, 369-375.

Beloglazova, N.V., De Boevre, M., Goryacheva, I.Y., Werbrouck, S., Guo, Y., & De Saeger, S. (2013). Immunochemical approach for zearalenone-4-glucoside determination. *Talanta, 106*, 422-430.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Blankson, G.K., & Mill-Robertson, F.C. (2016). Aflatoxin contamination and exposure in processed cereal-based complementary foods for infants and young children in greater Accra, Ghana. *Food Control, 64*, 212-217.

Broekaert, N. (2015). Disposition of 3- and 15-Acetyl-Deoxynivalenol and Deoxynivalenol-3-β-D-Glucoside in broiler chickens and pigs. Dissertation of thesis, University of Ghent.

Bryła, M., Roszko, M., Szymczyk, K., Jedrzejczak, R., & Obiedziński, M.W. (2016). Fumonisins and their masked forms in maize products. *Food Control, 59*, 619-627.

Cano-Sancho, G., Sanchis, V., Marín, S., & Ramos, A.J. (2013). Occurrence and exposure assessment of aflatoxins in Catalonia (Spain). *Food and Chemical Toxicology, 51*, 188-193.

Cunha, S.C., & Fernandes, J.O. (2012). Development and validation of a gas chromatography-mass spectrometry method for determination of deoxynivalenol and its metabolites in human urine. *Food and Chemical Toxicology, 50*, 1019-1026.

Dall'Asta, C., Galaverna, G., Mangia, M., Sforza, S., Dossena, A., & Marchelli, R. (2009a). Free and bound fumonisins in gluten-free food products. *Molecular Nutrition and Food Research, 53*, 492-499.

Dall'Asta, C., Mangia, M., Berthiller, F., Molinelli, A., Sulyok, M., Schuhmacher, R., Krska, R., Galaverna, G., Dossena, A., & Marchelli, R. (2009b). Difficulties in fumonisin determination: The issue of hidden fumonisins. *Analytical and Bioanalytical Chemistry, 395*, 1335-1345.

De Boevre, M., Landschoot, S., Audenaert, K., Maene, P., Di Mavungu, D., Eeckhout, M., Haesaert, G., & De Saeger, S. (2014). Occurrence and within field variability of *Fusarium* mycotoxins and their masked forms in maize crops in Belgium. *World Mycotoxin Journal, 7*, 91-102.

De Girolamo, A., Lattanzio, V.M.T., Schena, R., Visconti, A., & Pascale, M. (2016). Effect of alkaline cooking of maize on the content of fumonisins B1 and B2 and their hydrolysed forms. *Food Chemistry, 192*, 1083-1089.

Elias-Orozco, R., Castellanos-Nava, A., Gaytán-Martínez, M., Figueroa-Cárdenas, J.D., & Loarca-Piña, G. (2002). Comparison of nixtamalization and extrusion processes for a reduction in aflatoxin content. *Food Additives and Contaminants, 19*, 878-885.

European Commission, 2006. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal European Union, L364*, 5–24.

European Food Safety Authority (EFSA). 2007. Definition and description of “emerging risks” within EFSA’s mandate. ESAF/SC/415 Final. Available at: <http://www.efsa.europa.eu/> (accessed 21.09.16).

García-Moraleja, A., Font, G., Mañes, J., & Ferrer, E. (2015). Comparison of different cooking process for the emerging mycotoxins reduction in fresh pasta. *Revista de Toxicologia, 32*, 131-134.

Grabley, S., Gareis, M., Bockers, W., & Thiem, J. (1992). Glycosylation of mycotoxins. *Synthesis, 11*, 1078-1080.

Hahn, I., Nagl, V., Schwartz-Zimmermann, H.E., Varga, E., Schwarz, C., Slavik, V., Reisinger, N., Malachová, A., Cirlini, M., Generotti, S., Dall'Asta, C., Krska, R., Moll, W.-D., & Berthiller, F. (2015). Effects of orally administered fumonisin B1 (FB1), partially hydrolysed FB1, hydrolysed FB1 and N-(1-deoxy-D-fructos-1-yl) FB1 on the sphingolipid metabolism in rats. *Food and Chemical Toxicology, 76*, 11-18.

Heyndrickx, E., Sioen, I., Huybrechts, B., Callebaut, A., De Henauw, S., & De Saeger, S. (2015). Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study. *Environment International, 84*, 82-89.

Hove, M., De Boevre, M., Lachat, C., Jacxsens, L., Nyanga, L.K., & De Saeger, S. (2016). Occurrence and risk assessment of mycotoxins in subsistence farmed maize from Zimbabwe. *Food Control, 69*, 36-44.

Humer, E., Lucke, A., Harder, H., Metzler-Zebeli, B.U., Böhm, J., & Zebeli, Q. (2016). Effects of citric and lactic acid on the reduction of deoxynivalenol and its derivatives in feeds. *Toxins, 8*, 285.

Huong, B.T.M., Tuyen, L.D., Do, T.T., Madsen, H., Brimer, L., & Dalsgaard, A. (2016). Aflatoxins and fumonisins in rice and maize staple cereals in Northern Vietnam and dietary exposure in different ethnic groups. *Food Control, 70*, 191-200.

IARC (International Agency for Research on Cancer). 2002. Monograph on the evaluation of carcinogenic risk to humans: Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol. 82. Lyon, France, 1–556.

Jager, A.V., Tedesco, M.P., Souto, P.C.M.C., & Oliveira, C.A.F. (2013). Assessment of aflatoxin intake in São Paulo, Brazil. *Food Control, 33*, 87-92.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Leblanc, J.-C., Tard, A., Volatier, J.-L., & Verger, P. (2005). Estimated dietary exposure to principal food mycotoxins from The First French Total Diet Study. *Food Additives and Contaminants, 22*, 652-672.

Lee, J., Her, J.-Y., & Lee, K.-G. (2015). Reduction of aflatoxins (B1, B2, G1, and G2) in soybean-based model systems. *Food Chemistry, 189*, 45-51.

Lin, Y.C., Ayres, J.C., & Koehler, P.E. (1980). Influence of temperature cycling on the production of aflatoxins B1 and G1 by *Aspergillus parasiticus*. *Applied and Environmental Microbiology, 40*, 333-336.

Luciano, F.B., Meca, G. Manyes, L., & Mañes, J. (2014). A chemical approach for the reduction of beauvericin in a solution model and in food systems. *Food and Chemical Toxicology, 64*, 270-274.

Matumba, L., Monjerezi, M., Van Poucke, C., Biswick, T., Mwatseteza, J., & De Saeger, S. (2013). Evaluation of the bright greenish yellow fluorescence test as a screening technique for Aflatoxin-contaminated maize in Malawi. *World Mycotoxin Journal, 6*, 367-373.

Malachová, A., Štočková, L., Wakker, A., Varga, E., Krska, R., Michlmayr, H., Adam, G., & Berthiller, F. (2015). Critical evaluation of indirect methods for the determination of deoxynivalenol and its conjugated forms in cereals. *Analytical and Bionalytical Chemistry*, Article in press.

Moreno-Pedraza, A., Valdés-Santiago, L., Hernández-Valadez, L.J. Rodríguez-Sixtos Higuera, A., Winkler, R., & Guzmán-de Peña, D.L. (2015). Reduction of aflatoxin B1 during tortilla production and identification of degradation by-products by direct-injection electrospray mass spectrometry. *Salud Publica de Mexico, 57*, 50-57.

Muñoz, K., Blaszkewicz, M., & Degen, G.H. (2010a). Simultaneous analysis of ochratoxin A and its major metabolite ochratoxin alpha in plasma and urine for an advanced biomonitoring of the mycotoxin. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 878*, 2623-2629.

Muñoz, K., Campos, V., Blaszkewicz, M., Vega, M., Alvarez, A., Neira, J., & Degen, G.H. (2010b). Exposure of neonates to ochratoxin A: First biomonitoring results in human milk (colostrum) from Chile. *Mycotoxin Research, 26*, 59-67.

Nagl, V., Woechtl, B., Schwartz-Zimmermann, H.E., Hennig-Pauka, I., Moll, W.-D., Adam, G., & Berthiller, F. (2014). Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. *Toxicology Letters, 229*, 190-197.

Njumbe Ediage, E., Van Poucke, C., & De Saeger, S. (2015). A multi-analyte LC-MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: The forgotten sample matrix. *Food Chemistry, 177*, 397-404.

Oliveira, M.S., Diel, A.C.L., Rauber, R.H., Fontoura, F.P., Mallmann, A., Dilkin, P., & Mallmann, C.A. (2015). Free and hidden fumonisins in Brazilian raw maize samples. *Food Control, 53*, 217-221.

Pena, A., Seifrtová, M., Lino, C., Silveira, I., & Solich, P. (2006). Estimation of ochratoxin A in portuguese population: New data on the occurrence in human urine by high performance liquid chromatography with fluorescence detection. *Food and Chemical Toxicology, 44*, 1449-1454.

Rastegar, H., Shoeibi, S., Yazdanpanah, H., Amirahmadi, M., Khaneghah, A.M., Campagnollo, F.B., & Sant'Ana, A. (2017). Removal of aflatoxin B1 by roasting with lemon juice and/or citric acid in contaminated pistachio nuts. *Food Control, 71*, 279-284.

Rodrigues, I., Handl, J., & Binder, E.M. (2011). Mycotoxin occurrence in commodities, feeds and feed ingredients sourced in the middle East And Africa. *Food Additives and Contaminants: Part B Surveillance, 4*, 168-179.

Rodrigues, I., & Chin, L.J. (2012). A comprehensive survey on the occurrence of mycotoxins in maize dried distillers' grain and solubles sourced worldwide. *World Mycotoxin Journal, 5*, 83-88.

Rychlik, M, Humpf, H.-U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., & Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including “masked” mycotoxins. *Mycotoxin Research, 30*, 197-205.

Saalia, F.K., & Dixon Phillips, R. (2010). Degradation of aflatoxins in aqueous buffer in the presence of nucleophiles*. Food Control, 21*, 1066-1069.

Šarkanj, B., Warth, B., Uhlig, S., Abia, W.A., Sulyok, M., Klapec, T., Krska, R., & Banjari, I. (2013). Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. *Food and Chemical Toxicology, 62*, 231-237.

Spanjer, M.C., Rensen, P.M., & Scholten, J.M. (2008). LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25*, 472-489.

Solfrizzo, M., Gambacorta, L., Lattanzio, V.M.T., Powers, S., & Visconti, A. (2011). Simultaneous LC-MS/MS determination of aflatoxin M 1, ochratoxin A, deoxynivalenol, de-epoxydeoxynivalenol, α and β-zearalenols and fumonisin B 1 in urine as a multi-biomarker method to assess exposure to mycotoxins. *Analytical and Bioanalytical Chemistry, 401*, 2831-2841.

Simsek, S., Burgess, K., Whitney, K. L., Gu, Y., & Qian, S. Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287–292.

Scientific Cooperation on Questions Relating to Food Projects (SCOOP). 1996, Working Document in Support of a SCF Risk Assessment of Aflatoxin. Task 3.2.1 (SCOOP/CNTM/1), Task Coordinator, UK.

Thieu, N.Q., & Pettersson, H. (2009). Zearalenone, deoxynivalenol and aflatoxin B1 and their metabolites in pig urine as biomarkers for mycotoxin exposure. *Mycotoxin Research, 25*, 59-66.

Thuvander, A., Möller, T., Enghardt Barbieri, H., Jansson, A., Salomonsson, A.-C., & Olsen, M. (2001). Dietary intake of some important mycotoxins by the Swedish population. *Food Additives and Contaminants, 18*, 696-706.

Tran, S.T., & Smith, T.K. (2011). Determination of optimal conditions for hydrolysis of conjugated deoxynivalenol in corn and wheat with trifluoromethanesulfonic acid. *Animal Feed Science and Technology, 163*, 84-92.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016a). The fate of deoxynivalenol through wheat processing to food products. *Current opinion in Food Science, 11*, 34-39.

Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016b). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, 208*, 288-296.

Vidal, A., Cano-Sancho, G., Marín, S., Ramos, A.J., & Sanchis, V. (2016c). Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain. *World Mycotoxin Journal, 9*, 597-612.

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Frohlich, J., Krska, R. 2012. Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins. *Rapid Communication Mass Spectrometry. 26*, 1533–1540.

Welsch, T., & Humpf, H.-U. (2012). HT-2 toxin 4-glucuronide as new T-2 toxin metabolite: Enzymatic synthesis, analysis, and species specific formation of T-2 and HT-2 toxin glucuronides by rat, mouse, pig, and human liver microsomes. *Journal of Agricultural and Food Chemistry, 60*, 10170-10178.

Wu, L., & Wang, B. (2016). Transformation of deoxynivalenol and its acetylated derivatives in Chinese steamed bread making, as affected by pH, yeast, and steaming time. *Food Chemistry, 202*, 149-155.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

Zhou, B., Schwarz, P., He, G.-Q., Gillespie, J., & Horsley, R. (2008). Effect of enzyme pretreatments on the determination of deoxynivalenol in Barley. *Journal of the American Society of Brewing Chemists, 66*, 103–108.

9. Study IV. Stability of DON and OTA during the breadmaking process and determination of process and performance criteria.

Food Control (2014) 40, 234-242.

Authors: Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S.

9. Stability of DON and OTA during the breadmaking process and determination of process criteria and performance criteria.

Arnau Vidal, Hector Morales, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*.

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

9.1. Abstract

The fate of deoxynivalenol (DON) and ochratoxin A (OTA) during the breadmaking process was studied. In particular, toxin content was analysed in mixed baking ingredients before kneading, after fermentation and proofing, and finally after baking. Fermentation and proofing were carried out at 30 ºC for 1h, while baking was performed at different temperature levels (from 170 to 210 ºC) and baking times from 45 to 135 min, in a full factorial design. The trend showed level of DON increased from unkneaded mix to fermented dough, and decreased due to baking; this trend depended on the initial concentration of DON in the flour. The level in the bread was significantly lower than in the initial mix of ingredients. In contrast, deoxynivalenol-3-glucoside (DON-3-glucoside) content increased both during kneading and fermentation, and also during baking. Moreover, the results confirmed the high stability of OTA as no significant change in its content could be observed as a result of the breadmaking process. As conclusion, the design of bakery product processes may help to control DON in final products, because although quite stable, its levels can be reduced to some extent. However, high levels of DON-3-glucoside were released during baking, and this point should be further investigated. Mycotoxins have been always considered as stable compounds; however, in depth knowledge of the processing steps that may lead to some reduction (although limited) and those which can stimulate their release from conjugated forms, will definitely help in their control in finished foodstuffs.

Keywords: bread, fermentation, baking, deoxynivalenol, ochratoxin A, deoxynivalenol-3-glucoside

9.2. Introduction

Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins (Samar, Fontán, Resnik, & Pacin, 2003; Pleadin et al., 2013). To date, over 300 mycotoxins have been identified, however, not all of them represent a risk in food. Mycotoxins are produced by fungi, the main mycotoxin-producing fungi in foods belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Different studies show the high presence of mycotoxins, especially deoxynivalenol (DON) and ochratoxin A (OTA), in products of high consumption like beer and bakery products (Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Vendl, Crews, Macdonald, Krska, & Berthiller, 2010; Anli & Alkis, 2010; Cano-Sancho et al., 2011a). One of these products is bread. Wheat bread provides more nutrients to the world population than any other single food source (Peña, 2002). Bread is particularly important as a source of carbohydrates, proteins and vitamins B and E (Pomeranz, 1987). Moreover, the commonest cereal used for leavened bread is wheat (Betschart, 1982; Chaven & Kadam, 1989).

DON, also known as vomitoxin, is one of the most regular contaminants in cereals (Jelinek, Pohland, & Wood, 1989). Although DON is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010a,b). At the molecular level, DON disrupts normal cell function by inhibiting protein synthesis, affecting cell signaling, differentiation, and proliferation. An acute and high dose of DON can induce vomiting, whereas chronic dietary exposure to DON causes reduced food intake, decreased nutritional efficiency, reduced weight gain, and immune dysregulation (Pestka, 2010a,b). OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (SCF, 1998). This mycotoxin has been classified as a possible human carcinogen, in the group 2B, by IARC (IARC, 2002). OTA has been reported in cereals, coffee, grape juice, wine, beer, spices, and meat based foodstuffs.

The European Commission has set maximum permitted levels in processed cereal products for direct human consumption in OTA of 3 $\mu g \cdot kg^{-1}$, and a maximum concentration of 500 µg/kg for DON in bread (European Commission, 2006, 2010).

Flour processing into bread may affect DON and OTA content. Some studies reported a significant increase in DON levels during dough fermentation (Bergamini et al., 2010), while others showed a reduction of DON in the process (Samar, Neira, Resnik, & Pacin, 2001) (Table 9.1). Regarding baking, some studies reported that DON seems to be reduced and such reduction is affected by increasing time and temperature. However, other studies reported lower reduction of DON during baking which may have to do with the loaf size or to a mild heat treatment (Bergamini et al., 2010; Simsek, Burgess, Whitney, Gu, & Qian, 2012) (Table 9.1). On the other side, OTA is stable at high temperatures and its concentration does not decrease during baking (Scudamore, Banks, & MacDonald, 2003).

Table 9.1. Effect of bakery processing on DON contamination in wheat products.

Reference	DON level in flour (µg/kg)	Loaf size	Fermentation + proofing conditions	Use of improvers	Baking conditions	%DON reduction referred to flour	Results referred to equal basis (in general dw basis)
Neira et al. (1997)	1370 (natural)	Industrial	5-11h 25 ºC	-	10-40 min 210 ºC	21.6 (fermented dough) 44.3 (baked product) Both referred to kneaded dough	No
Samar et al. (2001)	150 (natural)	40 g	40-60 min, 30-50 ºC	Malt flour	-	0-41% (fermented dough) Referred to kneaded dough	Yes
	150 (natural)	40 g	60-90 min, 30-50 ºC	No	-	25-56% (fermented dough) Referred to kneaded dough	Yes
Lancova et al. (2008)	40/52/413/1223 (natural)	70g	95 min, 30 ºC	No	14 min 240 ºC	54-62% (fermented dough) -32-(-45)% (proofed dough) 0-4% (baked product)	Yes
Valle-Algarra et al. (2009)	200/750/1500 (spiked)	80g	1h, 30 ºC	No	30-50 min 190-240 ºC	None (fermented dough) 47.9% (baked product)	No
Scudamore et al. (2009)	60-284 (natural)	Bread	60 min, 40-45 ºC	Ascorbic acid, α-amylase	21 min 210 ºC	4-57% (baked product)	No
	60-284 (natural)	Cakes		No	15-30min 150-170 ºC	41-79% (baked product)	No
	60-284 (natural)	Biscuits		No	3-5 min 245-280 ºC	11-25% (baked product)	No
Bergamini et al. (2010)	100/160/967 (natural)	140 g (pilot scale)	45-85 min, 30-40 ºC	Malt flour, other	8-16min 180-210 ºC	-77-5% (fermented dough) -88-3% (baked product)	Yes
	100/160/967 (natural)	140 g (industrial scale)	45-85 min, 30-40 ºC	Malt flour, other	8-16min 180-210 ºC	-39% (fermented dough) -61% (baked product)	Yes
Pacin et al. (2010)	72 (natural)	35-65 g (industrial)	-	-	-	42-58% (baked product)	No
Kostelanska et al. (2011)	48-1049 (natural)	55g	95 min, 30 ºC	No	14 min 240 ºC	0% (fermented dough) 3% (baked product) Referred to kneaded dough	No
Simsek et al. (2012)	48-182 (natural)	>100 g	180min, 30 ºC	α-amylase	25 min 220 ºC	-99% (fermented dough) -69% (baked product) Referred to kneaded dough	Yes
Zachariasova et al. (2012)	729 (natural)	500 g	95 min, 30 ºC	Yes	14 min 240 ºC	28% (fermented dough) -4% (baked product)	No
Suman et al. (2012)	238-633 (natural, from bran)	Biscuits	2-4 min, 20-30 ºC	Flour malt, enzymes	1.5-5 min 225-270 ºC	-14-10% (fermented dough) -17-42% (baked product) Referred to kneaded dough	Yes
De Angelis et al. (2013)	816/954/1824 (natural)	500 g	60 min, ambient temp.	No	55 min, 200ºC	-18% (baked product)	Yes

'natural' refers both to contaminated flour from naturally contaminated and field inoculated wheat, while 'spiked' refers to directly spiked flour with toxin.

Although the published studies indicate that DON and OTA are largely stable and survive the breadmaking process, when concentrations are determined on an 'as is' basis as stipulated in the legislation, levels in finished products are usually lower than in the starting flour due to the dilution effect of other ingredients such as fat, sugar and water (Scudamore, Hazel, Patel & Scriven, 2009). Thus knowing the dynamics of reduction of DON and OTA during each stage in the process would allow adjusting the processing conditions and the quality of the incoming raw materials to obtain a safe product, setting then suitable performance criteria (PC) in order to achieve the desired Food Safety Objectives (FSO), which in the case of mycotoxins usually take the values of the legislated maximum permitted levels (García-Cela, Ramos, Sanchis, & Marin, 2012).

The aim of the study was to know the stability of DON and OTA in the two stages of bread making which are fermentation and baking of the dough and their effects of mycotoxins fate. The results obtained can be useful to calculate performance criteria (PC) and process criteria (PO) in order to achieve a desired food safety objective (FSO).

9.3. Materials and methods

9.3.1. Obtaining DON and OTA contaminated flours

In order to obtain DON contaminated flour and OTA contaminated flour, two strains of either *Fusarium graminearum* or *Aspergillus ochraceus* were used. They were previously proved to be DON and OTA producers in wheat flour. The initial flour did not contain OTA and DON and DON-3-glucoside levels were 250 ± 76.14 and 45.12 ± 15.34 µg/kg respectively.

The strains were inoculated and incubated in MEA at 25 ºC until strong sporulation. A spore suspension of each strain was made in water and Tween 80 (0.005% v/v). Five milliliters of either *F. graminearum* or *A. ochraceus* spore suspension were inoculated in glass flasks containing 250 g of flour and 50 mL of water. In total, 3 kg of flour were inoculated with each strain. The flasks were stored at 25 ºC for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus* with periodic shaking. Then, each kind of flour (3 kg) was properly ground and homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of 12,500 ± 1,235 µg/kg and 75.5 ± 15.2 µg/kg respectively, in each contaminated flour, while DON-3G level remained unchanged.

9.3.2. Dough preparation and bread making

Each loaf bread was made with 156 g of wheat flour, 2.3 g of salt, 4.7 g of sucrose, 4.7 of lard, 6.2 of commercial compressed yeast (*Saccharomyces cerevisiae*), 83 mL of water, 3.1 g of flour improver (containing calcium carbonate, wheat flour, soya flour, lecithin, ascorbic acid and enzymes). The initial flour was prepared by mixing uninoculated flour, DON contaminated flour and OTA contaminated flour. Three different combinations of mycotoxin concentrations were assayed in the initial flour: a) high, b) medium and c) low. For each of the three initial concentrations and 20 baking conditions (see bellow) three different loaves were prepared (Table 9.2). A sample of the initial mix of solid ingredients was taken and stored at -20 ºC until mycotoxin analysis.

Table 9.2. The calculated distribution of the toxin levels (mean±SD) in the initial flours.

Mycotoxin	High concentration(µg/kg)	Half concentration(µg/kg)	Low concentration(µg/kg)
DON	2090±525	1459±444	1012±269
DON-3-glucoside	48±27	43±47	46±41
OTA	9.5±0.2	5.1±0.1	0.8±0.1

Dough was manually kneaded until held together with a non-sticky, smooth and satiny appearance and optimum handling properties. Rounded pieces weighing 260 g each were prepared. From this point, thermoprobes were always used in the dough to record fermentation and baking temperatures, in particular, probes were placed in the centre of the loaf and close to the surface. Doughs were covered with a damp cloth and fermentation was carried out at 30 ºC for 15 minutes. Then the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 ºC. After the fermentation, a sample of 20 g was taken from every proofed dough which was stored at -20 ºC until mycotoxin analysis (results for the finished bread were corrected accordingly). The proofed dough was then baked. Five temperature levels (210, 200, 190, 180 and 170 ºC) and 4 baking times (45, 75, 105 and 135 minutes) were assayed in a full factorial design. Thus 3 initial toxin concentrations x 5 baking temperatures x 4 baking times x 3 replicates made 180 bread loaves. These conditions were established on the basis of previous experiments to obtain properly baked bread which was organoleptically acceptable.

After baking, bread pieces were stored at –20 ºC until analysis. For this purpose bread was sliced. Additionally, some slices (those baked at 210 ºC for 135 and 105 min and at 200 ºC for 135 min) were separated in three portions, the crumb central part, the section 0.5 cm under the crust and the crust and all of them were stored at –20 ºC until analysis.

9.3.3. Chemicals and reagents

Mycotoxin standards were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) and OTA (OCHRAPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). 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, Montcada i Reixach, Spain), potassium dihydrogen phosphate (0.2 g) (Sigma), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (J.T Baker) in 1 L of pure water; the pH was brought to 7.4 with hydrochloric acid 1 M.

9.3.4. Preparation of standard solutions

The standard of OTA was dissolved in methanol at a concentration of 5.0 mg/mL and stored at 4 ºC in a sealed vial until use. The concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis, chapter 49 (Horwitz & Latimer, 2006). Working standard solutions (0.5, 0.01, 0.005, 0.001 and 0.0005 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriated chromatographic system. The standard of DON was dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. Working standards (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriate chromatographic system. The standard of DON-3-glucoside was dissolved in acetonitrile at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriate chromatographic system.

9.3.5. Mycotoxins extraction, detection and quantification

Prior to extraction, all samples were dried at 40 ºC for 24 hours and weight loss recorded in order to present the results in a ‘as is’ basis (µg/kg) or in total content of toxin (µg or ng).

9.3.5.1. OTA

Briefly, for OTA analysis, 5 g of ground sample were extracted with 30 mL of extractant solution (60% acetonitrile, 40% water) by magnetically stirring for 10 min. and filtered with filter Whatman number 1. Four mL of filtered solution was diluted with 44 mL of PBS solution and drained through the IAC column. After this, the column was washed with 20 mL of PBS and OTA was eluted by applying 1.5 mL of methanol grade HPLC (three times back flushing) and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under nitrogen stream. Each dried sample was resuspended with acetonitrile:water:acetic acid (57:41:2).

OTA was determined by HPLC coupled with a Multi λ Fluorescence Detector Waters 2475®, an analytical column Water Spherisorb® 5 µm ODS2, 4.6 x 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. Mobile phase consisted of acetonitrile, water and acetic acid (57:41:2). The mobile phase flow rate was 1 $mL \cdot min^{-1}$. The injection volume was 100 µL. The retention time was 15 minutes.

9.3.5.2. DON and DON-3-glucoside

Regarding DON and DON-3-glucoside, 5 g of ground sample were extracted with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 g. Supernatant was filtered through a glass microfiber filter. Five milliliters of filtered sample was drained through the IAC column and the column washed with 10 mL of distilled water. DON was eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. The purified extracts were dried under nitrogen stream. Each dried sample was resuspended with the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON was determined by HPLC coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The mobile phase flow rate was 1.2 $mL \cdot min^{-1}$. The injection volume was 100 µL. The retention time for DON and DON-3-glucoside were 20 and 23 min, respectively.

9.3.6. Methods performance

The analytical methods used were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 were 0.97 and 0.99 for DON and OTA, respectively). Precision was established by determining OTA and DON levels in bread and DON and DON-3-glucoside in flour samples at least by triplicate, in those samples fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and OTA are summarized in Table 9.3.

Table 9.3. Method performances for ochratoxin A (OTA). deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-glucoside) determination in flour and bread.

Mycotoxin	Product	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery (%)[c]	RSDr[d](%)
OTA	Bread	0.14	0.42	5	0.5	103.90±15.57	4
				5	1.5	110.13±13.04	2
				5	3.0	99.65±12.57	1
DON	Bread	60.00	180.00	5	100	100.01±16.27	16
				5	500	98.84±9.01	9
				5	1000	102.33±5.26	5
	Flour	60.00	180.00	3	500	87.36±8.58	7
				3	300	123.26±30.29	41
DON-3-G	Flour	14.00	42.00	555	50	80.01±9.59	12
					250	79.71±4.84	6
					500	66.71±11.19	18

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

9.3.7. Statistical analysis

The results obtained were processed in two different ways: a) DON or OTA content in each step (µg of DON or ng of OTA) and, b) concentration 'as is' (µg/kg of DON and OTA in wet basis). In the first case it was possible to assess the real impact of the processing steps in the mycotoxins, while in the second one it is possible to draw conclusions on either compliance of maximum levels or calculation of PC. For the 'unkneaded mix' results obtained for dry ingredients from the mycotoxin analysis were corrected for the amount of water to be added. Kruskal-Wallis test and multifactorial ANOVA were applied to assess the significance of sample traits in the observed mycotoxin levels. Finally, multiple linear regressions were applied to assess the temperature/time effect of DON reduction during baking.

9.4. Results

9.4.1. DON content

The level of DON increased from unkneaded mix to fermented dough, and decreased due to baking ($p<0.05$); this trend depended on the initial concentration of DON in the flour. The level in the bread was significantly lower than in the initial mix of ingredients ($p<0.05$).

9.4.1.1. Fermentation

DON content increased after dough proofing (Table 9.4). Average increase was of 16, 10 and 30% for high, medium and low DON content in the initial mixture, respectively (Figure 9.1). However, according to Kruskal-Wallis test, this increase was only statistically significant ($p<0.05$) for the low initial DON concentration in the mix.

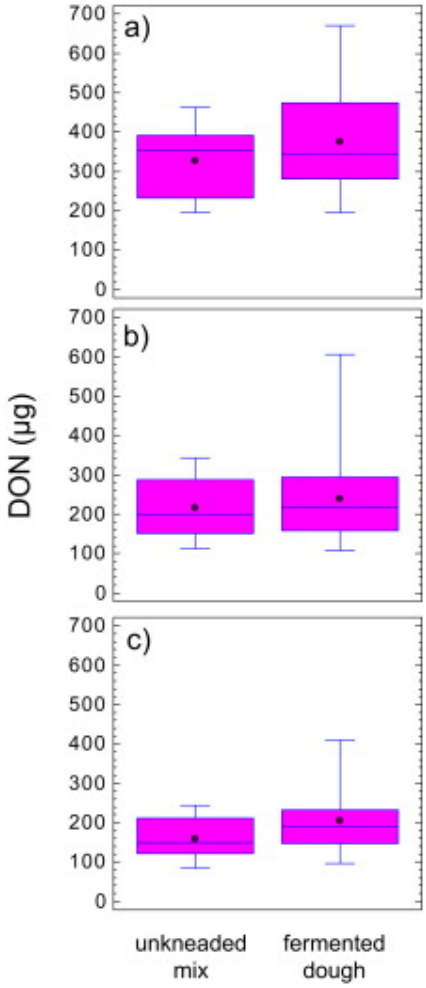


Figure 9.1. Distribution of DON content (µg) in the initial mixture and fermented dough at the high (a), medium (b) and high (c) initial concentration.

Regarding DON-3-glucoside, as the experiment was designed taking only into account DON levels, but not DON-3-glucoside levels, the initial concentration in flour of this mcyotoxins in the experiments with high/medium/low levels of DON was not significantly different, thus all results were pooled for a single initial concentration (mean 45.4 $\mu g \cdot kg^{-1}$, assuming LOD for the negative samples). According to Kruskal-Wallis test, fermentation led to a significant increase in DON-3-glucoside content, from median = 2.5 µg, to median = 3.6 µg ($p<0.05$).

Table 9.4. Evolution of DON mean content (µg) ±SD in the different breadmaking steps at the three initial concentration levels.

	High Concentration(µg)	Half Concentration(µg)	Low Concentration(µg)
Untreated mix	326±82	217±70	158±50
Fermented dough	376±121	239±107	205±80
Bread	239±107	202±103	130±53

9.4.1.2. Baking

Overall, baking led to a significant reduction in DON content ($p<0.05$) according to Kruskal-Wallis test, with mean percentages of 30, 17 and 33% for high, medium and low initial flour concentration, respectively, compared to fermented dough (Table 9.4).

Baking for 75-135 min at 180-210 ºC led to DON reduction ($p<0.05$) for the three assayed initial concentrations in the mix (Table 9.5), while in some treatments for 45 min or at 170ºC some increase in DON could be observed (data not shown). The statistical analysis suggested that the assayed levels of baking time had a much important effect than temperature levels in DON stability. Slight differences were observed among temperature levels over 170 ºC. While for the higher and medium initial concentration, both the effect of time and its interaction with temperature levels had a significant effect ($p<0.05$) in the reduction of DON content, for the lower concentration the effects of temperature and time were not significant.

The limited effect of temperature can be attributed to the similar temperatures reached inside the loaf bread even if oven temperatures were high. Figure 9.2a shows the temperature profiles recorded in the centre of loaves when baked at 170, 190 and 210 ºC for 105 minutes. The maximum temperature reached in the centre of the crumb was 98 ºC, such temperature was independent of the oven temperature, then baking time becomes more relevant. For example, at 210 ºC, for a baking time of 75 min, the crumb was 60 min over 90 ºC, and 90 min and 120 min, for baking times of 105 and 135 min, respectively. Moreover, recording of

temperature near to the surface of the loaves revealed temperatures in the crust up to 30 ºC higher than in the crumb (Figure 9.2b). DON analyses of the different parts of the bread (the crust, 0.5 cm below the crust and the crumb) showed a higher reduction in the crust compared to the other two fractions (data not shown), but the difference was not significant. Thus the major fraction of crumb compared to crust might determine the low effect of temperature on DON recorded in the whole bread.

On the other hand, a significant increase of DON-3-glucoside was observed in the bread compared to the fermented dough (from 8.8 to 25.4 µg). Although an increasing trend of DON-3-glucoside to increase with temperature levels was observed, temperature and baking time levels did not significantly affect the extent of such increase, which accounted for a mean value of 189% (data not shown).

Table 9.5. DON content reduction (mean%±SD) as affected by baking time and temperature.

	Temp (ºC)	170	180	190	200	210
	Initial DON conc.					
75 min	High	10±59	34±21	24±11	43±26	33±24
	Medium	-45±57	34±71	10±23	47±53	83±31
	Low	35±16	42±31	34±41	48±29	40±35
105 min	High	22±6	34±22	35±23	38±9	61±2
	Medium	18±45	-0.9±18	14±11	22±12	26±34
	Low	-2±62	73±7	28±11	32±37	39±32
135 min	High	46±11	32±24	38±21	35±9	48±10
	Medium	26±4	4±71	8±26	6±28	16±39
	Low	28±35	31±48	23±18	24±15	34±1

9.4.2. OTA content

Results confirmed the high stability of OTA as no significant change in its content could be observed as a result of the breadmaking process. While kneading and fermentation and baking had no significantly effect separately in OTA content, the final content in bread was significantly higher than in the initial mix of ingredients (41%). Although a mean increase of 19% was recorded during kneading and fermentation, it was not statistically significant. Unkneaded mix

contained 1483, 801 and 123 ng for the three concentration levels, high, medium and low, respectively, and the fermented dough 1501, 1174 and 132 ng of OTA, respectively. Regarding baking, as no significant reduction was observed in the loaves baked at 210 ºC for 105 and 135 min, and at 200 ºC for 135 min, the remaining loaves were not analysed. The average result was an overall increase of OTA of 24%. Thus as a conclusion the total amount of OTA in the flour remained finally in the resulting bread.

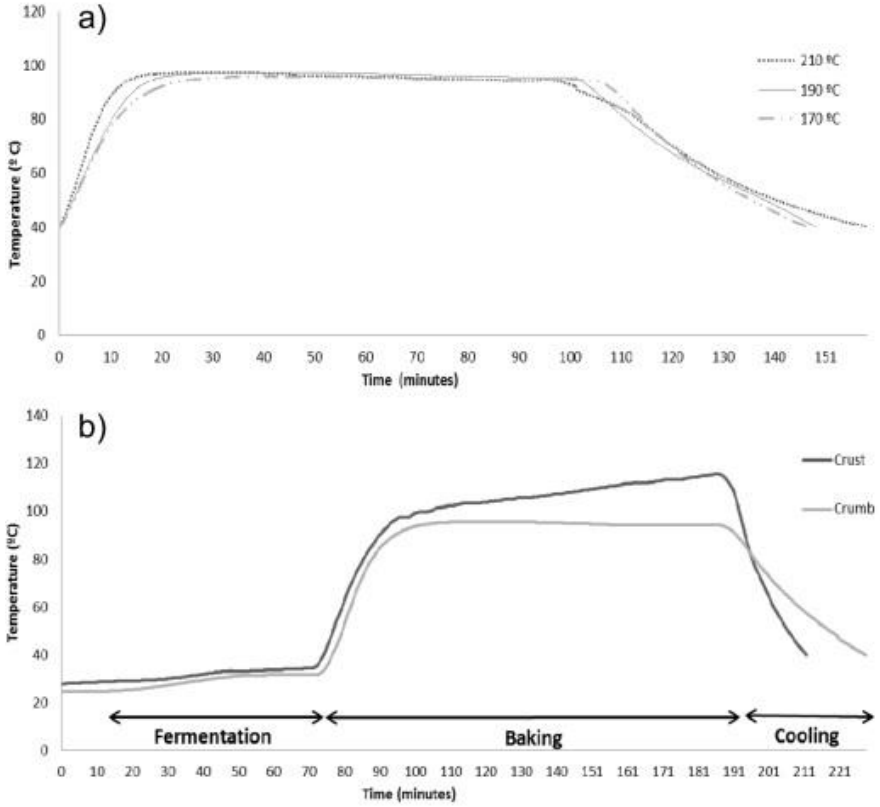


Figure 9.2. Recorded temperatures a) in the centre of the loaves at 170, 190 and 210 ºC for 105 minutes, and b) in the crumb and in the crust of the loaves after 105 minutes of baking.

9.4.3. DON concentration in flour and bread 'as is'

DON concentration in bread produced from a given flour batch will depend on the 'dilution' of the toxin with the addition of ingredients, on to a minor extent to water, carbon dioxide and ethanol balance, and finally to the stability of the toxin through the process, which was explained in the previous sections.

Combined 'dilution' plus increase in DON content due to fermentation led to significantly higher ($p<0.05$) DON concentration in flour than in fermented dough. The reductions obtained in the three different concentrations were 31, 37 and 22% for the high, medium and low concentration respectively (Table 9.6).Moreover, a significant reduction in DON concentration

from proofed dough to bread was observed for baking times of 75 min and over. For the higher and medium initial concentration in the flour, the percentage of reduction depended on temperature and baking time levels assayed (Figure 9.3a, r^2=0.695, %reduction=-229.081+0.532·temperature+2.850·time-0.012·t^2; Figure 9.3b, r^2=0.523, %reduction=-1180.62+5.130·temperature+11.986·time-0.040·T·t-0.021·t^2), while at the lower concentrations the effect was not significant, thus a single value of reduction (26 ± 18%) could be considered in this case. The lower percentages of reduction compared to those in DON content may be attributed to the evaporation of water and other dough components in the oven (mean weight loss of 8%), and consequent concentration of toxin. A 25% decrease in DON-3- glucoside was observed from flour to fermented dough, and a mean further increase of 224% from fermented dough to final bread (Table 9.6).

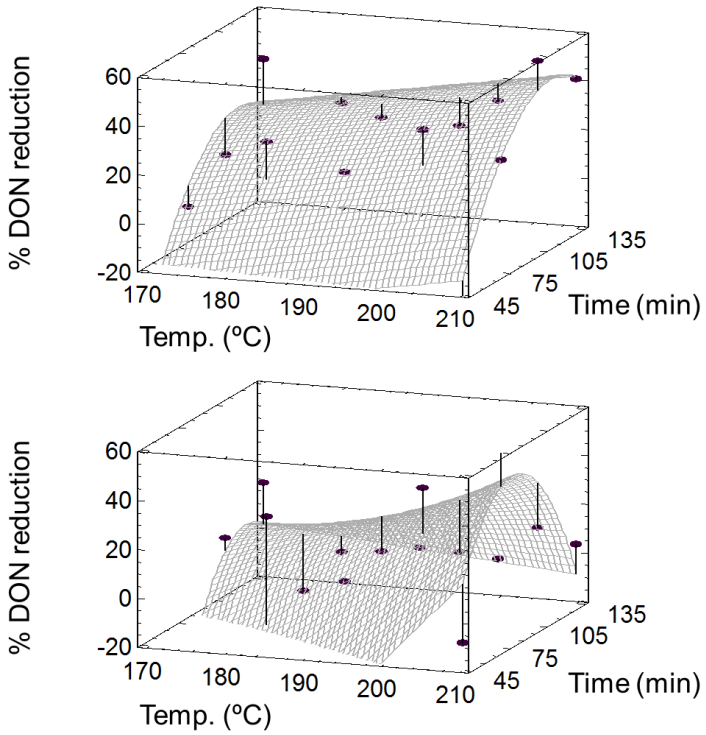
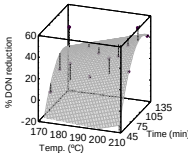
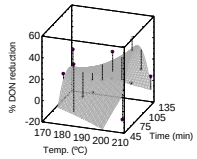


Figure 9.3. Response surface model for %reduction of DON concentration from fermented dough to bread as a function of temperature and baking time. a) high b) medium initial DON concentration in the flour.

Table 9.6. Evolution of mycotoxin concentration (µg/kg) in the different steps of breadmaking process (flour, fermented dough and bread).

	Flour (µg/kg)	Fermented dough 'as is' (µg/kg)	Bread 'as is' (µg/kg)	% Reduction (kneading +fermentation+proofing) 30ºC 75 min	% reduction baking
DON	2090	1445 (*1254)	variable	31% (*40%)	
	1459	921 (*875)	variable	37% (*40%)	
	1012	790 (*607)	585	22% (*40%)	26%
DON-3-G	45.4	34.03 (*27.3)	110.3	25% (*40%)	-224%
OTA	9.5	5.8 (*5.7)	8.9	39% (*40%)	-50%
	5.1	3.5 (*3.0)	5.0	31% (*40%)	-43%
	0.8	0.5 (*0.5)	0.6	37% (*40%)	-20%

* calculated value taking into account only dilution by recipe

9.4.4. OTA concentration in flour and bread 'as is'

OTA concentration in bread produced from a given flour will depend only on the 'dilution' of the toxin with the addition of ingredients, and on a minor extent to water, carbon dioxide and ethanol balance due to OTA stability during the breadmaking process, as explained in the previous sections. Thus OTA concentration (wet basis) was reduced by average in a 36% from flour to fermented dough (theoretical decrease, assuming complete stability would be 40%).

On the other hand, the average increase from fermented dough to bread was 27% (wet basis), although not significant (theoretical increase, assuming complete thermostability of OTA and 8% weight loss in the bread would be 9%).

9.5. Discussion

Some studies dealt in the past with the fate of DON during breadmaking, and although some trends were pointed out, only a few reported statistically significant effects on equal basis for each studied stage (Scudamore, Hazel, Patel, & Scriven, 2009). Similarly, in the present study a great variability was observed among repeated experiments. Apart from known heterogeneous distribution of mycotoxins in foodstuffs, enzymatic or microbial processes that take place during breadmaking, could be the cause of additional variability.

In the present study, DON increased with fermentation; as the initial analysis was carried out before kneading, such increase was due to the joint contribution of kneading, fermentation and proofing. This increasing trend has been mainly previously observed in those studies in which the results were given by comparison of concentration in fermented dough to flour, thus including kneading. Lancova et al. (2008) reported an increase in DON concentration due to kneading, which could confirm this hypothesis. High increase in DON content was also reported in those studies in which enzymes (mainly α-amylase) were used as dough improvers (Table 9.1). Both circumstances coexisted in the present study. By contrast, studies at higher than 30ºC fermentation temperatures reported a reduction in DON concentration from kneaded dough to fermented dough (Samar et al., 2001), while no DON reduction was observed at 30ºC. Confirming our results, Young, Fulcher, Hayhoe, Scott, & Dexter (1984) and Bergamini et al. (2010) observed an increase in DON content during fermentation at 30ºC. The authors suggested that wheat flour contained DON precursors (3-acetyl-deoxyinivalenol and 15-acetyl-deoxynivalenol), which were converted to DON by the active yeast. In the present study, 3-ADON and 15-ADON were analysed in some samples at random (data not shown), and it was concluded that although the precursors disappeared during fermentation, their concentration in the flour was too low (4-5% of DON concentration for the sum of both precursors) to account for the increase in DON. Bergamini et al. (2010) hypothesised an enzymatic release of DON from its unknown bound forms. This hypothesis was confirmed by Kostelanska et al. (2011) and Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova (2012) who indeed reported a much significant increase in DON-3-glucoside than in DON due to the use of bread improvers, as was confirmed in the present study.

The level of DON-3-glucoside in the uninoculated flour was similar or higher than those reported in naturally contaminated (or field inoculated) wheat flour intended for breadmaking (Kostelanska et al., 2011; Simsek et al., 2012). In the literature, DON-3-glucoside has been attributed to DON conjugation at plant level or due to heat treatments (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006). In our study, while both DON and DON-3-glucoside increased during kneading and fermentation, DON decreased during baking but DON-3-glucoside increased. By contrast, previous studies reported DON-3-glucoside reduction during baking (Kostelanska et al., 2011; Simsek et al., 2012).

The reduction in DON during baking was consistent with previous studies which always reported reduction at temperature over than 170 ºC as long as baking time was longer than 30 min (Table 9.1). Little difference was observed among the impact of the different temperature levels, thus for a target reduction level, several binomial temperature/time values could be used, satisfying at the same time the sensorial traits of the baked product. Few studies focused in the past on the combined effect of baking time and temperature in DON content in bakery products (Valle-Algarra et al., 2009; Bergamini et al., 2010; Suman, Manzitti, & Catellani, 2012), and in most cases no significant differences were observed or were not reported. Baking temperatures between 170-240 ºC have been assayed with small differences in their impact. However, in general, oven temperatures were reported and, as presented in the present study, the temperature levels inside the baked loaves may not be significantly different. Placing temperature probes in the loaves allowed us to confirm that fermentation temperature in the dough was 3-4 ºC under 30 ºC (as also measured by Samar et al., 2001), and that maximum attained temperature in the crumb during baking was 98 ºC, regardless of the oven temperature, similarly, Bergamini et al. (2010) reached a maximum of 86 ºC in the loaf when baking at 225 ºC. As a consequence of this, the size of the loaf may be determinant for the calculation of the extent of mycotoxin reduction during baking, as pointed out by Valle-Algarra et al. (2009). Looking at table 9.1, this can be the reason for some contradictory presented results. Some studies reporting higher reduction, included small size loaves, cakes and biscuits (Suman et al., 2012; Samar et al., 2001), while, interestingly, Zachariosova et al. (2012) using a 500 g loaf weight did not report any reduction when sampling the whole bread for analysis. Unfortunately, from our results, although higher DON levels were in general found in the crumb, no significant difference could be confirmed between crust and crumb, while this point could be clearly confirmed by Valle-Algarra et al. (2009). DON thermodegradation products (norDONs A-F and DON lactones) occur in bread crust (Kostelanska et al., 2011); such degradation products are less toxic than DON itself. The losses that cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions (Bretz et al., 2006). In general, higher reductions were observed during baking with higher DON concentration, but the trend was not significant as concluded before (Neira, Pacin, Martínez, Moltó, & Resnik, 1997; Bergamini et al., 2010). The increased DON levels observed sometimes at 45 min or 170ºC could be attributed to the prolongation of the enzymatic activity to the initial stages of baking, before enzyme inactivation.

The studies on OTA during the breadmaking process are scarce. In the present study little effect was observed, confirming the work of Scudamore, Banks, & MacDonald (2003). Baking led to an increase in OTA level that, although not significant, had never been reported before. Oppositely, baking of biscuits resulted in about two-thirds of the toxin being destroyed or immobilized (Subirade, 1996). The higher diminishing of OTA content in biscuits can be explained by the higher temperature reached when compared to bread and for the lower water content. On the other hand, Valle-Algarra et al. (2009) found an OTA reduction near to 30%, but they worked with OTA-spiked flour, which may be differently affected. In other fermented foods,

reduction in OTA caused by the degradation of OTA or the adsorption in the yeast cell walls has been detected, especially in wine (Abrunhosa, Serra, & Venâncio, 2002).

This work was developed in an attempt to investigate the possible PC to be attributed to the breadmaking steps, and then to be able to calculate the toxin concentration in the final bread (performance objective) produced from a given flour with H_o initial concentration of toxin. Looking at DON results and literature, it is clear that standard fermentation temperature, plus the widespread use of improvers leads to a minimising of the 'dilution' effect due to ingredients mixture to levels of 22-37% (PC). An alternative to increase this criterion would be to modify process criteria from 30 ºC 1 h to higher fermentation temperature, if applicable, which may lead to DON degradation, as suggested by Samar et al. (2001). The baking stage led to a minimum reduction of 20% (PC) as long as time was over 75 min and temperature over 180ºC (process criteria), thus temperature/time of baking can be adjusted for a desired process criterion. Overall, a minimum 38% reduction can be expected for the breadmaking process from flour to bread (in a 260 g bread loaf, with improvers added), which is a bit higher than the 33% assumed by Commission Regulation 1881/2006 setting a maximum level of 750 µg/kg for flour and 500 µg/kg for bread (in our case the only dilution effect would account for 40% reduction, but different recipes for bakery products could lead to much lower reduction, as suggested by Scudamore et al. (2003)). On the other hand, DON-3G concentration greatly increased with baking, although its increase could not be linked to baking temperature/time levels. The reason could be the glycosidation of DON in the initial stages of baking, before enzyme inactivation. This hypothesis is reinforced by the fact that DON-3-glucoside also increased during fermentation, and was previously suggested by Zachariasova et al. (2012). As a consequence, higher DON-3-glucoside concentration could be found in bread than in the initial flour (opposite to what reported by De Angelis et al., 2013). This point is of outmost importance as part of DON can be *in vivo* released from its conjugate (Berthiller et al. 2013) and should be further investigated. Moreover, a joint regulation of DON+DON-3G maximum levels might be proposed. Finally, for OTA the PC for fermentation at 30 ºC was near 40% which is the theoretical dilution factor by recipe, thus the fermentation process itself had no effect on OTA content. Although not significant, an increase in OTA during baking was observed, which resulted in final bread with similar OTA concentration to the initial flour. This confirms the suitability of an equal maximum level for both flour and bread (3 µg/kg), as set in the Commission Regulation 1881/2006.

As conclusion, the design of bakery products processes may help to control DON in final products, because although quite stable, its levels can be reduced to some extent. It was confirmed that DON release occurs from flour to fermented dough. By contrast, OTA was highly stable. Finally, high levels of DON-3-glucoside were released during baking, and this point should be further investigated. Mycotoxins have been always considered as thermostable compounds; however, in depth knowledge of the processing steps that may lead to some reduction (although limited) and those which can stimulate their release from conjugated forms will definitely help in their control in finished foodstuffs. Finally, the high stability of the

mycotoxins observed in the assay leads to give a lot of importance to the initial flour to obtain safe products.

9.6. Acknowledgements

The authors are grateful to the Spanish government (projects AGL2010-22182-C04-04 and AGL2011-24862) for the financial support. A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant. H. Morales is grateful to the Portuguese Government. (Ministério da Ciêcia, Tecnologia e Ensino Superior; FCT Fundação para a Ciência e a Tecnologia) Grant ref. SFRH/BPD/38011/2007.

9.7. References

Abrunhosa, L., Serra, R., & Venâncio, A. (2002). Biodegradation of ochratoxin A by fungi isolated from grapes. *Journal of Agricultural and Food Chemistry, 50(25)*, 7493-7496.

Anli, E., & Alkis, I.M. (2010). Ochratoxin A and brewing technology: A review. *Journal of the Institute of Brewing, 116(1)*, 23-32.

Bergamini, E., Catellani, D., Dall'asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27(5)*, 677-687.

Betschart, A. A. (1982). World food and nutrition problems. *Cereal Food World*, *27*, 562.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S.D., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, Speijers, G., & Stroka, J. 2013. Masked mycotoxins: A review. *Molecular Nutrition and Food Research, 57*(1), 165-186.

Bretz M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal Agricultural Food Chemical, 54*, 6445–6451.

Cano-Sancho, G., Gauchi, J.P., Sanchis, V., Marín, S., & Ramos, A. J. (2011b). Quantitative dietary exposure of the Catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives and Contaminants part A*, *28*, 1098−1109.

Cano-Sancho, G., Valle-Algarra, F. M., Jiménez, M., Burdaspal, P., Legarda, TM., Ramos, A. J., Sanchis, V., & Marín, S. (2011a). Presence of tricothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22*, 490−495.

Chaven, J. K., & Kadam, S. S. (1989). Nutritional improvement of cereals by fermentation. *CRC Critical Reviews in Food Science and Technology, 28*(5), 349.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: An investigation by high-performance liquid chromatography high-resolution mass spectrometry. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *30*(2), 345-355.

European Commission (EC). (2006). Commission Regulation (EC) No. 1881/2006 of 19/12/2006, setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L364, 5–24.

European Commission (EC). (2010). Commission Regulation (EC) No. 165/2010 of 26/2/2010, setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L446, 1–127.

García-Cela, E., Ramos, A.J., Sanchis, V., & Marin, S. (2012). Emerging risk management metrics in food safety: FSO, PO. How do they apply to the mycotoxin hazard?. *Food Control, 25* (2), 797-808.

Horwitz, W., & Latimer, G.W. (2006). *Official methods of analysis of AOAC International*. (18th ed.). AOAC Gaithersburg, Maryland.

IARC (International Agency for Research on Cancer). (1993). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins In IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 56. Lyon, France.

IARC (International Agency for Research on Cancer). (2002). *Monograph on the evaluation of carcinogenic risk to humans*, vol. 82. Lyon, France.

Jelinek, C.F., Pohland, A.E., & Wood, G.E. (1989). Worldwide occurrence of mycotoxins in foods and feeds an update. *Journal AOAC International*, *72*, 223–230.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*(17), 9303-9312.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer. *Food Additives and Contaminants*, *25*, 732–744.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37*(1), 21-25.

Pacin, A., Ciancio Bovier, E., Cano, G., Taglieri, D., & Hernandez Pezzani, C. (2010). Effect of the bread making process on wheat flour contaminated by deoxynivalenol and exposure estimate. *Food Control. 21*(4), 492-495.

Pacin, A.M., Resnik, S.L., Neira, M.S., Moltó, G., & Martínez, E. (1997). Natural occurrence of deoxynivalenol in wheat, wheat flour and bakery products in Argentina. *Food Additives and Contaminants, 14*(4), 327-331.

Peña, R.J. Wheat for Bread and Other Foods. In Curtis, B.C., Sajaram, S., Gómez Macpherson, H. (Eds.), *FAO Plant Production and Protection Series: Wheat Bread*. FAO, Rome, 2002. Available in http://www.fao.org/DOCREP/006/Y4011E/Y4011E00.HTM. Accessed 21.01.13.

Pestka, J.J. (2010a). Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal, 3*, 323–347.

Pestka, J.J. (2010b). Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology*, *84*, 663–679.

Pleadin, J., Vahčić, N., Perši, N., Ševelj, D., Markov, K., & Frece, J. (2013). *Fusarium* mycotoxins' occurrence in cereals harvested from Croatian fields. *Food Control, 32*(1), 49-54.

Pomeranz, Y. (1987). Bread around the world. In Y. Pomeranz (Eds.), *Modern cereal science and technology* (pp. 258-333). New York, NY, USA: VCH Publishers.

Samar, M.M., Fontán, C.F., Resnik, S.L., Pacin, A.M., & Castillo, M.D. (2003). Distribution of deoxynivalenol in wheat, wheat flour, bran, and gluten, and variability associated with the test procedure. *Journal of AOAC International, 86*(3), 551-556.

Samar, M.M., Neira, M.S., Resnik, S.L. & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants, 18,* 1004-1010.

Scientific Committee on Food (SCF). (1998). Commission of the European Communities, Outcome of discussions 14. Directorate General XXIV. Expressed 17.09.88.

Scudamore, K.A., Banks, J., & MacDonald, S.J. (2003). Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. *Food Additives and Contaminants, 20*(12), 1153-1163.

Scudamore, K.A., Hazel, C.M., Patel, S., & Scriven, F. (2009). Deoxynivalenol and other *Fusarium* mycotoxins in bread, cake, and biscuits produced from UK-grown wheat under commercial and pilot scale conditions. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*(8), 1191-1198.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of Deoxynivalenol and Deoxynivalenol-3-glucoside in wheat. *Food Control, 26*(2), 287-292.

Subirade, I. (1996). Fate of ochratoxin A during breadmaking. *Food Additives and Contaminants*, 13(SUPPL.), 25-26.

Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*(3), 241-249.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *26*(6), 896-906.

Vendl, O., Crews, C., Macdonald, S., Krska, R., & Berthiller, F. (2010). Occurrence of free and conjugated *Fusarium* mycotoxins in cereal-based food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *27*(8), 1148-1152.

Young, J.C., Fulcher, R.G., Hayhoe, J.H., Scott, P.M., & Dexter, J.E. (1984). Effect of milling and baking on deoxynivalenol (vomitoxin) content of eastern Canadian wheats. *Journal of Agriculture and Food Chemistry, 32,* 659-664.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*(36), 9280-9291.

10. Study V. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.

Food and Chemical Toxicology (2014) 68, 53-60.

Authors: Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V.

10. The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.

Arnau Vidal[1], Sonia Marín[1]*, Hector Morales[2], Antonio J. Ramos[1] and Vicente Sanchis[1]

[1]Applied Mycology Unit, Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, 25198 Lleida, Spain.

[2]IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

10.1. Abstract

Deoxynivalenol (DON) and ochratoxin A (OTA) are mycotoxins produced by fungal species which can contaminate, alone or simultaneously, cereal-based products such as bread. Due to the increasing interest in the beneficial effects of dietary bran, bran bread has attained high consumption. Usually, the higher mycotoxin concentrations in cereals are found in the external layers of the grain (bran), leading to higher concentration of DON and OTA in breads with added bran. Moreover, the use of sourdough in breadmaking is increasing, but no studies about its effect in the mycotoxins content exist. The objective of this study was to determine the variation of concentration of these mycotoxins during the breadmaking process including the following factors: two initial mycotoxin concentrations in the initial mix of ingredients, four different bran contents, and use of sourdough. OTA was confirmed to be quite stable during the breadmaking process, regardless of the assayed factors. DON concentration during breadmaking was not significantly affected by bran content of bread. However, it was significantly affected by kneading and fermentation steps in different way depending on sourdough use and flour contamination level: if DON reduction occurs during fermentation, this leads to a safer situation, but the possible increase in DON should be considered with care, as it can compensate the expected dilution effect by recipe. Finally, the results on deoxynivalenol-3-glucoside (DON-3-glucoside), although preliminar, suggest an increase of this toxin during fermentation, but mainly during baking.

Keywords: deoxynivalenol, ochratoixn A, masked mycotoxins, baking process.

10.2. Introduction

Wheat bread provides more nutrients to the world population than any other single food source (Peña, 2002). Bread is particularly important as a source of carbohydrates, proteins and vitamins B and E (Pomeranz, 1987). Consumption of bread prepared with whole grain flours is currently increasing in developed countries, due to the increasing interest in the beneficial effects of dietary fiber (Faridi and Faubion, 1995). Dietary fiber is defined as carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence (European Comission, 2011). The benefits of dietary fiber intake are numerous, ranging from improved large bowel function to slowed digestion and absorption of carbohydrate and fat and reduced risk for certain diseases. Based on the effects on bowel function, the EFSA Panel considered bran intake of 25 $g \cdot d^{-1}$ to be adequate for normal laxation in adults (EFSA, 2010).

Cereals and cereal based products like bread may contain mycotoxins, particularly deoxynivalenol (DON) and ochratoxin A (OTA). Wheat and wheat containing products (like bread and pasta) represent the major source of intake for DON (Cano-Sancho et al., 2011a; SCOOP, 2003). 55% of samples (raw cereals and derived products) were reported to be OTA positive (SCOOP, 2002) and cereals are considered one of the major sources of intake for OTA (Coronel et al., 2012).

DON, also known as vomitoxin, is one of the most regular contaminants in cereals (Alexa et al., 2013; Cano-Sancho et al., 2011b; EFSA, 2006a; Jelinek et al., 1989; Li et al., 2014). Although DON is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010a,b). At the molecular level, DON disrupts normal cell function by inhibiting protein synthesis, affecting cell signaling, differentiation, and proliferation. An acute and high dose of DON can induce vomiting, whereas chronic dietary exposure to DON causes reduced food intake, decreased nutritional efficiency, reduced weight gain, and immune dysregulation. OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (EFSA, 2006b). This mycotoxin has been classified as a possible human carcinogen in the group 2B by the IARC (IARC, 2002). OTA has been reported in cereals, coffee, grape juice, wine, beer and meat based foodstuffs (Coronel et al., 2012).

The European Commission has set a maximum permitted level for OTA in processed cereal products for direct human consumption of 3 $\mu g \cdot kg^{-1}$, and a maximum concentration of 500 µg/kg for DON in bread (European Commission, 2006).

Deoxynivalenol-3-glucoside (DON-3-glucoside) is a plant metabolite from DON, and then considered a masked mycotoxin. Berthiller et al. (2005) showed its high presence in wheat. Some studies pointed out a possible conversion of DON-3-glucoside to DON during baking (De

Angelis et al., 2013). Moreover, Berthiller et al. (2011) showed that DON-3-glucoside can be hydrolised by several lactic acid bacteria. Thus the Joint European Commission FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside as an additional contributing factor of the total dietary exposure to DON (Codex, 2011; JEFCA, 2010).

Nishio et al. (2010) studied the level of *Fusarium graminearum* infection in wheat and observed that the level of DON production was directly related to the incidence of fungal growth, and that fungal infection was greatest at or near the kernel surface. Generally, cereal bran is the part of the grain with the highest concentration of mycotoxins (Thammawong et al., 2010). L'Vova et al. (1998) reported that up to 60% of the initial DON content in grain passed into bran and that mycotoxin content in the bran exceeded that in the grain. Scudamore (2005) reported that by-products such as 'cleanings' or bran may contain high concentrations of OTA. Vidal et al. (2012) assessed the content of several mycotoxins in wheat and oat based bran supplements sold in the Spanish market; DON was present in 42% of the samples, with levels above the EU legislation in 19% of samples, whereas OTA was present in 25% of the samples.

Thermal treatment of contaminated cereals, flour and bran may decrease the levels of mycotoxins. However, some studies with OTA contaminated flour reported that this mycotoxin is stable at high temperatures, and that its concentration does not decrease during baking (Scudamore, 2005; Vidal et al., 2014). Also, the studies of Bullerman and Bianchini (2007) and Kabak (2009) reported that DON is stable in processing steps involving high temperatures. By contrast, Scott et al. (1984) reported a reduction of DON up to 74% in white flour bread, although other studies reported lower reduction of DON during baking (Lancova et al., 2008; Pacin et al., 2012; Vidal et al., 2014). DON stability during the baking process can be influenced by temperature, time and loaf size (Vidal et al., 2014). Finally, DON degradation may take place mostly in the bread crust, which reaches the highest temperature, whereas the content of DON in the breadcrumbs scarcely decreases (Kostelanka et al., 2011). Thus, the inactivation of DON and OTA during baking and the influence of bran content are not clear yet. It is obvious that the initial content of bran influences on the final content of mycotoxins in bread. Moreover, the effects of bran content of bread in toxin thermal stability may lead to different dynamics of the mycotoxin during baking.

The interest in the use of sourdough in the manufacture of bakery products is increasing as a traditional added value; sourdough is a very complex biological ecosystem where yeasts and lactic acid bacteria contribute to the dough fermentation (Gobbetti, 1998).

The objective of the assay presented herein was to assess the effect of dough fermentation and baking on DON and OTA content in bran bread combined with sourdough addition. For an additional experiment, OTA in the initial mixture came either from contaminated bran or contaminated flour, in order to study whether the source of the mycotoxin influences on its dynamics during dough fermentation and baking. Finally, DON-3-glucoside natural contamination was followed through the breadmaking process.

10.3. Materials and methods

10.3.1. DON and OTA contaminated flour and bran

In order to obtain DON or OTA contaminated flour, two strains of either *Fusarium graminearum* (TA 3.234) or *Aspergillus ochraceus* (TA 3.201) were used. Both of them are kept in the Food Technology Dept. collection, University of Lleida, Spain. They were previously proved to be DON and OTA producers when cultured on wheat flour. The initial flour did not contain OTA, and DON and DON-3-glucoside levels (n=3) were 250±76 and 45.1±15.3 $\mu g \cdot kg^{-1}$, respectively.

The strains were inoculated and incubated in MEA (malt extract agar) at 25 ºC until strong sporulation. A spore suspension of each strain was made in water and Tween 80 (0.005% v/v). Five milliliters of either *F. graminearum* or *A. ochraceus* spore suspension were inoculated in glass flasks containing 250 g of wheat flour and 50 mL of water. In total, 3 kg of flour were inoculated with each strain. The flasks were incubated at 25 ºC for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus*, with periodic shaking. Then, each contaminated flour (3 kg) was properly homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of 12,500±1,235 µg/kg and 75.5±15.2 µg/kg respectively (n=3), in each contaminated flour, while DON-3-glucoside level remained unchanged.

Commercial wheat bran was used to obtain mycotoxin contaminated bran. The initial analysis of this bran showed that it was naturally contaminated (n=3) with DON (2,070 ±49 µg/kg). Analogously to the flour contamination procedure described above, a sublot of DON naturally contaminated bran was further contaminated with OTA by inoculation with *A. ochraceus* and subsequent incubation. Thus bran contaminated with both OTA and DON was obtained. The concentration of OTA (n=3) reached in such bran was 85.7±19.6 µg/kg.

10.3.2. Dough preparation and baking

Pieces of dough (260 g) were made with different contents of bran (0, 100, 200 and 300 g/kg flour) and either with addition of sourdough or not. The desired DON and OTA concentration was achieved by adding either DON/OTA contaminated bran as well as DON/OTA contaminated flour and uninoculated flour.

Different flour/bran mixes were prepared, depending on the bran content and toxin levels to be achieved: a) dough without bran, DON and OTA was added through contaminated flour, b) dough with 100 or 200 g bran/kg flour, which source of OTA was contaminated flour,

while DON contamination came from both DON contaminated flour and commercial bran, c) dough with 200 g bran/kg flour, which OTA contamination source was contaminated bran, while DON contamination came from both DON contaminated flour and commercial bran; d) dough 1with 300 g bran/kg flour, where DON contamination source was commercial bran whereas OTA contamination came from flour. Table 10.1 summarizes all the initial mixtures prepared. In all cases, two levels of mycotoxin contamination were assayed: High mycotoxin contaminated (HMC) dough (n=30), with 1,197±319 µg/kg of DON, 20.6±14.8 µg/kg of DON-3-glucoside and 9.7±1.8 µg/kg of OTA, and low mycotoxin contaminated (LMC) dough (n=30), with 565±247 µg/kg of DON, 23.9±16.9 µg/kg DON-3-glucoside and 0.9±0.2 µg/kg of OTA. These results come from the initial mixes prepared for the breadmaking processes of the assay.

To each flour/bran mix (156 g), 2.3 g of salt, 4.7 g of sucrose, 4.7 g of lard, 6.2 g of commercial compressed yeast (*Saccharomyces cerevisiae*), and 3.1 g of flour improver (containing calcium carbonate, wheat flour, soya flour, lecithin, ascorbic acid and enzymes) were added. The dough was obtained by adding ca. 83 mL of water to the mixture.

The sourdough was obtained from a bakery, and was refreshed every 8 days and stored at 4 ºC. For refreshment, one half of the sourdough was removed and the other half was mixed with water and wheat flour (50:50). Sourdough was added in 10% w/w total dough. Initial flour/bran mixtures were recalculated taking into account the amount of flour/water contained in the sourdough in order to make results as much comparable as possible. The addition of lactic acid bacteria (LAB) in the form of sourdough has been reported to have positive effects on wheat bread quality and shelf-life (Corsetti et al., 2000; Crowley et al., 2002).

Dough was manually kneaded until held together with a non-sticky, smooth and satiny appearance and optimum handling properties. Rounded pieces weighing 260 g each were prepared. From this point, thermoprobes were always used in the dough to record fermentation and baking temperatures; in particular, probes were placed in the centre of the loaf and close to the surface. Doughs were covered with a damp cloth and fermentation was carried out at 30 ºC for 15 minutes. Then the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 ºC. After the fermentation a sample of 25 g was taken from every proofed dough and stored at -20 ºC until mycotoxin analysis (results for the finished bread were corrected accordingly). The proofed dough was then baked in an oven (Eurofred PE46SVR, Eurofred, Spain). Baking conditions were 180 ºC and 105 min. Such conditions were established on the basis of previous experiments to obtain suitable bread. After baking, a representative sample was taken and stored at -20 ºC until analysis.

Table 10.1. Initial prepared flour/bran mixes to assay the factors: initial toxin concentration, bran concentrations, sourdough use and source of OTA, on DON and OTA fate during breadmaking.

Level of DON and OTA concentration	Source of DON	Source of OTA	g of bran/1000 g of flour	Sourdough use
High concentration	Flour	Flour	0	Yes
				No
	Flour and bran	Flour	100	Yes
				No
	Flour and bran	Flour	200	Yes
				No
	Flour and bran	Bran	200	Yes
				No
	Bran	Flour	300	Yes
				No
Low concentration	Flour	Flour	0	Yes
				No
	Flour and bran	Flour	100	Yes
				No
	Flour and bran	Flour	200	Yes
				No
	Flour and bran	Bran	200	Yes
				No
	Bran	Flour	300	Yes
				No

10.3.3. Chemicals and reagents

Mycotoxin standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile (purity 99.99%), methanol (purity 99.99%) and ethanol (purity 99.5%) were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) and OTA (OCHRAPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (purity 98-100%, Panreac, Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (purity 99%, Panreac, Castellar del Vallès, Spain) and sodium chloride (8.0 g) (purity >99,5%, Fisher Bioreagents, New Jersey, USA) in 1 L of pure water; the pH was brought to 7.4 with hydrochloric acid 1 M.

10.3.4. Preparation of standard solutions

OTA standard solution was dissolved in methanol at a concentration of 5.0 mg/mL and stored at 4 ºC in a sealed vial until use. The concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis (Horwitz and Latimer, 2006). Working standard solution (0.5, 0.01, 0.005, 0.001 and 0.0005 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system.

DON standard solution was dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 ºC. The concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis (Horwitz and Latimer, 2006). Working standards (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared as for OTA, as well as calibration curves.

DON was dissolved in acetonitrile at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL) were prepared as for OTA, as well as calibration curves.

10.3.5. Mycotoxins extraction, detection and quantification

Prior to extraction, all samples were dried at 40 ºC for 24 hours and weight loss recorded in order to present the results in an ‘as is’ basis (µg/kg) or in total content of toxin (µg or ng).

Briefly, for OTA analysis, 5 g of ground sample (Ika, A11B) were extracted with 30 mL of extractant solution (60% acetonitrile, 40% water) by magnetically stirring for 10 min and filtered with filter Whatman number 1. 4 mL of filtered solution was diluted with 44 mL of PBS solution and loaded on the IAC column. After this, the column was washed with 20 mL of PBS and OTA was eluted by applying 1.5 mL of methanol grade HPLC (three times back flushing) and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of acetonitrile:water:acetic acid (57:41:2). OTA was determined by HPLC (Waters 2695®) coupled with a Multi λ Fluorescence Detector Waters 2475®, an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. HPLC mobile phase consisted of acetonitrile, water and acetic acid (57:41:2). The mobile phase flow rate was 1 mL·min^{-1}, the injection volume was 100 µL, and the retention time was 15 minutes.

Regarding DON and DON-3-G, 5 g of ground sample (Ika, A11B) were extracted with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 g. Supernatant was filtered through a glass microfiber filter. Five milliliters of

filtered sample were loaded on the IAC column and the column washed with 10 mL of distilled water. DON and DON-3-glucoside were eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. Zachariasova et al. (2012) confirmed the good cross-reactivity of DON-3-glucoside with the IAC DONPREP® columns. The purified extracts were dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON and DON-3-glucoside were determined by HPLC (Waters 2695®) coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The HPLC mobile phase flow rate was 1.2 mL·min^{-1}, the injection volume was 100 µL, and the retention time for DON and DON-3-glucoside were 20 and 23 min, respectively.

10.3.6. Methods performance

The analytical methods used were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 were 0.97, 0.96 and 0.99 for DON, DON-3-glucoside and OTA, respectively). Precision was established by determining OTA and DON levels in bread and DON and DON-3-glucoside in flour samples at least by triplicate, in those samples fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and OTA are summarized in Table 10.2.

Table 10.2. Method performances for ochratoxin A (OTA). deoxynivalenol-3-glucoside (DON-3-glucoside) and deoxynivalenol (DON) determination in some analyzed substrates.

Mycotoxin	Product	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery (%)[c]	RSDr[d](%)
OTA	Bread	0.14	0.42	5	0.5	103.9±15.6	4
				5	1.5	110.1±13.0	2
				5	3.0	99.6±12.6	1
DON	Bread	60	180	5	100	100.0±16.3	16
				5	500	98.8±9.0	9
				5	1000	102.3±5.3	5
	Flour	60	180	3	300	123.3±30.3	41
				3	500	87.4±8.6	7
DON-3-G	Flour	14	42	5	50	80.0±9.6	12
				5	250	79.7±4.8	6
				5	500	66.7±11.2	18

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

10.3.7. Statistics

Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin concentration levels as well as in the calculated percentages of increase/reduction at a $p<0.05$. Moreover, HSD-Tukey tests were applied to establish significant differences among levels of factors (the significance level was set at 5%). The results obtained were processed in two different ways: a) DON, DON-3-glucoside or OTA content in each step (µg of DON and DON-3-glucoside or ng of OTA) and, b) concentration 'as is' (µg/kg of DON, DON-3-glucoside and OTA in wet basis). In the first case it was possible to assess the real impact of the processing steps in the mycotoxins, while in the second one it is possible to draw conclusions on compliance of maximum permitted levels. For the 'no kneaded mix' results obtained for dry ingredients from the mycotoxin analysis were corrected for the amount of water to be added.

10.4. Results and discussion

10.4.1. Recorded temperatures

The temperature probes placed in the inner part of the dough and near the surface recorded higher temperatures near the surface (Figure 10.1). In the fermentation step, the temperature reached a maximum of 31 ºC and no differences existed between the internal and external part. During the baking process, the dough reached in the centre the maximum temperature (97 ºC) within the first 30 minutes of baking and slightly decreased afterwards; this decrease was probably due to the formation of the crust, which may hinder the penetration of heat. Near the surface the temperature kept on increasing and reached almost 120 ºC at the end of baking (far from the 180 ºC oven baking temperature).

10.4.2. Effect of dough fermentation and baking on DON and DON-3-glucoside content

Average DON content in the initial mixture and proofed dough was not significantly different (142.9 µg vs 122.7 µg for the overall mean, respectively) ($p>0.05$), although this difference depended on the other assayed factors. However, when percentages of reduction were calculated, and their variance analysed, the contribution of the sourdough and the initial concentration was significant ($p<0.05$), as well as their interactions. When sourdough was

added, a mean increase of DON of 24% was observed, with lower values with increasing bran content and initial DON concentration. On the other hand, doughs prepared without sourdough suffered a mean reduction of DON of 7%, such reduction was higher with 300 g/kg bran and HMC (Figure 10.2a, 2b). Overall, doughs prepared with HMC suffered a mean 17% decrease in DON concentration, while those with LMC an increase of 33%.

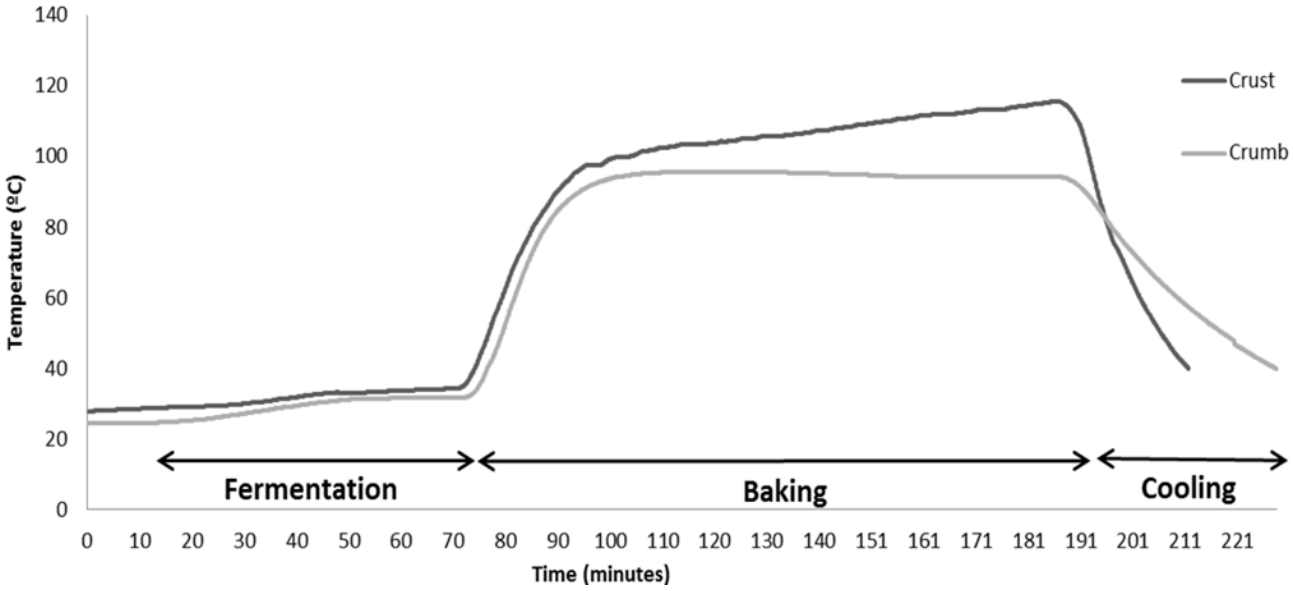


Figure 10.1. Mean temperatures recorded during the bread making process in the center and near the crust.

As the experiment was not designed for different levels of DON-3-glucoside in the initial flour mix, the natural levels detected in the mix of solid ingredients were not different in those treatments with high/low DON content, and the data were pooled, with a mean value before kneading of 3.5 µg, with most of the data under the limit of detection (the LOD value was used to calculate the mean). After fermentation, DON-3-glucoside was still undetectable in most of the treatments, thus although a mean 79% increase in DON-3-glucoside occurred, the significance of the different factors was not determined for this step.

Published studies on the effect of fermentation on DON content generally showed an increase in DON content when fermented dough was compared to the kneaded dough and enzymes were used as flour improvers (Simsek et al., 2012; Suman et al., 2012; Vidal et al., 2014). By contrast, when no improvers were used, no changes or reduction in DON concentration were observed (Konstelanska et al., 2011; Neira et al., 1997; Samar et al., 2001). In our case, although improvers were used and DON release was observed when sourdough was included in the recipe, reduction could be observed at HMC and high added bran. Some

authors (Kostelanska et al., 2011; Zachariasova et al., 2012) concluded that the use of bakery improvers containing enzymes may lead to a higher relative increase in DON-3-glucoside (consistent with our results), than in DON. When proofed/fermented dough was compared to initial flour, no change or increase in DON was reported (Bergamini et al., 2010; Valle-Algarra et al., 2009), this fact may be explained by the results of Lancova et al. (2008) who suggested that a release of DON took place during kneading, it decreased during fermentation and further increased during proofing; in those studies in which proofed dough was compared directly to flour, these sub-steps were not considered and thethe averaged results may be contradictory.

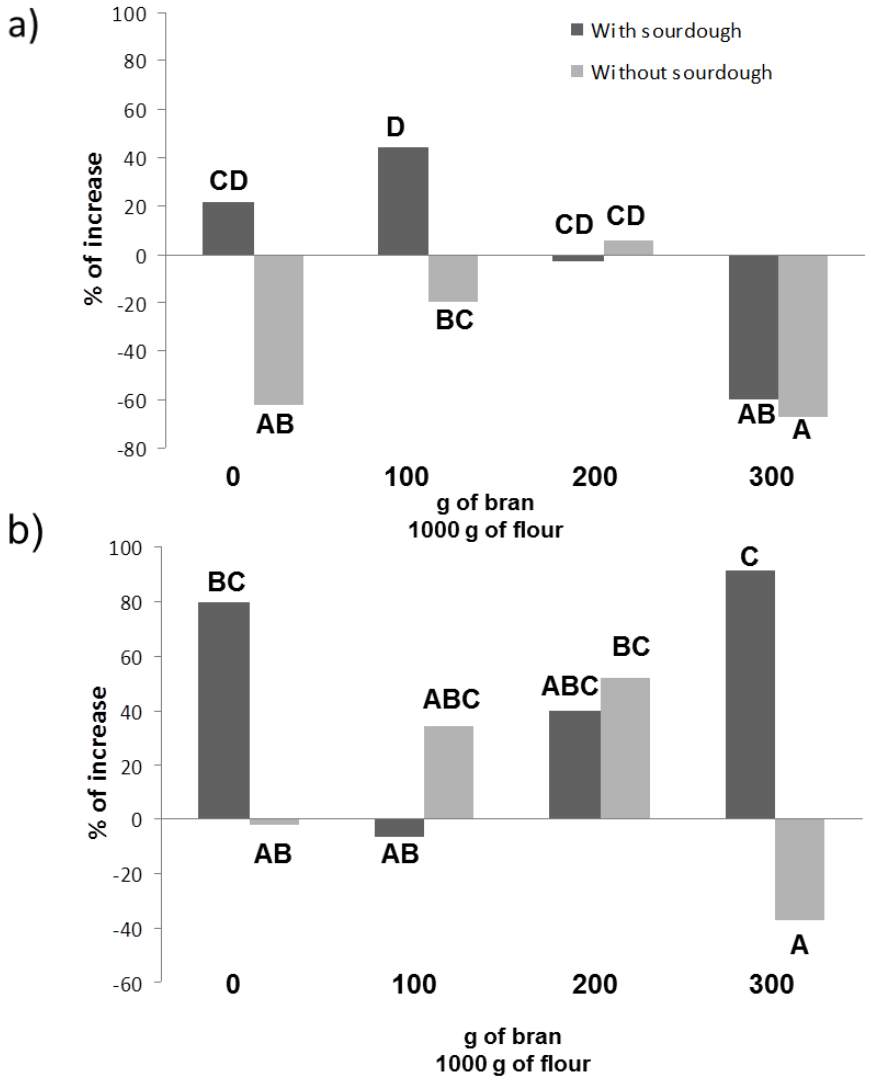


Figure 10.2. Percentage of increase in mean DON content (µg) ± SD due to dough kneading, fermentation and proofing as a function of bran level and presence of sourdough at the high (a) and low (b) initial DON concentration.

[A,B,C,D] Different groups of letters next to the bars indicate significant differences among treatments, same groups of letters mean no significant difference (Tukey's test $p < 0.05$).

In our assay an increase of DON content was observed in sourdough fermented samples ($p<0.05$). Bergamini et al. (2010) studied the fate of DON during the fermentation step using sourdough powder as well as bread making promoting agents. The authors reported a significant increase in DON level after fermentation (30-40 ºC for 45 to 85 minutes) and suggested that such increase could be due to the release of bound DON. Such release might be attributed to both enzymes contained in the improver and to the metabolism of sourdough bacteria which may be able to either transform DON precursors into DON or to release bound DON. In addition, Suman et al. (2012) prepared crackers with the sponge technique (fermentation for 2-4min at 20-30 ºC) and reported from 10% decrease to 14% increase in DON concentration compared to kneaded dough, depending on fermentation time and temperature and $NaHCO_3$ concentration.

Bran content did only affect the fermentation effect when added at 300 $g \cdot kg^{-1}$, leading to a more marked DON reduction; no studies exist in this point, only Scudamore et al. (2009) found similar global percentages of reduction for the whole breadmaking process from flour to bread of 35% for white bread and of 39% for wholemeal bread.

Average DON content in the fermented dough and bread was not significantly different (122.7 µg vs 126.2 µg, overall mean). Regarding the percentage of reduction/increase in DON content, it was independent of the factors assayed (DON concentration in flour, levels of bran and use of sourdough) and accounted for a mean value of 3% increase, although a trend existed for a certain release in sourdough containing samples and DON reduction without sourdough (Figure 10.3a, 3b).

As described before, DON-3-glucoside results for HMC and LMC samples were pooled, with most of the data under the limit of detection (the LOD value was used to calculate the mean) for kneaded+fermented dough. A significant increase of 229% (this value should be taken with care as only a few data for the fermented dough showed detectable values; and calculated values close the LOD may be inaccurate) was observed during baking (Figure 10.4), which was independent of the assayed factors: bran concentration and sourdough addition. Similarly, Vidal et al. (2014) observed an increase of DON-3-glucoside after baking; however it could not be related to DON reduction. By contrast, previous studies reported DON-3-glucoside reduction during baking (Kostelanska et al., 2011; Simsek et al., 2012). The possible increase of DON-3-glucoside during baking is of concern; although little knowledge exists in the toxicological effects of DON-3-glucoside, an important cleavage of DON-3-glucoside has been observed in the intestinal tract (Berthiller et al., 2011; Gratz et al., 2013). Thus DON-3-glucoside should be considered as an additional contributing factor of the total dietary exposure to DON (Codex, 2011; JEFCA, 2010).

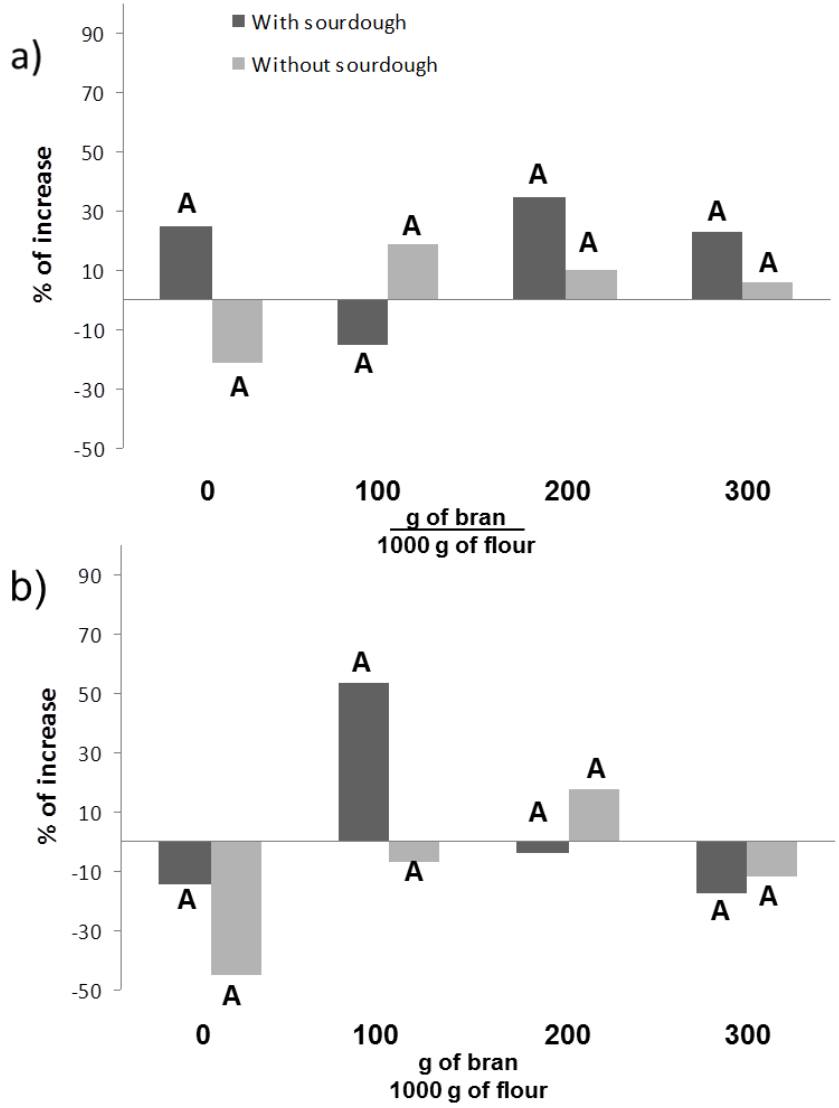


Figure 10.3. Percentage of increase in mean DON content (µg) ± SD due to baking as a function of bran level and presence of sourdough at the high (a) and low (b) initial DON concentration. [A] Same letter next to the bars indicate no significant differences among the treatments (Tukey's test $p < 0.05$).

Contradictory reports exist regarding the fate of DON during the baking step. Most studies reported DON reduction during baking (El-Banna et al., 1983; Scott et al., 1984; Valle-Algarra et al., 2009), while others (Bergamini et al., 2010; Simsek et al., 2012; Zachariasova et al., 2012) reported an increase in DON concentration after baking; two reasons could lead to such increase: i) shorter baking times together with lower temperatures and ii) bigger bread sizes, as in general those studies reporting reduction were usually at laboratory level with small size loaves (40-80 g). In our case, loaf size was bigger than in most published studies, and according to our temperature records the inner part of the dough did not reach more than 97 ºC. Probably, DON degradation took place exclusively in the crust but such degradation is not

enough to detect a significant decrease in DON content in the whole sample. Finally, baking temperature in our study was lower than average studies and baking time longer, thus the absence of effect of baking does not contradict the general trend of the existing studies

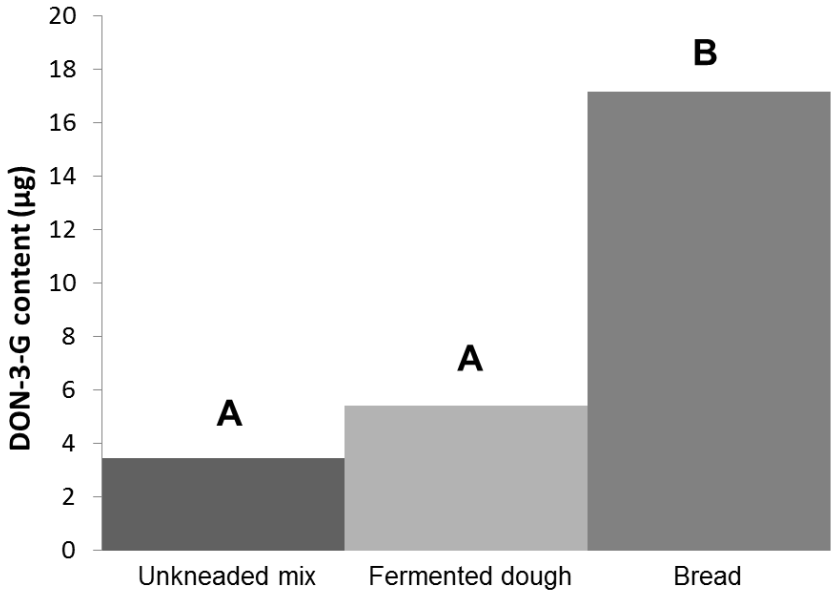


Figure 10.4. DON-3-glucoside content (µg) through the breadmaking process (Unkneaded mix, fermented dough and bread). [A,B] Different letters next to the bars indicate significant differences among the treatments, same letter no significant difference (Tukey's test p < 0.05).

10.4.3. Effect of dough fermentation and baking on OTA

Due to the high OTA stability observed in preliminary studies, only those samples with 200 g bran/kg flour were analysed. First of all, no significant differences were observed in the results when OTA was added either as contaminated flour or as contaminated bran. The total content of OTA (ng) did not significantly change during fermentation ($p<0.05$, mean of 1518 vs 1581 ng at HMC, and mean of 143 vs 129 ng at LMC). No significant effect of sourdough addition was observed.

The analysis of the sourdough used in this investigation resulted in a high concentration of lactic acid bacteria ($1 \cdot 10^7$ CFU/g) which have been reported to be able to degrade OTA to ochratoxin α (OTα) (Varga et al., 2000). However, the time required to cause OTA degradation should be probably longer than that assayed herein.

Although plant metabolism of OTA has been described in wheat, and OTα, (4R)- and (4S)-4-hydroxy-ochratoxin A and glucosides of both isomers have been found in large amounts,

no studies exist either on their fate or their conversion from or to the parent molecule during processing of wheat derivatives.

The total content of OTA (ng) tended to increase during baking although the trend was not significant (p=0.07, mean of 1581 vs 2291 ng at the HMC, and mean of 129 vs 245 ng at the LMC). Such increase (mean of 75%) did not depend on any of the assayed factors (initial OTA concentration in flour/bran and sourdough addition).

The high OTA stability through baking observed in the present study is in accordance with Scudamore et al. (2003) and Vidal et al. (2014), under similar conditions. Some studies, however, reported OTA reduction during baking. Valle-Algarra et al. (2009) reached over 20% reduction in the baking bread process. Subirade (1996) also reported OTA reduction (66%) during baking process of wheat biscuits. However, due to the size and shape of such product the penetration of the heat may be higher. Moreover, studies on OTA stability in coffee showed very important reductions (over 90% in some assays) (Pérez de Obano et al., 2005; Van der Stegen et al., 2001), but the temperatures used are much higher than those used in cereal processing (approximately 400 ºC). In summary, OTA in bread baking process seems to be stable and no reduction is observed.

10.4.4. DON and DON-3-glucoside concentration in flour and bread 'as is'

DON concentration in bread produced from a given flour batch will depend on the 'dilution' of the toxin with the addition of other ingredients, which depends on the recipe, to a minor extent on water, carbon dioxide and ethanol balance, and finally on the stability of the toxin through the process, which was explained in the previous sections.

The results suggest that from flour with a contamination of 1197 µg/kg it is possible to produce bread with a significantly lower contamination (44-63%) depending on whether sourdough is used or not (Table 10.3), while the theoretical calculation taking into account the dilution effects of the process leads to a 40% reduction. On the other hand, from a lot of flour containing 565 $\mu g \cdot kg^{-1}$, the final bread would contain DON levels 31% lower. This suggests that the diluting effect of the recipe may be either increased or limited, and even higher concentration of DON than expected thorough ingredients mixing can be encountered depending on initial DON concentration in the flour. This limited reduction is attributed to a certain increase in DON concentration during kneading and fermentation. Moreover, increase in DON concentration from proofed dough to bread was not significant, thus similar concentration can be observed in Table 10.3.

While DON-3-glucoside level in the fermented dough was not significantly different to that in the flour suggesting that the dilution effect was balanced by DON-3-glucoside increase during kneading and fermentation, a significant increase was observed from fermented dough to

bread (Table 10.3). The resulting bread from flour containing 19.6 μg/kg might contain a concentration more than three times higher. Further studies are required to assess the significance of this fact, and the consequences it might have.

Looking at DON results and literature, it seems that standard fermentation temperature, plus the widespread use of improvers might lead to a minimising of the 'dilution' effect due to recipe. According to Samar et al. (2001) higher fermentation temperatures, by contrast, may lead to DON degradation. The baking stage led to no significant reduction and, overall, a minimum 17% reduction (mean 35%) can be expected for the breadmaking process from flour to bread (in a 260 g bread loaf, with improvers added), which is lower than the 33% assumed by Commission Regulation 1881/2006 setting a maximum level of 750 μg/kg for flour and 500 μg/kg for bread. In our case the only dilution effect would account for 40% reduction, but different recipes for bakery products could lead to much lower reduction, as suggested by Scudamore et al. (2003). On the other hand, DON-3-glucoside concentration greatly increased with baking; the increase could be the glycosidation of DON during the initial stages of baking, before enzyme inactivation (Vidal et al., 2014). This hypothesis is reinforced by the fact that DON-3-glucoside also increased during fermentation, and was previously suggested by Zachariasova et al. (2012). As a consequence, higher DON-3-glucoside concentration could be found in bread than in the initial flour (opposite of what reported by De Angelis et al., 2013). This point is of outmost importance as part of DON can be *in vivo* released from its conjugate (Berthiller et al., 2013) and should be further investigated. Moreover, a joint regulation of DON+DON-3-glucoside maximum levels might be proposed.

Table 10.3. Evolution of mycotoxin concentration (μg/kg) in the different steps of breadmaking process (flour, fermented dough and bread).

	Sourdough use	Flour (μg/kg)	Fermented dough 'as is' (μg/kg)*	Bread 'as is' (μg/kg)	% Reduction (kneading +fermentation+proofing)* 30ºC 75 min	% reduction baking
DON	Yes	1101	613 (661)	747	44 % (40%)	-22 %
		563	389 (338)	469	31 % (40%)	-21 %
	No	1293	474 (776)	588	63 % (40%)	-24 %
		566	390 (340)	363	31 % (40%)	7%
DON-3-glucoside		19.6	20.8 (11.8)	73.8	-6 % (40%)	-255 %
OTA		8.6	6.1 (5.2)	9.9	29 % (40%)	-62 %
		0.8	0.5 (0.5)	1.1	38 % (40%)	-54 %

*values in parentheses are calculated taking into account only dilution by recipe

10.4.5. OTA concentration in flour and bread 'as is'

OTA concentration in bread produced from a given flour will depend only on the 'dilution' of the toxin with the addition of ingredients, and on a minor extent to water, carbon dioxide and ethanol balance due to OTA stability during the breadmaking process, as explained in the previous sections, although a trend to increase during baking was observed. Thus OTA concentration (wet basis) was reduced significantly by 29-38% from flour to fermented dough (theoretical decrease, assuming complete stability would be 40%). On the other hand, the average increase from fermented dough to bread was 58% (wet basis), although not significant (theoretical increase, assuming complete thermostability of OTA and 8% weight loss in the bread would be 9%). The whole breadmaking process could end in a concentration 1.4 times higher in bread than in the initial flour, which is in the line of an equal maximum level for both flour and bread (3 µg/kg), as set in the EC (2006).

10.5. Conclusions

As conclusion, OTA was confirmed to be quite stable during the breadmaking process. DON concentration was significantly affected by kneading+fermentation steps in different way depending on sourdough use and flour contamination level; while DON reduction by fermentation leads to a safer situation, the possible increase in DON should be considered with care, as it can compensate the expected dilution effect by recipe. Finally, the results on DON-3-G, although preliminary because a high percentage of samples were <LOD, suggest a marked release of this toxin during fermentation, but mainly during baking. This point, in particular, requires further investigation.

10.6. Acknowledgements

The authors are grateful to the Spanish government (projects AGL2010-22182-C04-04 and AGL2011-24862) for the financial support. A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant. H. Morales is grateful to the Portuguese Government (Ministério da Ciência, Tecnologia e Ensino Superior; FCT Fundação para a Ciência e a Tecnologia) Grant ref. SFRH/BPD/38011/2007.

10.7. References

Abrunhosa, L., Paterson, R.R.M. , Venâncio, A. Biodegradation of ochratoxin a for food and feed decontamination. Toxins 2010, 2, 1078-1099.

Alexa, E., Dehelean, C.A., Poiana, M.-A., Radulov, I., Cimpean, A.-M., Bordean, D.-M., Tulcan, C., Pop, G., 2013. The occurrence of mycotoxins in wheat from western Romania and histopathological impact as effect of feed intake. Chem. Cent. J. 7, 99.

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., Suman, M., 2010. Fate of F*usarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. Food Addit. Contam. 27, 677-687.

Berthiller, F., Dall'Asta, C., Schuhmacher, R., Lemmens, M., Adam, G., Krska, A.R., 2005. Masked mycotoxins: Determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. 53, 3421-3425.

Berthiller F., Krska R., Domig K.J., Kneifel W., Juge N., Schuhmacher R., Adam G., 2011. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion . Toxicol. Lett. 206, 264-267.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S.D., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, W.h, Speijers, G., Stroka, J., 2013. Masked mycotoxins: A review. Mol. Nutr. Food Res. 57, 165-186.

Bullerman, L.B., Bianchini, A., 2007. Stability of mycotoxins during food processing. Int. J. Food Microbiol.119, 140-146.

Cano-Sancho, G., Gauchi, J.-P., Sanchis, V., Marín, S., Ramos, A.J., 2011a. Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. Food Addit. Contam. 28, 1098-1109.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., Marín, S., 2011b. Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). Food Control 22, 490-495.

Codex Alimentarius Comission. 2011. Proposed draft maximum levels for deoxynivalenol (DON) and its acetylated derivatives in cereals and cereal-based products. The Hague, The Netherlands. Available at: ftp://ftp.fao.org/codex/meetings/cccf/cccf5/cf05_06e.pdf (accessed 07.01.13).

Coronel, M.B., Marín, S., Cano-Sancho, G., Ramos, A.J., Sanchis, V., 2012. Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. Food Addit. Contam. 29, 979-993.

Corsetti, A., Gobbetti, M., De Marco, B., Balestrieri, F., Paoletti, F., Russi, L., Rossi, J., 2000. Combined effect of sourdough lactic acid bacteria and additives bread firmness and staling. J. Agric. Food. Chem. 48, 3044-3051.

Crowley, P., Schober, T.J., Clarke, C.I., Arendt, E.K., 2002. The effect of storage time on textural and crumb grain characteristics of sourdough wheat bread. Eur. Food Res. Technol. 214, 489-496.

De Angelis, E., Monaci, L., Pascale, M., Visconti, A., 2013. Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: An investigation by high-

performance liquid chromatography high-resolution mass spectrometry. Food Addit. Contam. 30, 345-355.

El-Banna, A.A., Lau, P.Y., Scott, P.M., 1983. Fate of mycotoxins during processing of foodstuffs II. Deoxynivalenol (vomitoxin) during making of Egyptian bread. J. Food Prot. 46, 484-486.

European Commission (EC), 2006. Commission Regulation (EC) No. 1881/2006 of 19/12/2006, setting maximum levels for certain contaminants in foodstuffs. Official J. Eur. Union L364, 5-24.

European Commission (EC), 2011. Commission Regulation (EC) No. 1169/2011 of 25/10/2011, on the provision of food information to consumers. Official J. Eur. Union L304, 18-63.

European Food Safety Authority (EFSA). 2006. Deoxynivalenol in food and feed: occurrence and exposure. EFSA J. 11, 3379.

European Food Safety Authority (EFSA). 2006. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to ochratoxin A in food. The EFSA J., 365, 1-56. Available at: <http://www.efsa.europa.eu/> (accessed 04.04.13).

European Food Safety Authority (EFSA). 2010. Scientific opinion on dietary reference values for carbohydrates and dietary fibre. EFSA J., 8, 1462. Available at: <http://www.efsa.europa.eu/> (accessed 21.01.13).

Faridi, H., Faubion, J.M., 1995. Wheat usage in North America. In H. Faridi and J.M. Faubion, (Eds), Wheat end uses around the world (1-41). St Paul, MN, USA: American Association of Cereal Chemists.

Gobbetti, M., 1998. The sourdough microflora: Interactions of lactic acid bacteria and yeasts. Trends Food Sci. Tech. 9, 267-274.

Gratz, S.W., Duncan, G., Richardson, A.J., 2013. The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. Appl. Environ. Microbiol. 79, 1821-1825.

Horwitz, W., Latimer, G.W., 2006. Official methods of analysis of AOAC International. (18th ed.). AOAC Gaithersburg, Maryland. Chapter 49, 1-49.

IARC (International Agency for Research on Cancer). 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins In IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 56. Lyon, France.

IARC (International Agency for Research on Cancer). 2002. Monograph on the evaluation of carcinogenic risk to humans, vol. 82. Lyon, France.

Jelinek, C.F., Pohland, A.E., Wood, G.E., 1989. Worldwide occurrence of mycotoxins in foods and feeds an update. Journal AOAC International 72, 223–230.

JEFCA, 2010. WHO Technical Report Series 959: JEFCA, 2010: Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives: evaluation of certain contaminants in food. WHO Technical Report Series. 959.

Kabak, B., 2009. The fate of mycotoxins during thermal food processing. J. Sci. Food Agric. 89, 549-554.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., Hajslova, J., 2011. Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. J. Agric. Food. Chem. 59, 9303-9312.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., Sachambula, L., 2008. Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. Food Addit. Contam. 25, 732-744.

Li, R., Wang, X., Zhou, T., Yang, D., Wang, Q., Zhou, Y., 2014. Occurrence of four mycotoxins in cereal and oil products in Yangtze Delta region of China and their food safety risks. Food Control 35, 117-122.

L'vova, L.S., Kizlenko, O.I., Shul'gina, A.P., Omel'chenko, M.D., Bystryakova, Z.K., 1998. Distribution of deoxynivalenol in products of processing *Fusarium*-affected soft and hard wheats and barley. Appl. Biochem. Biotechnol. 34, 404-408.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., Resnik, S.L., 1997. The effects of bakery processing on natural deoxynivalenol contamination. Int. J. Food Microbiol. 37, 21-25.

Nishio, Z., Kanenori Takata, Ito, M., Tanio, M., Yamauchi, H., Tabiki, T., Ban, T., 2010. Deoxynivalenol distribution in flour and bran of spring wheat lines with different levels of *Fusarium* head blight resistance. Plant Dis. 94, 335-338.

Peña, R.J. Wheat for bread and other foods. In Curtis, B.C., Sajaram, S., Gómez Macpherson, H. (Eds.), *FAO Plant Production and Protection Series: Wheat Bread*. FAO, Rome, 2002. Available at: <http://www.fao.org/DOCREP/006/Y4011E/Y4011E00.htm> (accessed 21.01.13.)

Pacin, A., Ciancio Bovier, E., Cano, G., Taglieri, D., Hernandez Pezzani, C., 2010. Effect of the bread making process on wheat flour contaminated by deoxynivalenol and exposure estimate. Food Control 21, 492-495.

Pérez de Obanos, A., González-Peñas, E., López de Cerain, A., 2005. Influence of roasting and brew preparation on the ochratoxin A content in coffee infusion. Food Addit. Contam. 22, 463-471.

Pestka, J.J., 2010a. Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. World Mycotoxin J. 3, 323–347.

Pestka, J.J., 2010b. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Arch. Toxicol. 84, 663–679.

Pomeranz, Y., 1987. Bread around the world. In Y. Pomeranz (Eds.), *Modern cereal science and technology* (pp. 258-333). New York, NY, USA: VCH Publishers.

Samar, M.M., Neira, M.S., Resnik, S.L., Pacin, A., 2001. Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. Food Addit. Contam.18, 1004-1010.

Scientific Committee on Food (SCF), 1998. Commission of the European Communities, Outcome of discussions 14. Directorate General XXIV. Expressed 17.09.88.

SCOOP, 2002. *Assessment of dietary intake of ochratoxin A by the population of EU member states.* Reports on Tasks for Scientific Cooperation, task 3.2.7. http://ec.europa.eu/food/fs/scoop/3.2.7.pdf

SCOOP, 2003. *Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states*. Report on Tasks for Scientific

Cooperation task 3.2.10. http://europa.eu.int/comm/food/fs/scoop/task3210.pdf. Accessed 13.10.13

Scott, P.M., Kanhere, S.R., Dexter, J.E., Brennan, P.W., Trenholm, H.L., 1984. Distribution of the trichothecene mycotoxin deoxynivalenol (vomitoxin) during the milling of naturally contaminated hard red spring wheat and its fate in baked products. Food Addit. Contam. 1, 313-323.

Scudamore, K.A., Banks, J., MacDonald, S.J., 2003. Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. Food Addit. Contam. 20, 1153-1163.

Scudamore, K.A., 2005. Prevention of ochratoxin A in commodities and likely effects of processing fractionation and animal feeds. Food Addit. Contam. 22, 17-25.

Scudamore, K.A., Hazel, C.M., Patel, S., Scriven, F., 2009. Deoxynivalenol and other *Fusarium* mycotoxins in bread, cake, and biscuits produced from UK-grown wheat under commercial and pilot scale conditions. Food Addit. Contam. 26, 1191-1198.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., Qian, S.Y., 2012. Analysis of Deoxynivalenol and Deoxynivalenol-3-glucoside in wheat. Food Control 26, 287-292.

Subirade, I., 1996. Fate of ochratoxin A during breadmaking. Food Addit. Contam. 13, 25-26.

Suman, M., Manzitti, A., Catellani, D., 2012. A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. World Mycotoxin J. 5, 241-249.

Thammawong, M., Okabe, M., Kawasaki, T., Nakagawa, H., Nagashima, H., Okadome, H., Nakajima, T., Kushiro, M., 2010. Distribution of deoxynivalenol and nivalenol in milling fractions from *Fusarium*-infected Japanese wheat cultivars. J. Food Prot. 73, 1817-1823.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., Jiménez, M., 2009. Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. Food Addit. Contam. 26, 896-906.

Van der Stegen, G.H.D., Essens, P.J.M., Van der Lijn, J., 2001. Effect of roasting conditions on reduction of ochratoxin A in coffee. J. Agric. Food. Chem. 49, 4713-4715.

Varga, J., Péteri, Z., Tábori, K., Téren, J., Vágvölgyi, C., 2005. Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. . Int. J. Food Microbiol. 99, 321-328.

Vidal, A., Marín, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. Food Chem. Toxicol. 53, 133-138.

Vidal, A., Morales, H., Sanchis, V., Ramos, A.J. Marín, S. 2014. Stability of DON and OTA during the breadmaking process and determination of process and performance criteria.Food Control 40, 234-242.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., Hajslova, J., 2012. Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. J. Agric. Food. Chem. 60, 9280-9291.

11. Study VI. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.

Food Chemistry (2015) 178, 276-286.

Authors: Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S.

11. Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.

Running title: Kinetics of DON and OTA during baking of wheat products.

Arnau Vidal, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*.
Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

11.1. Abstract

The stability of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-glucoside), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), de-epoxy-deoxynivalenol (DOM-1) and ochratoxin A (OTA) during thermal processing has been studied. Baking temperature, time and initial mycotoxin concentration in the raw materials were assayed as factors. An improved UPLC-MS/MS method to detect DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 in wheat baked products was developed in the present assay. The results highlighted the importance of temperature and time in mycotoxin stability in heat treatments. OTA is more stable than DON in a baking treatment. Interestingly, the DON-3-glucoside concentrations increased (>300 %) under mild baking conditions. On the other hand, it was rapidly reduced under harsh conditions. The 3-ADON decreased during the heat treatment; while DOM-1 increased after the heating process. Finally, the data followed first order kinetics for analysed mycotoxins and thermal constant rates (*k*) were calculated. This parameter can be a useful tool for prediction mycotoxin levels.

Keywords: baking, wheat, deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, de-epoxy-deoxynivalenol, ochratoxin A and kinetics.

11.2. Introduction

Mycotoxins are produced by fungi and can contaminate various agricultural commodities either before harvest or under post-harvest conditions. The main mycotoxin-producing fungi in food commodities belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins. Different studies show the high presence of mycotoxins, mainly deoxynivalenol (DON), in wheat products (Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Vidal, Marín, Ramos, Cano-Sancho, & Sanchis, 2013). Moreover, cereal products represent one of the main sources of exposure to DON and ochratoxin A (OTA) (Marín, Ramos, Cano-Sancho, & Sanchis, 2013).

DON is not classified as to its carcinogenicity to human by IARC (1993), and it is linked with human gastroenteritis. OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties. This mycotoxin has been classified, as a possible human carcinogen, in the group 2B, by the International Agency for Research on Cancer (IARC, 1993).

Processing of cereals at high temperatures may affect DON and OTA content. However, the extent of DON and OTA reduction during thermal food processing seems to be quite variable and dependent on the processing conditions applied: temperature, time, type of mycotoxin, and size of cereal product. For bakery products, some studies reported a significant decrease in DON levels during baking (Numanoglu, Gökmen, Uygun, & Koksel, 2012; Valle-Algarra, Mateo, Medina, Mateo, Gimeno-Adelantado, & Jiménez, 2009). By contrast, the studies of De Angelis, Monaci, Pascale and Visconti (2013) and Zachariasova, Vaclavikova, Lacina, Vaclavik and Hajslova (2012) reported that DON is stable in processing steps involving high temperatures. Similarly, OTA seems to be quite more stable at high temperature than DON through baking (Vidal, Morales, Sanchis, Ramos, & Marín, 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b). Only results on coffee roasting show a clear reduction of OTA, although, the temperature achieved in the product is higher than in bakeries (Castellanos-Onorio et al., 2011, Scudamore, Banks, & MacDonald, 2003; Valle-Algarra et al., 2009). Sometimes, the contradictory published results may be due to the different size of assayed products, which affects heat transfer and favours gradients of temperature inside the products. Some studies have been carried out in aqueous systems in order to avoid the temperature gradient (Jackson, Hlywka, Senthil, Bullerman, & Musser, 1996). All of them showed effective mycotoxin reductions over 150 ºC; however their results cannot be extrapolated to solid food products.

On the other hand, unaltered mycotoxins might not be the only source of health hazard for consumers, because exists a group of metabolites called conjugates mycotoxins which cannot be detected in the rutinary mycotoxins analysis. The mycotoxin conjugates are mycotoxins attached with functional groups (masked mycotoxins) such as glycosyl residues or sulfates or attached with polymeric carbohydrate or protein matrices (bound mycotoxins)

(Berthiller et al., 2009). The conjugates can be classified in function of their origin, producing 4 differentiate groups: from plants, fungi, mammalian and food processing. The co-occurrence of conjugated DON forms has been documented in wheat, especially deoxynivalenol-3-glucoside (DON-3-glucoside) (Simsek, Burgess, Whitney, Gu, & Qian, 2012), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (Yang, Geng, Yao, Zhang, Zhang, & Ma, 2013). The fate of DON-3-glucoside through breadmaking has been hardly studied. While some authors point out to some reduction (De Angelis et al., 2013), others have seen marked increases (Vidal et al., 2014a). On the other hand, 3-ADON, 15-ADON and de-epoxy-deoxynivalenol (DOM-1) behavior has not been studied before.

HPLC-MS is usually applied for simultaneous detection of DON and its conjugates (Vendl, Berthiller, Crews, & Krska, 2009), specially DON-3-glucoside (Berthiller et al., 2009). Due to the low concentration of DON conjugates found in wheat products, the methods of analysis require low limits of detection. Lately, the ultra-high performance liquid chromatography (UPLC) has demonstrated to be highly effective for the quantification of DON conjugates in cereal products, such as malt and beer (Zachariasova et al., 2012). No previous studies exist on simultaneous analysis of DON, DON-3-glucoside, 3-ADON, 15-ADON, and DOM-1 in bakery products.

The current study aimed to investigate DON and DON conjugates (DON-3-glucoside, 3-ADON, 15-ADON and DOM-1) and OTA kinetics during baking in a short size model bakery product, small enough to avoid temperature gradients in it. Temperature, time and initial mycotoxin concentration were assayed as factors. Moreover an optimised method to quantify DON conjugates in bakery products is presented.

11.3. Materials and methods

11.3.1. DON and OTA contaminated flours

In order to obtain DON or OTA contaminated flour, one strain each of *Fusarium graminearum* (TA 3.234) and *Aspergillus ochraceus* (TA 3.201) were used. Both of them are kept in the Food Technology Dept. collection, University of Lleida, Spain. They were previously proved to be DON and OTA producers when cultured on wheat flour. The concentration of DON and DON-3-glucoside in the initial uninoculated flour (n=5) was 250 ± 44.78 and 45.1±15.3 µg/kg, respectively, while OTA could not be detected. The remaining DON conjugates were not analysed in the initial flour.

The strains were inoculated and incubated in MEA (malt extract agar) at 25 ºC until strong sporulation. A spore suspension of each strain was made in water and Tween 80 (0.005% v/v). Five millilitres of either *F. graminearum* or *A. ochraceus* spore suspension were

inoculated in glass flasks containing 250 g of flour and 50 mL of water. In total, 3 kg of flour were inoculated with one of the two strains. The flasks were stored at 25 ºC for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus,* with periodic shaking. Then, each kind of flour (3 kg) was properly powdered and homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of 12,500 ± 1,235 µg/kg and 75.5 ± 15.2 µg/kg respectively (n=3), in each contaminated flour. DON conjugates were not analysed in the flour at this stage.

11.3.2. Bakery analogue preparation

The bakery analogue was prepared for each 100 g of mix with 27 g of wheat flour, 26 g of sugar, 26 g of eggs, 21 g of sunflower oil and adding to the 100 g of mix 0.5 g of baking powder (maize starch, sodium bicarbonate and disodium diphosphate). The flour used was previously prepared by mixing the uninoculated flour with the DON contaminated flour and the OTA contaminated flour depending on the desired initial mycotoxin concentration: high mycotoxin concentration (HMC) or low mycotoxin concentration (LMC). The analysed toxin levels in the initial mixed flours (n=3) were: a) HMC, 1042 ± 170 µg/kg of DON and 3.01 ± 0.24 µg/kg of OTA; and b) LMC, 550 ± 98 µg/kg of DON and 2.11 ± 0.30 µg/kg of OTA. The levels were chosen to be close to real values in food samples.

The mix was manually mixed and 3 g aliquots were poured in small paper moulds. From this point, thermoprobes (Proges Plus, Pluck&Track, Thermo bouton) were always used in some of them to register the baking temperatures; probes were placed in the centre of the moulds. Four oven temperature levels (200, 180, 160 and 140 ºC) and 8 baking times (every five minutes starting at minute 5 and finishing at minute 40) were assayed in a full factorial design. These conditions were established on the basis of previous experiments. Thus 2 initial toxin concentrations x 4 baking temperatures x 8 baking times x 3 replicates made 192 different runs (9 equal cakes weighing 3 g each conformed each of the 192 runs). From the 9 cakes, 3 were pooled and used for OTA analysis, other 3 for DON analysis, and the remaining 3 were kept at -20 ºC. All samples were lyophilised for 72 h, and then the samples were stored at –20 ºC until analysis.

11.3.3. Chemicals and reagents

Mycotoxin standard solution of OTA was supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). DON, DON-3-glucoside, 3-ADON, 15-ADON, DOM-1 and isotolabeled ($^{13}C_{15}$) DON were supplied by Biopure (Tulln, Austria). ($^{13}C_{15}$) DON was used as internal standard for UPLC-MS/MS. Acetonitrile (99.9%), methanol (99.9%) and ethanol (99.5%) were purchased from J.T. Baker (Deventer, The Netherlands). Dichloromethane (≥ 99.8%) and ammonium acetate (≥98%) were purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain).

All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) and OTA (OCHRAPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (98-100%, Panreac, Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (99%, Panreac, Castellar del Vallès, Spain) and sodium chloride (8.0 g) (≥99.5%, Fisher Bioreagents, New Jersey, USA) in 1 L of milli-Q water; the pH was brought to 7.4 with hydrochloric acid 1 M.

11.3.4. Preparation of standard solutions

The standard solution of OTA was dissolved in methanol at a concentration of 5.0 µg/mL and stored at 4 ºC in a sealed vial until use. From this, a stock solution was prepared and checked by UV spectroscopy according to AOAC Official methods of analysis. Working standard solutions (0.5, 0.01, 0.005, 0.001 and 0.0005 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system. The standard solutions of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 were dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. DON concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis. Working standard solutions (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared as for OTA, as well as the calibration curves for all the standard solutions.

11.3.5. Sample preparation and analysis

11.3.5.1. DON and OTA extraction for analysis with HPLC-UV and HPLC-FL

For DON, 5 g of ground sample was extracted with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 g. Supernatant was filtered through a glass microfiber filter. Five millilitres of filtered sample was loaded on the IAC column and the column washed with 10 mL of distilled water. DON was eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. The purified extracts were dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON was determined by HPLC (Waters 2695®) coupled with a UV/Visible dual λ absorbance Detector Waters 2487. The column an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm. Absorption wavelength was set at 220 nm. The HPLC mobile phase flow rate was 1.2 mL/min, the injection volume was 100 µL and the column temperature was 40 ºC. The retention time for DON was 20 min.

Briefly for OTA, 5 g of ground sample were extracted with 30 mL of extracting solution (60% acetonitrile, 40% water) by magnetically stirring for 10 min and filtered with number 1 filter Whatman. 4 mL of filtered solution was diluted with 44 mL of PBS solution and loaded on the IAC column. After this, the column was washed with 20 mL of PBS and OTA was eluted by applying 1.5 mL of methanol grade HPLC (three times back flushing) and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of mobile phase (acetonitrile, water and acetic acid, 57:41:2). OTA was determined by HPLC (Waters 2695®) coupled with a Multi λ Fluorescence Detector Waters 2475®, and an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. The mobile phase flow rate was 1 mL/min, column temperature 40 ºC, the injection volume was 100 µL and the retention time was 15 minutes.

11.3.5.2. Extraction of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 for UPLC-MS/MS analysis.

The samples with the high mycotoxin initial concentration (96 samples) were selected for further UPLC-MS/MS analysis with the objective of quantifying not only DON but its conjugates: DON-3-glucoside, 3-ADON, 15-ADON and DOM-1.

1 g of ground sample was extracted with 7 mL of extracting solution (54 % water, 45 % acetonitrile and 1 % acetic acid) by magnetically stirring for 20 min. Then the sample was centrifuged for 10 min at 1780 g. 2 mL of the supernatant were mixed with 2 mL of dichloromethane and the mixture was stirred for 10 min and then it was centrifuged for 3 min at 500 g for the separation of the two phases. The upper layer was kept in a vial.

Chromatographic separation was performed by a 100 mm x 2.1 mm i.d., 1.8 µm, Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). Column temperature was held at 40 ºC. The mobile phases consisted of Acetonitrile (A) and 10 mM $AcNH_4$ in water (B). The gradient was as follows: start with 20 % A, then from 0.5 min a linear increase to 50 % for 2.5 min, then at min 3 it is 50 %. At min 3.01 the mobile phase changes to 100 % A till min 4.5; and at min 4.51 it switches to 20 % A, and then column is equilibrated for 1.5 min before the next injection. The flow rate was 300 µL/min, and an injection volume of 2.5 µL was enabled.

For mass spectrometric detection, ultrahigh-resolution orbitrap technology was used. The operation parameters of the orbitrap MS were optimized for heated electrospray interface in both positive and negative ionization modes. Three individual transitions were monitored for each analyte, except for 3-ADON and 15-ADON, where four transitions were measured. One chromatographic run consisted of two MS/MS periods. The first period monitored analytes in positive mode (DON, DON-3-glucoside and DOM-1), whereas in the second period the negative mode was used (ADONs), and both ionization modes were run simultaneously by polarity switching.

All measurements were done with the following settings: source temperature 150 ºC, desolvation temperature 350 ºC, cone gas flow was 2.5 L/min, desolvation gas flow was 16.7 L/min, collision gas flow 0.17 mL/min and the capillary was 3000 V. The analyte-dependent MS/MS parameters were optimized via direct infusion of reference standard solutions; the resulting parameters are displayed in supplementary material (supplementary material, Table 11.1).

11.3.6. Methods performance

The analytical methods used were assessed for linearity, precision and recovery.

11.3.6.1. HPLC-FL and HPLC-UV

Standard curves were generated by linear regression of peak areas against concentration (r^2 were 0.97 and 0.99 for DON and OTA, respectively). Precision was established by determining OTA and DON levels in bakery products at least by triplicate, in those samples fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and OTA are summarized in Table 11.1.

11.3.6.2. UPLC-MS/MS

As in the previous section, standard curves were generated by linear regression of peak areas against concentration (all r2 ≥ 0.996). Precision was established by determining mycotoxin levels in bakery products at least by triplicate, in those samples fortified in order to calculate the recovery rates. A calibration curve for external calibration was generated for each analysis based on five concentration levels. For preparation of the standard concentrations, the multi-standard working solution was diluted (v/v) with solvent. Selectivity, sensitivity and stability of the measured product ions were evaluated throughout method development and validation by analysis of the blank food matrixes. The LOD was considered to be three fold the signal of blank noise, and the limit of quantification LOQ was calculated as 3 x LOD. Method performance characteristics for DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 are summarized in Table 11.1.

Table 11.1. Method performances for DON, deoxynivalenol-3-glucoside, 3-ADON, 15-ADON and DOM-1 determination in matrix food baked using acetonitrile (79%)/water (19%)/acetic acid (1%) as extraction solution with UPLC MS/MS.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d](%)
DON	0.7	2.1	3	74.53	75.1±10.6	11.1
			3	501.35	88.5±8.7	8.8
			5	1002.70	72.9±8.8	8.9
DON-3-glucoside	1.6	4.8	3	5.61	38.3±5.7	5.9
			3	37.74	54.1±10.1	10.9
			5	75.48	48.5±3.2	3.3
3-ADON	1.5	4.5	3	5.61	87.4±4.7	5.1
			3	37.74	85.4.±6.2	6.5
			5	75.48	88.2±8.6	8.9
15-ADON	1.5	4.5	3	5.61	89.0±7.2	7.5
			3	37.74	89.6±9.0	9.6
			5	75.48	78.1±2.3	2.9
DOM-1	0.5	1.5	3	5.56	82.8±4.6	5.8
			3	37.37	84.3±8.4	8.1
			5	74.74	78.4±10.5	10.8

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

11.3.7. Statistical analysis

Multifactorial ANOVA were applied to assess the significance of sample traits in the observed mycotoxin levels. Moreover, multiple linear regressions were applied to assess the temperature/time effect of DON, DON conjugates and OTA reduction during the baking process.

11.3.8. Kinetic calculations

Reaction order and kinetic constants were calculated by graphic evaluation. Based on the integration of kinetic equations for zero-, first-, and second-order, C_A, $\ln(C_A/C_0)$, and $1/C_A$ were plotted against residence time, where C_0 and C_A refer to the initial and remaining mycotoxin concentration (micrograms per gram), respectively, after time t (seconds). The reaction rate constant was calculated from the slope of the linearized rate law equation. The half-life was calculated from the rate law equation by allowing C_A to equal 0.5 C_0.

11.4. Results and discussion

11.4.1. Optimization of the sample preparation for UPLC-MS/MS analysis

For analysis of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1, a simple analytical method allowing low detection limits was used. Previous studies on DON and DON conjugates in bakery products have used acetonitrile/water (different percentages) and in some cases acetonitrile/water/acetic acid (≤ 1 % acetic acid) for extraction (Malachova, Dzuman, Veprikova, Vaclavikova, Zachariasova, & Hajslova, 2011; Zachariasova et al., 2012). Our aim was avoiding the clean-up step, but the high concentration of carbohydrates and fat can interfere. Therefore, extracting the target compounds from the matrix with weak interference and high recovery was a critical point. Due to the different polarity and solubility of the compounds to be extracted, in the optimization phase of the analytical protocol, spiked bakery products were extracted with mixtures consisting of different acetonitrile/water/acetic acid ratios. First, the extraction phase, acetonitrile (84%)/water (16%) (Malachova et al., 2011) was tested without good recovery results (data not shown). The mixture of acetonitrile (79%)/water (19%)/acetic acid (1%) used in some works for DON and its conjugates (De Boevre et al., 2012), led to low percentage of recovery for DON-3-glucoside (supplementary material, Table 11.2).

The extraction mixture of water (54%)/acetonitrile (45%)/acetic acid (1%) got an acceptable percentage of recovery for all the analysed mycotoxins (between 74 and 109%, Table 11.1) and it was used for sample analyses. To reach these recovery levels, an additional step was required after centrifugation, which was not reported in previous studies: 2 mL of dichloromethane were added to 2ml of sample and shaken to obtain a more purified sample in the organic phase. This helped to improve the specificity of the method.

Previous researchers analysed either DON and DON-3-glucoside (De Angelis et al., 2013; Malachova et al., 2011; Vidal et al., 2014a) or DON and 3-ADON and 15-ADON (Yang et al., 2013) in bakery products. However, both DON-3-glucoside and ADONs from DON can be present at significant concentrations in wheat products (Simsek et al., 2012; Yang et al., 2013), thus a suitable method of analysis able to simultaneously detect DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 was required.

11.4.2. Optimisation of UPLC-MS/MS conditions

The MS parameters (declustering potential, collision energy, and cell exit potential) were optimized by means of syringe infusion on each compound separately. This showed a better response in the ESI+ for DON, DON-3-glucoside, DOM-1 and ($^{13}C_{15}$) DON while ADONs had a better response in the ESI-. Once the precursor ions were selected, product scans were

recorded to test different values of declustering potential and collision energies. Declustering potential was set according to the sensitivity of precursor ions, whereas collision energies were selected to give the maximum intensity of the obtained fragment ions (supplementary material, Table 11.1).

The selectivity study demonstrated that interfering peaks at the retention time of analytes only occurred in those samples baked for too long (almost burnt), which were consequently not analysed through this technique. On the other hand, in regular samples, no interfering peaks were observed. The typical Multiple Reaction Monitoring (MRM) chromatogram of spiked bakery product samples is shown in supplementary material (supplementary material, Figure 11.1).

Different mobile phases from previous publications were tested to achieve an optimal separation, including mixtures of water with a volatile organic acid (formic acid, acetic acid) or ammonium acetate with an organic solvent (methanol or acetonitrile) (Yang et al., 2013; Zachariasova et al., 2012). The results showed that the best sensitivity for the mycotoxins analysed was achieved with acetonitrile (mobile phase A) and 10 mM of $AcNH_4$ in water (mobile phase B).The composition finally chosen is very similar to that in Xu et al. (2014), who analysed DON, 3-ADON,15-ADON and DOM-1 using methanol and 0.1 % formic acid in water, or Simsek et al. (2012), who analysed DON and DON-3-glucoside using 0.01 % acetic acid in water and 0.01 % acetic acid in acetonitrile, in bakery products.

Finally, the suitable recovery of DON-3-glucoside (mean 96%), compared to previously reported methods (around 60%, Malachova et al., 2011; Zachariasova et al., 2012), was the most important achievement of the used method. On the other hand, the LOD achieved is low enough (1.6 µg/kg) for cereal DON-3-glucoside analysis. Moreover, the other DON conjugates could be simultaneously analysed. Nowadays, due to the proven presence of DON-3-glucoside in cereals and the suggested transformation to DON during the digestion (Berthiller et al., 2011), a suitable method for analysis is required.

11.4.3. Temperature profiles during baking

Due to their small size, maximum temperature levels reached in the centre of the analogues paralleled those in the oven, after 15 min. Figure 11.1 shows the evolution of the temperature in the products and in the oven over time. The higher the temperature in the oven, the steepest the initial increase of temperature in the cakes.

11.4.4. Fate of DON during baking

DON was reduced by the baking process depending on baking temperature. In particular, the cake analogues baked at 160, 180 and 200 ºC had a significantly lower DON concentration than at the beginning of the process ($p < 0.05$), while at 140 ºC the reduction achieved was not significant. The percentages of reduction at 40 minutes varied from 29 % at 140 ºC to 81 % at 200 ºC. The higher the temperature the higher the visually observed inactivation rates (Figure 11.2). For instance, the reduction achieved at 200 ºC after 15 min, would require more than 30 min at other temperature levels.

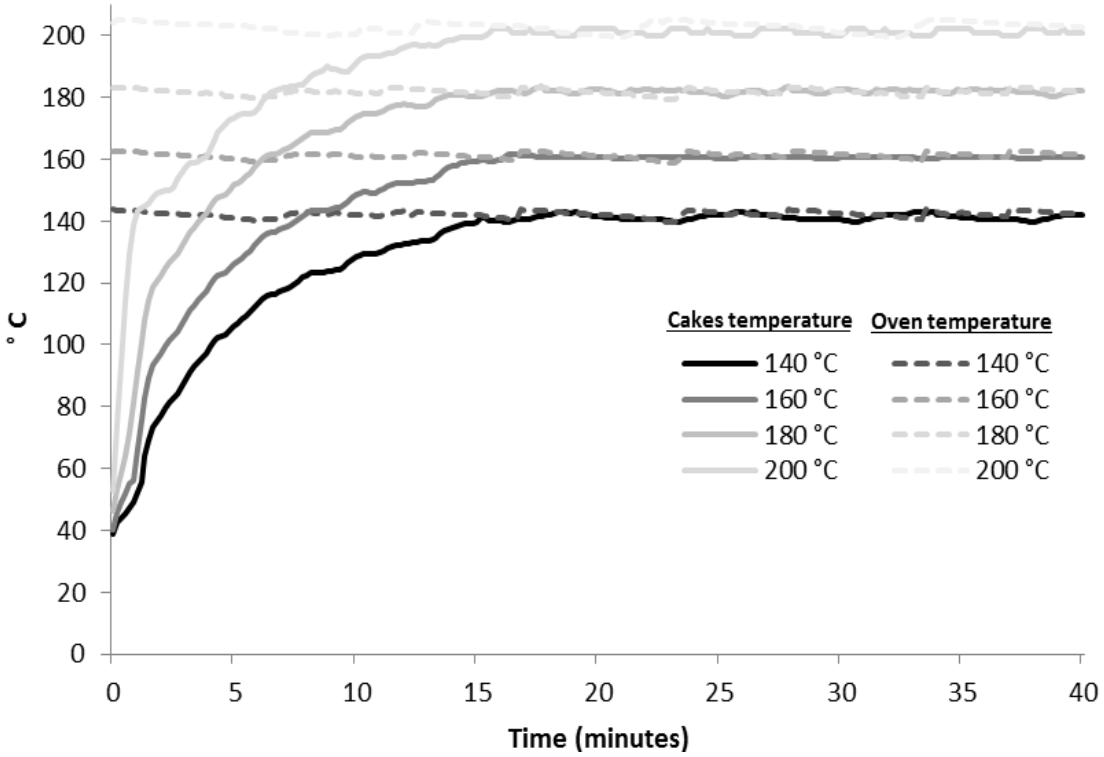


Figure 11.1. Recorded temperatures in the oven and in the centre of the baking cakes for 40 minutes at 140, 160, 180 and 200 ˚C.

Moreover, the cake analogues with higher initial DON concentration showed highest reduction results. At 200 ºC, the percentages of reduction were significantly different ($p < 0.05$), 89% at HMC and 67% at LMC. The differences between treatments with different initial mycotoxin concentration increased with increasing temperature levels.

Some studies dealt in the past with the stability of DON during the baking process and, although some trends were pointed out, only a few studies reported statistically significant effects (Lancova et al., 2008; Neira, Pacin, Martínez, Moltó, & Resnik, 1997; Numanoglu et al., 2012; Scudamore, Hazel, Patel, & Scriven, 2009; Valle-Algarra et al., 2009), while in other studies no significant reduction was observed (Kostelanska et al., 2011; Zachariasova et al.,

2012). The different size of the bakery product could be the factor which caused such different results. In some cases, the high percentage of reduction can be attributed to the use of mycotoxin spiked products, with lower effects of baking in naturally contaminated products.

In general, the present work presents higher DON reductions than those reported before, due to the easy heat transfer across the products. Analysing the previous studies, the most important reductions were obtained in small bakery pieces where the crust of the product accounted for a high proportion (Scudamore et al., 2009), although still the temperature in the centre of the pieces did not probably reach 100 ºC. Our approach, although theoretical due to the size of the cakes, allows assessing DON stability in a solid food matrix, and its reduction kinetics, but still in a much realistic approach than studies on mycotoxin solutions.

A few studies focused on the effect of different baking temperature levels in DON content in bakery products (Bergamini et al., 2010; Valle-Algarra et al., 2009; Vidal et al., 2014a), with not much significant differences among the effect of different temperature levels, and some contradictory results. The big size of the loaves, leading to a homogeneous temperature in the core area could be the reason. In the present study significant differences were found due to the tiny size of the baked product.

Comparing the present results with similar studies, it seems that when the initial concentration of DON is higher, more reduction is observed in the baking step, so the initial concentration is another factor which determines DON reduction, as observed Bergamini et al. (2010).

The time of treatment was the most determinant factor, as shown before (Lancova et al., 2008; Scudamore et al., 2009; Vidal et al., 2014a) the longer the baking time, the higher DON reduction.

Finally, the DON reduction could not produces a totally mycotoxin destruction because the detected DON reduction in the baked products could be caused by the binding of DON to the matrix or by the formation of DON conjugates.

11.4.5. Fate of DON conjugates during baking (further analysed with UPLC-MS/MS)

Selected samples were analysed by UPLC-MS/MS, in particular HMC samples excluding the harshest treatments, where the method of analysis did not perform optimally. DON concentrations obtained with UPLC-MS/MS confirmed those by HPLC–UV.

The initial flour contained also DON-3-glucoside, and the initial level in the unbaked cakes was 9.7 ± 3.0 µg/kg. DON-3-glucoside is commonly found in cereals, and almost all wheat samples analysed for DON also contain DON-3-glucoside. Simsek et al. (2012) detected

levels of 290 µg/kg in wheat flour. The levels of DON-3-glucoside vary among studies, however the ratio DON-3-glucoside/DON concentration is similar among the assays, from 10 to 30 % (Berthiller et al., 2009). In our study the ratio was 18 %, with an initial concentration of DON-3-glucoside in the flour was 45 µg/kg. Moreover, DON-3-glucoside significantly increased after baking ($p < 0.05$) (Table 11.2), although significant differences were only observed for certain treatments. The reason was that the levels seemed to increase under lower temperature levels and short time baking periods, while later the toxin was reduced (eg. at 180 ºC a significant increase was observed after 10-15 min, while later no toxin was detected). This DON-3-glucoside increase would confirm earlier reports (Vidal et al., 2014a), where a high increase (224 %) of DON-3-glucoside was also observed, although in that case the increase was reported also at high temperature and longer time, suggesting that the bigger size of the bread led to lower temperature levels, which were conducive for DON-3-glucoside release instead of reduction. By contrast, a reduction (40-50 %) in DON-3-glucoside was reported in De Angelis et al. (2013) and Simsek et al. (2012) at 200 and 220 ºC baking temperature where >100 g bread loaves were used. The DON-3-glucoside increases observed in breadmaking process have been also observed in brewing process; the increase has been hypothesised to be due to enzyme actuation. Enzymes could degrade the cell walls, membrane bound proteins, and starch depots in kernels, releaising DON-3-glucoside from insoluble forms (Lancova et al., 2008). The enzyme activity could explain the increase of DON-3-glucoside detected in some bakery products. In our case, the increase of DON-3-glucoside could be due to enzymatic release of bound forms from the matrix that would explain that as time increases enzymatic inactivation occurs and the increasing trend turns in a decreasing one.

In the present assay, DON-3-glucoside increased from 30 to 642 %, while a 100% reduction was observed at the higher T/t conditions. There was a positive significant correlation ($p<0.05$) between DON and DON-3-glucoside concentrations when all samples were included in the analysis, as in both cases degradation occurred at the higher T/t treatments. However, the samples with undetectable values were deleted, the correlation coefficient became negative and also significant, suggesting that in the milder treatments DON still decreased but DON-3-glucoside increased. It is uncertain whether DON-3-glucoside could be formed at expenses of DON in this step or as commented before, the increase of DON-3-glucoside could be due to enzymatic release of bound forms from the matrix.

The accumulation of DON-3-glucoside in bakery products is of concern. Although DON-3-glucoside is far less active as protein biosynthesis inhibitor than DON (Poppenberger et al., 2003), DON-3-glucoside likely will be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, microbial activity in the intestine as shown *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011), thus its presence is important for food safety.

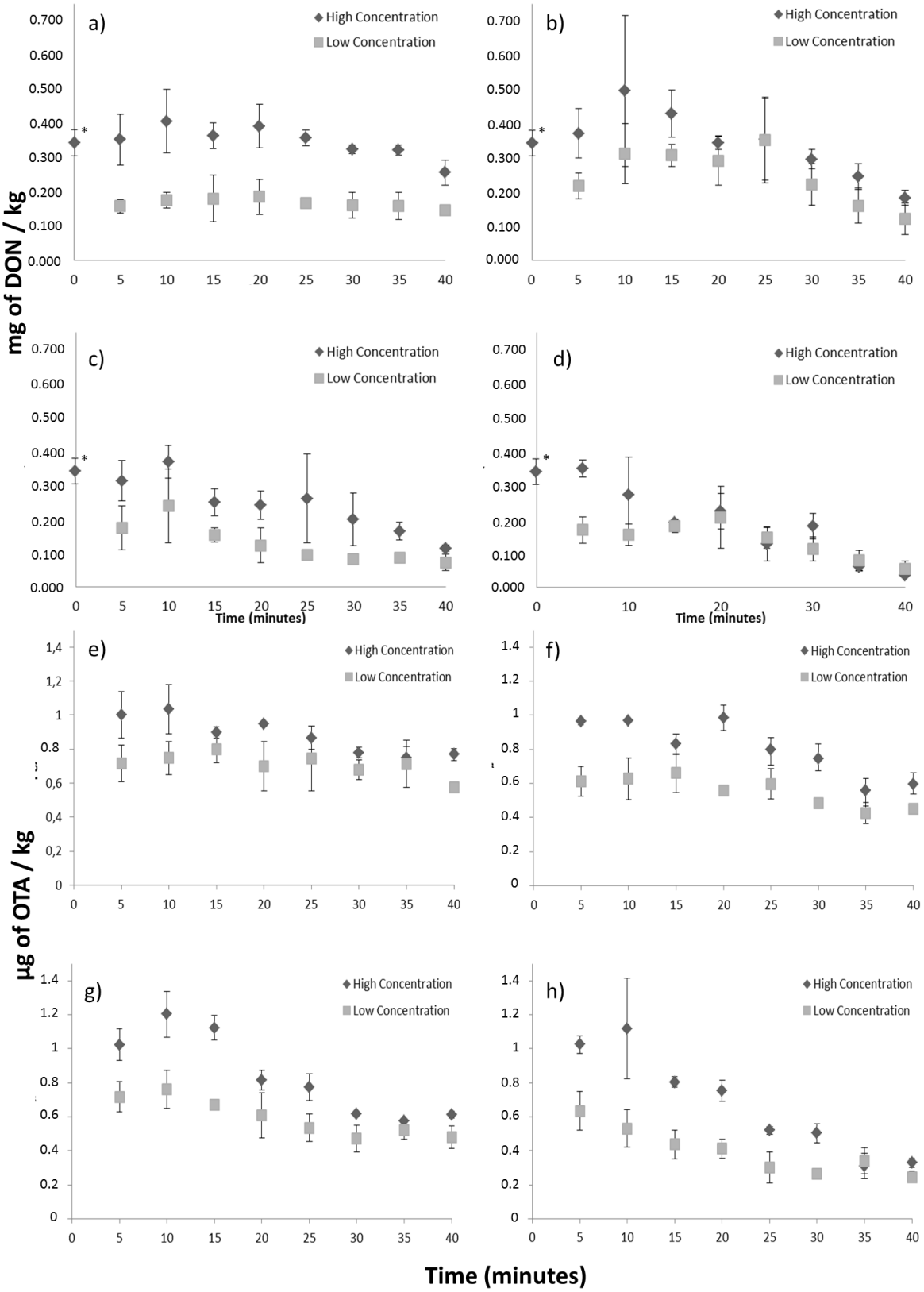


Figure 11.2. Concentration of DON (µg/kg) in cake analogues over time at 140 (a), 160 (b), 180 (c) and 200 (d) ºC at two different initial DON concentrations and concentration of OTA (µg/kg) in cake analogues over time at 140 (e), 160 (f), 180 (g) and 200 (h) ºC at two different initial OTA concentrations.

The concentration of 3-ADON in cake analogues at time 0 was 8.5±1.3 µg/kg, which means that if there was no change in its content during mixing, the concentration in the flour was approximately 30 µg/kg (27 parts of flour in the cake).The 3-ADON is a fungal metabolite which has been previously detected in wheat flour, but its presence is scarce and the contamination level can vary widely. Yang et al. (2013) found only one sample with 3-ADON from four analysed wheat grain samples. The study of scientific cooperation (SCOOP, 2003) task 3.2.10 pointed out that only 8% of the wheat samples contain 3-ADON, but 27% of the positive samples presented a concentration over 520 µg/kg. In our case 3-ADON could come both from naturally contaminated flour or be produced by the inoculated *F. graminearum.* Due to the lack of information about 3-ADON behaviour during baking, these results are important. The effects of temperature and the interaction temperature x time were significant on the 3-ADON concentration. The levels of 3-ADON tended to decrease with increasing temperature and baking time, although the differences among the levels of treatments were most times not significant (Table 11.2). The low initial concentration (8.5 µg/kg), close to the LOD, produced contradictory results in some analysed samples. The possible low stability of 3-ADON observed in our results agree with the only existing study on 3-ADON thermal stability, which pointed out a fast transformation of 3-ADON to DON, norDON A, norDON B, and norDON C, when 3-ADON was submitted either to alkali or heat (Young, 1986).

15-ADON was not quantifiable at time 0 (<4.5 µg/kg), and it was only detected in 11 % of the analysed cake analogues, just in some cases in 140 and 160 ºC treatments, and never at 180 and 200 ºC, thus no significant effect of either temperature or time could be assessed. Similarly to 3-ADON, 15-ADON is another fungal metabolite which has been detected in wheat grain (Yang et al., 2013). The major presence of 3-ADON versus 15-ADON is a phenotypic difference within *Fusarium* species. In southern of Europe the predominant 15-ADON genotype is more predominant (Somma et al., 2014); however, we used a *F. graminearum* strain from Argentina. Castañares et al. (2014) showed predominant presence of 3-ADON phenotypic *Fusarium* species in Argentina than in Europe. No studies exist on 15-ADON thermal stability; however, De Boevre et al. (2013) detected 15-ADON in processed food products, which suggests a certain stability of this mycotoxin.

DOM-1 is known to be a conjugated product from mammalian metabolism, and it is used as a biomarker for the DON exposition (Solfrizzo, Gambacorta, & Visconti, 2014). On the other hand, it has been also identified as a degradation product of DON after a heat process (Mishra, Dixit, Dwivedi, Pandey, & Das, 2014). In our case, at time 0 cakes contained 1.8±1.25 µg/kg of DOM-1, thus the flour could contain DOM-1. No studies analysing the presence of DOM-1 in flour exist, thus the origin of DOM-1 in our samples is unknown. Interestingly, temperature and time treatments had a significant effect on DOM-1 ($p<0.05$), which in general increased with heat treatments (Table 11.2). The increase was extremely high (285%) at the higher temperature tested (200 ºC, 20 min), which was the only treatment which was significantly different from the initial control, while at 200 ºC and 25 min, it was not detected.

Moreover, there was a significant negative correlation between DON and DOM-1 concentrations in those samples with quantifiable levels of both metabolites (Pearson correlation coefficient=-0.5884, p=0.0000), which confirm DOM-1 as a degradation product of DON caused by heat. In an aqueous system, Mishra et al. (2014) observed the formation of DOM-1 as a degradation product of DON after heating at 125 – 200 ºC.

Finally, more degradation products could be produced after the baking step because still the sum of DON-3-glucoside and DOM-1 increases is lower than the degraded DON. Bretz, Beyer, Cramer, Knecht, & Humpf (2006) detected other degradation products after heating DON, including norDON A, B and C, however the lack of standards for these products avoid the quantification of them in this assay.

Table 11.2. Concentration (µg/kg) of DON and DON conjugates (UPLC MS/MS) for the different time and temperature levels assayed.

	DON				DOM-1				DON-3-glucoside				3-ADON			
Temperature (ºC)	140	160	180	200	140	160	180	200	140	160	180	200	140	160	180	200
0 min	347.33^{a}	347.33^{a}	347.33^{a}	347.33^{a}	1.83^{a}	1.83^{a}	1.83^{a}	1.83^{a}	9.67^{a}	9.67^{a}	9.67^{a}	9.67^{a}	8.50^{a}	8.50^{a}	8.50^{a}	8.50^{a}
5 min	339.32^{ab}	348.45^{a}	318.74^{ab}	339.20^{ab}	1.63^{a}	1.74^{a}	1.49^{a}	1.75^{a}	14.27^{a}	12.05^{a}	18.01^{ab}	29.23^{ab}	8.66^{a}	9.49^{a}	5.39^{ab}	8.07^{ab}
10 min	346.48^{a}	325.49^{ab}	324.62^{ab}	263.47^{abc}	1.74^{a}	1.72^{a}	1.74^{ab}	<LOD$_{a}$	14.09^{a}	12.62^{a}	67.91^{c}	46.81$^{ab}_{c}$	8.14^{ab}	7.47^{ab}	8.69^{a}	1.08^{a}
15 min	298.86^{abc}	276.71^{abc}	246.92$^{abc}_{d}$	<LOD^{d}	<LOD$_{a}$	<LOD$_{a}$	2.84^{ab}	4.04^{ab}	15.14^{a}	19.65^{ab}	63.79$^{ab}_{c}$	<LOD^{a}	6.43^{ab}	7.98^{ab}	<LOD$_{ab}$	6.65^{ab}
20 min	307.70^{ab}	260.19^{abc}	144.62^{b}	<LOD^{d}	1.64^{a}	1.70^{a}	3.37^{ab}	5.12^{b}	13.85^{a}	59.56^{bc}	<LOD^{a}	<LOD^{a}	8.77^{a}	7.63^{ab}	3.04^{ab}	7.97^{ab}
25 min	291.51^{abc}	227.05^{bcd}	<LOD^{e}	<LOD^{d}	1.65^{a}	2.74^{ab}	3.92^{ab}	<LOD$_{a}$	23.30^{ab}	34.08$^{ab}_{c}$	<LOD^{a}	<LOD^{a}	8.14^{a}	4.85^{ab}	6.74^{ab}	7.88^{ab}
30 min	304.20^{ab}	181.09^{cd}	x	x	<LOD$_{a}$	3.07^{ab}	x	x	38.98$^{ab}_{c}$	41.56^{ab}	x	x	8.60^{a}	3.57^{ab}	x	x
35 min	278.70^{abc}	X	x	x	2.02^{ab}	x	x	x	40.88$^{ab}_{c}$	X	x	x	4.81^{ab}	X	x	x

^{abcd} Different letters next to the value indicate significant differences among the treatments, same letter, no significant difference (Tukey's test $p < 0.05$).
x, not analysed samples

11.4.6. Fate of OTA during baking

OTA was significantly affected by both baking temperature and time ($p < 0.05$). The maximum reduction was at 200 ºC for 40 minutes (64%), while at 140 ºC for the same time it was 21%. No significant differences in the reduction profiles were observed between the two different initial concentrations, although the inactivation was slightly higher at the higher concentration (Figure 11.2). Valle-Algarra et al. (2009) detected different level of reduction as a function of the initial OTA concentration (5 and 10 µg/kg flour), however, our initial concentrations were much closer (2 and 3 µg/kg flour).

Few works exist on OTA stability in bakery products and they showed conflicting results. Our results agree with those of Ferraz et al. (2010), Subirade (1996) and Valle-Algarra et al. (2009) who reported certain reduction in baking and roasting processes. On the other hand, some authors reported no changes or little changes in OTA content during baking (Scudamore et al., 2003; Vidal et al., 2014a); however, they used bigger size pieces of bread which probably led to lower temperatures in the inner part. In particular, Subirade (1996) showed a reduction of 66% in OTA in biscuits. Moreover, Valle-Algarra et al. (2009) found in the crust of the bread a reduction of 40% after 50 minutes at 190 ºC, which is very close to our results (37-68% reduction). Significant effects of temperature levels were reported by Boudra, Le Bars, & Le Bars (1995), however, other similar works observed none (Scudamore et al., 2003; Valle-Algarra et al., 2009). Although the effect of baking temperatures has been previously assayed, this is the first report on the effect of time.

As in DON, the possible reduction of OTA after baking does not mean a totally safer product if not OTA could be bound to the matrix or it could be transformed to degradation products. Although in our work OTA degradation products have not been studied, the literature points out to a partial isomerization of OTA in position C3 into a diastereomer (Studer-Rohr, Dietrich, Schlatter, & Schlatter, 1995), which results in the formation of 14-(R)-ochratoxin A and 14-decarboxy-ochratoxin A. These degradation products are less toxic than original OTA (Cramer, Königs, & Humpf, 2008), so it is important because the final baked products may be safer. They have not been analysed because no standards exist and moreover they have not been detected in cereals products.

11.4.7. Kinetic study

Kinetic data were studied for DON, DON-3-glucoside, DOM-1 and OTA.

11.4.7.1 Determination of DON inactivation kinetics

DON concentrations from HPLC-UV analysis were taken, as more results were available.

First of all, the kinetic order was determined. As the log-transformed concentrations showed a linear trend versus time (*1*), the degradation reaction was assumed to be of first-order ($dC / dt = k \cdot C$).

$$ln\ C = ln\ C_0 - k \cdot t \quad (1)$$

C = concentration of DON at time t (µg/kg).

C_0 = initial concentration of DON at time 0 (µg/kg).

k = thermal degradation rate constant (min^{-1}).

t = processing time (min).

The equation (*1*) is the result from the integration of the differential equation dC / dt = k·C. The slope of the line is the thermal degradation rate constant (*k*). Both first order degradation constants and half-lives are presented in Table 11.3. The half-life ($t_{½}$) is a timescale by which the initial concentration is decreased by half of its original value; when the half-life is smaller it means the reaction is faster. It is calculated from the rate law equation by allowing C to equal 0.5 C_0.

The half-lives and first order reaction constants confirmed the faster reduction for the high mycotoxin initial concentration and at 200 ºC ($t_{½}$ = 12.16 s and *k* = 0.057 min^{-1}). The slowest was at 140 ºC with the low mycotoxin concentration ($t_{½}$ = 231.05 s and *k* = 0.003 min^{-1}).

Other studies about kinetics of mycotoxin inactivation also used the first order kinetic model (Castells, Pardo, Ramos, Sanchis, & Marín, 2006; Jackson et al., 1996; Numanoglu et al., 2012). There is only a previous work on kinetics of inactivation of DON in a solid matrix, where authors used maize bread disks containing over 4500 µg/kg (Numanoglu et al., 2012). The estimated *k* at 150 ºC was 0.026 min^{-1}, while our *k* at 160 ºC was 0.023 min^{-1}, confirming the trend for faster inactivation when DON concentration is higher.

Knowing the reaction order of the system and k, it is possible to find the energy of activation for the degradation of DON from the Arrhenius equation (*2*). Logarithmic transformation of equation (2) results in equation (*3*).

$$k = A\, e^{-(Ea/RT)} \qquad (2)$$

k = thermal degradation rate constant (min^{-1}).
A = frequency factor.
Ea = degradation activation energy (kJ/mol).
R = gas constant 8.31 kJ/(mol·K).
T = absolute temperature (K).

$$ln\ k = -(Ea/RT) + \ln A \qquad (3)$$

Based on equation (*3*) Arrhenius graphs were built with ln *k* versus 1/T (Figure 11.3). The r^2 obtained shows a high correlation between temperature and thermal degradation rate constant (Figure 11.5a and b for the high and low mycotoxin initial concentration respectively). From the slope of the line it is possible to estimate the *Ea* which resulted to be 46.29 and 62.39 kJ/mol for the high and low concentration respectively, which means that activation of the degradation reaction is easier when the toxin concentration is higher.

Table 11.3. Estimated reaction rate constants (*k*) and half-lives ($t_{1/2}$) for DON, 3-ADON, DON-3-glucoside, DOM-1 and OTA.

Mycotoxin	Temperature (ºC)	Initial concentration	*k* (min^{-1})	$t_{1/2}$ (*min*)	r
DON	140	High	0.009	77.02	0.777
		Low	0.003	231.05	0.547
	160	High	0.023	30.14	0.880
		Low	0.019	36.48	0.649
	180	High	0.028	24.75	0.919
		Low	0.032	21.66	0.929
	200	High	0.057	12.16	0.910
		Low	0.031	22.36	0.835
DON-3-glucoside	140	-	-0.042	16.23	0.890
	160	-	-0.058	11.81	0.834
	180	-	-0.126	5.50	0.844
	200	-	-0.094	7.37	1.000
DOM-1	140	-	-0.016	42.78	0.847
	160	-	-0.023	30.01	0.961
	180	-	-0.063	11.00	0.979
	200	-	-0.110	6.26	0.921
OTA	140	High	0.010	69.31	0.936
		Low	0.005	138.63	0.660
	160	High	0.016	43.32	0.888
		Low	0.012	57.76	0.874
	180	High	0.022	31.51	0.918
		Low	0.014	49.51	0.932
	200	High	0.038	18.24	0.967
		Low	0.025	27.72	0.928

11.4.7.2. Determination of DON conjugates inactivation/release kinetics

The kinetics for DON conjugates were calculated as for DON (see section 3.7.1), but for DON-3-glucoside and DOM-1 the values for which inactivation was reported after increasing concentrations over time were omitted. In this case, the reactions (degradation or increase) were also assumed to be of first-order.

The obtained r values suggested that the release of DON-3-glucoside and DOM-1 followed a first order reaction (Table 11.3). Firstly, the *k* values for DON-3-glucoside and DOM-1 are negative which indicates that these mycotoxins increased during baking. The increase of DON-3-glucoside was faster than in DOM-1, and both of them in general faster than DON degradation. The *k* absolute values in DON-3-glucoside increased with the temperature, so the increase is higher with the temperature, however the *k* at 180 ºC is bigger than k value at 200 ºC, this situation is produced because in our results the DON-3-glucoside at 200 ºC only was detectable until minute 10, so few data were available to build the kinetics. For DOM-1 absolute *k* always increased with the temperature.

Estimated Ea were 25.87 and 49.95 kJ/mol for DON-3-glucoside and DOM-1, respectively (Figure 11.5c and d), which suggests that more energy is required to trigger the release of DOM-1 from the matrix or from DON transformation than to release DON-3-glucoside from the matrix or from DON reaction.

This is the first report on kinetics of these conjugated forms through a thermal treatment. Knowing their kinetics can allow for an approximate estimation of their concentration in the final products. For further studies, it would be of interest to check the kinetics under different initial DON concentrations.

11.4.7.3. Determination of OTA inactivation kinetics

The degradation reaction followed a first-order kinetic model. The estimated k values increased with the temperature (Table 11.3), with higher k values with higher initial OTA level. Half-lives and first order reaction constants at 200 ºC were $t_{½}$ = 18.24 min and k = 0.038 min^{-1}, at the high mycotoxin concentration. The high r^2 showed in Figure 11.5e,f confirm a high correlation between temperature and thermal degradation rate constant (estimated *Ea,* 34.99 and 40.62 kJ/mol).

Kinetics of OTA degradation in solid substrates have only been studied in coffee (Castellanos-Onorio et al., 2011; Ferraz et al., 2010). The kinetic constants (*k*) in coffee works are higher, 0.11 min^{-1} at 200 ºC (Ferraz et al., 2010) and 0.38 min^{-1} at 230 ºC (Castellanos-Onorio et al., 2011) because the reduction is clearly faster as a result of more extreme roasting conditions in the coffee case.

Differences between the two parent mycotoxins studied are detected, mainly in the kinetics, where DON is less thermostable than OTA. *k* was higher for DON than for OTA, however, *Ea* for OTA was lower, leading to a higher increase in *k* with temperature. Despite this, the *k* value at high temperature was still higher for DON than for OTA. The bigger extent of reduction of DON than OTA in the baking step agrees with other works (Vidal et al., 2014a). The higher thermo stability of OTA than DON may be due to the structure of the molecules. While OTA has many double bonds between carbons, DON only has one. Maybe for this reason the main OTA degradation products known are made by isomerization at the C3 position (Cramer et al., 2008).

Kinetic studies on degradation of mycotoxins are essential to provide baking factories with applied knowledge about mycotoxins degradation, but nowadays scarce works exist about this (Castells et al., 2006; Ferraz et al., 2010; Numanoglu et al., 2012). The Arrhenius equations obtained in the present work allow for easy estimation of DON and OTA concentration after the baking process as a function of temperature and time used if the initial concentration is known.

Thus the knowledge of the mycotoxin kinetics can be crucial in the control of mycotoxins. For instance, bakeries could set their maximum allowable toxin levels in the flour (always under the legal limit) depending on the final product to be manufactured and its known reduction factor. Moreover, the study of conjugated mycotoxins is of much interest. The possible increases for some of them through food processing increases the importance of their monitoring and their control.

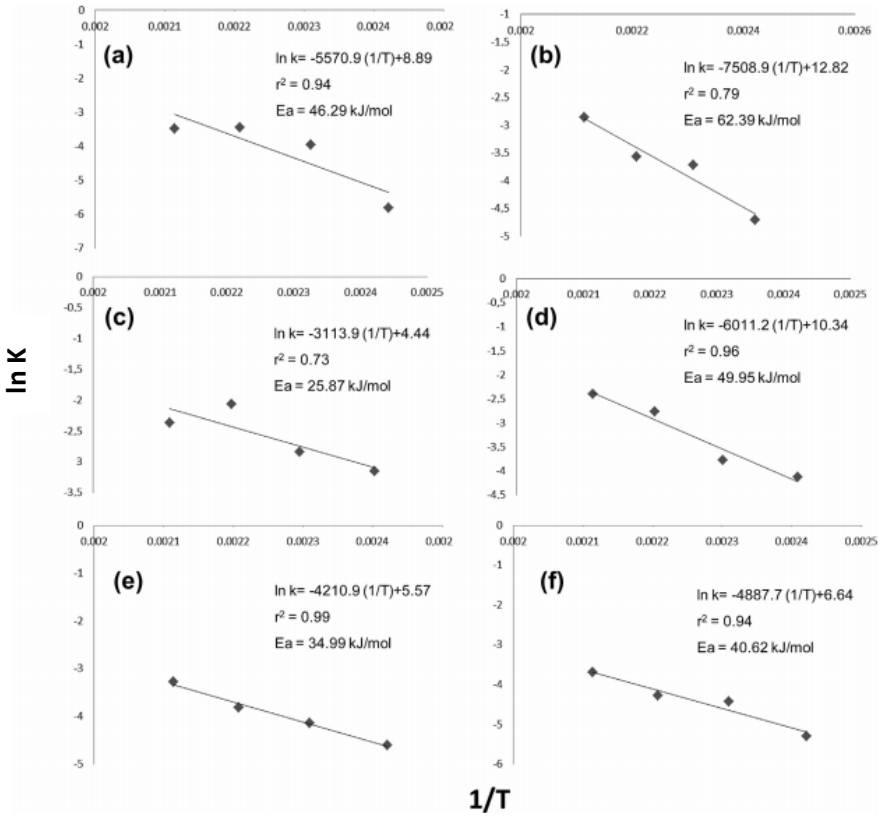


Figure 11.3. Arrhenius equations for DON in high (a) and low (b) initial mycotoxin concentration, DON-3-glucoside (c), DOM-1 (d) and OTA in high (e) and low (f) initial mycotoxin concentration.

11.5. Conclusions

DON and OTA can be highly reduced in baking processes, however, temperature, time and size of the product are critical factors. Moreover, OTA is more stable than DON in front of a baking treatment. The analysis of DON conjugates revealed that DON-3-glucoside, under mild baking conditions may be released, while it is erased fast in harsh conditions. The different behaviour as a function of the baking conditions might explain the contradictory existing reports. Moreover, DOM-1 increased during the heating process.

The use of kinetics developed in the work can be a useful tool for the control of DON, DON conjugates and OTA levels in the bakery industry, through the prediction of expected final levels once the mycotoxins concentration in the raw materials are known.

11.6. Acknowledgements

The authors are grateful to the Spanish government (projects AGL2010-22182-C04-04 and AGL2011-24862) for the financial support. A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant.

11.7. References

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of Fusarium mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Boudra, H., Le Bars, P., & Le Bars, J. (1995). Thermostability of ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology*, *61*, 1156-1158.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445–6451.

Castañares, E., Albuquerque, D.R., Dinolfo, M.I., Pinto, V.F., Patriarca, A., & Stenglein, S.A. (2014). Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina. *International Journal of Food Microbiology*, 179, 57-63.

Castellanos-Onorio, O., Gonzalez-Rios, O., Guyot, B., Fontana, T.A., Guiraud, J.P., Schorr-Galindo, S., Durand, N., & Suárez-Quiroz, M. (2011). Effect of two different roasting techniques on the ochratoxin A (OTA) reduction in coffee beans (*Coffea arabica*). *Food Control. 22*, 1184-1188.

Castells, M., Pardo, E., Ramos, A.J., Sanchis, V., & Marín, S. (2006). Reduction of ochratoxin A in extruded barley meal. *Journal of Food Protection*, *69*, 1139-1143.

Cramer, B., Königs, M., & Humpf, H.-U. (2008). Identification and *in vitro* cytotoxicity of ochratoxin A degradation products formed during coffee roasting. *Journal of Agricultural and Food Chemistry, 56*, 5673-5681.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: An investigation by high-performance liquid chromatography high-resolution mass spectrometry. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *30*, 345-355.

De Boevre, M., Di Mavungu, J.D., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut, A., Berthiller, F., Van Peteghem, C., & De Saeger, S. (2012). Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 819-835.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J.D., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B., & De Saeger, S. (2013). Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters*, *218*, 281-292.

Ferraz, M.B.M., Farah, A., Iamanaka, B.T., Perrone, D., Copetti, M.V., Marques, V.X., Vitali, A.A., & Taniwaki, M.H. (2010). Kinetics of ochratoxin A destruction during coffee roasting. *Food Control, 21*, 872-877.

IARC (International Agency for Research on Cancer). (1993). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56. Lyon, France.

Jackson, L.S., Hlywka, J.J., Senthil, K.R., Bullerman, L.B., & Musser, S.M. (1996). Effects of time, temperature, and pH on the stability of fumonisin B1 in an aqueous model system. *Journal of Agricultural and Food Chemistry*, *44*, 906-912.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer. *Food Additives and Contaminants*, *25*, 732–744.

Malachova, A., Dzuman, Z., Veprikova, Z., Vaclavikova, M., Zachariasova, M., & Hajslova, J. (2011). Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *Journal of Agricultural and Food Chemistry*, *59*, 12990-12997.

Marín, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*. *60*, 218-237.

Mishra, S., Dixit, S., Dwivedi, P.D., Pandey, H.P., & Das, M. (2014). Influence of temperature and pH on the degradation of deoxynivalenol (DON) in aqueous medium: Comparative cytotoxicity of DON and degraded product. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 121-131.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37,* 21-25.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. (2012). Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 423-430.

Pacin, A.M., Resnik, S.L., Neira, M.S., Moltó, G., & Martínez, E. (1997). Natural occurrence of deoxynivalenol in wheat, wheat flour and bakery products in Argentina. *Food Additives and Contaminants, 14*, 327-331.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

SCOOP. 2003. Collection of Occurrence Data of Fusarium Toxins in Food and Assessment of Dietary Intake by the Population of EU Member States. Report on Tasks for Scientific Cooperation task 3.2.10. <http://europa.eu.int/comm/food/fs/scoop/task3210.pdf>. Accessed 03.06.14.

Scudamore, K.A., Banks, J., & MacDonald, S.J. (2003). Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. *Food Additives and Contaminants, 20*, 1153-1163.

Scudamore, K.A., Hazel, C.M., Patel, S., & Scriven, F. (2009). Deoxynivalenol and other *Fusarium* mycotoxins in bread, cake, and biscuits produced from UK-grown wheat under commercial and pilot scale conditions. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 1191-1198.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Solfrizzo, M., Gambacorta, L., & Visconti, A. (2014). Assessment of multi-mycotoxin exposure in southern Italy by urinary multi-biomarker determination.*Toxins, 6*, 523-538.

Somma, S., Petruzzella, A.L., Logrieco, A.F., Meca, G., Cacciola, O.S., & Moretti, A. (2014). Phylogenetic analyses of *Fusarium graminearum* strains from cereals in Italy, and characterisation of their molecular and chemical chemotypes. *Crop and Pasture Science, 65*, 52-60.

Subirade, I. (1996). Fate of ochratoxin A during breadmaking. *Food Additives and Contaminants*, *13*, 25-26.

Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour during

dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *26*, 896-906.

Vendl, O., Berthiller, F., Crews, C., & Krska, R. (2009). Simultaneous determination of deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food by LC-MS-MS. *Analytical and Bioanalytical Chemistry, 395*, 1347-1354.

Vidal, A., Marín, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market. *Food and Chemical Toxicology, 53*, 133-138.

Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234-242.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology, 68,* 53-60.

Xu, L., Zhang, G., Guo, C., Zhang, Y., Zhang, Y., Zeng, J., Yang, H., Yang, D., He, L., Zeng, Z., & Fang, B. (2014). Simultaneous determination of major type-B trichothecenes and the de-epoxy metabolite of deoxynivalenol in chicken tissues by HPLC-MS/MS. *Journal of Separation Science, 37*, 642-649.

Yang, D., Geng, Z.M., Yao, J.B., Zhang, X., Zhang, P.P., & Ma, H.X. (2013). Simultaneous determination of deoxynivalenol, and 15- and 3-acetyldeoxynivalenol in cereals by HPLC-UV detection. *World Mycotoxin Journal, 6*, 117-125.

Young, J.C. (1986). Formation of sodium bisulfite addition products with trichothecenones and alkaline hydrolysis of deoxynivalenol and its sulfonate. *Journal of Agricultural and Food Chemistry, 34*, 919-923.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

11.8. Supplementary material

Table 11.1. The optimized ESI-MS/MS parameters for the confirmation and quantification of DON, DOM-1, DON-3-glucoside, 3-ADON, 15-ADON and $^{13}C_{15}$ DON.

Mycotoxin	Precursor ion (m/z)	Product ions [A](m/z)	DP[A,B](V)	CE[A,C] (eV)	CXP[A,D] (v)	Dwell time[A] (ms)
DOM-1	281.1	109.06/215.08/233.11	55	16/10/10	6.0/6.0/6.0	108/108/108
DON/DON-3-glucoside	297.09	203.10/231.09/249.06	60	16/10/10	2.0/2.0/2.0	52/52/52
$^{13}C_{15}$ DON	312.09	216.06/244.99/262.93	65	16/10/10	2.0/2.0/2.0	52/52/52
ADONs	337.30	150.30/173.30/277.30/307.00	62	14/14/14/14	16.0/16.0/16.0/16.0	80/80/80/80

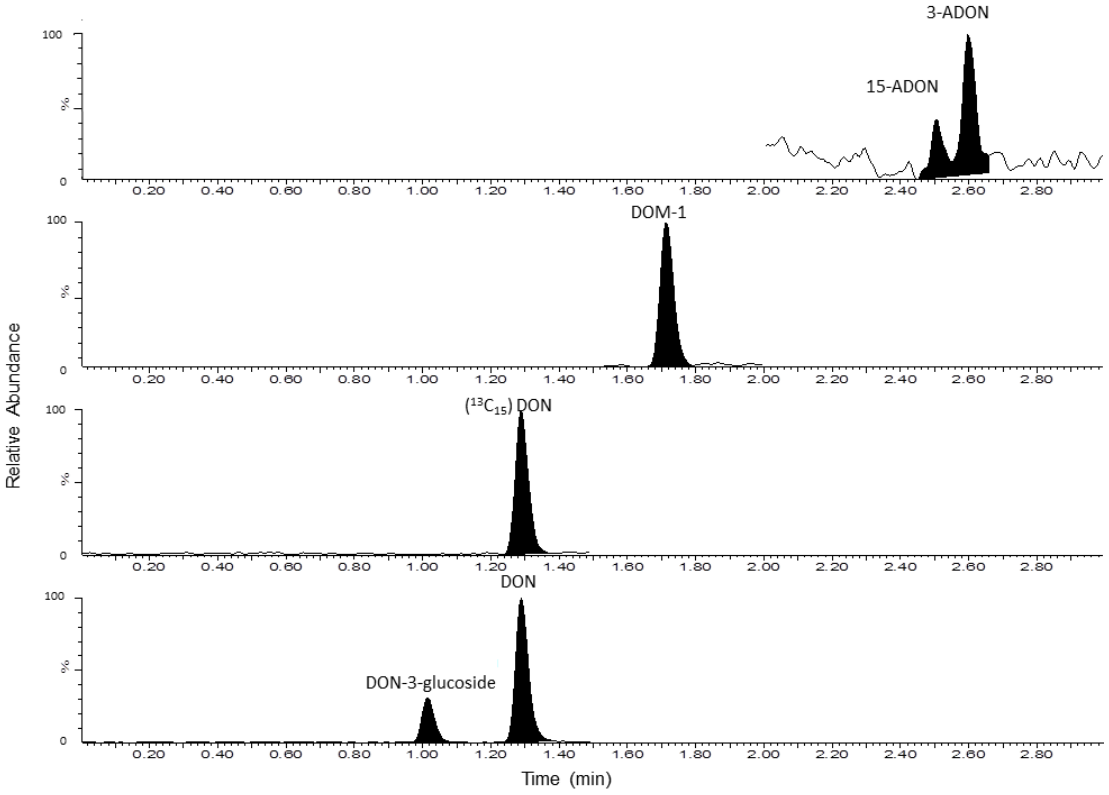
[A] Values are given in the order quantifier ion/ qualifier ion.
[B] DP: Declustering potential
[C] CE: Collision energy.
[D] CXP: Cell exit potential.

Table 11.2. Method performances for OTA, DON, deoxynivalenol-3-glucoside, 3-ADON and 15-ADON and DOM-1determination in bakery products.

Mycotoxin	Method used	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery [c] (%)	RSDr[d](%)
OTA	HPLC FL	0.02	0.06	3	0.6	94±3	3.2
				3	1.0	93±2	2.7
				5	1.5	90±1	1.9
DON	HPLC UV	50.00	150	3	150	108±15	14.2
				3	350	93±5	5.0
				5	750	99±1	1.6
DON	UPLC MS/MS	0.7	2.1	3	74.53	92.1±5.6	6.1
				3	501.35	92.5±9.1	9.9
				5	1002.70	87.9±8.3	9.5
DON-3-glucoside	UPLC MS/MS	1.6	4.8	3	5.61	104.1±6.1	5.9
				3	37.74	95.4±17.1	17.9
				5	75.48	87.4±7.2	8.3
3-ADON	UPLC MS/MS	1.5	4.5	3	5.61	79.4±13.5	17.1
				3	37.74	74.0±9.2	12.5
				5	75.48	78.3±8.3	10.6
15-ADON	UPLC MS/MS	1.5	4.5	3	5.61	80.0±10.2	11.2
				3	37.74	79.6±7.5	8.0
				5	75.48	87.1±6.3	7.1
DOM-1	UPLC MS/MS	0.5	1.5	3	5.56	109.8±3.8	3.5
				3	37.37	96.7±4.5	4.6
				5	74.74	80.6±11.1	8.2

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation

Figure 11.1. MRM chromatograms of spiked samples under the chromatographic conditions described in the text. Spiking level for DON and (13C15) DON 1002.70 µg/kg and 75.48 µg/kg for DON-3-glucoside, 3-ADON, 15-ADON and DOM-1.



12. Study VII. Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Food Chemistry (2016) 208, 288-296.

Authors: Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S.

12. Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Arnau Vidal, Asier Ambrosio, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*.

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

12.1. Abstract

The stability of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-glucoside) during the breadmaking process was studied. Some enzymes used in the bakery industry were examined to evaluate their effects on DON and DON-3-glucoside. The level of DON in breads without added enzymes was reduced (17-21%). Similarly, the addition of cellulase, protease, lipase and glucose-oxidase did not modify this decreasing trend. The effect of xylanase and α-amylase on DON content depended on the fermentation temperature. These enzymes reduced the DON content by 10-14% at 45ºC. In contrast, at 30ºC, these enzymes increased the DON content by 13-23%. DON-3-glucoside levels decreased at the end of fermentation, with a final reduction of 19-48% when no enzymes were used. However, the presence of xylanase, α-amylase, cellulase and lipase resulted in bread with greater quantities of DON-3-glucoside when fermentation occurred at 30ºC. The results showed that wheat bran and flour may contain hidden DON that may be enzymatically released during the breadmaking process when the fermentation temperature is close to 30ºC.

Keywords: Deoxynivalenol; Deoxynivalenol-3-glucoside; Ochratoxin A; baking process; enzymes; flour improvers.

12.2. Introduction

Deoxynivalenol (DON), also known as vomitoxin, is one of the most common contaminants in cereals (Cano-Sancho et al., 2011a). DON can be found at relatively high concentrations in wheat and wheat-containing products (such as bread and pasta) (Cano-Sancho et al., 2011a). In addition, wheat and wheat-containing products are considered to be the major source of human intake of DON (Cano-Sancho, Gauchi, Sanchis, Marín, & Ramos, 2011b). Although DON is not classifiable by the International Agency for Research on Cancer (IARC, 1993) due to its carcinogenicity to humans, DON has been linked to human gastroenteritis (Pestka, 2010).

Wheat grains contaminated with DON may also contain deoxynivalenol-3-glucoside (DON-3-glucoside), a plant metabolite of DON (Berthiller et al., 2009). Reported levels of DON-3-glucoside are variable; however, the ratio of DON-3-glucoside/DON concentrations is similar among assays, ranging from 10 to 30 % (Berthiller et al. 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013). Moreover, Berthiller et al. (2011) have shown that DON-3-glucoside can be hydrolysed to DON by several lactic acid bacteria that may be present in the intestines. Thus, the FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside to be an additional contributing factor to total dietary exposure to DON (JEFCA, 2010).

Due to the high presence of DON and DON-3-glucoside in raw wheat, studying the stability of DON and DON-3-glucoside during the breadmaking process is critical. Contradictory reports exist regarding the fate of DON during this process. First, results on the effect of fermentation on DON are contradictory: while some studies have suggested that DON concentrations are reduced during fermentation (Neira, Pacin, Martínez, Moltó, & Resnik, 1997; Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012), other studies have shown that DON concentrations significantly increase after fermentation (Bergamini et al., 2010; Lancova et al., 2008; Vidal, Morales, Sanchis, Ramos, & Marín, 2014a). Studies that have examined DON concentrations after baking are also contradictory: some studies have observed reductions in DON levels (Neira et al., 1997; Bergamini et al., 2010), while other studies have reported no changes or even increases in DON levels (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Zachariasova et al., 2012). However, inconsistencies may exist because these studies have been conducted on different scales: some studies have been conducted in laboratories, while other studies have been conducted at the industrial level (Bergamini et al., 2010). Moreover, Vidal, Sanchis, Ramos and Marín (2015), using small items, demonstrated that DON levels may be reduced only in the external part of the loaves due to the reduced heat transmission. Thus, the size of the baked items may also provide an explanation for the inconsistent results reported for baking studies (Vidal et al. 2014a). Four publications also report show also contradictory results of DON-3-glucoside after the fermentation and baking steps (Generotti et al., 2015; Suman, Manzitti, & Catellani, 2012; Vidal et al., 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b; Zachariasova et al., 2012). Recently, Vidal et al. (2015) showed that DON-3-

glucoside may be released under mild baking conditions of temperature and time (for instance, 140 ºC for 35 minutes or 200 ºC for less than 10 minutes) but reduced under harsher baking conditions (i.e., longer periods of time and higher temperatures).

The increases in DON and DON-3-glucoside concentrations reported during breadmaking may be due to enzymatic activity (Simsek et al., 2012; Vidal et al., 2014a). Enzymes may hydrolyse mycotoxins bound to carbohydrates or to other components related to the ingredients of the recipe formulations causing an increase in mycotoxin concentrations at the end of the breadmaking process. For example, Zhou, Schwarz, He, Gillespie and Horsley (2008) detected higher DON levels in barley samples after treatment with protease, xylanase and cellulase. Simsek et al. (2012) obtained the same result for wheat samples after the samples were treated with xylanase. Finally, Zachariasova et al. (2012) found that α-amylase caused no changes in DON-3-glucoside levels when malt samples were treated for more than six hours.

Given that hydrolytic enzymes may affect the release of DON during breadmaking, the objective of this study was to assess the effects of different enzymes (xylanase, α-amylase, cellulase, protease, lipase and glucose oxidase that are commonly used in breadmaking) on DON and DON-3-glucoside levels during bran bread production.

12.3. Materials and methods

12.3.1. Initial levels of DON and DON-3-glucoside in flour and bran

Flour and bran wheat were purchased from a flour mill in Lleida (Spain) and were analysed for natural DON and DON-3-glucoside contaminations. The initial DON concentration in the flour was 251 ± 30 µg/kg (n = 3); DON-3-glucoside was not detected. In the bran, DON and DON-3-glucoside concentrations were 2004 ± 72 and 579 ± 61 µg/kg (n = 3), respectively.

12.3.2. Dough preparation and baking

A flour + bran mix was prepared (200 g of bran/1000 g of flour) and used for the bread experiments. Therefore, the concentrations of DON and DON-3-glucoside were 651 ± 13 and 137 ± 23 µg/kg (n = 6), respectively, in the mix of flour + bran.

To each flour + bran mix (156 g), 2.3 g of salt, 4.7 g of sucrose, 4.7 g of lard and 6.2 g of commercial compressed yeast (*Saccharomyces cerevisiae*, Levanova, Lesaffre Ibérica, S.A., Spain) were added. The dough was obtained by adding 83 mL of water to the mixture. Different doughs were prepared, containing six different enzymes (xylanase, α-amylase, cellulase, protease, lipase and glucose oxidase) plus a control. The enzyme concentrations were adjusted according to breadmaking standards: 1 U of xylanase/g flour (Oliveira, Telis-Romero, Da-Silva, & Franco, 2014), 10 U of α-amylase/g flour (Kim, Maeda, & Morita, 2006), 35 mU of cellulase/g flour (Haros, Rosell, & Benedito, 2002), 10 U of protease/g flour (Harada, Lysenko, & Preston, 2000), 1 U of lipase/g flour (Moayedallaie, Mirzaei, & Paterson, 2010) and 10 U of glucose oxidase/g flour (Hanft & Koehler, 2006). The enzymes were added in powder form. Moreover, the second fermentation was conducted separately at 30 or 45 ºC. Thus, 14 treatments were tested in the study, and the experiment was repeated three times.

Dough was manually kneaded until it was held together with a non-sticky, smooth and satiny appearance and had optimum handling properties. Rounded pieces weighing 250 g each were prepared. From this point, thermoprobes (Thermo Bouton, Proges Plus, France) were used in the dough to record fermentation and baking temperatures; specifically, probes were placed in the centre of the loaf and close to the surface. Doughs were covered with a damp cloth, and the first fermentation was conducted at 30 ºC for 15 minutes. Next, the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 or 45 ºC. After fermentation, a sample of 25 g was taken from each proofed dough. Samples were lyophilized and stored at -20 ºC until mycotoxin analysis. The proofed doughs were then baked in an oven (Eurofred PE46SVR, Eurofred, Spain). Baking conditions were 180 ºC and 75 min. Such conditions were used to obtain suitable bread based on previous experiments. After baking, a representative sample was taken, lyophilized and stored at -20 ºC until the analyses were performed.

12.3.3. Chemicals, reagents and enzymes

Mycotoxin (DON and DON-3-glucoside) standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. 3,5-Dinitrosalicylic acid (DNS) (≥ 98 %), sodium azide (≥ 99.5 %), starch (from potato), *o*-dianisidine (peroxidase substrate), Triton X-100 (laboratory grade), copper (II) acetate (≥ 99.5 %), caseinate (from bovine milk) and trichloroacetic acid (≥ 99.0 %) were supplied by Sigma. Malic acid (≥ 99 %), 2,2,4-trimethylpentane (≥ 99.5 %) and acetic acid (100 %) were supplied from VWR Prolabo (Llinars del Vallès, Spain). Sodium hydroxide (≥ 99.5 %), sulphuric acid (≥ 96 %) and sodium chloride (≥ 99.5 %) were supplied by Fisher Bioreagents (New Jersey, USA). Sodium and potassium tartrate (≥ 99 %) and Tris buffer (reagent grade) were supplied by Scharlau (Barcelona, Spain). Sodium carbonate (≥ 99.5 %) and Folin's reagent were supplied by Panreac (Castellar del

Valles, Spain). Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) extract clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA). The six enzymes used in the study, namely, xylanase (from *Trichoderma longibrachiatum*), α-amylase (*Aspergillus oryzae*), cellulase (*Aspergillus niger*), protease (*Aspergillus oryzae*), lipase (*Aspergillus niger*) and glucose oxidase (*Aspergillus niger*), were purchased from Sigma.

12.3.4. Preparation of mycotoxin standard solutions

DON standard solution was dissolved in ethanol at a concentration of 10.0 µg/mL and stored at 4 ºC. The concentration in the stock solution was confirmed by UV spectrometry according to the AOAC Official methods of analysis. Working standards (2.5, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with the mobile phase and were used to obtain calibration curves in the appropriated chromatographic system. The DON-3-glucoside standard was dissolved in acetonitrile at a concentration of 10.0 µg/mL and stored at 4 ºC in a sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL) and calibration curves were prepared for DON.

12.3.5. Enzyme activity quantification

To quantify the enzymatic activity in each sample, a calibration curve was required. Moreover, a blank reference was required to set the spectrophotometer to zero absorbance. The following common steps were used in each enzyme protocol:

- The blank reagent was prepared (with neither substrate nor enzyme, only reagents). The blank reagent was used to set the spectrophotometer to zero absorbance.
- Calibration samples were prepared using substrates with known added enzyme concentrations plus a zero sample with no added enzyme. Calibration curves were built for fermented dough and for bread.

12.3.5.1. Xylanase, α-amylase and cellulase activities

The activities of the three enzymes were analysed by following the reduction of DNS to 3-amino-5-nitrosalicylic acid (ANS) spectrophotometrically at 540 nm (Miller, 1959). The extraction solution was the same for the three enzymes (250 mL of 0.2 M malic acid, 250 mL of 0.35 M sodium hydroxide, 250 mL of 0.2 M sodium chloride and 250 mL of 0.003 M sodium azide; all of these components constituted 1 L of extraction solution). DNS reagents were

prepared with 1 g of DNS, 20 mL of 2 M sodium hydroxide, 10 mL of 10.6 M sodium and potassium tartrate and 70 mL of water.

12.3.5.1.1. Xylanase activity

Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL of extraction solution without shaking. A total of 0.5 mL of the supernatant was added to 1.5 mL of the substrate (5 g of wheat fibre in 80 mL of water, boiled for 15 min with agitation, followed by 15 minutes of agitation at room temperature; 10 mL of 1 M sodium acetate was then added; the solution then reached a volume of 100 mL with water). The tubes were heated for 8 minutes at 50 ºC, and 3 mL of the DNS reagent was added at the end of the heating step. Subsequently, the tubes were placed in boiling water for 5 minutes. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath. Cooling to ambient temperature was necessary because of the effect of temperature on the absorbance of the coloured reaction product. After cooling, the absorbance at 540 nm was measured using the spectrometer. To generate calibration curves, xylanase calibration samples (5, 2.5, 1, 0.5, 0.1 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.75 and 0.99 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

12.3.5.1.2. α-Amylase activity

Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL of extraction solution without shaking. Then, 0.5 mL of the supernatant was added to 1.5 mL of the substrate (1 g of starch in 20 mL of 1 M sodium hydroxide; 10 mL of 10.6 M sodium and potassium tartrate and 70 mL of water). The tubes were kept at 25 ºC for 3 minutes, and then 1 mL of the DNS reagent was added. The tubes were boiled for 5 minutes in boiling water. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath, and 10 mL of water were then added. Finally, absorbance was measured in the spectrometer at 540 nm. α-amylase calibration samples (50, 10, 5, 1, 0.5 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.86 and 0.96 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

12.3.5.1.3. Cellulase activity

Tubes containing 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL of extraction solution without shaking. A 0.5-mL aliquot of the supernatant was added to 0.25 g of filter paper (Whatman no 1). The tubes were kept at 50 ºC for 60 minutes, and 3 mL of DNS reagent was then added. The tubes were boiled for 5 minutes in boiling water. After 5 minutes in boiling water, the tubes were cooled to room temperature. The absorbance was measured in the spectrometer at 540 nm. Cellulase calibration samples (50,

10, 5, 2.5, 1, 0.5 and 0.1 mU/g) were prepared with fermented dough/bread samples and used to obtain calibration curves (r^2 values were 0.81 and 0.94 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

12.3.5.2. Protease activity

The protease assay followed the methods described by Ladd and Butler (1971). Briefly, 2 g of fermented dough/bread samples was weighed in a tube and blended with 5 mL of caseinate solution (10 mg/mL) in 0.1 M Tris-buffer, pH 8.1. The tubes were heated for 60 minutes at 50 ºC without shaking. Next, 1 mL of 1.0 M trichloroacetic acid was added. The mixture was centrifuged, and 2 mL of the supernatant was mixed with 3 mL of 1.4 M sodium carbonate and 1 mL of 1.3 M Folin's reagent. After incubating for 10 minutes at 25 ºC, the sample was measured spectrophotometrically at 700 nm. Protease calibration samples (15, 10, 7.5, 5, 2.5 and 1 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.95 and 0.84 for fermented dough and bread, respectively) by following the same protocol for the analysis described above.

12.3.5.3. Lipase activity

The method described by Duncombe (1963) was used to measure lipase activity in the samples. A total of 0.5 g of fermented dough/bread samples was weighed and mixed with 0.3 mL of olive oil and 0.5 mL of Tris-HCl solution (100 mL of 0.05 M Tris-HCl with 1 mL of Triton-X, pH = 7.5). The samples were incubated for 60 minutes at 37 ºC without shaking. A total of 0.1 mL of 1 M HCl and 5 mL of isooctane were added to each sample. Next, the samples were placed in boiling water for 5 minutes. A total of 2.5 mL of copper reagent (copper acetate 0.28 M, pH = 6.1) was added. The solution was then centrifuged for a few minutes to separate the phases, and the upper layer was taken for measurements. Lipase activity was measured with the spectrophotometer at 540 nm. Lipase calibration samples (2.5, 1.5, 1, 0.5 and 0.1 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.96 and 0.99 for fermented dough and bread, respectively) by following the same protocol for the analysis described above.

12.3.5.4. Glucose-oxidase activity

To measure glucose-oxidase activity, we followed the methods described by Bergmeyer, Gawehn and Grassl (1974). A total of 3 g of fermented dough/bread samples was placed in a tube, and 5 mL of extraction solution (20 mL of 8.3 M sodium acetate and 2.5 mL of acetic acid in water, up to 1 L) plus 2.5 mL of 1 M glucose were added. The mixture was kept at

30 ºC for 5 minutes without shaking. Next, 1 mL of *o*-dianisidine was added. The samples were kept at 30 ºC for 5 more minutes, and then 4 mL of the supernatant was mixed with 2 mL of sulphuric acid (9 M). The samples were measured at 540 nm with a spectrophotometer. Glucose-oxidase calibration samples (15, 10, 5, 2.5,1 and 0.5 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.74 and 0.79 for fermented dough and bread, respectively) by following the same protocol for the analysis described above.

12.3.6. Mycotoxin extraction, detection and quantification

DON and DON-3-glucoside were extracted from 5 g of lyophilized ground sample (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany) with 30 mL of distilled water by magnetically stirring for 10 min. Next, the sample was centrifuged for 8 min at 1780 × *g*. The supernatant was filtered through a glass microfiber filter. Five millilitres of filtered sample was loaded on the DONPREP® IAC column, and the column was washed with 10 mL of distilled water. DON and DON-3-glucoside were eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of Milli-Q water, consecutively. Zachariasova et al. (2012) confirmed the robust cross-reactivity of DON-3-glucoside using IAC DONPREP® columns (99-102 % recovery for DON and DON-3-glucoside when less than 500 ng of these toxins was loaded). The purified extracts were dried under a stream of nitrogen at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON and DON-3-glucoside were quantified using a HPLC Waters 2695® system with an analytical column (Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm, coupled with a UV/Visible dual λ absorbance Detector Waters 2487). The absorption wavelength was set to 220 nm. The HPLC mobile phase flow rate was 0.6 mL/min. The injection volume was 100 µL. The column temperature was 40 ºC. The retention times for DON and DON-3-glucoside were 20 and 23 min, respectively.

12.3.7. Performance of the methods

The analytical methods for DON and DON-3-glucoside were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 values were 0.99 and 0.96 for DON and DON-3-glucoside, respectively). Precision was estimated by determining DON and DON-3-glucoside levels in flour and DON levels in bread samples, in triplicate, in fortified samples to calculate recovery rates. Recovery was not tested in dough, as the dough was considered to be similar in composition to both flour and bread. The limit of detection (LOD) was considered to be threefold greater than the signal of blank noise, and the limit of quantification (LOQ) was calculated to be 3 x LOD.

Characteristics of the method performance for DON and DON-3-glucoside are summarized in Table 12.1.

Table 12.1. Performance of the DON and DON-3-glucoside determination in flour and bread.

Mycotoxin	Product	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d] (%)
DON	Bread	60	180	5	100	100±16	16
				5	500	99±9	9
				5	1000	102±5	5
	Flour	60	180	3	300	123±30	41
				3	500	87±9	7
DON-3-glucoside	Bread	15	30	-	-	-	-
	Flour	15	30	5	50	80±10	12
				5	250	80±5	6
				5	500	67±11	18

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

12.3.8. Statistics

The results are shown in terms of dry weight. Multifactorial ANOVA was used to detect significant differences in enzymatic activity between treatments. Additionally, multifactorial ANOVA was used to assess the significance of sample traits in the observed mycotoxin concentration levels and in the calculated percentages of the increases and decreases in mycotoxin concentrations.

12.4. Results and discussion

12.4.1. Impact of enzyme addition on the presence of DON

12.4.1.1. Fate of DON in bread without added enzymes

Wheat flour naturally contains several technologically important enzymes, such as amylases, proteases, lipoxygenase, polyphenol oxidase and peroxidase. Although these

enzymes are inactive during storage of grain and flour, when water is added, they become active and play a significant role in determining the functional attributes of flour (Rani, Prasada Rao, Leelavathi, Haridas, & Rao, 2001).

The unkneaded mix of the ingredients contained 594 ± 12 µg/kg of DON, while the mean concentrations in the fermented doughs were 562 ± 17 and 460 ± 24 µg/kg at a fermentation temperature of 30 and 45 ºC, respectively (Table 12.2). Both reductions (5 and 23 %) were significant, and there was a significant difference between each treatment ($p < 0.05$). The reduction of DON levels during fermentation has been observed in several other studies. For instance, Neira et al. (1997) observed a 21.6 % reduction in DON levels. Other studies have reported lower DON concentrations in fermented dough than in the initial flour, but this reduction may also be due to the dilution required in the recipe (Lancova et al., 2008). Conversely, an increase in DON levels at the end of fermentation has been observed in other studies (Bergamini et al., 2010; Lancova et al, 2008; Vidal et al., 2014b). In some cases, when enzymes (especially α-amylase) were added, an increase in DON levels was detected. Simsek et al. (2012) detected an increase of up to 99 % in DON after fermentation using α-amylase. Suman et al. (2012) detected an increase of up to 14 % in DON using non-specific enzymes. Moreover, Vidal et al. (2014a), using flour improvers with non-specific enzymes, detected a 30 % increase in DON during fermentation. Moreover, sourdough use also led to increases in DON content during fermentation (Vidal et al. 2014b). In summary, studies in which either malt flour or other enzymes were added reported increases in DON levels (Simsek et al., 2012, Suman et al., 2012; Vidal et al., 2014a), while in the absence of added enzymes, reductions in DON content often occur (Neira et la., 1997). Thus, the presence of enzymes may determine the fate of DON during fermentation. The increase in DON content during fermentation has been associated with the release of DON from the wheat matrix through enzyme catalysis (Simsek et al., 2012; Vidal et al., 2014a).

Moreover, in the present study, DON reduction was higher at a fermentation temperature of 45 ºC than at 30 ºC ($p < 0.05$). However, few authors have considered fermentation temperature. Samar et al. (2001) assayed different temperatures of fermentation (from 30 to 50 ºC). They found that reductions in DON concentrations were greater as the temperature increased (from 0 to 56 %). The highest reduction in DON levels was observed at the highest temperature (50 ºC) and the longest time tested (60 min). This result is consistent with the results of our study. Additionally, Generotti et al. (2015) assayed different fermentation temperatures (from 26 to 46 ºC), and they found that that the stability of DON was reduced at higher fermentation temperatures. Thus, fermenting at high temperatures may be a feasible alternative to reduce DON content in bread, as long as bread quality is not affected. Although proofing temperature can be as high as 54 ºC (Pyler, 1973), most authors agree that a range from 27 to 46 ºC is optimal for bread production (Freilich, 1949; Hui, Corke, De Leyn, Nip, & Cross, 2007). A fermentation temperature of approximately 30 ºC maximizes taste due to the

high production of lactic acid. Conversely, fermentation above 40 ºC can reduce the quantity of lactic acid and result in tasteless breads; however, higher fermentation temperatures can also lead to improved baking volumes (Dobraszyk, Smewing, Albertini, Maesmans, & Schofield, 2003).

Table 12.2. Mean DON concentration (µg/kg) ± SD in fermented doughs and breads, percentage of reduction during fermentation compared to the initial mix (594±12), reduction in the baking step (%) and total reduction (%) in the final product compared to the initial mix (594±12) for each type of treatment.

Temperature of fermentation (°C)	30					45				
	Fermented dough	Reduction in fermentation (%)	Bread	Reduction in baking (%)	Total reduction (%)	Fermented dough	Reduction in fermentation (%)	Bread	Reduction in baking (%)	Total reduction (%)
No enzymes	562 ± 17	5	496 ± 27	12	17	460 ± 24	23	467 ± 12	-2	21
Xylanase	542 ± 61	9	732 ± 28*	-35	-23	531 ± 8*	11	509 ± 16*	4	14
α-Amylase	656 ± 12*	-10	670 ± 5*	-2	-13	447 ± 75	25	535 ± 15*	-20	10
Cellulase	576± 92	3	484 ± 67	16	19	752 ± 39*	-27	560 ± 107	26	6
Protease	596 ± 7	0	431 ± 123	28	27	804 ± 24*	-35	411 ± 42	49	31
Lipase	487 ± 48	18	522 ± 49	-7	12	570 ± 121	4	646 ± 82	-13	-9
Glucose oxidase	525 ± 59	12	568 ± 160	-8	4	821 ± 26*	-38	551 ± 4*	33	7

* There are significant differences compared to the same matrix sample without enzymes and at the same fermentation temperature ($p < 0.05$).

Bread fermented at 30 ºC had a final concentration of 496 ± 27 µg/kg, which meant that there was a significant reduction of 12 % during baking ($p < 0.05$). The final DON concentration of the bread fermented at 45 ºC was 467 ± 12 µg/kg (not significantly different from that of fermented dough). Thus, DON reduction in baking critically depended on the fermentation temperature ($p < 0.05$). Considering the entire breadmaking process, the DON concentration in bread was similar regardless of the fermentation temperature (mean reduction from beginning to end approximately 19 %). The reduction in DON content during baking is consistent with most previous studies, which have reported a reduction in DON content at temperatures over 170 ºC as long as the baking time was longer than 45 min (Vidal et al., 2014a). The results of this study are consistent with the response surface model for DON reduction in bread baking formulated by Vidal et al. (2014a). No effect of baking was observed in bread fermented at 45 ºC. This fact may be caused by the lower initial DON concentration in this case. Reduction of DON levels during baking has been shown to be higher at higher initial toxin concentrations and not significant at lower initial DON concentrations (Vidal et al. 2014a, 2015). Reduction of DON levels may result in thermodegradation products (norDONs A-F and DON lactones), which are less toxic than DON itself. The losses that cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006). Still, some existing studies have reported no reductions of DON levels or even slight increases during baking, which may be attributed to extended enzymatic activity duringat the early stages of baking (Bergamini et al. 2010; Simsek et al., 2012; Suman et al., 2012).

12.4.1.2. Fate of DON in bread with added enzymes

Xylanases are hydrolytic enzymes randomly cleave the β-1,4 backbone of plant cell wall xylans. Xylanases are of great value in baking, as they have been found to improve bread volume and crumb structure and to reduce stickiness. In our case, xylanase activity increased during fermentation with the addition of xylanase; however, the activity of the existing flour xylanase at 45 ºC was still higher than the activity observed at 30 ºC with added xylanase ($p < 0.05$) (Table 12.3). Xylanase addition had only a significant effect on DON variation during fermentation when xylanase was added at 45 ºC ($p < 0.05$, despite the increase in activity not being significant), where the final DON concentration in the fermented dough was 531 ± 8 µg/kg (a 15 % increase, Table 12.2). However, this level of activity was still lower than that before fermentation. The different behaviour of DON detected at 45 ºC may be linked to the fact that the optimum temperature for xylanase activity (45 ºC for xylanase produced by *T. longibrachiatum*; Chen, Chen, & Lin, 1997) was reached. Baking resulted in a 35 % increase in DON levels in the xylanase-containing dough fermented at 30 ºC ($p < 0.05$); the final concentration of DON in the bread was 732 ± 28 µg/kg. Baking did not significantly change the DON concentration when the fermentation temperature was 45 ºC. However, the final bread with xylanase had a higher DON concentration (509 ± 16 µg/kg) than the control bread ($p < 0.05$) due to the increases experienced during fermentation. In sum, DON levels increased with the presence of xylanase during fermentation at 45 ºC. DON levels also increased during baking when the fermentation temperature was 30 ºC. This pattern may be linked to the optimum temperature for xylanase activity, as mentioned above. In the dough fermented at 30 ºC, the majority of the xylanase activity probably occurred during the early stages of baking before enzymes became inactivated over 55 ºC (Irfan, & Syed, 2012). Thus, xylanase-added breads contained higher levels of DON at the end of the breadmaking process than control breads, regardless of the fermentation temperature ($p < 0.05$); however, only breads fermented at 30 ºC experienced an increase in DON concentrations. Similarly, Simsek et al. (2012) have reported an increase in DON (13 %) in xylanase-treated wheat at 50 ºC for 18 hours. Zhou et al. (2008) found a tendency for DON levels to increase after treatment of barley grains with xylanase/cellulase (5 hours at 50 ºC). Xylanases cause the hydrolysis of cell wall material (arabinoxylan) in the dough, resulting in the release of DON bound to the polysaccharides of cereal cell walls.

α-amylase is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch. In our study, α-amylase activity was not detected in the fermented doughs (when the enzyme was not externally added), and the activity of α-amylase was more similar in amylase-added doughs fermented at 30 than at 45 ºC. However, some residual activity was still detected in the resulting breads (Table 12.3). Different studies have shown that α-amylase has high thermostability. For instance, Raviyan, Tang and Rasco (2003) have shown that α-amylase is stable above 75 ºC. Inside the bread during baking, the temperature is always below 100 ºC. The tested α-amylase produced an increase in DON

concentration during fermentation at 30 ºC, with a final concentration in the fermented dough of 656 ± 12 µg/kg (Table 12.2, 10 % increase compared to the unkneaded mix, $p < 0.05$). Baking resulted in no reduction in DON levels in breads previously fermented at 30 ºC (670 ± 5 µg/kg). Conversely, the baking step caused an increase in DON (20 %) in breads fermented at 45 ºC, and the final DON concentration in bread was 535 ± 15 µg/kg. Thus, breads with added α-amylase contained higher concentrations of DON than the controls ($p < 0.05$). Even in bread fermented at 30 ºC, the concentration of DON was higher than that in the initial ingredient mix. The more marked effect on DON at 30 ºC was related to the activity of the α-amylase from *A. oryzae* (Evstatieva, Nikolova, Ilieva, Getov, & Savov, 2010). α-amylase is one of the most used enzymes in the breadmaking process, and our results at 30 ºC demonstrated that the release of DON described in previous studies may be caused by the use of α-amylase. Moreover, in the literature, the longer the fermentation time with α-amylase is, the higher the increase in DON that is reported. For instance, Simsek et al. (2012) have reported an increase in DON concentrations at the end of the fermentation (180 minutes at 30 ºC) of nearly 100 %. Bergamini et al. (2010) have reported a 38 % increase in DON (85 minutes at 40 ºC), while Suman et al. (2012) have reported a 10-14 % increase in DON in biscuits (4 minutes at 30 ºC). These results suggest that α-amylase has an impact on DON balance during the breadmaking process, as DON may be released from forms bound to starch.

Cellulase added to bread hydrolyses non-starch polysaccharides, leading to an improvement of the rheological properties of dough, bread loaf volume and crumb firmness. Low activity of cellulase was detected in doughs without added cellulose, while the detected activity in cellulose-added doughs was higher when the fermentation temperature was 45 ºC than at 30 ºC. No cellulase activity was detected in any of the breads (Table 12.3). No effect of cellulase addition was observed when fermentation was conducted at 30 ºC (Table 12.2). Cellulase addition, however, caused changes in DON concentrations when the fermentation temperature was 45 ºC ($p < 0.05$), with a DON concentration of 752 ± 39 µg/kg (63 % higher than that of the control) in the fermented dough and 560 ± 107 µg/kg in the final bread. In this case, the higher reduction in DON levels during baking observed in breads fermented at 45 ºC may be caused by the higher DON concentration found at the end of the fermentation, as mentioned previously. Thus, the final bread in this case contained a level of DON not significantly different from that in the unkneaded mix. The optimum temperature of cellulase produced by *A. niger* is close to 45 ºC (Coral et al., 2002). This optimum temperature may explain the differences observed between temperatures. An increase in DON levels was observed as a result of the activity of a xylan/cellulase mix in barley samples (5 hours at 50 ºC) (Zhou et al. 2008). This finding indicates that DON may be bound to the cellulose of the cell wall of cereals.

High levels of protease activity break up gluten, destroying the network that forms during kneading. Conversely, low levels of protease activity soften the dough and make the dough more workable. In addition, proteases affect the flavour of bread. Protease activity

generates single amino acids when the last peptide bond of the protein chain is broken. These amino acids can participate in the flavour and browning reactions that occur in the crust during baking. No protease activity was detected in our samples, except for fermented dough at 45 ºC (Table 12.3). This finding was consistent with the DON results, where protease-added samples only differed from the controls in DON concentration in fermented doughs at 45ºC (Table 12.2). The optimum temperature for protease activity is at 60 ºC (Yin et al., 2013), and the highest protease activity was after fermentation at 45 ºC. No protease effect was detected by Simsek et al. (2012) when whole wheat grains were treated. This finding may stemmed from the location of the proteins in the endosperm, which were poorly accessible in their experiment (Veraverbeke, & Delcour, 2002). No differences in cellulose activity were found in the final loaves compared to the control, as the increase in DON levels observed during fermentation was compensated by the decrease in DON that occurred during baking.

Table 12.3. Mean enzymatic activity (units) ± SD in fermented doughs and breads.

Temperature of fermentation (˚C)	30				45			
	Fermented dough		Bread		Fermented dough		Bread	
	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added
Xylanase (U/g)	0.16 ± 0.06	0.35 ± 0.01*	<0.05	<0.05	0.51 ± 0.09	0.62 ± 0.03	<0.05	<0.05
α-Amylase (U/g)	<0.05	34.1 ± 22.17*	<0.05	3.89 ± 0.51*	<0.05	21.09 ± 17.18*	<0.05	2.97 ± 0.31*
Cellulase (mU/g)	<0.1	7.16 ± 0.63*	<0.1	<0.1	0.80 ± 0.75	9.01 ± 0.52*	<0.1	<0.1
Protease (U/g)	<1	<1	<1	<1	<1	3.94 ± 1.94*	<1	<1
Lipase (U/g)	0.25 ± 0.02	0.31 ± 0.03*	<0.1	<0.1	0.27 ± 0.03	0.35 ± 0.09	<0.1	<0.1
Glucose oxidase (U/g)	<0.5	8.76 ± 1.30*	<0.5	<0.5	<0.5	5.58 ± 3.10*	<0.5	<0.5

* There are significant differences compared to the sample without added enzymes at the same fermentation temperature ($p < 0.05$).

Lipases are particularly effective in retarding bread staling. Lipase activity was similar in control and lipase-added doughs. No lipase activity was detected in breads (Table 12.3). The use of lipase in fermentation did not have any effect on DON concentration. After baking, DON concentrations slightly increased ($p < 0.05$). A low interaction of DON with the lipid fraction of bread ingredients is a possibility.

Glucose oxidase catalyses the oxidation of *β*-D-glucose to glucono-*δ*-lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide. The use of glucose oxidase results in stronger and more elastic doughs with a dry surface. Glucose oxidase activity was only detected in those doughs where the enzyme was intentionally added and the fermentation temperature was 30 and 45 ºC (Table 12.3). However, no impact on DON concentration was observed at 30 ºC (Table 12.2). But, at 45 ºC, it led to the highest DON concentration (821 ± 26 µg/kg, 38 % increase) in a fermented dough, although it dropped to 551 ± 4 µg/kg due to baking, but still higher than the control ($p < 0.05$). This point could be linked with DON-3-

glucoside presence, as discussed later. The optimum temperature of glucose oxidase is a 35-40 ºC (Bhatti, Madeeha, Asgher, & Batool, 2006), and glucose-oxidase activity was detected in fermented dough at both 30 and 45 ºC.

The enzyme activities presented here for the fermented dough were determined at the end of the fermentation step. Thus, the values presented in table 12.3 are not indicative of the levels of activity that may have occurred previously during fermentation or later in the early stages of baking.

In sum, the presence of some enzymes (α-amylase, cellulase, protease and glucose oxidase) led to the release of DON during fermentation, whereas xylanase promoted lower DON concentrations during fermentation. However, the effect of xylanase on DON depended on the fermentation temperature. At 30 ºC, only the presence of α-amylase promoted higher DON content after the kneading + fermentation process compared to the DON content in the unkneaded mix. The increase in DON content detected in xylanase- and α-amylase-added breads fermented at 30 ºC after baking is the main concern because the DON content in this treatment was higher than in the initial unkneaded mix. Conversely, fermentation at 45 ºC led to an increase in DON content relative to the initial unkneaded mix when cellulose, protease and glucose oxidase were used at the end of fermentation. The DON content in xylanase-added fermented dough was higher than in the control fermented dough but lower than that in the initial unkneaded mix. For doughs fermented at 45 ºC, baking led to lower levels of DON, resulting in breads that were not significantly different from the controls, except for xylanase-, α-amylase- and glucose oxidase-treated breads. However, the addition of the three enzymes did not cause higher DON contents in the final bread than in the unkneaded mix.

In sum, only xylanase- and α-amylase-added breads fermented at 30 ºC experienced DON concentration in the final product that were greater than the DON content in the flour mix. In these two particular cases, an additional risk should be considered. The ability of some enzymes to release bound DON from flour during the breadmaking process was shown. Few studies have dealt with food-processing enzymes and mycotoxin behaviour, but the few studies that have been conducted have generated results that are consistent with this hypothesis (Lancova, et al., 2008; Simsek et al., 2012; Zhou et al., 2006).

12.4.2. Impact of enzyme addition on the presence of DON-3-glucoside

The DON-3-glucoside concentration (that is, dry concentration) in the unkneaded mix was 125 ± 28 µg/kg, and the DON-3-glucoside concentration at the end of fermentation without added enzymes decreased to <LOQ (30 µg/kg), regardless of the fermentation temperature (unlike the pattern observed in DON). After baking, the DON-3-glucoside concentration was restored to 101 ± 17 µg/kg and 65 ± 39 µg/kg at 30 and 45 ºC respectively (Table 12.4). The

detected reduction in DON-3-glucoside after dough-proofing without enzymes is consistent with previous studies (Kostelanska et al., 2011).

When enzymes were added to the dough, a clear decrease in DON-3-glucoside during fermentation was observed, regardless of the temperature. Despite a reduction in the DON-3-glucoside concentration at the end of fermentation, xylanase and protease at 30 ºC and cellulase and lipase at 45 ºC led to less pronounced reductions in DON-3-glucoside concentrations than control fermented doughs (Table 12.4). Conversely, the exception to DON-3-glucoside reduction at the end of fermentation was for glucose oxidase activity, which led to a significant increase in DON-3-glucoside at both temperatures (360 ± 59 µg/kg at 30 ºC). The presence of glucose oxidase generated an important increase in DON-3-glucoside in the fermented dough compared to the initial mix.

The effects of enzymes during baking were more noticeable (Table 12.4), mostly in doughs that had been fermented at 30 ºC. Xylanase, α-amylase, cellulase and lipase led to significant increases in DON-3-glucoside. The activities of these enzymes during baking presumably promoted increases in the DON-3-glucoside content in the final breads compared to the initial mix. Glucose oxidase-added breads exhibited significant decreases in DON-3-glucoside concentrations compared to fermented dough, the unkneaded mix and control breads. However, in doughs fermented at 45 ºC, baking had no significant effect on the DON-3-glucoside concentration ($p < 0.05$), and the resulting breads did not differ from control breads.

The use of enzymes as improvers in past studies caused increases in DON-3-glucoside during fermentation; however, the exact enzymes responsible for this increase were not described (Kostelanska et al. 2011; Vidal et al., 2014a, 2014b). Simsek et al. (2012) used only α-amylase and detected a reduction in DON-3-glucoside at the end of fermentation at 30 ºC (5 %) (similar to the present study).

The changes in DON-3-glucoside after baking observed in this study are consistent with the results from previous studies on wheat products (Vaclavikova et al., 2013; Vidal et al., 2014b, 2015); however, some studies have shown a reduction in DON-3-glucoside after baking (Kostelanska et al., 2011; Simsek et al., 2012). Vidal et al. (2015) showed that DON-3-glucoside exhibits different behaviours in thermal treatments as a function of the size of the product, temperature and time. The mild baking conditions tested in this assay (especially due to the large size of the product) promoted an increase in DON-3-glucoside during baking, while harsher treatments would have led to a reduction in DON-3-glucoside. Our results demonstrate the large effect that enzymes have in the release of DON-3-glucoside during baking in breads fermented at 30 ºC with added xylanase, amylase, cellulase, protease and lipase. DON-3-glucoside may be bound to flour components and released during baking. In general, no significant correlation was found between increases in DON-3-glucoside and decreases in DON or vice versa. Only in breads that had been fermented at 45 ºC was there a slight inverse relationship between changes in DON-3-glucoside and DON. Similarly, Kostelanska et al.

(2011) concluded that the behaviours of the two mycotoxins were not linked because, in their study, the concentration of DON did not change. These authors suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may occur. This proposal is consistent with our observation of large increases in DON-3-glucoside during baking, especially when xylanase and cellulase were present in the matrix. More studies that examine the relationship between DON and DON-3-glucoside are necessary to fully understand the interactions between parent and conjugated mycotoxins in food processes. Finally, the increase in DON-3-glucoside during baking is of concern because, although DON-3-glucoside is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003), DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more importantly, microbial activity in the intestines. The activity of chemical hydrolases and intestinal microbes has been demonstrated *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011); thus, the presence of these enzymes and microbes is important for food safety.

Table 12.4. Mean DON-3-glucoside concentration (µg/kg) ± SD in fermented doughs and breads. The DON-3-glucoside concentration in the initial mix was 125 ± 28 µg/kg.

Temperature of fermentation (˚C)	30		45	
	Fermented dough	Bread	Fermented dough	Bread
No enzymes	<LOQ	101 ± 17	<LOQ	65 ± 39
Xylanase	62 ± 28	563 ± 65*	<LOQ	<LOQ
α-Amylase	<LOQ	568 ± 131*	<LOQ	<LOQ
Cellulase	<LOQ	629 ± 290*	30 ± 0	36 ± 10
Protease	37 ± 5	1835 ± 1472	<LOQ	126 ± 41
Lipase	<LOQ	275 ± 19*	45 ± 9	<LOQ
Glucose oxidase	360 ± 59*	56 ± 12*	51 ± 0*	76 ± 10

* There are significant differences compared to the sample without enzymes subjected to the same temperature ($p<0.05$).

12.5. Conclusion

In conclusion, DON concentrations could be lowered in the breadmaking process if no enzymes are added. For example, while flour without added enzymes exhibits a decrease in the DON concentration during breadmaking (fermentation at 30ºC), the presence of xylanase and α-amylase can promote an increase in DON at the end of the breadmaking process. Aside from the different optimum temperatures for the tested enzymes, this fact may also imply that DON is more likely to be bound to starch and arabinoxylans than to other polysaccharides, fats and proteins. Moreover, if fermentation is conducted at 45 ºC, the presence of glucose oxidase may promote an increase in DON levels in the final bread with respect to the initial DON

concentration. The presence of xylanase, α-amylase, cellulase and lipase resulted in breads with increased concentrations of DON-3-glucoside when fermentation was conducted at 30 ºC compared to the initial mycotoxin concentration.

12.6. Acknowledgements

The authors are grateful to the Spanish government (project AGL2014-55379-P) for financial support. A. Vidal thanks the Spanish government (Ministry of Education) for the pre-doctoral grant.

12.7. References

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Bergmeyer, H.U., Gawehn, K., & Grassl, M. (1974). *Methods of Enzymatic Analysis.* (2nd ed.). New York: Academic Press, Inc.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Bhatti, H.N., Madeeha, M., Asgher, M., & Batool, N. (2006). Purification and thermodynamic characterization of glucose oxidase from a newly isolated strain of *Aspergillus niger*. *Canadian Journal of Microbiology, 52*, 519-524.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445–6451.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., & Marín, S. (2011a). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22,* 490-495.

Cano-Sancho, G., Gauchi, J.-P., Sanchis, V., Marín, S., & Ramos, A.J., (2011b). Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1098-1109.

Chen, C., Chen, J.-L., & Lin, T.-Y. (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. *Enzyme and Microbial Technology, 21,* 91-96.

Coral, G., Arikan, B., Ünaldi, M.N., & Güvenmez, H.K. (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 Wild-Type Strain. *Turkish Journal Biology, 26*, 209-213.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal*, *6*, 83-91.

Dobraszczyk, B.J., Smewing, J., Albertini, M., Maesmans, G., & Schofield, J.D. (2003). Extensional rheology and stability of gas cell walls in bread doughs at elevated temperatures in relation to breadmaking performance. Cereal Chemistry, 80, 218-224.

Duncombe, W. G. (1963). The colorimetric micro-determination of long-chain fatty acids. B*iochemistry Journal, 88*, 7.

Evstatieva, Y., Nikolova, D., Ilieva, S., Getov, L., & Savov, V. (2010). Identification and characteritzation of α-amylase and endoxylanase, produced by *Aspergillus* mutant strains. *Biotechnology & Biotechnological Equipment, 24,* 613-617.

Freilich, J. (1949). Time, temperature and humidity: Factors in dough proofing. *Baker's Dig, 33*, 27-30.

Generotti, S., Cirlini, M., Malachova, A, Sulyok, M, Berthiller, F, Dall'Asta, C., & Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins, 7,* 2773-2790.

IARC (International Agency for Research on Cancer). (1993). *Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 56). Lyon, France.

JEFCA. 2010. WHO Technical Report Series 959: *JEFCA, 2010: Seventy-second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food.* WHO Technical Report Series. 959.

Kim, J.H., Maeda, T., & Morita, N. (2006). Effect of fungal α-amylase on the dough properties and bread quality of wheat flour substituted with polished flours. *Food Research International, 39,* 117-126.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Hanft, F., & Koehler, P. (2006). Studies on the effect of glucose oxidase in bread making. *Journal of the Science of Food and Agriculture, 86*, 1699-1704.

Harada, O., Lysenko, E.D., & Preston, K.R. (2000). Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics. *Cereal Chemistry, 77*, 70-76.

Haros, M., Rosell, C.M., & Benedito, C. (2002). Effect of different carbohydrases on fresh bread texture and bread staling. *European Food Research and Technology, 215*, 425-430.

Hui Y.H., Corke, H., De Leyn, I., Nip, W.K., & Cross, N. (2006). *Bakery Products Science and Technology*. (eds). Ames, IA: Blackwell Publishing.

Irfan, M., & Syed, Q. (2012). Partial purification and characterization of Xylanase from *Trichoderma viride* produced under SSF. *International Journal of Applied Research in Natural Products, 5*, 7-11.

Ladd, J.N., Butler, J.H.A. (1971). Inhibition by soil humic acids of native and acetylated proteolytic enzymes. *Soil Biology and Biochemistry, 3*, 157-160.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer*. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *25*, 732–744.

Moayedallaie, S., Mirzaei, M., & Paterson, J. (2010). Bread improvers: Comparison of a range of lipases with a traditional emulsifier. *Food Chemistry, 122*, 495-499.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry, 31*, 426-428.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37,* 21-25.

Oliveira, D.S., Telis-Romero, J., Da-Silva, R., & Franco, C.M.L. (2014). Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread quality. *Food Chemistry, 143,* 139-146.

Pestka, J.J. (2010). Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal*, *3*, 323–347.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Pyler, E.J. (1973). *Baking Science & Technology. (2nd ed.).* Chicago: Siebel Publishing.

Rani, K.U., Prasada Rao, U.J.S.,Leelavathi, K., Haridas, & Rao, P. (2001). Distribution of enzymes in wheat flour mill streams. *Journal of Cereal Science, 34,* 233-242.

Raviyan, P., Tang, J., & Rasco, B.A. (2003). *T*hermal stability of α-amylase from *Aspergillus oryzae* entrapped in polyacrylamide gel . *Journal of Agricultural and Food Chemistry, 51*, 5462-5466.

Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants, 18*, 1004-1010.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). “Emerging” mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry, 136,* 750-757.

Veraverbeke, W.S., & Delcour, J.A. (2002). Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. *Critical Reviews in Food Science and Nutrition, 42,* 179-208.

Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234-242.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology, 68,* 53-60.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry, 178*, 276-286.

Yin, L.-J., Lin, H.-H., & Xiao, Z.-R. (2010). Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology, 18*, 466-471.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

Zhou, B., Schwarz, P., He, G.-Q., Gillespie, J., & Horsley, R. (2008). Effect of enzyme pretreatments on the determination of deoxynivalenol in Barley. *Journal of the American Society of Brewing Chemists, 66*, 103-108.

13. Study VIII. Effect of xylanase and α-amylase in DON and his conjugates during breadmaking process.

Food Control (sent)

Authors: Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S.

13. Effect of xylanase and α-amylase in DON and its conjugates during the breadmaking process.

Arnau Vidal, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

13.1. Abstract

Deoxynivalenol (DON) is one of the most frequently occurring mycotoxins in wheat crops worldwide and it poses a risk to human and animal health due to its wide range of adverse effects. DON-3-glucoside is a DON plant conjugate which is widely found in cereal products. As their accumulation at field seems to be unavoidable, it is very important to investigate all conditions affecting their stability during food processing. Fermentation temperature and time were assayed to assess the mycotoxin stability. Moreover different α-amylase and xylanase concentrations were added to the dough to be fermented. DON was reduced during fermentation and baking however the reduction for each step is related to fermentation temperature. α-amylase and xylanase presence caused increases of DON during fermentation and at early baking. DON-3-glucoside was slightly reduced after fermentation and it was widely increased (> 80 %) after baking. DOM-1 increased during the breadmaking process.

Keywords: deoxynivalenol, deoxynivalenol-3-glucoside, breadmaking, α-amylase and xylanase.

13.2. Introduction

Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins (Samar, Fontán, Resnik, & Pacin, 2003; Pleadin et al., 2013). To date, over 300 mycotoxins have been identified, however, not all of them represent a risk in food. Mycotoxins are produced by fungi, the main mycotoxin-producing fungi in foods belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Different studies show the high presence of mycotoxins, especially deoxynivalenol (DON), in products of high consumption like beer and bakery products (Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Cano-Sancho et al., 2011). DON is one of the most regular contaminants in cereals (Jelinek, Pohland, & Wood, 1989). Although it is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010 a,b).

On the other hand, some DON conjugates have been identified in cereals and the co-occurrence of free and modified DON forms has been docuemented. Thus, DON-3-glucoside, a plant conjugate, is the most common DON conjugate found in cereals and the ratio of DON-3-glucoside/DON concentrations in unprocessed cereals is similar among assays, ranging from 0.1 to 0.3 (Berthiller et al., 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013; De Boevre et al., 2012). 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), fungal conjugates, are also very common in cereals however the concentrations are usually lower than DON-3-glucoside (Amarasinghe, Simsek, Brûlé-Babel, & Fernando, 2016; De Boevre et al., 2012; Tibola, Fernandes, & Guarienti, 2015). The presence of DON conjugates in cereals is of concern because Berthiller et al. (2011) showed that some lactic acid bacteria hydrolyse DON-3-glucoside *in vitro* and 3-ADON and 15-ADON are rapidly converted to DON during digestion (Broekaert, 2015; Veršilovskis et al., 2012).

Due to the high presence of DON and ts conjugates in raw wheat, it is important to study their stability during food processing. Processing of cereals at high temperatures may affect DON and its conjugates content. However, the extent of DON and its conjugates reduction during the bread making process seems to be quite variable and dependent on the processing conditions applied: temperature, time, type of mycotoxin, enzymes, and size of cereal product. For bakery products, some studies reported a significant decrease in DON levels during bread baking process (Numanoglu, Gökmen, Uygun, & Koksel, 2012; Valle-Algarra, Mateo, Medina, Mateo, Gimeno-Adelantado, & Jiménez, 2009). By contrast, the studies of De Angelis, Monaci, Pascale and Visconti (2013) and Zachariasova, Vaclavikova, Lacina, Vaclavik and Hajslova (2012) reported that DON is stable in processing steps. Moreover a possible release of DON from the flour could occur (Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016) resulting in an increase of DON after baking.

Similar to DON, DON-3-glucoside stability during baking is also affected by the different size of assayed products, causing variable results in past studies (Generotti et al., 2015; Suman, Manzitti, & Catellani, 2012; Vidal Morales, Sanchis, Ramos, & Marín, 2014; Vidal,

Marín, Morales, Ramos, & Sanchis, 2014; Zachariasova et al., 2012). However, Vidal et al. (2015) showed that DON-3-glucoside may be released under mild baking conditions of temperature and time (for instance, 140 ºC for 35 minutes or 200 ºC for less than 10 minutes),but reduced under harsher baking conditions (i.e., longer periods of time and higher temperatures).

One important factor affecting DON and DON-3-glucoside stability during the bread making process may be enzyme presence. Vidal et al. (2016) showed that some enzymes (xylanse, cellulase, α-amylase, etc.) could also produce a change in the mycotoxin concentration. Besides, the effect of enzymes presence is related to temperature fermentation.

The current study aimed to investigate the fate of DON and his conjugates during fermentation at different times and temperatures and baking of bread making process. The effect of xylanase and α-amylase was checked during breadmaking process.

13.3. Materials and methods

13.3.1. Initial levels of DON and DON-3-glucoside in flour and bran

Flour and bran wheat were purchased from a flour mill in Lleida (Spain) and were analysed for natural DON and DON-3-glucoside contaminations. The initial DON concentration in the flour was 251 ± 30 µg/kg (n = 3); DON-3-glucoside was not detected. In the bran, DON and DON-3-glucoside concentrations were 2004 ± 72 and 579 ± 61 µg/kg (n = 3), respectively.

13.3.2. Dough preparation and baking

A flour + bran mix was prepared (200 g of bran/1000 g of flour) and used for the bread experiments. Concentrations of DON and DON-3-glucoside found were 402.6 ± 25.7 and 117 ± 21 µg/kg (n = 6), respectively, in the mix of flour + bran.

To each flour + bran mix (156 g), 2.3 g of salt, 4.7 g of sucrose, 4.7 g of lard and 6.2 g of commercial compressed yeast (*Saccharomyces cerevisiae*, Levanova, Lesaffre Ibérica, S.A., Spain) were added. The dough was obtained by adding 83 mL of water to the mixture. Different doughs were prepared, containing two different enzymes (xylanase and α-amylase) at two different concentration plus a control. The enzyme concentrations were adjusted according to breadmaking standards: 1 and 2.5 U of xylanase/g flour (Oliveira, Telis-Romero, Da-Silva, &

Franco, 2014) and 10 and 20 U of α-amylase/g flour (Kim, Maeda, & Morita, 2006). The enzymes were added in powder form. Moreover, the fermentation was conducted separately at 30 or 45 ºC. Thus, 10 treatments [(4 enzyme treatments+control) x 2 temperature levels] were tested in the study, and the experiment was repeated three times.

Dough was manually kneaded until it was held together with a non-sticky, smooth and satiny appearance and had optimum handling properties. Rounded pieces weighing 250 g each were prepared. From this point, thermoprobes (Thermo Bouton, Proges Plus, France) were used in the dough to record fermentation and baking temperatures; specifically, probes were placed in the centre of the loaf and close to the surface. Next, the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 or 45 ºC. Samples of 25 g were taken from each dough before fermentation, and after 20, 40 and 60 minutes (final point). Samples were lyophilized and stored at -20 ºC until mycotoxin analysis. The proofed doughs were then baked in an oven (Eurofred PE46SVR, Eurofred, Spain). Baking conditions were 180 ºC and 75 min. Such conditions were used to obtain suitable bread based on previous experiments. A 25 g sample was taken after 15 minutes of baking, lyophilized and stored at -20 ºC. After baking, a representative sample was taken, lyophilized and stored at -20 ºC until the analyses were performed.

13.3.3. Chemicals and reagents

Mycotoxin standard solution of DON, DON-3-glucoside, 3-ADON, 15-ADON, DOM-1 and isotolabeled ($^{13}C_{15}$) DON were supplied by Biopure (Tulln, Austria). ($^{13}C_{15}$) DON was used as internal standard for UPLC-MS/MS. Acetonitrile (99.9%), methanol (99.9%) and ethanol (99.5%) were purchased from J.T. Baker (Deventer, The Netherlands). Dichloromethane (≥ 99.8%) and ammonium acetate (≥98%) were purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain). All solvents were LC grade.

13.3.4. Preparation of standard solutions

The standard solutions of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 were dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 ºC in a sealed vial until use. DON concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis. Working standard solutions (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the chromatographic system.

13.3.5. Sample preparation and analysis

13.3.5.1. Extraction of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 for UPLC-MS/MS analysis

The method followed is described in Vidal et al. (2015) but it is briefly summarised: 1 g of ground sample was extracted with 7 mL of extracting solution (54 % water, 45 % acetonitrile and 1 % acetic acid) by magnetically stirring for 20 min. Then the sample was centrifuged for 10 min at 1780 g. 2 mL of the supernatant were mixed with 2 mL of dichloromethane and the mixture was stirred for 10 min and then it was centrifuged for 3 min at 500 *g* for the separation of the two phases. The upper layer was kept in a vial.

Chromatographic separation was performed by a 100 mm x 2.1 mm i.d., 1.8 µm, Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). Column temperature was held at 40 ºC. The mobile phases consisted of Acetonitrile (A) and 10 mM $AcNH_4$ in water (B). The gradient was as follows: start with 20 % A, then from 0.5 min a linear increase to 50 % for 2.5 min, then at min 3 it is 50 %. At min 3.01 the mobile phase changes to 100 % A till min 4.5; and at min 4.51 it switches to 20 % A, and then column is equilibrated for 1.5 min before the next injection. The flow rate was 300 µL/min, and an injection volume of 2.5 µL was enabled.

For mass spectrometric detection, ultrahigh-resolution orbitrap technology was used. The operation parameters of the orbitrap MS were optimized for heated electrospray interface in both positive and negative ionization modes. Three individual transitions were monitored for each analyte, except for 3-ADON and 15-ADON, where four transitions were measured. One chromatographic run consisted of two MS/MS periods. The first period monitored analytes in positive mode (DON, DON-3-glucoside and DOM-1), whereas in the second period the negative mode was used (ADONs), and both ionization modes were run simultaneously by polarity switching.

All measurements were done with the following settings: source temperature 150 ºC, desolvation temperature 350 ºC, cone gas flow was 2.5 L/min, desolvation gas flow was 16.7 L/min, collision gas flow 0.17 mL/min and the capillary was 3000 V. The analyte-dependent MS/MS parameters were optimized via direct infusion of reference standard solutions. Method performance characteristics for DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 are summarized in Table 13.1.

Table 13.1. Method performances for DON, deoxynivalenol-3-glucoside, 3-ADON, 15-ADON and DOM-1.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d](%)
DON	0.7	2.1	3	74.53	75.1±10.6	11.1
			3	501.35	88.5±8.7	8.8
			5	1002.70	72.9±8.8	8.9
DON-3-glucoside	1.6	4.8	3	5.61	38.3±5.7	5.9
			3	37.74	54.1±10.1	10.9
			5	75.48	48.5±3.2	3.3
3-ADON	1.5	4.5	3	5.61	87.4±4.7	5.1
			3	37.74	85.4.±6.2	6.5
			5	75.48	88.2±8.6	8.9
15-ADON	1.5	4.5	3	5.61	89.0±7.2	7.5
			3	37.74	89.6±9.0	9.6
			5	75.48	78.1±2.3	2.9
DOM-1	0.5	1.5	3	5.56	82.8±4.6	5.8
			3	37.37	84.3±8.4	8.1
			5	74.74	78.4±10.5	10.8

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation

13.3.5.2. Enzyme activity quantification

To quantify the enzymatic activity in each sample, a calibration curve was required. Moreover, a blank reference was required to set the spectrophotometer to zero absorbance. The following common steps were used in each enzyme protocol:

- The blank reagent was prepared (with neither substrate nor enzyme, only reagents). The blank reagent was used to set the spectrophotometer to zero absorbance.

- Calibration samples were prepared using substrates with known added enzyme concentrations plus a zero sample with no added enzyme. Calibration curves were built for fermented dough and for bread.

The activities of the two enzymes were analysed by following the reduction of DNS to 3-amino-5-nitrosalicylic acid (ANS) spectrophotometrically at 540 nm (Miller, 1959). The extraction solution was the same for the two analysed enzymes (250 mL of 0.2 M malic acid, 250 mL of 0.35 M sodium hydroxide, 250 mL of 0.2 M sodium chloride and 250 mL of 0.003 M sodium azide; all of these components constituted 1 L of extraction solution). DNS reagent was prepared with 1 g of DNS, 20 mL of 2 M sodium hydroxide, 10 mL of 10.6 M sodium and potassium tartrate and 70 mL of water.

13.3.5.2.1. Xylanase activity

Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL of extraction solution without shaking. A total of 0.5 mL of the supernatant was added to 1.5 mL of the substrate (5 g of wheat fibre in 80 mL of water, boiled for 15 min with agitation, followed by 15 minutes of agitation at room temperature; 10 mL of 1 M sodium acetate was then added; the solution then reached a volume of 100 mL with water). The tubes were heated for 8 minutes at 50 ºC, and 3 mL of the DNS reagent was added at the end of the heating step. Subsequently, the tubes were placed in boiling water for 5 minutes. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath. Cooling to ambient temperature was necessary because of the effect of temperature on the absorbance of the coloured reaction product. After cooling, the absorbance at 540 nm was measured using the spectrometer. To generate calibration curves, xylanase calibration samples (5, 2.5, 1, 0.5, 0.1 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.75 and 0.99 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

13.3.5.2.2. α-Amylase activity

Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL of extraction solution without shaking. Then, 0.5 mL of the supernatant was added to 1.5 mL of the substrate (1 g of starch in 20 mL of 1 M sodium hydroxide; 10 mL of 10.6 M sodium and potassium tartrate and 70 mL of water). The tubes were kept at 25 ºC for 3 minutes, and then 1 mL of the DNS reagent was added. The tubes were placed for 5 minutes in boiling water. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath, and 10 mL of water were then added. Finally, absorbance was measured in the spectrometer at 540 nm. α-amylase calibration samples (50, 10, 5, 1, 0.5 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves (r^2 values were 0.86 and 0.96 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

13.3.6 Statistical analysis

Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin levels. Moreover, multiple linear regressions were applied to assess the temperature/time effect of DON and DON conjugates during the bread making process.

13.4. Results and discussion

13.4.1. DON stability during bread making process

DON concentration was reduced after fermentation without enzymes ($p < 0.05$) moreover the DON reduction was larger when the fermentation temperature was 45 º C (19.47 ± 6.45 %) than 30 ºC (7.18 ± 2.43 %) ($p < 0.05$). A gradual DON reduction through the fermentation is observed (Table 13.2). DON was slightly reduced after 15 minutes of baking when enzymes were not present in the recipe (10.97 ± 2.79 %). At the end of all bread making process the DON concentration was reduced 25.67 ± 7.15 %. A tendency of a major reduction in baking after fermentation at 30 ºC (18.87 %) compared to 45 ºC (8.45 %) was observed. α-amylase prevented from DON reduction during fermentation, specially, when the α-amylase concentration used was higher and the fermentation temperature was 30 ºC (Table 13.2), in the same way, DON concentration was not reduced after 15 minutes of baking and a small reduction was got after baking. So, DON concentration in the breads with α-amylase presence was higher than in control ones (Table 13.2). Xylanase caused the increase of DON during fermentation, especially when the fermentation was at 45 ºC ($p < 0.05$) (Table 13.2). DON reduction after baking was similar with the presence of xylanase, however the increase of DON detected during fermentation caused a higher DON concentration in the final breads with xylanase than in control ones ($p < 0.05$).

DON was reduced during fermentation and it has been observed in several other studies (Neira et al., 1997) while other studies reported DON increases at the end of fermentation (Bergamini et al., 2010; Lancova et al., 2008; Vidal et al., 2014). It seems that enzyme presence affects DON stability during fermentation leading to an increase of this toxin. Simsek et al. (2012) detected an increase of up to 99 % in DON after fermentation using α-amylase. Suman et al. (2012) detected an increase of up to 14 % in DON using non-specific enzymes. Moreover, Vidal et al. (2014a), using flour improvers with non-specific enzymes, detected a 30 % increase in DON during fermentation. Moreover, sourdough use also led to increases in DON content during fermentation (Vidal et al. 2014b). Vidal et al. (2016) showed the importance of xylanase, α-amylase and cellulase in the increase of DON during fermentation; the present results fully agree with those presented in this publication. The increase of DON due to enzyme activity could be associated with the release of DON from the wheat matrix through enzyme catalysis.

Vidal et al. (2016) also observed the bigger DON reduction at higher temperatures. Samar et al. (2001) assayed different temperatures of fermentation (from 30 to 50 ºC). They found that reductions in DON concentrations were greater as the temperature increased (from 0 to 56 %). The highest reduction in DON levels was observed at the highest temperature (50 ºC) and the longest time tested (60 min). Besides, Generotti et al. (2015) assayed different

fermentation temperatures (from 26 to 46 ºC), and they found that that the stability of DON was reduced at higher fermentation temperatures. Thus, fermenting at high temperatures may be a feasible alternative to reduce DON content in bread, as long as bread quality is not affected. Although proofing temperature can be as high as 54 ºC (Pyler, 1973), most authors agree that a range from 27 to 46 ºC is optimal for bread production (Hui, Corke, De Leyn, Nip, & Cross, 2007). A fermentation temperature of approximately 30 ºC maximizes taste due to the high production of lactic acid. Conversely, fermentation above 40 ºC can reduce the quantity of lactic acid and result in tasteless breads; however, higher fermentation temperatures can also lead to improved baking volumes (Dobraszyk, Smewing, Albertini, Maesmans, & Schofield, 2003). When enzymes are present, fermentation temperature is also important because differences have been detected in function of temperature and DON stability. Thus, DON increases have been detected due to α-amylase presence when the fermentation temperature was at 30 ºC. On the other hand, DON increases have been found with xylanase presence when the fermentation temperature was at 45 ºC. These differences related to the optimum enzyme activity temperature (Table 13.3). The more marked effect on DON when α-amylase was present at 30 ºC was related to the activity of the α-amylase from *Aspergillus oryzae* (Evstatieva, Nikolova, Ilieva, Getov, & Savov, 2010). Xylanase caused a bigger DON increase at 45 ºC because the optimum temperature for xylanase activity is at 45 ºC (xylanase produced by *Trichoderma longibrachiatum*; Chen, Chen, & Lin, 1997). The enzyme concentration was also important because the breads with higher enzyme concentration showed higher DON increase. In summary, DON is reduced at the end of fermentation if there is not enzyme presence and the reduction depends on the fermentation temperature. On the other hand, α-amylase and xylanase minimize DON reduction and even a DON increase can be detected at certain temperatures and enzyme concentration.

Fifteen minutes after baking DON reduction was detected in breads without enzymes and DON concentration remained similar till the end of baking. Thus almost all DON reduction occurred in the first stage of baking. Probably, DON is only reduced in the external layers of bread because inside of the bread temperatures were lower than 100 ºC and external layers reached high temperatures very soon. On the other hand, α-amylase and xylanase presence minimised this reduction and DON increases were detected at the end of this first baking stage. The DON increase was specially marked when xylanase was used after fermentation at 30 ºC. Probably, xylanase activity reached its optimum during this first stage of baking.

DON reduction in the absence of added enzymes during baking critically depended on the fermentation temperature ($p < 0.05$). Considering the entire breadmaking process, the DON concentration in bread was similar regardless of the fermentation temperature (mean reduction from beginning to end approximately 25 %). The reduction in DON content during baking is consistent with most previous studies, which have reported a reduction in DON content at temperatures over 170 ºC as long as the baking time was longer than 45 min (Vidal et al., 2014a). The results of this study are consistent with the response surface model for DON

reduction in bread baking formulated by Vidal et al. (2014a). A slight effect of baking was observed in bread fermented at 45 ºC. This fact may be caused by the lower initial DON concentration in this case. Reduction of DON levels during baking has been shown to be higher at higher initial toxin concentrations and not significant at lower initial DON concentrations (Vidal et al. 2014a, 2015). Enzymes avoided the DON reduction due to baking process, however, this was caused by the increase in the first stage of baking, because a reduction was observed afterwards. Reduction of DON levels may result in thermodegradation products (norDONs A-F and DON lactones), which are less toxic than DON itself. The losses that cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006). Still, some existing studies have reported no reductions of DON levels or even slight increases during baking, which may be attributed to extended enzymatic activity at the early stages of baking (Bergamini et al. 2010; Simsek et al., 2012; Suman et al., 2012).

13.4.2. DON-3-glucoside stability during bread making process

DON-3-glucoside concentration was slightly reduced after fermentation, 7 % approximately without differences due to fermentation temperature (Table 13.4). But an extremely high increase of DON-3-glucoside was detected after baking (> 80 %), the increase happened in the final stage of baking. The presence of α-amylase and xylanase did not affect the DON-3-glucoside concentration during fermentation although some slight increases were detected with α-amylase presence. The baking step caused a similar DON-3-glucoside increase with the presence of enzymes with a DON-3-glucoside increaseof 60 % in all treatments (Table 13.4).

Detected reduction in DON-3-glucoside after dough-proofing without enzymes is consistent with previous studies (Kostelanska et al., 2011; Vidal et al., 2016). Despite a reduction in the DON-3-glucoside concentration at the end of fermentation, α-amylase and xylanase at optimum temperatures 30 and 45 ºC respectively and at high enzyme concentrations led to slight increases in DON-3-glucoside concentrations (Table 13.4).

Use of enzymes as improvers in past studies caused increases in DON-3-glucoside during fermentation; however, the exact enzymes responsible for this increase were not described (Kostelanska et al. 2011; Vidal et al., 2014a, 2014b). Simsek et al. (2012) used only α-amylase and detected a reduction in DON-3-glucoside at the end of fermentation at 30 ºC (5 %) (similar to the present study).

Changes in DON-3-glucoside after baking observed in this study are consistent with the results from previous studies on wheat products (Vaclavikova et al., 2013; Vidal et al., 2014b, 2015); however, some studies have shown a reduction in DON-3-glucoside after baking (Kostelanska et al., 2011; Simsek et al., 2012). Vidal et al. (2015) showed that DON-3-glucoside

exhibits different behaviours in thermal treatments as a function of the size of the product, temperature and time. The mild baking conditions tested in this assay (especially due to the large size of the product) promoted an increase in DON-3-glucoside during baking, while harsher treatments would have led to a reduction in DON-3-glucoside. Our results demonstrate the increase occurs at the end of baking process because after 15 minutes of baking DON-3-glucoside concentration did not differ from that after fermentation. This fact could be linked with the temperature inside the bread, which increases slowly. DON-3-glucoside may be bound to flour components and released during baking. In general, no significant correlation was found between increases in DON-3-glucoside and decreases in DON or vice versa. Similarly, Kostelanska et al. (2011) concluded that the behaviours of the two mycotoxins were not linked because, in their study, the concentration of DON did not change. These authors suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may occur. More studies that examine the relationship between DON and DON-3-glucoside are necessary to fully understand the interactions between parent and conjugated mycotoxins in food processes. Finally, the increase in DON-3-glucoside during baking is of concern because, although DON-3-glucoside is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003), DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more importantly, microbial activity in the intestines. The activity of chemical hydrolases and intestinal microbes has been demonstrated *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011); thus, the presence of these enzymes and microbes is important for food safety.

13.4.3. Other DON conjugates stability during bread making process

Acetyldeoxynivalenol conjugates (3-ADON and 15-ADON) were not detected during breadmaking process. On the other hand, DOM-1 was detected in all the analysed samples. DOM-1 was not reduced during fermentation and even increases of it have been detected during this step. Fermentation temperature affects the DOM-1 stability because the DOM-1 increase was always higher at 30 ºC than at 45 ºC ($p < 0.05$). Baking caused an important DOM-1 increase (> 20 %) and it resulted in a higher DOM-1 concentration after breadmaking process in bread.

Acetyldeoxynivalenol conjugates are fungal conjugates and their concentration is normally very scarce in wheat flour, for instance, Yang et al. (2013) found only one sample with 3-ADON from four analysed wheat grain samples. The study of scientific cooperation (SCOOP, 2003) task 3.2.10 pointed out that only 8% of the wheat samples contain 3-ADON. In our case 3-ADON could come both from naturally contaminated flour or be produced by the inoculated *F. graminearum.* The information about 3-ADON stability during bread making is scarce, however, the levels of 3-ADON tended to decrease with increasing temperature and baking time (Vidal et

la., 2015) and other studies pointed out a fast transformation of 3-ADON to DON, norDON A, norDON B, and norDON C, when 3-ADON was submitted either to alkali or heat (Young, 1986).

Table 13.2. Mean DON concentration (µg/kg) ± SD in fermented doughs and breads, percentage of reduction compared to the unfermented dough (%), reduction in the baking step (%) and total reduction (%) in the final product compared to the unfermented dough for each type of treatment.

			Time (minutes)								
			Fermentation					Baking			Bread
			0	20	40	60		75	135		
Treatment	Enzyme concentration	Fermentation temperature (°C)	Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Fermentation reduction (%)	Conc. (µg/kg)	Conc. (µg/kg)	Baking reduction (%)	Total reduction (%)
Control		30	339.4 ± 31.2	324.7 ± 30.7	334.0 ± 9.5	314.7 ± 24.0	7.2	272.6 ± 17.3	254.9 ± 31.4	19.0	24.9
		45	353.1 ± 30.2	297.7 ± 3.6	307.1 ± 10.9	283.6 ± 22.0	19.5	259.1 ± 19.3	258.8 ± 28.6	8.7	26.7
α-amylase	High	30	287.0 ± 2.6	302.7 ± 10.5	297.5 ± 7.4	312.5 ± 24.1	-8.9	316.3 ± 10.8	314.5 ± 9.7	-0.6	-9.6
		45	311.1 ± 6.9	287.8 ± 12.1	286.3 ± 14.2	302.9 ± 18.9	2.6	309.2 ± 9.6	321.6 ± 10.4	-6.2	-3.4
	Low	30	280.6 ± 21.2	289.5 ± 10.9	294.9 ± 32.9	286.5 ± 10.8	-2.1	317.0 ± 8.7	313.2 ± 20.5	-9.3	-11.6
		45	312.4 ± 24.9	292.0 ± 12.1	305.9 ± 8.9	311.9 ± 2.7	0.0	317.9 ± 21.0	316.3 ± 4.6	-1.4	-1.2
Xylanase	High	30	290.5 ± 5.9	325.2 ± 14.7	304.9 ± 3.8	293.4 ± 38.2	-1.0	312.9 ± 11.1	306.6 ± 20.4	-4.5	-5.5
		45	290.7 ± 18.9	295.9 ± 8.7	313.3 ± 31.7	306.5 ± 28.9	-5.4	314.4 ± 16.4	304.0 ± 24.9	1.9	-4.5
	Low	30	307.1 ± 22.2	282.9 ± 36.5	286.7 ± 19.9	294.5 ± 3.9	4.1	308.1 ± 25.8	304.5 ± 26.2	-3.4	1.5
		45	307.5 ± 3.7	291.2 ± 15.4	301.1 ± 18.5	311.2 ± 24.1	-1.2	306.1 ± 34.8	294.1 ± 15.3	6.5	4.4

Table 13.3. Mean enzymatic activity (units) ± SD for α-amylase and xylanase in fermented doughs and breads.*

Temperature of fermentation (°C)		30				45			
		Fermented dough		Bread		Fermented dough		Bread	
	Concentration	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added
α-Amylase (U/g)	20	<0.05	56.5± 6.25*	<0.05	5.26± 1.21*	<0.05	50.6 ± 12.97*	<0.05	4.81 ± 1.4
	10		29.2 ± 11.32*		3.25 ± 0.76*		22.97 ± 10.18*		2.68 ± 0.49*
Xylanase (U/g)	2.5	0.16 ± 0.06	1.2 ± 0.34*	<0.05	<0.05	0.51 ± 0.09	1.45± 0.12	<0.05	<0.05
	1		0.42 ± 0.12*		<0.05		0.58 ± 0.10		<0.05

* There are significant differences compared to the sample without added enzymes at the same fermentation temperature ($p < 0.05$).

Table 13.4. Mean DON-3-glucoside concentration (µg/kg) ± SD in fermented doughs and breads, percentage of reduction compared to the unfermented dough, reduction in the baking step (%) and total reduction (%) in the final product compared to the unfermented dough for each type of treatment.

			Time (minutes)								
			Fermentation						Baking		
Treatment	Enzyme concentration	Fermentation temperature (°C)	0	20	40	60		75	135		
			Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Fermentation reduction (%)	Conc. (µg/kg)	Conc. (µg/kg)	Baking reduction (%)	Total reduction (%)
Control		30	82.3 ± 1.7	73.0 ± 6.1	68.6 ± 5.3	77.1 ± 5.8	6.3	72.9 ± 5.9	159.9 ± 21.1	-107.4	-94.3
		45	82.8 ± 9.0	77.6 ± 11.4	73.6 ± 4.7	75.6 ± 1.5	8.7	90.1 ± 9.8	151.3 ± 5.0	-100.1	-82.7
α-amylase	High	30	65.8 ± 7.4	77.3 ± 0.9	77.2 ± 11.8	77.2 ± 4.6	-17.3	85.7 ± 4.5	151.6 ± 19.1	-96.4	-130.4
		45	77.6 ± 4.5	69.6 ± 9.4	74.0 ± 5.0	75.2 ± 5.8	3.1	88.7 ± 8.4	173.9 ± 9.7	-131.2	-124.1
	Low	30	72.2 ± 6.4	76.7 ± 4.1	71.8 ± 6.7	78.5 ± 4.1	-8.7	80.9 ± 9.9	158.3 ± 5.8	-101.6	-119.2
		45	77.7 ± 1.6	70.6 ± 6.8	78.9 ± 10.6	73.1 ± 3.0	5.9	89.7 ± 8.1	169.9 ± 14.3	-132.4	-117.5
Xylanase	High	30	66.8 ± 4.6	62.9 ± 7.2	63.7 ± 8.1	66.0 ± 4.3	1.2	62.6 ± 6.4	123.9 ± 17.1	-87.7	-85.5
		45	65.6 ± 10.6	64.2 ± 9.4	65.2 ± 5.2	69.5 ± 6.4	-5.9	78.8 ± 16.4	114.2 ± 49.8	-64.3	-74.1
	Low	30	74.0 ± 18.2	57.8 ± 8.8	66.8 ± 6.8	56.2 ± 7.4	24.0	65.8 ± 11.1	128.0 ± 8.2	-127.7	-72.9
		45	64.7 ± 6.4	58.6 ± 4.6	61.9 ± 9.8	58.7 ± 6.5	9.3	70.1 ± 8.4	131.8 ± 6.9	-158.6	-103.7

Table 13.5. Mean DOM-1 concentration (µg/kg) ± SD in fermented doughs and breads, percentage of reduction compared to the unfermented dough, reduction in the baking step (%) and total reduction (%) in the final product compared to the unfermented dough for each type of treatment.

			Time (minutes)								
			Fermentation						Baking		
Treatment	Enzyme concentration	Fermentation temperature (°C)	0	20	40	60		75	135		
			Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Conc. (µg/kg)	Fermentation reduction (%)	Conc. (µg/kg)	Conc. (µg/kg)	Baking reduction (%)	Total reduction (%)
Control		30	18.7 ± 4.1	19.2 ± 3.7	18.3 ± 0.6	23.8 ± 4.1	-27.7	24.5 ± 3.6	24.5 ± 2.5	-2.9	-31.0
		45	19.7 ± 3.2	20.4 ± 4.4	20.2 ± 3.8	19.9 ± 3.2	-1.0	27.4 ± 3.8	26.4 ± 1.7	-32.6	-34.0
α-amylase	High	30	15.3 ± 0.8	21.1 ± 3.2	20.3 ± 5.5	20.4 ± 4.2	-33.3	22.7 ± 4.4	28.4 ± 2.6	-39.2	-85.6
		45	16.9 ± 2.8	19.5 ± 5.3	21.4 ± 4.4	17.1 ± 0.5	-1.2	23.5 ± 6.1	25.1 ± 5.1	-46.8	-48.5
	Low	30	14.3 ± 0.6	17.9 ± 3.4	18.2 ± 0.9	19.6 ± 3.9	-37.1	24.4 ± 2.3	28.8 ± 3.5	-46.9	-101.4
		45	18.8 ± 4.4	19.7 ± 4.1	19.9 ± 7.2	21.5 ± 2.9	-14.4	20.3 ± 1.0	28.6 ± 2.1	-33.0	-52.1
Xylanase	High	30	19.6 ± 7.7	16.4 ± 0.2	20.1 ± 4.2	19.9 ± 4.7	-1.5	20.2 ± 5.3	23.6 ± 5.4	-18.6	-20.4
		45	18.9 ± 4.9	18.8 ± 4.4	18.4 ± 5.6	15.5 ± 2.5	18.0	20.5 ± 3.6	25.8 ± 4.8	-66.4	-36.5
	Low	30	15.6 ± 4.6	17.2 ± 2.4	19.6 ± 5.2	19.5 ± 5.5	-25	25.6 ± 1.5	23.7 ± 4.7	-21.5	-51.9
		45	16.9 ± 3.7	19.4 ± 4.6	23.3 ± 0.2	19.9 ± 3.2	-17.7	24.3 ± 1.6	23.4 ± 3.1	-17.5	-38.5

Similarly to 3-ADON, 15-ADON is another fungal metabolite which has been detected in wheat grain (Yang et al., 2013). The major presence of 3-ADON versus 15-ADON is a phenotypic difference within *Fusarium* species. In southern of Europe the predominant 15-ADON genotype is more predominant (Somma et al., 2014). No studies exist on 15-ADON thermal stability; however, De Boevre et al. (2013) detected 15-ADON in processed food products, which suggests a certain stability of this mycotoxin.

DOM-1 is known to be a conjugated product from mammalian metabolism, and it is used as a biomarker for the DON exposure (Solfrizzo, Gambacorta, & Visconti, 2014). On the other hand, the detected increase in our results mainly after baking agrees with other studies which identified it as a degradation product of DON after a heat process (Mishra, Dixit, Dwivedi, Pandey, & Das, 2014 and Vidal et al., 2015). In other studies a significant negative correlation between DON and DOM-1 concentrations in those samples with quantifiable levels of both metabolites was found (Pearson correlation coefficient=-0.5884, p=0.0000), which confirms DOM-1 as a degradation product of DON caused by heat. In an aqueous system, Mishra et al. (2014) observed the formation of DOM-1 as a degradation product of DON after heating at 125 – 200 ºC. The effect on DOM-1 of xylanase and α-amylase was not studied in other studies but they did not change the behaviour of DOM-1 although if DOM-1 is a DON degradation product and DON stability is linked to enzymes, DOM-1 could be affected indirectly by the presence of enzymes. However, we were not able to detect this possible relation.

In conclusion, DON can be reduced during breadmaking process both during fermentation and baking. However the presence of α-amylase and xylanase could avoid the reduction of it. It is important to know the effect of enzymes during bread making process because the DON concentration could change in the final bread which could be over the legislation limit. So, it is important to know the features of breadmaking process before to select the wheat flour and anticipate the final DON concentration. DON-3-glucoside is slightly reduced after fermentation but a worrying increase is detected after baking (> 80 %) and enzymes have poor affect in his behaviour. The obtained results confirmed the increase of DOM-1 during baking.

13.5. Acknowledgements

The authors are grateful to the Spanish government (project AGL2014-55379-P) for financial support. A. Vidal thanks the Spanish government (Ministry of Education) for the pre-doctoral grant.

13.6. References

Amarasinghe, C.C., Simsek, S., Brûlé-Babel, A., & Fernando, W.G.D. (2016). Analysis of deoxynivalenol and deoxynivalenol-3-glucosides content in Canadian spring wheat cultivars inoculated with *Fusarium graminearum*. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 33*, 1254-1264.

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445–6451.

Broekaert, N., Devreese, M., De Mil, T., Fraeyman, S., Antonissen, G., De Baere, S., De Backer, P., Vermeulen, A., & Croubels, S. (2015). Oral Bioavailability, Hydrolysis, and Comparative Toxicokinetics of 3-Acetyldeoxynivalenol and 15-Acetyldeoxynivalenol in Broiler Chickens and Pigs. *Journal of Agricultural and Food Chemistry, 63*, 8734-8742.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., & Marín, S. (2011). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22,* 490-495.

Chen, C., Chen, J.-L., & Lin, T.-Y. (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. *Enzyme and Microbial Technology, 21,* 91-96.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal*, *6*, 83-91.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: An investigation by high-

performance liquid chromatography high-resolution mass spectrometry. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 345-355.

De Boevre, M., Di Mavungu, J.D., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., & De Saeger, S. (2012). Natural occurrence of mycotoxins and their masked forms in food and feed products. *World Mycotoxin Journal, 5*, 207-219.

Dobraszczyk, B.J., Smewing, J., Albertini, M., Maesmans, G., & Schofield, J.D. (2003). Extensional rheology and stability of gas cell walls in bread doughs at elevated temperatures in relation to breadmaking performance. Cereal Chemistry, 80, 218-224.

Evstatieva, Y., Nikolova, D., Ilieva, S., Getov, L., & Savov, V. (2010). Identification and characteritzation of α-amylase and endoxylanase, produced by *Aspergillus* mutant strains. *Biotechnology & Biotechnological Equipment, 24,* 613-617.

Freilich, J. (1949). Time, temperature and humidity: Factors in dough proofing. *Baker's Dig, 33*, 27-30.

Generotti, S., Cirlini, M., Malachova, A, Sulyok, M, Berthiller, F, Dall'Asta, C., & Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins, 7,* 2773-2790.

Harada, O., Lysenko, E.D., & Preston, K.R. (2000). Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics. *Cereal Chemistry, 77*, 70-76.

Hui Y.H., Corke, H., De Leyn, I., Nip, W.K., & Cross, N. (2006). *Bakery Products Science and Technology*. (eds). Ames, IA: Blackwell Publishing.

IARC (International Agency for Research on Cancer). (1993). *Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 56). Lyon, France.

Jelinek, C.F., Pohland, A.E., & Wood, G.E. (1989). Worldwide occurrence of mycotoxins in foods and feeds--an update. *Journal of the Association of Official Analytical Chemists, 72*, 223-230.

Kim, J.H., Maeda, T., & Morita, N. (2006). Effect of fungal α-amylase on the dough properties and bread quality of wheat flour substituted with polished flours. *Food Research International, 39,* 117-126.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and

its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *25*, 732–744.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry, 31*, 426-428.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37,* 21-25.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. (2012). Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 423-430.

Oliveira, D.S., Telis-Romero, J., Da-Silva, R., & Franco, C.M.L. (2014). Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread quality. *Food Chemistry, 143,* 139-146.

Pacin, A.M., Resnik, S.L., Neira, M.S., Moltó, G., & Martínez, E. (1997). Natural occurrence of deoxynivalenol in wheat, wheat flour and bakery products in Argentina. *Food Additives and Contaminants, 14*, 327-331.

Pestka, J.J. (2010a). Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal*, *3*, 323–347.

Pestka, J.J. (2010b). Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology, 84*, 663-679.

Pleadin, J., Vahčić, N., Perši, N., Ševelj, D., Markov, K., & Frece, J. (2013). *Fusarium* mycotoxins' occurrence in cereals harvested from Croatian fields. *Food Control, 32*, 49-54.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Pyler, E.J. (1973). *Baking Science & Technology. (2nd ed.).* Chicago: Siebel Publishing.

Raviyan, P., Tang, J., & Rasco, B.A. (2003). *T*hermal stability of α-amylase from *Aspergillus oryzae* entrapped in polyacrylamide gel . *Journal of Agricultural and Food Chemistry, 51*, 5462-5466.

Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants, 18*, 1004-1010.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Tibola, C.S., Fernandes, J.M.C., Guarienti, E.M., & Nicolau, M. (2015). Distribution of *Fusarium* mycotoxins in wheat milling process. *Food Control, 53*, 91-95.

Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). “Emerging” mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry, 136,* 750-757.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 896-906.

Veršilovskis, A., Huybrecht, B., Tangni, E.K., Pussemier, L., de Saeger, S., & Callebaut, A. (2011). Cross-reactivity of some commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns to DON- and ZEN-conjugated forms and metabolites. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1687-1693.

Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234-242.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology, 68,* 53-60.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry, 178*, 276-286.

Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, 208*, 288-296.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

14. Study IX. Stability of DON and DON-3-glucoside with food additives presence.

Food international research (sent)

Authors: Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S.

14. Stability of DON and DON-3-glucoside during baking as affected by food additives presence.

Arnau Vidal, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*.

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

14.1. 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 like wheat flour. Parent mycotoxin can not be the only danger in mycotoxin exposure; conjugated mycotoxins can be also present in cereals products. Thus deoxynivalenol-3-glucoside (DON-3-glucoside) is a common DON plant conjugate. Mycotoxins concentration could be affected by food process and the stability mentioned mycotoxin during baking process was studied. Small wheat flour doughs were baked to assess the stability of the mentioned mycotoxins. Moreover, a range of common food additives were added to asses any possible interference: ascorbic acid (E300), citric acid (E330), sorbic acid (E200), calcium propionate (E282), lecithin (E322), diacetiltartaric acid esters of mono and diglycerids of fatty acids (E472a), calcium phosphate (E341), disodium diphosphate (E450i), xanthan gum (E415), polidextrose (E1200), sorbitol (E420i), sodium bicarbonate (E500i), and gluten of wheat and malt flour. After baking for 20 minutes at 180 ºC, DON content was reduced (40 %) and DON-3-glucoside concentration increased. However, the presence of food additives did not have any effect.

Keywords: deoxynivalenol, deoxynivalenol-3-glucoside, baking, additives.

14.2. Introduction

Deoxynivalenol (DON), also known as vomitoxin, is one of the most regular contaminants in cereals, and it can be found at relatively high concentrations in wheat and wheat containing products (like baking products) (Cano-Sancho et al., 2011a). In addition, cereals are considered the major source of human intake for DON (Cano-Sancho, Gauchi, Sanchis, Marín, & Ramos, 2011b). Although DON is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010).

Contaminated wheat grains with DON may also contain deoxynivalenol-3-glucoside (DON-3-glucoside), a plant metabolite from DON (Berthiller et al., 2009). The reported levels of DON-3-glucoside are variable, however the ratio DON-3-glucoside/DON concentration is similar among the assays, from 10 to 30 % (Berthiller et al. 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013). Moreover, Berthiller et al. (2011) showed that DON-3-glucoside can be hydrolysed to DON by several lactic acid bacteria that may be present in the intestine. Thus the FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside as an additional contributing factor of the total dietary exposure to DON (JEFCA, 2010).

Due to the high presence of DON and DON-3-glucoside in raw wheat, it is important to study their stability during baking process. Processing of cereals at high temperatures may affect DON and DON-3-glucoside content. However, the extent of DON and DON-3-glucoside reduction during thermal food processing seems to be quite variable and dependent on the processing conditions applied: temperature, time, type of mycotoxin, ingredients, and size of cereal product. For bakery products, some studies reported a significant decrease in DON levels during baking (Numanoglu, Gökmen, Uygun, & Koksel, 2012; Valle-Algarra, Mateo, Medina, Mateo, Gimeno-Adelantado, & Jiménez, 2009). By contrast, the studies of De Angelis, Monaci, Pascale and Visconti (2013) and Zachariasova, Vaclavikova, Lacina, Vaclavik and Hajslova (2012) reported that DON is stable in processing steps involving high temperatures. Sometimes, the contradictory published results are due to the different size of assayed products, which affects heat transfer and favours gradients of temperature inside the products (Vidal, Sanchis, Ramos, & Marin, 2015). Moreover a possible release of DON from the flour could be due to the presence of some added enzymes (Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016). Similar to DON, DON-3-glucoside is also affected by the different size of assayed products, causing variable results in past studies (Generotti et al., 2015; Suman, Manzitti, & Catellani, 2012; Vidal Morales, Sanchis, Ramos, & Marín, 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b; Zachariasova et al., 2012). However, Vidal et al. (2015) showed that DON-3-glucoside may be released under mild baking conditions of temperature and time (for instance, 140 ºC for 35 minutes or 200 ºC for less than 10 minutes) but reduced under harsher baking conditions (i.e., longer periods of time and higher temperatures). Furthermore, some enzymes

(xylanase, cellulase, α-amylase …) could also produce changes in the DON-3-glucoside behaviour during baking (Vidal et al., 2016).

Other additives might also produce some effect in mycotoxins stability. They are used by food industry to improve the flavour or to extend shelf life of bakery products. Some of them change the pH of the food, and DON is sensitive to pH variations (Mishra, Dixit, Dwivedi, Pandey, and Das 2014). Recent studies have tested the possible effect of additives in the DON stability, for instance, L-cysteine (E920) did not cause any variation in DON stability (Chang et al., 2015).

The current study aimed to investigate the effect of commonly used food additives in the food industry (ascorbic acid (E300), citric acid (E330), sorbic acid (E200), calcium propionate (E282), lecithin (E322), diacetiltartaric acid esters of mono and diglycerids of fatty acids (E472a), calcium phosphate (E341), disodium diphosphate (E450i), xanthan gum (E415), polidextrose (E1200), sorbitol (E420i), sodium bicarbonate (E500i), gluten of wheat and malt flour) in DON and DON-3-glucoside stability during baking.

14.3. Materials and methods

14.3.1. DON and DON-3-glucoside contaminated flours

Wheat flour was purchased in a flour mill in Lleida (Spain), and was analysed for natural DON and DON-3-glucoside contamination. The initial DON and DON-3-glucoside concentrations in the flour (n = 5) were 251.5 ± 30.3 and 60.1±15.3 µg/kg, respectively.

In order to obtain a more DON contaminated flour, one strain of *Fusarium graminearum* (TA 3.234) was used. It is kept in the Food Technology Dept. collection, University of Lleida, Spain. It was previously proven to be DON producer when cultured on wheat flour. The strain was inoculated and incubated in MEA (malt extract agar) at 25 ºC until strong sporulation. A spore suspension of the mould was made in water and Tween 80 (0.005% v/v). Five millilitres of *F. graminearum* spore suspension were inoculated in glass flasks containing 250 g of flour and 50 mL of water. In total, 3 kg of flour were inoculated with the strain. The flasks were stored at 25 ºC for 19 days with periodic shaking. Then, the content of the flasks was properly powdered and homogenized and underwent DON analysis. The content of DON was of 12,500 ± 1,235 µg/kg (n=3). DON-3-glucoside (a plant metabolite) did not change the concentration.

14.3.2. Bakery analogue preparation

Firstly, the used flour was previously prepared by mixing the uninoculated flour with the DON contaminated flour. Therefore, the concentrations of DON and DON-3-glucoside were 1210.4 ± 55.1 and 61.5 ± 13.6 µg/kg (n = 5), respectively. The level was chosen to be close to real values in food samples. The bakery product analogue was prepared for each 100 g of mix with 27 g of wheat flour, 26 g of sugar, 26 g of eggs, 21 g of sunflower oil and adding to the 100 g of mix 0.5 g of baking powder (maize starch, sodium bicarbonate and disodium diphosphate). Besides the described ingredients, for each treatment, we added a different additive/ingredient to the mix, and we did three repetitions for each additive (Table 14.1). The tested additives are regularly used in the baking industry and their concentration was consulted in references: ascorbic acid (E300), citric acid (E330), sorbic acid (E200), calcium propionate (E282), lecithin (E322), diacetiltartaric acid esters of mono and diglycerids of fatty acids (E472a), calcium phosphate (E341), disodium diphosphate (E450i), xanthan gum (E415), polidextrose (E1200), sorbitol (E420i), sodium bicarbonate (E500i), gluten of wheat and malt flour.

Table 14.1. Method performances for DON, deoxynivalenol-3-glucoside, 3-ADON, 15-ADON and DOM-1 determination in matrix food baked using acetonitrile (79%)/water (19%)/acetic acid (1%) as extraction solution with UPLC MS/MS.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d](%)
			3	150	108±15	14.2
DON	50	150	3	350	93±5	5.0
			5	750	99±1	1.6
			3	50	80±9	12
DON-3-glucoside	50	150	3	250	89±5	6
			5	500	67±11	18

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

The mix was manually mixed and 20 g aliquots were poured in small paper moulds. From this point, thermoprobes (Proges Plus, Pluck&Track, Thermo bouton) were always used in some of them to register the baking temperatures; probes were placed in the centre of the moulds. Then, they were located in the oven at 180 ºC for 20 minutes. These conditions were established on the basis of previous experiments and used conditions in the bakery industry. After baking, all samples were lyophilised for 72 h, and then the samples were stored at –20 ºC until analysis.

14.3.3. Chemicals, reagents and additives

Mycotoxin (DON and DON-3-glucoside) standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. Sorbic acid (> 98.5 %), citric acid (> 99.5 %), disodium diphosphate (> 98 %) and calcium phosphate (> 98 %) were purchased from Panreac (Castellar del Valles, Spain). Ascorbic acid (> 99 %) was purchased from Merck (Madrid, Spain). Calcium propionate (> 99 %), xanthan gum (> 99 %) sodium bicarbonate (> 99 %), sorbitol (> 98 %), diacetyltartaric acid esters of mono and diglycerids of fatty acids(> 99%) and polydextrose (> 99%) were purchased from Sigma. Lecithin was purchased from Santiveri (Barcelona, Spain). Malt flour was purchased from Puratos (Girona, Spain). Gluten of wheat was purchased from El Granero Integral (Madrid, Spain).

Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA).

14.3.4. Preparation of mycotoxin standard solutions

DON standard solution was dissolved in ethanol at a concentration of 10.0 µg/mL and stored at 4 ºC. The concentration in the stock solution was checked by UV spectrometry according to the AOAC Official methods of analysis. Working standards (2.5, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system. DON-3-glucoside standard was dissolved in acetonitrile at a concentration of 10.0 µg/mL and stored at 4 ºC in a sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL) were prepared as for DON, as well as calibration curves.

14.3.5. Mycotoxins extraction, detection and quantification

DON and DON-3-glucoside were extracted from 5 g of lyophilised ground sample (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany) with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 *g*. The supernatant was filtered through a glass microfiber filter. Five milliliters of filtered sample were loaded on the DONPREP® IAC column and the column washed with 10 mL of distilled water. DON and DON-3-glucoside were eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. Zachariasova et al. (2012) confirmed the good cross-reactivity of DON-3-glucoside with the IAC DONPREP® columns (99-

102 % recovery for DON and DON-3-glucoside when less than 500 ng of these toxins were loaded). The purified extracts were dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON and DON-3-glucoside were determined in a HPLC Waters 2695® system, with an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm, and coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The HPLC mobile phase flow rate was 0.6 mL/min, the injection volume was 100 µL, the column temperature was 40 ºC and the retention times for DON and DON-3-glucoside were 20 and 23 min, respectively.

14.3.6. Methods performance

The analytical methods for DON and DON-3-glucoside were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 were 0.98 and 0.97, for DON and DON-3-glucoside, respectively). Precision was established by determining DON and DON-3-glucoside levels in flour and DON in bakery product analogue samples at least by triplicate, in those samples fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and DON-3-glucoside are summarized in Table 14.2.

14.3.7. Statistics

The results are given in dry weight basis. Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin concentration levels as well as in the calculated percentages of increase/reduction.

14.4. Results and discussion

14.4.1. DON stability

The uncooked food matrix had a DON concentration of 350.1 ± 42.8 µg/kg. DON concentration in flour was 1108.4 ± 55.1 µg/kg but mixing ingredients produced a reduction of DON (68 %) due to dilution effect of flour. A DON concentration reduction (42 %) was achieved in control samples after baking for 20 min at 180 ºC and an average DON concentration of

201.4 ± 28.2 µg/kg was found. On the other hand, additives did not cause any significant change in DON concentration compared with control ($p > 0.05$).

Due to the high presence of DON in cereal based products (Marín, Ramos, Cano-Sancho, & Sanchis, 2013), the stability of DON during cereal processing has been widely studied, however a high variability of results exists among published studies. Fermentation in some bakery products can produce changes in DON content and contradictory results are shown in different studies (Vidal, Sanchis, Ramos, & Marín, 2016). While some of them have suggested that DON concentrations are reduced during fermentation (Neira, Pacin, Martínez, Moltó, & Resnik, 1997), others have shown that DON concentration significantly increases after fermentation (Bergamini et al., 2010; Lancova et al., 2008). It seems that fermentation temperature, enzyme addition (Vidal et al., 2016), sourdough (Vidal et al., 2014b), fermenting yeast (Generotti et al., 2016) and presence of 3-acetyldeoxinivalenol (3-ADON) and 15-acetyldeoxinivalenol (15-ADON) which are converted easily to DON (Wu and Wang, 2015) are the important factors affecting DON behaviour during fermentation. However, our bakery products were not fermented, they were sponge cakes, placed in the oven just after mixing of ingredients.

Baking caused reduction of DON in the baked products (42 %). Mycotoxins are considered highly thermostable compounds, however they can be reduced during baking but a huge amount of factors affect their stability. For this reason studies are contradictory and some studies have observed reductions in DON levels (Neira et al., 1997; Bergamini et al., 2010), while other studies have reported no changes or even increases in DON levels (Vidal et al., 2014a; Zachariasova et al., 2012). Size of the product is a very important factor, small products as used in this study allow for a fast heat transmission (Vidal et al. 2015). On the other hand, inside bread loaves the temperature is always below 100 ºC, regardless the baking temperature (Vidal et al., 2014a), contrary to our products where higher temperatures have been registered (Figure 14.1). Presence of enzymes could affect the stability of DON and xylanase and α-amylase produced increases of DON after baking (Vidal et al., 2016), because enzymes released embedded DON from the wheat matrix.

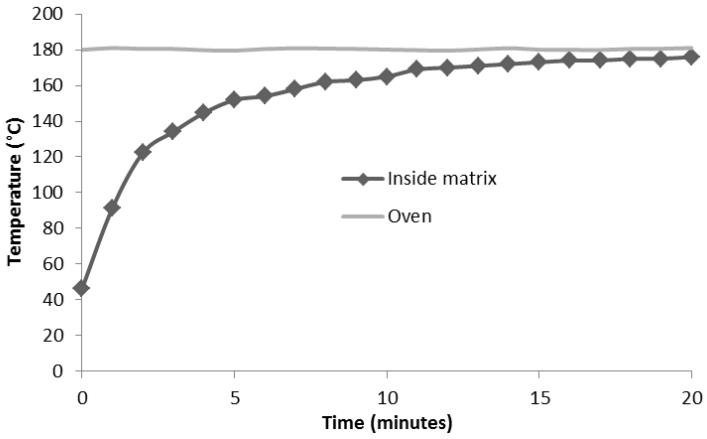


Figure 14.1. Registered temperatures inside the baked products and in the oven.

Additives added to the products did not produce any change in DON concentration compared to control ones and similar reduction was observed (Table 14.2). Some additives caused pH decreases until pH = 5.2 (ascorbic acid). But the variation of pH was not correlated with the observed percentages of reduction. Mishra et al. (2014) showed DON concentration declined sharply at pH = 3, much lower than the values recorded here Chang et al. (2015) did not get differences in the final DON concentration during bread making process when they used L-cysteine hydrochloride (E920). Boyacioglu, Heitiarachchy, and D'appolonia (1993) examined the effect of potassium bromate (E924) and ascorbic acid (E300) and these additives did not change the DON concentration during the bread making process compared with breads without additives. On the other hand, they detected drops of up to 40 % due to additive presence compared with breads withouth additive when they used sodium bisulphite (E222), l-cysteine (E920) and ammonium phosphate (E342). However, European legislation did not permit to use ammonium phosphate (E343). Sodium bisulphite (E222) and l-cysteine (E920) cause the break of disulphide bonds. Disulphide bonds are covalent bonds between the sulphur atoms of two cysteine amino acids. So, DON could be linked to these covalent bonds of the wheat matrix.

Finally, DON reduction cause the apparition of DON reduction products. Mishra et al. (2014) and Vidal et al. (2016) observed the formation of DOM-1 as a degradation product of DON after heating at 125 – 200 ºC. Moreover Bretz, Beyer, Cramer, Knecht, and Humpf (2006) detected other degradation products after heating DON, including norDON A, B and C, however the lack of standards for these products avoid the quantification of them in this assay.

14.4.2. DON-3-glucoside stability

The DON-3-glucoside concentration was below the LOD (50 µg/kg) in the uncooked food matrix. The dilution of the flour (60.1 µg/kg) during the mix caused the reduction of DON-3-glucoside concentration. However, we were able to detect DON-3-glucoside in the baked products due to the increase during the baking step. Hence, DON-3-glucoside concentration in the control baked product was 57.2 µg/kg.

DON-3-glucoside is a DON conjugate mycotoxin produced by plant as a detoxification process where the hydroxyl group in position C13 of DON is replaced by a glucose unit. DON-3-glucoside in cereal grains has been known since some years ago; Sewald, Von Gleissenthall, Schuster, Müller, and Aplin (1992) were the first to detect this DON metabolite in maize plants. The assays show the high percentage of DON-3-glucoside presence in some crops, and sometimes its presence reaches 100 % of the analysed samples in wheat, maize, oat and triticale (Berthiller et al. 2009; Desmarchelier and Seefelder, 2010; Rasmussen, Storm, Rasmussen, Smedsgaard, and Nielsen, 2010 and Dall'asta et al. 2013). Because of its high presence in cereals, DON-3-glucoside stability has been studied during baking in the last years.

The observed increase of DON-3-glucoside after baking agrees with previous similar studies where DON-3-glucoside increased (Vaclavikova et al., 2013; Vidal et al., 2014b, 2015); however, some studies have shown a reduction in DON-3-glucoside after baking (Kostelanska et al., 2011; Simsek, Burgess, Whitney, Gu, & Qian, 2012). The presence of enzymes (xylanase, amylase, cellulase, protease and glucose-oxidase) (Vidal et al., 2016), size of the product, temperature and time (Vidal et al., 2015) seem to be important factors for the stability of DON-3-glucoside during baking. Therefore mild baking conditions (for instance, 140 ºC for 35 minutes or 200 ºC for less than 10 minutes) cause increases of DON-3-glucoside at the end of baking while harsher treatments (i.e. longer periods of time and higher temperatures) would led to a reduction in DON-3-glucoside. So, the increase of DON-3-glucoside in our case was

expected because we used mild baking conditions. This increment in toxin level during baking products is proven because the ratio DON-3-glucoside/DON concentration in cereals ranges from 10 to 30 % (Berthiller et al. 2009; Desmarchelier et al. 2010, Rasmussen et al., 2010 and Dall'asta et al. 2013), while this ratio in baked products increases and even in some cases the DON-3-glucoside concentration is the same than DON concentration (De Boevre et al., 2013).

Additives did not affect the DON-3-glucoside concentration after baking (Table 14.2). Unfortunately, there are not more studies about DON-3-glucoside stability with additive presence. No relation between DON decrease and DON-3-glucoside increase has been observed in the results and the fate of the two mycotoxins could be independent. Besides, Kostelanska et al. (2011) concluded that the behaviours of the two mycotoxins were not linked because, in their study, the concentration of DON did not change. These authors suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may occur. More studies that examine the relationship between DON and DON-3-glucoside are necessary to fully understand the interactions between parent and conjugated mycotoxins in food processes.

Finally, the increase in DON-3-glucoside during baking is of concern because, although DON-3-glucoside is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003), DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, microbial activity in the intestines. The activity of chemical hydrolases and intestinal microbes has been demonstrated in vivo in swine and in vitro using human intestinal microbiota (Berthiller et al., 2011).

In conclusion, DON and DON-3-glucoside are not affected by the presence of the food additives and ingredients tested (ascorbic acid (E300), citric acid (E330), sorbic acid (E200), calcium propionate (E282), lecithin (E322), diacetiltartaric acid esters of mono and diglycerids of fatty acids (E472a), calcium phosphate (E341), disodium diphosphate (E450i), xanthan gum (E415), polidextrose (E1200), sorbitol (E420i), sodium bicarbonate (E500i), gluten of wheat and malt flour) during baking process. Moreover, it is confirmed that DON and DON-3-glucoside

concentrations can vary during heating and even increases of DON-3-glucoside are detected after baking.

Table 14.2. List of all additives and additional ingredients tested, with the E identification, concentrations used, pH conditions and the average concentration (μg/kg) ± standard deviation with % of reduction referred to the control for DON (201.4 ± 28.2 μg/kg) and DON-3-glucoside (57.2 ± 11.4 μg/kg).

Name of additives	E identification	Quantity of added additive/ingredient (%)	pH	DON		DON-3-glucoside	
				Concentration (μg/kg)	Reduction (%)	Concentration (μg/kg)	Reduction (%)
Control	-	-	6.1	201.4 ± 28.2	-	57.2 ± 11.4	-
Ascorbic acid	E300	0.02	5.2	185.7± 18.8	7.8	61.5 ± 15.9	-7.5
Citric acid	E330	0.30	5.3	195.5± 25.0	2.9	50.8 ± 10.4	11.2
Sorbic acid	E200	0.20	5.3	191.8± 35.4	4.8	68.4 ± 9.7	-19.6
Calcium propionate	E282	0.3	5.4	190.0± 11.9	5.7	57.5 ± 18.8	-0.5
Lecithin	E322	0.40	6.1	203.5± 29.7	-1.0	59.7 ± 20.2	-4.4
Diacetiltartaric acid esters of mono and diglycerids of fatty acids	E472a	0.30	6.0	209.7± 20.4	-4.1	64.8 ± 14.5	-13.3
Calcium phosphate	E341	0.25	5.8	220.5 ± 31.8	-9.5	63.9 ± 25.6	-11.7
Disodium diphosphate	E450i	0.30	6.2	183.8 ± 23.8	8.7	51.0 ± 7.6	10.8
Xanthan gum	E415	0.2	6.0	236.9 ± 11.9	-17.6	58.9 ± 25.8	-3.0
Polydextrose	E1200	26 (sugar was replaced by polydextrose)	6.1	200.8 ± 22.6	0.3	61.5 ± 13.0	-7.5
Sorbitol	E420i	3	6.1	209.4 ± 39.6	-4.0	60.8 ± 19.1	-6.3
Sodium bicarbonate	E500i	1	6.3	238.2± 30.9	-18.3	55.6 ± 22.8	2.8
Gluten of wheat	-	2	6.0	196.4 ± 19.9	2.5	64.9 ± 12.1	-13.5
Malt flour	-	1	6.0	226.0 ± 32.3	-12.2	60.8 ± 17.2	-6.3

14.5. Acknowledgements

The authors are grateful to the Spanish government (project AGL2014-55379-P) for financial support. A. Vidal thanks the Spanish government (Ministry of Education) for the pre-doctoral grant.

14.6. References

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol

(DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Boyacioglu, D., Heitiarachchy, N.S., & D'appolonia, B.L. (1993). Additives Affect Deoxynivalenol (Vomitoxin) Flour during Breadbaking. *Journal of Food Science, 58*, 416-418.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445–6451.

Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., & Marín, S. (2011a). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22,* 490-495.

Cano-Sancho, G., Gauchi, J.-P., Sanchis, V., Marín, S., & Ramos, A.J., (2011b). Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 28*, 1098-1109.

Chang, J., Li, J., Zhao, Y., Zhu, Y., Ding, N., Liu, Y. (2015). Degradation effect of deoxynivalenol in bread processing. Journal of Chinese Institute of Food Science and Technology, 15, 199-205.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal*, *6*, 83-91.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: an investigation by high-performance liquid chromatography high-resolution mass spectrometry. *Food Additives and Contaminants e Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 345-355.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J.D., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B., & De Saeger, S. (2013). Human

exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281-292.

Desmarchelier, A., & Seefelder, W. (2010). Survey of deoxynivalenol and deoxynivalenol-3-glucoside in cereal-based products by liquid chromatography electrospray ionization tandem mass spectrometry. *World Mycotoxin Journal, 4*, 29–35.

Generotti, S., Cirlini, M., Malachova, A, Sulyok, M, Berthiller, F, Dall'Asta, C., & Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins, 7,* 2773-2790.

IARC (International Agency for Research on Cancer). (1993). *Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 56). Lyon, France.

JEFCA. 2010. WHO Technical Report Series 959: *JEFCA, 2010: Seventy-second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food.* WHO Technical Report Series. 959.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer*. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *25*, 732–744.

Marín, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology, 60*, 218-237.

Mishra, S., Dixit, S., Dwivedi, P. D., Pandey, H. P., & Das, M. (2014). Influence of temperature and pH on the degradation of deoxynivalenol (DON) in aqueous medium: comparative cytotoxicity of DON and degraded product. *Food Additives & Contaminants: Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 121-131.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37,* 21-25.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. (2012). Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 423-430.

Pestka, J.J. (2010). Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal*, *3*, 323–347.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Rasmussen, R. R., Storm, I. M. L. D., Rasmussen, P. H., Smedsgaard, J., & Nielsen, K. F. (2010). Multi-mycotoxin analysis of maize silage by LC-MS/MS. *Analytical and Bioanalytical Chemistry, 397*, 765–776.

Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants, 18*, 1004-1010.

Sewald, N., Von Gleissenthall, J.L., Schuster, M., Müller, G., & Aplin, R.T. (1992). Structure elucidation of a plant metabolite of 4-desoxynivalenol. *Tetrahedron-Asymmetry, 3,* 953-960.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). “Emerging” mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry, 136,* 750-757.

Valle-Algarra, F. M., Mateo, E. M., Medina, Á., Mateo, F., Gimeno-Adelantado, J. V., & Jiménez, M. (2009). Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants e Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 896-906.

Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234-242.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology, 68,* 53-60.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry, 178*, 276-286.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016). The fate of deoxynivalenol through wheat processing to food products. *Current Opinion in Food Science, 11,* 34-39.

Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, 208*, 288-296.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

15. Study X. Stability and kinetics of leaching of deoxynivalenol, deoxynivalenol-3-glucoside and ochratoxin A during boiling of wheat spaghettis.

Food Research International (2016), 85, 182-190.

Authors: Vidal, A., Bendicho, J., Sanchis, V., Ramos, A.J., & Marín, S.

15. Stability and kinetics of leaching of deoxynivalenol, deoxynivalenol-3-glucoside and ochratoxin A during boiling of wheat spaghettis.

Arnau Vidal, Jordi Bendicho, Vicente Sanchis, Antonio J. Ramos and Sonia Marín*.

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: smarin@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

15.1. Abstract

The stability of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-glucoside) and ochratoxin A (OTA) during spaghetti production and cooking was investigated. Initial mycotoxin concentration, boiling time and use of egg as ingredient were assayed as factors. DON was stable during kneading and drying, but a consistent reduction of DON (> 40 %) was observed in boiled spaghettis. According to our results, DON was transferred to broth, where it was not degraded, and boiling time determined the extend of the transfer. A DON leaching model was fitted to data with a high goodness fit (r^2 = 0.99). This model can be used for prediction of final DON concentration in cooked pasta, and a useful tool in risk assessment models. DON-3-glucoside is totally stable through the pasta making process; moreover DON-3-glucoside is slightly released from pasta components and it is leached to broth. Similarly, OTA is also stable during pasta making, however, it is scarcely transferred to broth during boiling. The presence of egg as ingredient did not affect the final mycotoxin concentration in pasta in any case.

Keywords: deoxynivalenol, deoxynivalenol-3-glucoside, ochratoxin A, boiling, durum wheat, leaching.

15.2. Introduction

Mycotoxins are produced by fungi and can contaminate various agricultural commodities either before harvest or under post-harvest conditions. The main mycotoxin-producing fungi in food commodities belong to the genera *Aspergillus, Penicillium* and *Fusarium*. Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins. Moreover, cereal products represent one of the main sources of exposure to deoxynivalenol (DON) and ochratoxin A (OTA) (Marín, Ramos, Cano-Sancho, & Sanchis, 2013). Different studies show the high presence of mycotoxins in durum wheat (Brockmeyer & Thielert, 2004; Covarelli et al, 2014; Lippolis, Pascale, Cervellieri, Damascelli, & Visconti, 2014). In addition, it has been shown that durum wheat is generally more contaminated with DON than common wheat (Covarelli et al., 2014). The high presence of DON is of concern, because although DON is not classified as to its carcinogenicity to human by IARC (International Agency for Research on Cancer) (1993), but it is linked with human gastroenteritis. On the other hand, OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties. This mycotoxin has been classified, by the International Agency for Research on Cancer (IARC, 1993) in the group 2B, as a possible human carcinogen. Unaltered mycotoxins might not be the only source of health hazard for consumers, because there is a group of metabolites called conjugated mycotoxins which cannot be detected in the routinary mycotoxins analysis. The co-occurrence of conjugated DON forms has been documented in raw wheat, especially deoxynivalenol-3-glucoside (DON-3-glucoside) (Berthiller et al. 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013; Rasmussen, Storm, Rasmussen, Smedsgaard, & Nielsen, 2010) and it is a plant metabolite of DON (Berthiller et al., 2009). Although DON-3-glucoside presence in durum wheat has been detected (Dall'Asta et al., 2013), few studies exist on its occurrence. Berthiller et al. (2011) showed that DON-3-glucoside can be hydrolysed to DON by several lactic acid bacteria. Thus, the Joint European Commission FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside as an additional contributing factor of the total dietary exposure to DON (Codex, 2011; JEFCA, 2010).

Processing of wheat at high temperatures might affect DON, DON-3-glucoside and OTA content. Up to now, few studies exist on the fate of DON during the cooking of durum wheat pasta (Table 15.1), but significant DON reductions have been reported. Such reduction levels may be affected by some factors like ingredients and boiling time. In this way, Visconti, Haidukowski, Pascale, & Silvestri (2004) showed the importance of the pasta/water ratio: the lower the ratio the greater the reduction. Regarding boiling time, Cano-Sancho, Sanchis, Ramos, & Marín (2013) observed increasing reduction with longer times. Although important DON reductions are detected in cooked pasta, most authors confirm they are mainly attributed to the high water-solubility of DON, thermal degradation playing a minor role; thus, analysis of broth results in high DON concentrations after the boiling step (Cano-Sancho et al., 2013; Nowicki, Gaba, Dexter, Matsuo, & Clear, 1988; Visconti et al., 2004). Moreover, some enzymes can also affect DON stability (Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016) causing

important increases (> 20 %) during the breadmaking process. Enzymes have not been studied in pasta making, however, eggs are a common ingredient in pasta and they contain abundant lysozyme (Alderton & Fevold, 1946), which was not studied in Vidal et al. (2016). Vidal et al. (2016) showed that DON and DON-3-glucoside could be bound to wheat components and enzymes may cleave them releasing DON and DON-3-glucoside. Moreover, egg contains some ovoinhibitors which are protease inhibitors (Liu, Means, & Feeney, 1971) and proteases, in their turn, can have an effect in DON and DON-3-glucoside stability during breadmaking process (Vidal et al., 2016). Although the thermo stability of DON-3-glucoside during baking of wheat products has been widely studied (Kostelanska et al., 2011; Vidal, Morales, Sanchis, Ramos, & Marín, 2014a; Vidal, Sanchis, Ramos, & Marín, 2015), few studies exist about DON-3-glucoside stability during boiling (Zhang & Wang, 2015). Concerning OTA, it showed higher thermo stability than DON during baking (Vidal et al., 2015). Looking at the few existing results, OTA, as well as DON, would be reduced in boiled pasta. For example, Sakuma et al. (2013) observed approximately a 34 % of OTA reduction after 6 min (10 g of pasta with 400 mL of water), and the authors also pointed out to the transfer of OTA to broth.

The existent literature about DON, DON-3-glucoside and OTA during boiling is scarce and more information is required, in particular for exposure assessments. The current study aims to investigate the stability of DON, DON-3-glucoside and OTA during boiling assaying different factors (boiling time, initial mycotoxin concentration and egg presence) in durum wheat pasta and modelling the kinetics of reduction of DON during boiling of pasta.

15.3. Materials and methods

15.3.1. DON and OTA contaminated semolina

In order to obtain DON or OTA contaminated semolina, one strain each of *Fusarium graminearum* (TA 3.234) and *Aspergillus ochraceus* (TA 3.201) were used, respectively. Both of them are kept in the Food Technology Dept. collection, University of Lleida, Spain. They were previously proven to be DON and OTA producers when cultured on wheat flour (Vidal et al., 2014a, 2014b, 2015). The concentration of DON and DON-3-glucoside in the initial uninoculated semolina (n=3) was 286.31 ± 21.91 and 72.15 ± 15.24 µg/kg, respectively, while OTA could not be detected.

Table 15.1. Effect of boiling in DON content in pasta.

Reference	Cereal	Product	Mycotoxin	Initial mycotoxin concentration (µg/g)	Cooked spaghetti quantity (g)	Pasta/water ratio	Boiling time (min)	NaCl in water (%)	% of mycotoxin reduction	% of mycotoxin in water	Recovered toxin in pasta+water (%)
Nowicki et al., 1988	Durum wheat semolina	Spaghettis	DON	3400-4330 (Natural)	75	1:10	12	0	49.5	39.8	90.3
					75	1:10	22	0	53.4	48.1	94.8
Visconti et al., 2004	Durum wheat semolina	Spaghettis	DON	190-6370 (Natural)	25	1:5	7	0.4	79.6	58.4	78.8
					25	1:4	7	0.5	50.4	55.3	91.56
Sugita-Konishi et al., 2006	Soft wheat flour	Noodles	DON	850 (Natural)	50	1:20	10	0.2	69.4	50.58	81.2
Brera et al., 2013	Durum wheat semolina	Spaghettis	DON	140-190 (Natural)	100	1:10	-	1.0	36.1	-	-
Cano-Sancho et al., 2013	Durum wheat flour	Spaghettis	DON	620 (Natural)	-	-	2	0	38.9	22.1	83.2
							6	0	56.5	58.5	102
							10	0	74.6	73.9	99.3
Sakuma et al., 2013	Soft wheat semolina	Noodles	OTA	5-10 (Spiked)	10	1:40	6	0.1	34.1	34.3	100.2
Zhang et al., 2015	Soft wheat flour	Noodles	DON	900-6870 (Natural)	100	1:10	5	0	52.0	-	-

- = data not provided.

The strains were inoculated and incubated in MEA (malt extract agar) at 25 ºC for 14 days until strong sporulation. For the inoculation of semolina we followed the method used by Jijakli & Lepoivre (1998). Briefly, a sterile inoculation loop was used to remove the conidia, suspending them in Tween 80 (0.005 %). A spore suspension of each strain was made. After homogenizing, five millilitres of either *F. graminearum* or *A. ochraceus* spore suspension were inoculated in glass flasks containing 250 g of semolina and 50 mL of water. In total, 3 kg of semolina were inoculated with each strain. The flasks were incubated at 25 ºC for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus,* with periodic shaking. The incubation times were calculated based on our previous knowledge in recent similar studies (Vidal et al., 2015), to achieve the desired mycotoxin contamination in the semolina. Anyway, before ending the incubation period the semolina was sampled to check the concentration attained. Then, each kind of semolina (3 kg) was properly powdered and homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of 3,212.32 ± 80.70 µg/kg and 10.5 ± 0.2 µg/kg respectively (n=3), in each contaminated semolina. DON-3-glucoside was not analysed in the semolina at this stage.

15.3.2. Spaghetti production

Spaghetti was prepared with 100 g of durum wheat semolina, and 50 g of egg or 40 mL of water. The semolina used was previously prepared by mixing uninoculated semolina with DON contaminated semolina and OTA contaminated semolina, depending on the desired initial mycotoxin concentration: high mycotoxin concentration (HMC) or low mycotoxin concentration (LMC). The analysed toxin levels in the initial mixed semolina (n=3) were: a) HMC, 1310.08 ± 51.63 µg/kg of DON, 60.74 ± 4.39 µg/kg of DON-3-glucoside and 3.52 ± 0.34 µg/kg of OTA; and b) LMC, 572.65 ± 21.51 µg/kg of DON, 70.08 ± 6.50 µg/kg of DON-3-glucoside and 1.58 ± 0.22 µg/kg of OTA. The levels were chosen to be close to real values in food samples (Juan, Covarelli, Beccari, Colasante, & Mañes, 2016). Moreover, the levels were around the maximum levels set by the European Union (European Comission 1881/2006) for processed cereals, such as semolina, which are 750 µg/kg and 3 µg/kg, for DON and OTA, respectively.The DON-3-glucoside concentration was not significantly different in both semolina batches.

The dough was manually mixed until held with a non-sticky, smooth and satiny appearance and optimum handling properties. Then, dough was transferred to a roller machine to get a thin dough sheet (approximately 5 mm), which was later cut into spaghetti (Imperia 650, Imperia & Monferrina SPA, Italy). The resulting spaghettis were hung on metal bars where they were allowed to dry for 12 hours. The water content of the final product was 12.6 ± 0.3 %. Spaghetti (100 g) were cooked for 9 different times (0, 1, 2, 3, 4, 6, 8, 10 and 12 minutes) in 500 mL of broth (2.5 g NaCl), so, the ratio pasta:water was 1:5. Thus 2 initial toxin concentrations x 9 boiling times x 3 replicates made 54 different runs. Additionally, egg pasta was made with the same two different toxin concentrations, however, egg spaghettis were tested only up to 10

minutes. From the 100 g cooked pasta, 25 g were used for OTA analysis, other 25 g for DON and DON-3-glucoside analysis, and the remaining 50 g were kept at - 20 ºC. All samples were lyophilised for 72 h, and then stored at - 20 ºC until the analyses were performed. Moreover, for each run, 30 mL of broth was kept and stored at - 20 ºC until the mycotoxins analyses were performed.

15.3.3. Chemicals and reagents

Mycotoxin standard solution of OTA was supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). DON and DON-3-glucoside were supplied by Biopure (Tulln, Austria). Acetonitrile (≥ 99.9 %), methanol (≥ 99.9 %) and ethanol (≥ 99.5 %) were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) and OTA (OCHRAPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). Fresh eggs were purchased from La Receta (Madrid, Spain). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (98-100 %, Panreac, Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (99 %, Panreac, Castellar del Vallès, Spain) and sodium chloride (8.0 g) (≥ 99.5 %, Fisher Bioreagents, New Jersey, USA) in 1 L of milli-Q water; the pH was brought to 7.4 with hydrochloric acid 1 M.

15.3.4. DON, DON-3-glucoside and OTA by HPLC

15.3.4.1. Preparation of standard solutions

The standard solution of OTA was dissolved in methanol at a concentration of 500 ng/mL and stored at 4 ºC in a sealed vial until use. From this, a stock solution was prepared and confirmed by UV spectroscopy according to AOAC Official methods of analysis (Horwitz & Latimer, 2006). Working standard solutions (5.0, 1.0, 0.5, 0.01 and 0.05 ng/mL) were prepared by appropriate dilution of known volumes of the stock solution with the mobile phase and were used to obtain calibration curves in the appropriated chromatographic system. The standard solutions of DON and DON-3-glucoside were dissolved in ethanol at a concentration of 10 µg/mL and stored at 4 ºC in a sealed vial until use. DON concentration in the stock solution was confirmed by UV spectroscopy according to AOAC Official methods of analysis (Horwitz & Latimer, 2006). Working standard solutions were 5.0, 1.0, 0.5, 0.1 and 0.05 µg/mL for DON and 1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL for DON-3-glucoside. They were prepared as for OTA, as well as calibration curves.

15.3.4.2. Sample preparation and analysis with HPLC-UV and HPLC-FL

For DON and DON-3-glucoside, 5 g of ground sample was extracted with 30 mL of distilled water by magnetically stirring for 10 min. Next, the sample was centrifuged for 8 min at 1780 x *g*. Supernatant was filtered through Whatman 1 filter. On the other hand, broth was centrifuged for 10 min at 1780 x *g* and then filtered through Whatman 1 filter. In both cases, five millilitres of filtered sample was cleaned-up using a IAC DONPREP® column (R-Biopharm). Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova (2012) confirmed the robust cross-reactivity of DON-3-glucoside with the IAC DONPREP® columns (99-102 % recovery for DON and DON-3-glucoside when less than 500 ng of these toxins was loaded). DON and DON-3-glucoside was eluted by applying 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water, consecutively. The purified extracts were dried under a stream of nitrogen at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON and DON-3-glucoside were quantified using a HPLC Waters 2695® system with an analytical column (Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm, coupled with a UV/Visible dual λ absorbance Detector Waters 2487). The absorption wavelength was set to 220 nm. The HPLC mobile phase flow rate was 0.6 mL/min. The injection volume was 100 µL. The column temperature was 40 ºC. The retention times for DON and DON-3-glucoside were 20 and 23 min, respectively.

Regarding OTA, 5 g of ground sample were extracted with 30 mL of extraction solution (60 % acetonitrile, 40 % water) by magnetically stirring for 10 min and filtered with Whatman 1 filter. On the other hand, the broth was centrifuged for 10 min at 1780 x *g* and then filtered through a Whatman 1 filter. In both cases, 4 mL of filtered solution was diluted with 44 mL of PBS solution and the resulting extract was cleaned-up using an IAC OCHRAPREP® column (R-Biopharm). OTA was eluted by applying 1.5 mL of methanol grade HPLC and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under a stream of nitrogen. Each dried sample was resuspended with 0.5 mL of mobile phase (acetonitrile:water:acetic acid, 57:41:2). OTA was determined by HPLC (Waters 2695®) coupled with a Multi λ Fluorescence Detector Waters 2475®, and an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. The mobile phase flow rate was 1 mL/min, column temperature was 40 ºC, the injection volume was 100 µL, and the retention time was 15 minutes.

15.3.4.3. Methods performance for HPLC-UV and HPLC-FL

The analytical methods used were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentration (r^2 values were 0.99, 0.97 and 0.99 for DON, DON-3-glucoside and OTA, respectively). Precision was estimated by determining DON, DON-3-glucoside and OTA levels in broth and spaghettis,

in triplicate, in fortified samples prepared to calculate recovery rates. The limit of detection (LOD) was considered to be three fold greater than the signal of blank noise, and the limit of quantification (LOQ) was calculated to be 3 x LOD. Characteristics of the method performance for DON, DON-3-glucoside and OTA are summarized in Table 15.2.

Table 15.2. Performances of the DON, DON-3-glucoside and OTA determination in spaghetti and broth.

Mycotoxin	Product	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	n	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d](%)
DON	Spaghetti	50.0	150.0	3	100	93±6	5.9
				5	500	81±3	3.2
				3	1000	92±7	7.2
	Broth	2.5	7.5	3	20	91±2	14.2
				5	100	87±2	1.9
				3	500	92±6	7.2
DON-3-glucoside	Spaghetti	25.0	75.0	3	50	93±6	5.9
				5	150	82±3	3.2
				3	500	92±7	7.2
	Broth	2.0	6.0	3	5	82±4	4.3
				5	15	84±5	6.5
				3	30	84±4	4.2
OTA	Spaghetti	0.02	0.06	3	0.1	87±13	15.4
				5	1.0	81±9	11.8
				3	5.0	96±1	1.4
	Broth	0.005	0.015	3	0.05	86±4	4.3
				5	0.5	108±2	1.3
				3	1.0	102±3	3.3

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

15.3.5. Statistical analysis

Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin levels; the software used for multifactorial ANOVA was Statistics 20.0 (IBM SPSS Statistics 20.0 Inc., Chicago, IL). Moreover, linear regression was applied to assess the rates of DON, DON-3-glucoside and OTA reduction during the boiling process.

15.3.6. Equations

15.3.6.1. Mass balance

A system of mass balance was developed for DON in the boiling process. The water mass balance was made with 4 products: uncooked pasta, water before boiling, pasta after boiling and broth. The water mass balance between pasta and broth resulted in:

$$H_0 + W_0 = H_t + W_t \qquad (1)$$

H_0 = Content of water in the uncooked pasta (g).

W_0 = Weight of water before to start the boiling step (g).

H_t = Content of water in the cooked pasta at time *t* (g).

W_t = Weight of broth at time *t* (g).

From eq. *1* the W_t is isolated and the weight of the broth at time *t* is known.

$$H_0 + W_0 - H_t = W_t \qquad (2)$$

Knowing W_t a DON mass balance can be made among uncooked pasta, initial water, and cooked pasta at time 12 minutes and broth at time 12 minutes. This balance was made under the assumption than no thermal degradation of DON occurred.

$$y_0 H_0 + x_0 W_0 = y_t H_t + x_t W_t \qquad (3)$$

y_0 = weight of DON in uncooked spaghettis (ng) / (weight of DON + weight of water in pasta in uncooked spaghetti) (g).

x_0 = weight of DON in initial boiling water (ng) / (weight of DON + weight of broth) (g).

y_t = weight of DON in pasta (ng) / (weight of DON + weight of water in pasta) at time t (g).

x_t = weight of DON in broth in balance conditions (ng) / (weight of DON in broth + weight of broth) at time t (g).

When equilibrium between DON in the spaghetti and DON in the broth is reached, y_t will equal x_t.

$y_t = x_t = b \qquad (4)$

From eq. 3, the value *b* can be calculated as:

$$b = \frac{y_0 H_0}{H_t + W_t} = \frac{y_0 H_0}{H_0 + W_0} \qquad (5)$$

15.3.6.2. Kinetic calculations

According to literature, several models can be used to explain the kinetics of sorption (e.g. first-order, pseudo-first, pseudo-second-order reaction model) (Ho & McKay, 1999). The studies on

the kinetics of leaching of water-soluble compounds have revealed that the pseudo-second-order model provides the best correlation (Ho, Harouna-Oumarou, Fauduet, & Porte, 2005).

$dp_t / dt = k \cdot (p_m - p_t)^2$ (6)

Where

p_t = percentage of DON leached at time t (%).

t = time (min).

p_m = maximum percentage of DON leached (%).

k = leaching rate constant (1/min %).

Accordingly, the pseudo-second-order reaction model was applied to our experimental data in order to determine the leaching rate constant. The integrated linear form of the pseudo-second order model is

$$\frac{t}{p_t} = \frac{\mathbf{t}}{p_m} + \frac{\mathbf{1}}{p_m^2 \cdot k} \quad (7)$$

The leaching rate constant (k) comes from the interception.

15.4. Results and discussion

15.4.1. DON

Kneading and drying did not cause any difference in DON concentration because DON concentrations in semolina and in uncooked spaghettis were very similar (Table 15.3). However, DON decreased along time in cooked spaghettis ($p < 0.05$) (Figure 15.1). Although DON content in pasta dropped during boiling, no further significant DON reduction occurred from minute 2 (Figure 15.1). A similar trend was observed regardless of the initial toxin concentrations, with percentages of reduction in spaghettis above 30 %. As a result, analysed broth showed a significant increase in DON through time till minute 3-6 (Figure 15.1), due to the leaching process from pasta to broth. The presence of egg did not affect DON content neither in the preparation nor in the boiling process.

Similar to what was observed here, the existing literature on DON fate during pasta making reported a high stability of DON during kneading. For example, Visconti et al. (2004) found a non-significant slight decrease of DON (10.8 %) after the kneading and drying process;

they used a pasta extruder (40 °C at 80-100 bars) and dried the pasta at 80 and 90 °C for almost 5 hours, thus their process was harsher than ours. Also in boiling step, the levels of DON reduction in boiled pasta found in our study agreed with other studies (Brera et al., 2013; Visconti et al., 2004; Zhang et al., 2015). The results show boiling time is a crucial factor in the level of reduction. Cano-Sancho et al. (2013) tested three different times (2, 6 and 12 minutes), with higher reduction with longer time, although the levels after 6 and 12 minutes were very similar (Table 15.1). Alike, similar DON reduction after boiling for 12 minutes (48.54 %) and 22 minutes (54.30 %) were obtained by Nowicki et al. (1988). This suggests that transfer of DON from pasta to water occurs till equilibrium is reached. This equilibrium point depends on the initial DON concentration because there was more DON in the broth when the initial DON concentration was higher. That way, some authors suggested the ratio pasta/water was an important factor in DON reduction during boiling. Hence, Visconti et al. (2004) showed increasing DON reduction in pasta with decreasing ratio pasta:water (Table 15.1). The amount of DON retained by cooked spaghettis consistently decreased by increasing the pasta:water ratio during cooking. Different ratios were not tested in the present assay. This suggests that DON reduction in pasta is explained by leaching to water during the boiling process. Previous studies observed DON leaching to water but few information exists on the kinetics of such leaching process. The amount of DON in water plus that in pasta was nearly constant (Figure 15.1), thus DON thermal stability was confirmed. In fact, boiling conditions (100 °C) are mild and boiling time is short, thus this result was expectable. Baking of bread and bakery products has shown that harsh conditions are required for DON inactivation (e.g. 40 minutes at 160 °C or 20 minutes at 200 °C (Vidal et al., 2015). The high stability of DON in broth agrees with Mishra, Dixit, Dwivedi, Pandey, & Das (2014), who observed DON was only unstable at 125-250 °C showing 16-100 % degradation. Enzymes present in wheat or artificially added to doughs have shown to be important for DON fate (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal et al., 2016). The presence of egg did not cause any change in DON content during spaghetti making process. Water represents more than 75 % of total egg, the rest are mostly lipids and proteins. Regarding enzymes, lysozyme is the main enzyme found in egg and its effect on DON has not been tested. However, the short time involved in kneading and pasta production may not allow for significant enzymatic activity. To our knowledge, this is the first time different ingredients are tested to study DON stability during the boiling of pasta, although some studies exist regarding other food processes, mainly baking (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal et al., 2016).

Table 15.3. Evolution of mycotoxin concentration (mean ± standard deviation) in the different steps of pasta making process: semolina (ng/g), uncooked spaghetti (ng/g), cooked spaghetti for 10 min (ng/g) and in broth (ng/mL).

Mycotoxin		High Initial Concentration				Low Initial Concentration			
		Semolina	Uncooked spaghetti	Cooked spaghetti	Broth	Semolina	Uncooked Spaghetti	Cooked spaghetti	Broth
DON	Egg	1310.08±51.63	1323.66±98.96	640.20±18.19*	172.32±15.22	572.65±21.51	562.33±32.23	331.00±45.58*	58.76±5.13
	Without egg		1389.14±18.05	772.82±140.34*	181.60±21.52		591.88±15.68	372.40±28.63*	75.18±14.41
DON-3-glucoside	Egg	60.74±4.39	59.18±11.02	103.65±31.32	8.26±2.37	70.08±6.50	75.03±3.78	73.28±2.77	7.28±1.68
	Without egg		62.99±15.97	85.06±27.56	8.66±3.72		73.45±1.04	82.65±12.47	9.46±2.04
OTA	Egg	3.52±0.34	3.69±0.47	3.51±0.23	0.23±0.00	1.58±0.22	1.47±0.15	1.61±0.27	0.09±0.00
	Without egg		4.26±0.42	4.47±0.22	0.33±0.04		1.69±0.10	1.97±0.53	0.14±0.01

* There are significant differences compared to the previous step ($p < 0.05$).

15.4.1.1. Mass balance

Initially, DON concentration in pasta decreased quickly till a plateau was reached after six minutes; a parallel increase occurred in the broth, suggesting that an equilibrium was reached (Figure 15.1). A water mass balance between pasta and broth resulted in the application of the eq. 1 (see section 2.6.1), and in our experiment, H_0 is 12.6 because it is the average moisture find in the uncooked spaghettis. W_0 is always 500 g because we always used 500 mL of water for boiling. H_t is 253.4 g, it was the average moisture of our spaghettis cooked for 12 minutes. Eq. 2 results in a W_t = 259.2 g. Knowing W_t a DON mass balance (eq. 3) can be made among uncooked pasta, initial water, and cooked pasta at time 12 minutes and broth at time 12 minutes. We used minute 12 but any time between 6 and 12 could have been used because all of them are in equilibrium. From the eq. 3, only y_0 and x_0 are known, with y_0 = 9616.16 ng/g and 4097.24 ng/g for high and low initial DON concentration, respectively, and x_0 always 0. Then from the eq. 3 we found the *b* values which are 236.84 ng/g and 101.91 ng/g, for high and low initial DON concentration respectively. y_t found in the analysis are 276.77 ± 46.97 ng/g and 114.75 ± 22.86 ng/g. The high similarity between predicted and experimental y_t confirms that the system was in equilibrium at minute 12. Experimental x_t were 189.76 ± 29.31 ng/g and 86.67 ± 11.19 ng/g for high and low initial DON concentration, so they are also similar to predicted x_t. Thus, if equilibrium is reached at the end of the boiling time, the eq. 5 can be used directly to find the final DON concentration in boiled pasta. It is only necessary to know the DON content in uncooked pasta, the humidity of uncooked pasta, the volume of broth and the final humidity of cooked pasta. The lack of thermal effects plus the equilibrium assumptions were also tested on data from Visconti, et al. (2004), who described all information required for DON balance (Table 15.4). The obtained concentrations experimentally parallel predicted concentrations, so at the end of boiling time the system is in balance and equations can be used to know the DON concentration in boiling spaghettis. The agreement between observed and calculated data confirms that there is not DON degradation during boiling, and that only a leaching process takes place. The amount of DON detected in pasta plus that in the broth at the end of the boiling process equals that in the pasta at the beginning.

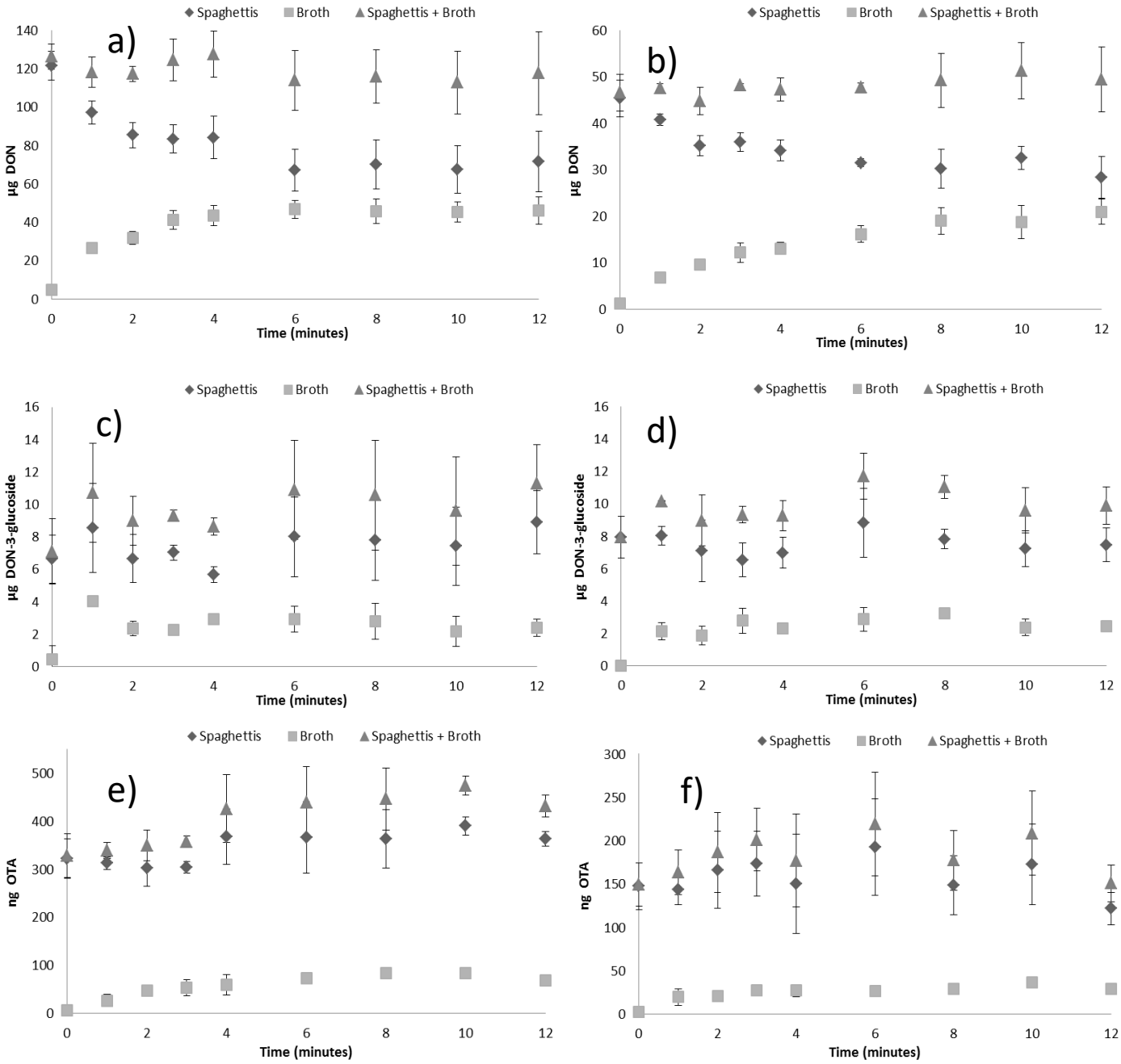


Figure 15.1. Content of DON (µg) in spaghettis (◆), broth (■) and sum of DON content in spaghettis and broth (▲) over time at high initial DON concentration (a) and low initial DON concentration (b), content of DON-3-glucoside (µg) in spaghettis (◆), broth (■)and sum of DON-3-glucoside content in spaghettis and broth (▲) over time at high initial DON-3-glucoside concentration (c) and low initial DON-3-glucoside concentration (d)and content of OTA (ng) in spaghettis (◆) and broth (■) and sum of OTA content in spaghettis and broth (▲) over time at high initial OTA concentration (e) and low initial OTA concentration (f) (bars indicate standard deviation).

15.4.1.2. Kinetics of DON leaching

As shown in section 3.1.1., DON leached from pasta to broth until an equilibrium point was reached, with some DON still remaining in the pasta. In order to know the remaining DON concentration in pasta at any time point the DON leaching process was studied. The equation described in section 2.6.2 was followed and a pseudo-second-order reaction model was applied to our experimental data in order to determine the leaching rate constant. When the eq. 6 was applied to our data (Figure 15.2) the slope of the straight line led to a maximum percentage of DON leached (p_m) at equilibrium of 45.45 %. The leaching rate constant (k) was 0.024 min (Table 15.5).

To our knowledge, there is no previous report on modelling DON leaching during boiling. However some differences in p_m and k could be found in other leaching situations because several factors can influence, mainly pasta:water ratio seems an important factor in DON reduction. It must be pointed out that modelling of mycotoxins behaviour during food processes is essential to provide an applied knowledge about mycotoxins intake by the population, but nowadays scarce works exist about this (Castells, Pardo, Ramos, Sanchis, & Marín, 2006; Ferraz et al., 2010; Numanoglu, Gökmen, Uygun, & Koksel, 2012; Vidal et al., 2015). In particular, exposure assessment studies could benefit from correction of the initial DON concentration in uncooked pasta.

Table 15.4. Comparison of DON concentration (ng/g) remaining in pasta boiled for 12 minutes without egg from mass balance equation (eq. 5) and experimental values at the end of boiling process for our experiments (high and low initial concentration) and Visconti et al. (2004) results with the DON concentration (ng/g) in the uncooked spaghettis.

			DON content remaining in pasta (ng/g)	
		Initial DON content (ng/g)	Calculated	Observed
High initial concentration		1389.14±18.05	698.91	820.28±180.93
Low initial concentration		591.88±15.68	297.15	325.31±51.32
Visconti et al., 2004	Sample 1	170±30	42.14	37.51±6.78
	Sample 2	230±0.00	58.46	47.00±22.14
	Sample 3	260±20	61.00	48.80±29.82
	Sample 4	500±30	205.51	280.61±35.25
	Sample 5	420±10	175.56	203.34±27.11
	Sample 6	790±70	339.44	389.06±27.11
	Sample 7	1850±60	873.99	993.67±93.09
	Sample 8	3280±410	1281.74	1619.97±150.93
	Sample 9	6970±100	3062.25	2816.99±311.79

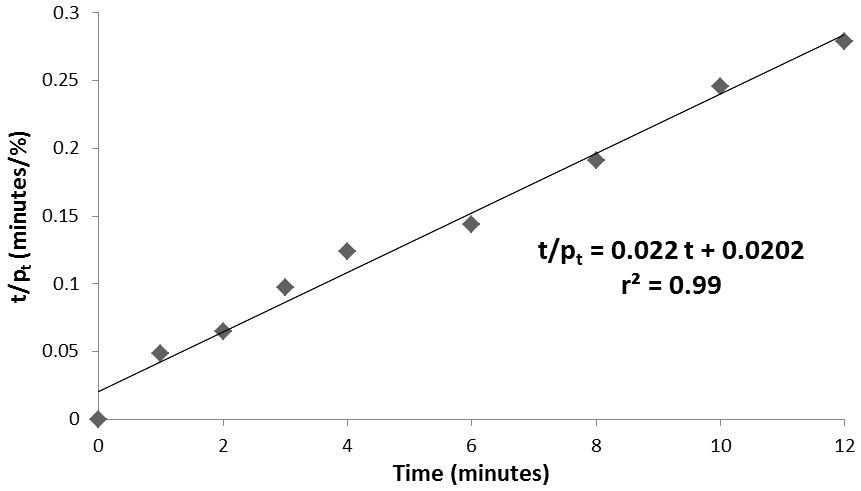


Figure 15.2. Linear model of DON leaching model through the time.

Table 15.5. Comparison of observed and predicted DON concentration in spaghetti without egg during boiling process using the kinetic model.

		High Concentration			Low concentration		
Time (minutes)	Predicted reduction (%)	Observed reduction (%)	Predicted concentration (ng/g)	Observed concentration (ng/g)	Observed reduction (%)	Predicted concentration (ng/g)	Observed concentration (ng/g)
0	0	-0.21±6.11	1389.14	1392.12±84.90	12.27±7.73	591.88	504.71±27.32
1	23.69	20.01±5.02	1060.05	1111.25±69.69	21.12±2.50	451.66	467.05±14.81
2	31.15	29.58±5.40	956.42	978.22±74.97	31.90±4.19	407.51	403.24±24.82
3	34.80	31.28±6.02	905.72	954.66±83.66	30.41±3.85	385.91	412.03±22.88
4	36.97	30.63±9.23	875.57	963.61±128.22	33.89±4.27	373.06	391.44±25.31
6	39.42	44.56±8.98	841.54	770.23±124.79	39.03±1.64	358.56	361.19±9.79
8	40.77	42.12±10.48	822.79	804.07±145.55	41.53±8.17	350.57	346.23±48.39
10	41.63	44.43±10.10	810.84	772.82±140.34	37.10±4.84	345.48	372.40±28.63
12	42.22	40.97±13.02	802.65	820.28±180.93	45.07±8.67	341.99	325.25±51.32

15.4.2. DON-3-glucoside

The initial semolina contained also DON-3-glucoside (Table 15.3). DON-3-glucoside content was the same in the two assayed batches because it is a plant conjugate (Berthiller et al., 2009) and till now there is no evidence that it can be produced by fungi. The levels of DON-3-glucoside vary among wheat studies, however the ratio DON-3-glucoside/DON concentration is similar among the assays, from 10 to 30 % (Berthiller et al., 2009; Dall'Asta et al., 2013; Desmarchelier & Seefelder, 2010; Rasmussen et al., 2010). Hitherto, few studies exist about DON-3-glucoside in durum wheat but the ratio DON-3-glucoside/DON in durum wheat could well be similar. We got a ratio of 25 % and Dall'Asta et al. (2013) also obtained ratios between 20 and 30 %. Moreover, DON-3-glucoside is not only found in raw cereals, because some studies indicate the high presence of DON-3-glucoside in cereal based products (De Boevre et al., 2012; Malachova, Dzuman, Veprikova, Vaclavikova, Zachariasova, & Hajslova, 2011). Thus,

although it seems it is important to study DON-3-glucoside stability during food processing, few investigations have been made about it and scarce knowledge exists for pasta making process.

The concentration of DON-3-glucoside did not change after kneading and drying pasta, thus the concentrations were similar in semolina and uncooked pasta (Table 15.3). Regarding boiling, DON-3-glucoside remained nearly constant in spaghettis (Figure 15.1) through the time. On the other hand, a slight and fast increase of DON-3-glucoside in broth was detected ($p < 0.05$) (Figure 15.1). This increase suggests that an increase in the total amount of DON-3-glucoside occurred during boiling (Figure 15.1). The DON-3-glucoside concentration in broth was the same regardless of the initial DON concentration. The presence of egg in formulation instead of water did not cause any change in DON-3-glucoside content (Table 15.3).

By contrast, Zhang et al. (2015), who studied DON-3-glucoside stability in noodles production detected a significant increase of DON-3-glucoside (69 %) in uncooked pasta. However, they used fermentation (30 minutes at room temperature) after mixing of the ingredients. Fermentation showed to cause an increase in DON-3-glucoside in breadmaking studies (Kostelanska et al., 2011; Vidal et al., 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b). The high stability of DON-3-glucoside found after boiling of pasta agrees with the results found by Zhang et al. (2015). They did not find any DON-3-glucoside reduction after boiling noodles for 5 minutes. Similarly, increases of DON-3-glucoside have been observed during baking (Vaclavikova, Malachova, Veprikova, Dzuman, Zachariasova, & Hajslova, 2013; Vidal et al., 2014b), although some studies showed important reductions after baking (De Angelis, Monaci, Pascale, & Visconti, 2013; Kostelanska et al., 2011; Simsek et al., 2012). Vidal et al. (2015) revealed that DON-3-glucoside could either increase under mild baking conditions (for instance 140 º for 35 minutes or 200 ºC for less than 10 minutes), or decrease under harsher temperature/time conditions. The mild conditions involved in boiling (100 °C and short times) may lead to DON-3-glucoside release instead of thermal degradation as in baking. The detected increase of DON-3-glucoside content could be caused by the release of DON-3-glucoside from the matrix due to the thermal treatment. DON-3-glucoside found in broth was not be linked to DON presence, because in one hand no change in the total amount of DON was detected and, in the other hand, DON-3-glucoside content found in broth was independent of the initial DON content. Other baking studies did not find any relation between both toxins (Kostelanska et al. 2011; Vidal et al., 2015); they pointed out to a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides. However, to our knowledge, their possible relation has not been studied in depth yet.

DON-3-glucoside presence in the broth confirms that leaching from pasta took place (Figure 15.1). The high solubility of DON-3-glucoside and other DON conjugates has been observed in malting and brewing process (Lancova et al., 2008). Thus during boiling, an increase of DON-3-glucoside content occurs in the pasta due to a release from its components, which is subsequently transferred to broth. Finally, the stability of DON-3-glucoside in spaghettis during boiling is of concern because, although DON-3-glucoside is far less active as protein

biosynthesis inhibitor than DON (Poppenberger et al., 2003), DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, to microbial activity in the intestine as shown *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011), thus its presence is important for food safety.

15.4.3. OTA

Although our semolina batch did not contain OTA, durum wheat has been shown to contain OTA in previous studies (Winnie, Mankotia, Pantazopoulos, Neil, Scott, & Lau, 2009), and some authors pointed out that durum wheat may be more contaminated by OTA than other types of wheat (Kuruc, Manthey, Simsek, & Wolf-Hall, 2014). So, it is important to study the fate of OTA during durum wheat processing to food products.

OTA showed a high stability during the entire studied process. Kneading, drying and boiling of spaghettis did not cause any significant change in OTA concentration. Thus, OTA concentration in semolina and cooked spaghetti was similar regardless of the two initial assayed concentrations (Table 15.3). However, slight increases of OTA through time were detected in broth (Figure 15.1) ($p < 0.05$). Furthermore, the level of OTA in the broth depended on the initial OTA concentration in spaghettis. So, transfers of OTA from spaghetti to water obviously occurred during boiling, although no significant changes in OTA concentration of cooked spaghettis were detected. On the other hand, no variations were detected when egg was used (Table 15.3).

There is limited information about food processing effects on OTA. OTA stability has been confirmed in the breadmaking process where kneading and fermentation of flour wheat did not cause differences in OTA content (Vidal et al., 2014a). An existing study on OTA fate after boiling of spaghetti showed, by contrast, a 35 % of OTA reduction after boiling 10 g during 6 minutes in 400 mL of water (Sakuma et al., 2013). However they worked with OTA spiked spaghettis, which may easily loose the toxin. In addition they used a high ratio water:pasta which may favour OTA leaching, nevertheless this factor has not yet been studied during boiling process in OTA. The transfer of OTA to water was suggested by Sakuma et al. (2013) because their OTA content in broth paralleled OTA losses in spaghettis. Thus a transfer of OTA to water is possible but not clearly observed in our study. An increase of OTA content in broth was observed when boiling time increased, and the transfer of OTA reached over 15 % in the last minutes of boiling. On the other hand, a 47 % of OTA transfer was reached after boiling for 3 hours in decoctions of herbal medicines (Shim, Ha, Kim, Kim, & Chung, 2014) and 1 % of OTA transfer occurred after 5 minutes of boiling infusion tea (Ariño, Herrera, Estopañan, & Juan, 2007). So, boiling time has an importance in the level of OTA transfer. Finally, the sum of OTA content in broth and boiled spaghetti showed no loss of OTA during the process, so the temperature used in boiling does not cause OTA degradation. OTA is thermo stable; baking

studies only showed some reduction under high temperatures (> 140 ºC) and long times (Vidal et al., 2015). The higher transfer of DON to broth could be caused by its higher solubility in water than OTA. DON is one of the more polar trichothecenes with a solubility of 11 g/L at 25 °C in water (Chemicaldictionary, 2009), whereas OTA is hardly soluble in water (1.31 mg/L at 25 °C) (SCR, 2010).

15.5. Conclusion

DON is stable during kneading and drying, but a high DON reduction (> 40 %) was observed in boiled spaghettis. DON is transferred to broth, where it is not degraded and boiling time determines the extent of the transfer. The use of the DON leaching model developed in the work can be a useful tool in risk assessment under different scenarios of pasta cooking when the initial mycotoxin concentrations in the raw materials are known. By contrast, DON-3-glucoside is totally stable through the pasta making process; moreover DON-3-glucoside is released from pasta components and it is leached to broth. OTA is also stable during pasta making, however it is scarcely transferred to broth during boiling.

15.6. Acknowledgements

The authors are grateful to the Spanish government (project AGL2014-55379-P) for the financial support. A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant.

15.7. References

Alderton, G, & Fevold, H.L. (1946). Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. *The Journal of Biological Chemistry, 164,* 1-5.

Ariño, A., Herrera, M., Estopañan, G., & Juan, T. (2007). High levels of ochratoxin A in licorice and derived products. *International Journal of Food Microbiology, 114,* 366-369.

Berthiller, F., Dall'Asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and

maize. *Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26,* 507–511.

Berthiller, F., Krska, R., Domig, K. J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206,* 264–267.

Brera, C., Peduto, A., Debegnach, F., Pannunzi, E., Prantera, E., Gregori, E., De Giacomo, M., & De Santis, B. (2013). Study of the influence of the milling process on the distribution of deoxynivalenol content from the caryopsis to cooked pasta. *Food Control, 31,* 309-312.

Brockmeyer, A., & Thielert, G. (2004). Deoxynivalenol (DON) in Durum wheat. *Mycotoxin Research, 20,* 37-41.

Cano-Sancho, G., Sanchis, V., Ramos, A.J., & Marín, S. (2013). Effect of food processing on exposure assessment studies with mycotoxins. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30,* 867-875.

Castells, M., Pardo, E., Ramos, A. J., Sanchis, V., & Marín, S. (2006). Reduction of ochratoxin A in extruded barley meal. *Journal of Food Protection, 69,* 1139–1143.

Chemicaldictionary, 2009. <http://www.chemicaldictionary.org/dic/D/Deoxynivalenol_842.html> (accessed: 07.01.2015).

Codex Alimentarius Comission. 2011. Proposed draft maximum levels for deoxynivalenol (DON) and its acetylated derivatives in cereals and cereal based products. The Hague, The Netherlands. <ftp://ftp.fao.org/codex/meetings/cccf/cccf5/cf05_06e.pdf> (accessed 07.01.13).

Covarelli, L., Beccari, G., Prodi, A., Generotti, S., Etruschi, F., Juan, C., Ferrer, E., & Mañes, J. (2014). *Fusarium* species, chemotype characterisation and trichothecene contamination of durum and soft wheat in an area of central Italy. *Journal of the Science of Food and Agriculture, 95,* 540-551.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal, 6,* 83-91.

De Angelis, E., Monaci, L., Pascale, M., & Visconti, A. (2013). Fate of deoxynivalenol, T-2 and HT-2 toxins and their glucoside conjugates from flour to bread: An investigation by high-performance liquid chromatography high-resolution mass spectrometry. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30,* 345-355.

De Boevre, M., Di Mavungu, J.D., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., & De Saeger, S. (2012). Natural occurrence of mycotoxins and their masked forms in food and feed products. *World Mycotoxin Journal, 5,* 207-219.

European Commission, 2006. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union, L364*, 5–24.

Ferraz, M. B. M., Farah, A., Iamanaka, B. T., Perrone, D., Copetti, M. V., Marques, V. X., Vitali, A.A., & Taniwaki, M.H. (2010). Kinetics of ochratoxin A destruction during coffee roasting. *Food Control, 21,* 872–877.

Ho, Y.S., & McKay, G. (1999). Pseudo-second order model for sorption processes. *Process Biochemistry, 34,* 451-465.

Ho, Y.S., Harouna-Oumarou, H.A., Fauduet, H., & Porte, C. (2005). Kinetics and model building of leaching of water-soluble compounds of *Tilia sapwood*. *Separation and Purification Technology, 45,* 169-173.

Horwitz, W., & Latimer, G. W. (2006). Official methods of analysis of AOAC International (18th ed.). Gaithersburg, Maryland: AOAC.

IARC (International Agency for Research on Cancer). (1993). Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56. Lyon, France.

JEFCA, 2010. WHO Technical Report Series 959: JEFCA, 2010: Seventy-second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food. WHO Technical Report Series. 959.

Jijakli, M.H., & Lepoivre, P. (1998). Characterization of an exo-β-1,3-glucanase produced by Pichia anomala strain K, antagonist of Botrytis cinerea on apples. *Phytopathology, 88*, 335-343.

Juan, C., Covarelli, L., Beccari, G., Colasante, V., & Mañes, J. (2016). Simultaneous analysis of twenty-six mycotoxins in durum wheat grain from Italy. *Food Control, 62,* 322-329.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59,* 9303–9312.

Kuruc, J.A., Manthey, F., Simsek, S., & Wolf-Hall, C. (2014). Survey of ochratoxin A in freshly harvested durum and hard red spring wheat in the United States, 2011 and 2012. *Journal of Food Protection, 77,* 1005-1009.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25,* 732-744.

Lippolis, V., Pascale, M., Cervellieri, S., Damascelli, A., & Visconti, A. (2014). Screening of deoxynivalenol contaminantion in durum wheat by MOS-based electronic nose and identification of the relevant pattern of volatile compounds. *Food Control, 37,* 263-271.

Liu, W. H., Means, G. E., & Feeney, R. E. (1971) The inhibitory properties of avian ovoinhibitors against proteolytic enzymes. *Biochimica et Biophysica Acta, 229,* 176–185.

Malachova, A., Dzuman, Z., Veprikova, Z., Vaclavikova, M., Zachariasova, M., & Hajslova, J. (2011). Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *Journal of Agricultural and Food Chemistry, 59,* 12990–12997.

Marin, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurence, toxicology, and exposure assessment. *Food and Chemical Toxicology, 60,* 218-237.

Mishra, S., Dixit, S., Dwivedi, P. D., Pandey, H. P., & Das, M. (2014). Influence of temperature and pH on the degradation of deoxynivalenol (DON) in aqueous medium: Comparative cytotoxicity of DON and degraded product. *Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31,* 121–131.

Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37,* 21-25.

Nowicki, T.W., Gaba, D. G., Dexter, J. E., Matsuo, R. R., & Clear, R. M. (1988). Retention of the *Fusarium* mycotoxin deoxynivalenol in wheat during processing and cooking of spaghetti and noodles. *Journal of Cereal Chemistry, 8,* 189-202.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. (2012). Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29,* 423–430.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by aUDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278,* 47905–47914.

Rasmussen, R.R., Storm, I.M.L.D., Rasmussen, P.H., Smedsgaard, J., & Nielsen, K.F. (2010). Multi-mycotoxin analysis of maize silage by LC-MS/MS. *Analytical and Bioanalytical Chemistry, 397,* 765-776.

Sakuma, H., Watanabe, Y., Furusawa, H., Yoshinari, T., Akashi, H., Kawakami, H., Saito, S., & Sugita-Konishi, Y. (2013). Estimated dietary exposure to mycotoxins after taking into account the cooking of staple foods in Japan*. Toxins, 5,* 1032-1042.

Shim, W.-B., Ha, K.-S., Kim, M.-G., Kim, J.-S., & Chung, D.-H. (2014). Evaluation of the transfer rate of ochratoxin A to decoctions of herbal medicines. *Food Science and Biotechnology, 23,* 2103-2108.

Simsek, S., Burgess, K., Whitney, K. L., Gu, Y., & Qian, S. Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26,* 287–292.

SRC. 2010. Interactive PhysProp Database Demo. Syracuse Research Corporation < https://esc.syrres.com/fatepointer/webprop.asp?CAS=303479> (accessed: 07.01.2015).

Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). 'Emerging' mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry, 136,* 750-757.

Vidal, A., Morales, H., Sanchis, V., Ramos, A. J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria*. Food Control, 40,* 234–242.

Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology, 68,* 53-60.

Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry, 178,* 276-286.

Vidal, A., Ambrosio, A., Sanchis V., Ramos, A.J., & Marin, S. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, Article in press.*

Visconti, A., Haidukowski, E.M., Pascale, M., & Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters, 153,* 181-189.

Winnie, N.G., Mankotia, M., Pantazopoulos, P., Neil, R.J., Scott, P.M., & Lau, B. P. Y. (2009). Survey of dry pasta for Ochratoxin A in Canada. *Journal of Food Protection, 72,* 890-893.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New ‘‘masked’’ *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60,* 9280–9291.

Zhang, H., & Wang, B. (2015). Fates of deoxynivalenol and deoxynivalenol-3-glucoside during bread and noodle processing. *Food Control, 50,* 754-757.

16. Study XI. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.

World Mycotoxin Journal (2016) 9, 597-612.

Authors: Vidal, A., Cano-Sancho, G., Marín, S., Ramos, A.J., & Sanchis, V.

16. Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia (Spain).

Running header: Multidetection of mycotoxins: pilot time-course study and risk assessment.

Arnau Vidal [a, 1], German Cano-Sancho [b, 1], Sonia Marín [a], Antonio J. Ramos [a] and Vicente Sanchis [a*].

[a] Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Lleida, Spain.

[b] Department of Environmental Toxicology. University of California at Davis, Davis, United States.

*Corresponding autor: Tel. +34-973-702535. Fax: +34-973-702596. E-mail address: vsanchis@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

[1] These authors contributed equally to this work and should be considered as co-first author.

16.1. Abstract

Presence of 2 main mycotoxins, ochratoxin A (OTA) and deoxynivalenol (DON), is widespread in cereal based foodstuffs marketed in Europe. The objectives of this study were to develop and validate a multi-detection analytical methodology to simultaneously assess the urinary concentrations of OTA, DON and their metabolites, and to apply this methodology in a preliminary follow-up trial in Catalonia (Spain). Hence, an ultra-performance liquid chromatography with tandem mass spectrometry method was developed to simultaneously assess the urinary levels of OTA, DON, deoxynivalenol-3-glucoside (DON-3-glucoside), deoxynivalenol-3-glucuronide (DON-3-glucuronide), 3-acetyldeoxynivalenol (3-ADON) and de-epoxy-deoxynivalenol (DOM-1). Urine mycotoxins levels and food dietary intake were prospectively monitored in a group of volunteers throughout a restriction period followed by a free-diet period. The proposed multi-detection methodology for urinary OTA and DON metabolites was validated, providing suitable recovery, linearity and precision. The results from the pilot trial showed that urinary OTA, DON and its metabolites were detected in most of background samples, displaying moderate reductions after the restriction period and subsequently recovering the background levels. Despite the restriction period, some DON metabolites, such as 3-ADON or DOM-1, were still found in urine samples, placing alternative sources of DON exposure other than the ones considered in the study under suspicion. DON and DON-3-glucuronide were significantly associated with consumption of bread, pasta and

pastries, while OTA was only associated with consumption of wine and breakfast cereals. The urinary levels of OTA were significantly correlated with plasmatic levels of OTA and OTα, supporting the results from the multidetection method in urine. The results also showed that the high exposure to DON could be held throughout the time by the same person, exceeding the tolerable daily intake (TDI) systematically instead of eventually. The estimates of OTA exposure through urine are largely higher than those obtained with the dietary approach. The background levels found in urine revealed that the exposure to DON and OTA could be of concern for the Catalonian population, thus, further studies applying this biomonitoring methodology in a larger sample of Catalonian population are needed to accurately characterize the human health risks at population level.

Keywords: OTA, DON, urine, risk assessment, biomarkers.

16.2. Introduction

Mycotoxins are produced by fungi and can contaminate various agricultural commodities, either before harvest or under post-harvest conditions (FAO, 1991). The main mycotoxin-producing fungi in foods belong to the genera *Aspergillus*, *Penicillium* and *Fusarium* (Pitt and Hocking, 2009). Ochratoxin A (OTA) is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (Scientific Committee on Food (SCF), 1998). This mycotoxin has been classified as a possible human carcinogen, in the group 2B, by the International Agency for Research on Cancer (IARC) (IARC, 2002). Deoxynivalenol (DON) is a mycotoxin that acts as a potent inhibitor of protein synthesis, stimulates the pro-inflammatory response, cause ribotoxic stress, cytotoxicity and apoptosis, resulting on the impairment of multiple physiological functions, such as the intestinal barrier, growth, immune regulation or reproduction. Typical acute effects include nausea, vomiting, abdominal pain, diarrhoea, headache, dizziness, or fever, and it has been linked with animal and human gastroenteritis outbreaks (Pestka, 2010a,b).

Due to the harmful effects of these mycotoxins and the widespread distribution in temperate countries, the European Commission (EC) introduced regulatory limits for OTA and DON concentrations in food marketed in the European Union country members (European Commission, 2006). Moreover, toxicological evaluation and risk assessment of OTA and DON were extensively addressed by the European Food Safety Authority (EFSA) and the joint FAO/WHO Expert Committee on Food Additives (JECFA), resulting in a maximum tolerable weekly intake (TWI) of 120 ng/kg bw for OTA (EFSA, 2006); and a maximum tolerable daily intake (TDI) of 1 µg/kg bw for DON and acetylated metabolites (JECFA, 2011). The dietary exposure to these mycotoxins has been estimated to be highly probable in most of the European populations (Marín *et al.*, 2013). The mean exposure to OTA in Catalonia (Spain) was estimated between 0.20 and 0.47 ng/kg bw/day (Coronel *et al.*, 2012), and the median exposure to DON in the same region was estimated in the range from 0.27 to 0.36 µg/kg bw/day (Cano-Sancho *et al.*, 2011). Furthermore, other DON metabolites and conjugated products have been lately highlighted by their large presence in some products, especially deoxynivalenol-3-glucoside (DON-3-glucoside), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (Edwards, 2009; Juan *et al.*, 2013; Simsek *et al.*, 2012; Yang *et al.*, 2013), however, little is known about their toxicity and interactions. Other metabolites, such us the de-epoxy-deoxynivalenol (DOM-1) are released by intestinal anaerobic bacteria in the lumen previously to the enterocyte absorption (Maresca, 2013).

The routine exposure assessment approaches merge dietary contamination and consumption data from a sub-sample of population to extrapolate a distribution of exposure estimates on the whole population, using broad assumptions and missing inter-individual variability. The biomonitoring approach provides an internal measure of the individual exposure to pollutants which accounts for all intake routes and inter-individual variability (Clewell *et al.*, 2008). However there are still a lot of uncertainties and limitations to fully validate the mycotoxin

biomarkers, for instance biomonitoring data may depend on the moment in time when the sample is collected (Clewell *et al.,* 2008). For this reason, the biomarker approach is not replacing the indirect method for risk assessment of mycotoxins yet. Urine and plasma are biological specimens commonly used as biomarkers of OTA exposure (Coronel *et al.*, 2011), urine being the most convenient for large scale studies due to its higher accessibility. In case of DON and considering the short excretion half-life, only the urinary levels of DON or its glucuronide forms have been proposed as reliable biomarkers. A preliminary approach was developed based on the breakage of DON-glucuronides and subsequent determination of "total DON" (sum of free DON and DON released by hydrolysis) (Turner *et al.*, 2010a). Afterwards, a direct method for quantification of DON metabolites such as deoxynivalenol-3-glucuronide (DON-3-glucuronide) was developed by Warth *et al.* (2011), supported by the high proportion of this metabolite in urine (Shephard *et al.*, 2013). Recent advances in the application of high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for multidetection of mycotoxins have been extended to the biomonitoring studies, appearing as a promising tool to obtain general pictures with a wide range of mycotoxins and metabolites in the same sample (Warth *et al.*, 2013).

Considering the substantial exposure of Catalonian population to OTA and DON, the aims of this study were: 1) to develop and validate a multi-detection analytical methodology to simultaneously assess the urinary concentrations of OTA, DON, DON-3-glucoside, DON-3-glucuronide, 3-ADON and DOM-1, and 2) to apply this methodology in a preliminary pilot study to assess the time-course of these mycotoxins in urine from a sample of volunteers from Catalonia whose diet was temporarily restricted.

16.3. Materials and methods

16.3.1. Chemical analysis

16.3.1.1. Chemicals and reagents

Acetonitrile (99.9 %), methanol (99.9 %) and ethanol (99.5 %) were purchased from J.T. Baker (Deventer, The Netherlands). Dichloromethane (≥ 99.8 %), ammonium acetate (≥ 98 %), picric acid moistened with water (≥ 98 %) and creatinine (≥ 98 %) were purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain). Isopropanol (99.9 %), sodium hydroxide (≥ 99.5 %), sodium bicarbonate (100 %), phosphoric acid (85.4 %) and chloroform (≥ 99.99 %) were purchased from Fisher (Fisher, Bioreagents, New Jersey, USA). All solvents were LC grade. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Pure water was

obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (98-100 %, Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (98-100 %, Panreac, Castellar del Vallès, Spain), disodium phosphate anhydrous (1.16 g) (99 %, Panreac, Castellar del Vallès, Spain) and sodium chloride (8.0 g) (≥ 99.5 %, Fisher Bioreagents, New Jersey, USA) in 1 L of milli-Q water; the pH was brought to 7.4 with hydrochloric acid 1 M. Hydrolysis buffer for the enzymatic treatment of plasma samples was prepared with 13.6 g sodium acetate trihydrate (99 %), 0.1 g EDTA (99 %) both purchased from Sigma-Aldrich (Alcobendas, Spain), 1.0 g ascorbic acid (99 %, Merck, Darmstadt, Germany), in 100 mL of pure water, adjusted to pH 5.0 with acetic acid (100 %, Normapur VWR Prolabo, Llinars del Vallès, Spain). Enzymatic hydrolysis of the plasma samples was done using *β-glucuronidase* (5·10^6 units/g) from *Escherichia coli* (Type IX-A, Sigma-Aldrich, Alcobendas, Spain). Standards of OTA and OTα were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). DON, DON-3-glucoside, 3-ADON, DOM-1 and isotope-labelled ($^{13}C_{15}$) DON were supplied by Biopure (Romer Labs, Tulln, Austria). ($^{13}C_{15}$) DON was used as internal standard. DON-3-glucuronide was kindly supplied by Dr. Franz Berthiller who recently developed a novel methodology to isolate this compound (Fruhmann *et al.*, 2012).

16.3.1.2. Detection of OTA, DON, DON-3-glucoside, DON-3-glucuronide, 3-ADON and DOM-1 in urine by ultra-performance liquid chromatography with mass spectrometry (UPLC-MS/MS)

One mL of each urine sample was centrifuged at 1000 *g* for 3 min at 4 ºC and 0.1 mL from the upper layer was diluted 1:4 with ammonium acetate 1 % before direct injection. No enzymatic treatment of samples was made. Mass spectrometric analysis was carried out on a Waters ACQUITY TQD tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation was performed by a 100 mm x 2.1 mm i.d., 1.8 µm, Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). The mobile phases consisted of acetonitrile (A) and 10 mM ammonium acetate in water (B). The gradient was as follows: start with 10 % A, at 1.5 min, a linear increase to 75 % was achieved in 2.5 min. At min 4.01, it switched to 10 % A, and the column was equilibrated for 2 min before the next injection. The flow rate was 300 µL/min, and an injection volume of 7.5 µL was enabled.

One chromatographic run consisted of two MS periods. The first period monitored analytes in positive mode (OTA, DOM-1, DON, DON-3-glucoside, ($^{13}C_{15}$) DON), in the second period the negative mode was used (DON-3-glucuronide and 3-ADON), and both ionization modes were run simultaneously by polarity switching. All measurements were done with the following settings: source temperature 150 ºC, desolvation temperature 350 ºC, cone gas flow 2.5 L/min, desolvation gas flow 16.7 L/min, collision gas flow 0.17 mL/min and the capillary was 3000 V. The analyte-dependent MS/MS parameters were optimized via direct infusion of reference standard solutions; the resulting parameters are displayed in Table 16.1.

16.3.1.3. OTA and OTα detection in plasma by high-performance liquid chromatography with fluorescence detection (HPLC-FLD)

In order to compare the performance of the urinary multidetection method to assess OTA levels, we also monitored the OTA and OTα concentration in plasma from a subsample of volunteers throughout the intervention period. The analytical methodology for extraction, clean-up and determination of OTA and OTα in urine has been described in detail elsewhere (Coronel *et al.*, 2011). Briefly, an enzymatic cleavage of conjugates in plasma was made to release glucuronide and sulphated conjugates, where 1 mL of plasma sample was mixed with 0.1 mL hydrolysis buffer solution and 10 µL enzyme. The enzyme treated sample was mixed with 5 mL of 1 % sodium bicarbonate in water, followed by a pH adjustment with 1 M phosphoric acid to pH 3. Then OTA and OTα were extracted using 3 mL chloroform/isopropanol (97:3, v/v). OTA and OTα were determined by HPLC (Waters 2695®) coupled with a Multi λ Fluorescence Detector Waters 2475®, an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm.

Table 16.1. The optimized ESI-MS/MS parameters for the confirmation and quantification of OTα, OTA, DON/DON-3-glucoside, DON-3-glucuronide, 3-ADON, DOM-1 and $^{13}C_{15}$ DON in urine

Mycotoxin	MRM transitions	CE[A,B] (eV)	CXP[A,C] (v)	Dwell time[A] (ms)
OTα	257.00 > 221.00	25/15	12.0/12.00	63/63
	257.00 > 239.00			
OTA	404.00 > 239.00	25/10	20/20	46/46
	404.00 > 358.00			
DON/DON-3-glucoside	297.09 > 203.10	16/10/10	2.0/2.0/2.0	52/52/52
	297.09 > 231.09			
	297.09 > 249.06			
DON-3-glucuronide	471.20 > 175.00	40/20	20.0/10.0	56/56
	471.20 > 265.00			
3-ADON	337.30 > 150.30	14/14/14/14	16.0/16.0/16.0/16.0	80/80/80/80
	337.30 > 173.30			
	337.30 > 277.30			
	337.30 > 307.00			
DOM-1	281.10 > 109.06	16/10/10	6.0/6.0/6.0	108/108/108
	281.10 > 215.08			
	281.10 > 233.11			
$^{13}C_{15}$ DON	312.09 > 216.06	16/10/10	2.0/2.0/2.0	52/52/52
	312.09 > 244.99			
	312.09 > 262.93			

[A] Values are given in the order quantifier ion/ qualifier ion.
[B] CE: Collision energy.
[C] CXP: Cell exit potential.

16.3.1.4. Validation of analytical method

Validation of the developed methods was carried out following the FDA guideline for bioanalytical method validation (U.S. Department of Health and Human Services Food and Drug Administration, 2001) with minor modifications. The parameters investigated include accuracy,

precision, recovery, selectivity, sensitivity, and stability. Accuracy, precision and apparent recovery of all analyst were evaluated by measurements of blank urine samples spiked at five concentration levels Blank urine previously analysed for all analytes with no detectable levels of OTA, DON, DON-3-glucoside, DON-3-glucuronide, 3-ADON and DOM-1, was used as blank to generate validation analysis. For plasma analysis, plasma previously analysed for OTA content at levels lower than 0.02 ng/mL for OTA was used as blank to generate validation analysis. Accuracy was evaluated for all analytes by measurement of blank urine and plasma samples spiked at five mycotoxin concentration levels. The chosen spiking ranges for urine were: 0.16 - 0.50 ng/L (OTA) and 10 - 75 ng/mL (DON, DON-3-glucoside, DON-3-glucuronide, 3-ADON and DOM-1). The chosen spiking range for plasma was: 0.07 - 1.5 ng/mL (OTA and OTα) (Table 16.2). The sensitivity of the method was assessed by the limits of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were the lowest matrix matched calibration providing signal-to-noise ratios greater than 3 and 10, respectively, at both quantitation and confirmation transitions, and matching the intensity ratio observed for the particular compound in the standard solution, using the according analyst software tool and the quantifier transition. Standard calibration curves were generated by linear regression of peak areas against concentrations. Precision was established by determining mycotoxins levels in fortified urine at least by triplicate, in order to calculate the recovery rates.

A calibration curve for calibration was generated for each analysis based on five concentration levels. For preparation of the standard calibration solutions, the multi-standard working solution was diluted (v/v) with solvent. Selectivity, sensitivity and stability of the measured product ions were evaluated throughout method development and validation by analysis of the blank matrices. On the other hand, several chromatographic conditions were tested until a suitable option was reached. Method performance characteristics for analysed mycotoxins are summarized in Table 16.2.

16.3.1.5. Creatinine analysis

Creatinine was determined in urine samples to correct the different inter-individual excretion rates which may affect analyte concentrations in urine samples. Analyses were performed with a spectrophotometric method for the direct measurement of creatinine in urine as described by Bader *et al.* (2007).

Table 16.2. Results of validation for urine and plasma samples.

Product	Mycotoxin	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	n	Spiking level (ng/mL)	Recovery[c] (%)	RSDr[d] (%)	Linear Range
Plasma (HPLC FL)	OTA	0.02	0.07	3	0.07	113.6.±9.7	10	0.02-10.00
				3	0.75	109.8±2.6	3	
				5	1.50	100.4.±5.7	6	
	OTα	0.02	0.07	3	0.07	115.2±2.5	2	0.02-10.00
				3	0.75	85.4±3.5	4	
				5	1.50	71.4±2.1	3	
Urine (LC MS/MS)	OTA	0.003	0.008	3	0.16	64.2±5.5	4	0.05-0.750
				3	0.33	65.6±2.6	4	
				5	0.50	67.7±1.2	2	
	OTα	0.053	0.160	3	0.16	129.9±6.7	5	0.05-0.750
				3	0.33	133.9±8.2	6	
				5	0.50	139.6±3.9	3	
	DON	0.5	1.7	3	10.0	87.2±11.9	14	1.5-150.0
				3	30.0	96.1±0.4	1	
				5	75.0	90.2±3.9	4	
	DON-3-glucoside	0.6	1.8	3	10.0	85.5±1.0	1	1.5-150.0
				3	30.0	85.1±13.0	5	
				5	75.0	85.3±7.3	9	
	DON-3-glucuronide	3.3	10.0	3	10.0	106.5±24.5	23	1.5-150.0
				3	30.0	86.7±3.5	4	
				5	75.0	75.3±2.6	4	
	3-ADON	0.1	0.3	3	10.0	104.1±5.1	5	1.5-150.0
				3	30.0	115.4±4.0	3	
				5	75.0	118.7±2.5	2	
	DOM-1	0.6	1.7	3	10.0	88.6±1.4	2	1.5-150.0
				3	30.0	81.4±1.7	2	
				5	75.0	89.4±4.1	5	

[a] LOD = Limit of detection.
[b] LOQ = Limit of quantification.
[c] Mean value ± standard deviation.
[d] RSDr = relative standard deviation.

16.3.2. Pilot trial

16.3.2.1 Study design and sample collection

The analytical method was piloted in a group of volunteers throughout an interventional and longitudinal trial. The study was conducted with 23 healthy adults (mean age 35 years old, range 18-59 years old), 14 women and nine men, recruited from Lleida city (Catalonia, Spain). The biomarkers were evaluated at different exposure scenarios, thus, the volunteers consumed a restricted diet over a period of five days followed by seven days of normal diet (Figure 16.1). The diet restricted those products susceptible of DON and/or OTA contamination: cereals and cereal based foods and beverages, chocolate, coffee, wine, beer, cayenne, dried pepper, peanuts, pistachios, nuts and raisins. During the subsequent 7 days (Day 0-6), the volunteers consumed their common diet, registering the food intake by means of a 7-day dietary record.

The restriction diet did not entail additional risks for the participants and they signed an informed consent. The study was made in winter starting on the 20th of January in 2014.

	Restricted Diet[a]					Normal Diet						
Days	**-5**	**-4**	**-3**	**-2**	**-1**	**0[b]**	**1**	**2**	**3**	**4**	**5**	**6**
Urine Sampling	✓					✓	✓	✓		✓		✓
Plasma Sampling			✓		✓	✓		✓		✓		✓
Dietary record						✓	✓	✓	✓	✓	✓	✓

[a] During the restriction period the following foods were not allowed: cereals and cereal based foods and beverages, chocolate, coffee, wine, beer, cayenne, dries pepper, peanuts, pistachios, nuts and raisins.
[b] The samples from the day 0 were collected in the morning, before resuming the normal diet.

Figure 16.1. Scheme from the experimental setup and sampling strategy.

The levels of OTA, DON and its metabolites were monitored in urine over the whole period with special focus to those days after restriction. The samples at the beginning of the restriction provided information about the biomarker in normal conditions (without intervention), at day 0 provided information about the adherence to the restriction and the following-up samples informed about the recovery of normal levels and dietary fluctuations. Furthermore the levels of OTA and OTα were monitored in plasma from a sub-group of 13 volunteers in order to compare the estimates from both matrices.

Participants provided 30 mL of urine collected at first hour in the morning in a sterile disposable container. The urine samples were collected the days -5, 0, 1, 2, 4 and 6 (Figure 16.1). Urine samples were stored at -20 ºC on the same day. The sub-group of volunteers provided the blood at day -3, -1, 0, 2, 4 and 6 of the experiment (Figure 16.1). The blood volunteers were the urine volunteers which also volunteered to participate in the plasma experiment. There were not any type of selection or exclusion criteria. Plasma (10 mL) was obtained from whole blood centrifugation at 1000 *g* for 10 min at 4 ºC. Then the samples were stored at -20 ºC until the analysis. Blood was withdrawn in vacutainers containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant.

16.3.2.2. Food intake, dietary exposure, data analysis and statistical anaylsis

Dietary food intake after the restriction period was requested from each volunteer by means of a daily dietary record of the following seven days. The volunteers were previously trained to fill the dietary record with each consumed food and beverage, by means of weighting the portions or referring the dishes to household sizes. The volunteers were also asked to report in the data form any deviation on the adherence to the diet during the restriction period. At the

beginning of the intervention, information about gender, age (years), weight (kg), height (cm), total fat (by impedance, %) and hip (cm) and waist perimeter (cm), was gathered by a certified nutritionist using calibrated instrumentation.

We estimated the probable daily intake (PDI) of DON and OTA using the methodology implemented by Solfrizzo *et al.* (2014) with some modifications. The urinary levels (C; in μg/L), individual body weight (W, in kg), mean 24 h human urine volume (V, assumed to be 1.5 L), and the mean urinary excretion rate in 24 h (E, assumed to be 72% for DON (Turner *et al.*, 2010a) and 50% for OTA (Heyndrickx *et al*., 2015)), were combined by applying the following equation (the excretion rates are subject of assumptions):

$$PDI = C \times \frac{V}{W} \times \frac{100}{E}$$

The respective PDIs were compared with the TDIs of 17 ng/kg bw for OTA (EFSA, 2006) and 1 μg/kg bw for DON and acetylated derivatives (JECFA, 2011) to characterize the human health risks.

Normality of raw datasets of mycotoxin levels in plasma or urine was tested by the Kolmogornov-Smirov test. Wilcoxon-Mann-Whitney rank sum test was used to compare two groups of data, and the Kruskal-Wallis test in comparisons among more than two groups. Food consumption estimates were normalised with the body weight. The time-course of mycotoxins and their metabolites was represented graphically by means of Box plots. The non-detects were used with the value of LOD in all cases. Associations between independent and dependent variables were assessed by using univariate and multivariable linear regression models, including the biomarker levels normalized by creatinine. Independent variables included the food intake (bread, pasta, pastries, beer, wine in g/kg bw), age (years), BMI (kg/m^2) and body fat (%). A backward elimination approach was used to improve the model deleting non explanatory variables. The statistical analysis was performed using the software Microsoft Excel® 2010 and SPSS® v15.0.

16.4. Results and discussion

16.4.1. Dietary intake and anthropometry of volunteers

Table 16.3 summarizes the anthropometric measurements of volunteers. The volunteers presented body mass indexes (BMI) between 18.6 and 34.4 kg/m^2 and body fat content between 18 and 34 %. Food consumption data reported by the 7-days dietary record were tabulated, normalised by the individual body weight and displayed in a database. Only the food items highly susceptible of OTA and DON contamination were finally retained. None of the

participants reported lack of adherence to the restriction diet during the restriction period. The main food categories reported during the free-diet period were bread, pasta, pastries, snacks, breakfast cereals, wine, beer, coffee, nuts or chocolate. As shown in the Table 16.4, the most consumed categories were bread and pastries with percentage of consumers ranging between 88 and 65 %, respectively and normalised mean consumption of 2 g/kg bw/day. Other items, for example beer, were consumed by a reduced percentage of volunteers (21 %) and during few days (two out of seven), but at high amounts (8.2 mL/kg bw/day). We did not detect any difference regarding sexes, age or different dietary habits, probably due to the low statistical potency.

Table 16.3. Anthropometric parameters of volunteers measured by a certified nutritionist.

Parameters	Units	Mean	SD	Median	Min	Max
Age	(years)	34.91	12.88	28.00	17	59
Weight	(kg)	66.86	14.79	64.30	50.40	104.00
Height	(m)	1.60	0.35	1.66	1.53	1.82
Body mass index	(kg/m^2)	23.70	4.46	22.74	18.60	34.35
Total fat	(%)	25.13	8.73	23.70	7.90	45.10
Waist circumference	(cm)	80.30	13.76	78.50	61.00	113.00
Hip circumference	(cm)	97.80	8.55	98.00	80.00	117.00

16.4.2. Optimization of the multidetection method

The current multidetection method was based on the procedure known as "*dilute and shoot*" previously developed by Warth *et al.* (2012), with minor modifications. A solution 1:4 (instead of 1:9) of ammonium acetate 1 % in H_2O (mobile phase of LC-MS/MS) was selected as the best option for the analysis of OTA, DON, DON-3-glucoside, DON-3-glucuronide, 3-ADON and DOM-1 in urine, as the analytes were better identified and separated in the chromatogram (Figure 16.2). The modification on solvent proportion allowed a lower LOD for some analytes such as OTA and DOM-1, improving the detection rates on naturally contaminated human samples. The MS parameters (declustering potential, collision energy, and cell exit potential) were optimized by means of syringe infusion on each compound separately. The results showed a better response in the ESI+ for OTA, DON, DON-3-glucoside, DOM-1, and ($^{13}C_{15}$) DON while 3-ADON and DON-3-glucuronide had a better response in the ESI-. Declustering potential was set according to the sensitivity of precursor ions, whereas collision energies were selected to give the maximum intensity of the obtained fragment ions (Table 16.1). The selectivity study demonstrated that no interfering peaks were observed at the retention time of analytes in the urine. The typical Multiple Reaction Monitoring (MRM) chromatogram of spiked urine is shown in Figure 16.2. The RSD% (Table 16.2) confirmed an acceptable precision of the

method. The mobile phase composed of acetonitrile (mobile phase A) and 10 mM of ammonium acetate in water (mobile phase B) was preliminarily tested providing good sensitivity.

Table 16.4. Summary of mycotoxin-related foodstuffs consumption during the diet free period.

	Bread	Pasta	Pastries	Snacks	Corn flakes	Wine	Beer	Coffee	Nuts	Chocolate
Consumers (%)	87.6	32.9	64.6	8.1	19.9	18.0	21.1	63.4	6.2	43.5
Mean days/week consumption (days)	6	3	5	3	4	3	2	6	2	4
Normalised daily consumption (g or mL/kg bw/day)										
Mean	1.9	1.5	2.0	0.7	0.7	2.8	8.2	1.4	0.5	0.6
SD	0.3	0.2	0.5	0.5	0.1	0.7	4.1	0.1	0.1	0.3
Median	2.0	1.5	2.0	0.6	0.7	2.6	8.7	1.4	0.6	0.7
Maximum	6.6	2.4	11.7	1.5	1.4	7.0	41.6	3.6	0.8	4.0

16.4.3. Background levels of OTA, DON and its metabolites in urine

OTA was detected in all urine samples collected before the restriction period with an average value of 0.042 ng/mL (range from 0.039 to 0.056 ng/mL) (see Table 16.5); LOD was used in all the non-detected samples. As expected, these values were lower than those values previously reported in Catalonia using an enzymatic method to cleave OTα glucuronide forms, estimating an average value and concentration range of 0.237 ng/mL and 0.057-0.562 ng/mL, respectively (Coronel *et al.*, 2011). Nevertheless, these values were similar to other studies performed in other close regions such as Portugal where the authors found an average OTA level of 0.019 ng/mL (Manique *et al.*, 2008) and 0.022 ng/mL (Duarte *et al.*, 2010). Moreover, in Valencia (Spain), Manique *et al.* (2008) reported a mean of 0.032 ng/mL and Pascale and Visconti (2001) found in positive samples a range from 0.012 to 0.046 ng/mL in Italy.

DON-3-glucuronide was detected in 74 % of samples and represented the main excretion DON analysed metabolite in background urine. The average and maximum concentrations were 56.98 and 219.46 ng/mL, respectively. The high occurrence and concentration of DON-3-glucuronide in urine was previously reported by other studies, whose authors reported glucuronidation ratios between 79 and 98 % (Turner *et al.*, 2011; Warth *et al.*, 2012). DON-15-glucuronide seems to be the main DON urine metabolite (Warth et al., 2012), however we did not analyse it because there are no DON-15-glucuronide standards in the market. The lack of information about DON-15-glucuronidation in our samples could lead to an important underestimation of total DON found in urine. The latest studies pointed out that the

rate of DON-15-glucuronidation within glucuronide conjugates seems high, over 65 % (Warth et al., 2012; Warth *et al.,* 2013; Warth *et al.,* 2014). Otherwise, despite the high occurrence of DON (91 %) in background urine, the concentration levels were commonly low (mean 2.9 ng/mL). DON levels accounted for the 4 % of the total sum of DON (Free DON + DON-3-glucuronide). This low concentration of DON in urine is in agreement with other previous studies using non-enzymatic methods; see Table 16.6. The major presence of DON (91 %) than DON-3-glucuronide (74 %) in the analysed samples could be because the LOD for DON-3-glucuronide was higher than the LOD for DON. So, it could be that DON-3-glucuronide was present at the same percentage as DON, but we could not detect it. Surprisingly, DOM-1 was present in all samples (100 %), showing also notorious concentration levels (average 13.0 ng/mL). In contrast, this metabolite was poorly detected in previous studies (34 %, 0.2-2.8 ng/mL) (Turner *et al.*, 2010b; Rodríguez-Carrasco *et al.*, 2014). On the other hand, DON-3-glucoside was detected in 74 % of samples (1.0-3.6 ng/mL) and 3-ADON was detected in all background samples (0.1-2.5 ng/mL).

The co-occurrence of OTA, DON and its metabolites in urine was very frequent, confirming the simultaneous exposure to both mycotoxins through the Catalonian diet. For instance, OTA and DON were simultaneously present in 91 % of the samples, while complex mixtures involving OTA, DON with the rest of metabolites were present in 39 % of samples. This finding confirms the need of further research on the still unknown field of toxicological interactions of mycotoxin cocktails, especially those of high probability of occurrence, as the case of OTA and DON.

16.4.4. Time-course of DON and its metabolites in urine

The time-course of DON and its metabolites was characterized by a high inter-individual variability, so it was difficult to capture a common time-course pattern for all subjects. The largest decrease of concentration after the restriction period was observed for DON, DON-3-glucoside and DON-3-glucuronide (Figure 16.3), but the differences were not statistically significant ($p < 0.05$). The detected levels, close to the LOD, likely caused the lack of statistical significance. After the restriction, DON was reduced in most of cases (83 %) but samples from some volunteers showed still some detectable levels. Regarding DON-3-glucoside, a concentration of 1.53 ng/mL at day 0 was detected, so a reduction of 15 % from day -5 was observed, and a similar concentration from day -5 was detected at day 1, after one day without diet restriction (1.86 ng/mL). At day -5, the mean DON-3-glucuronide concentration was 56.98 ng/mL, and after the restriction diet the concentration was 37.42 ng/mL. Moreover, the day after the end of

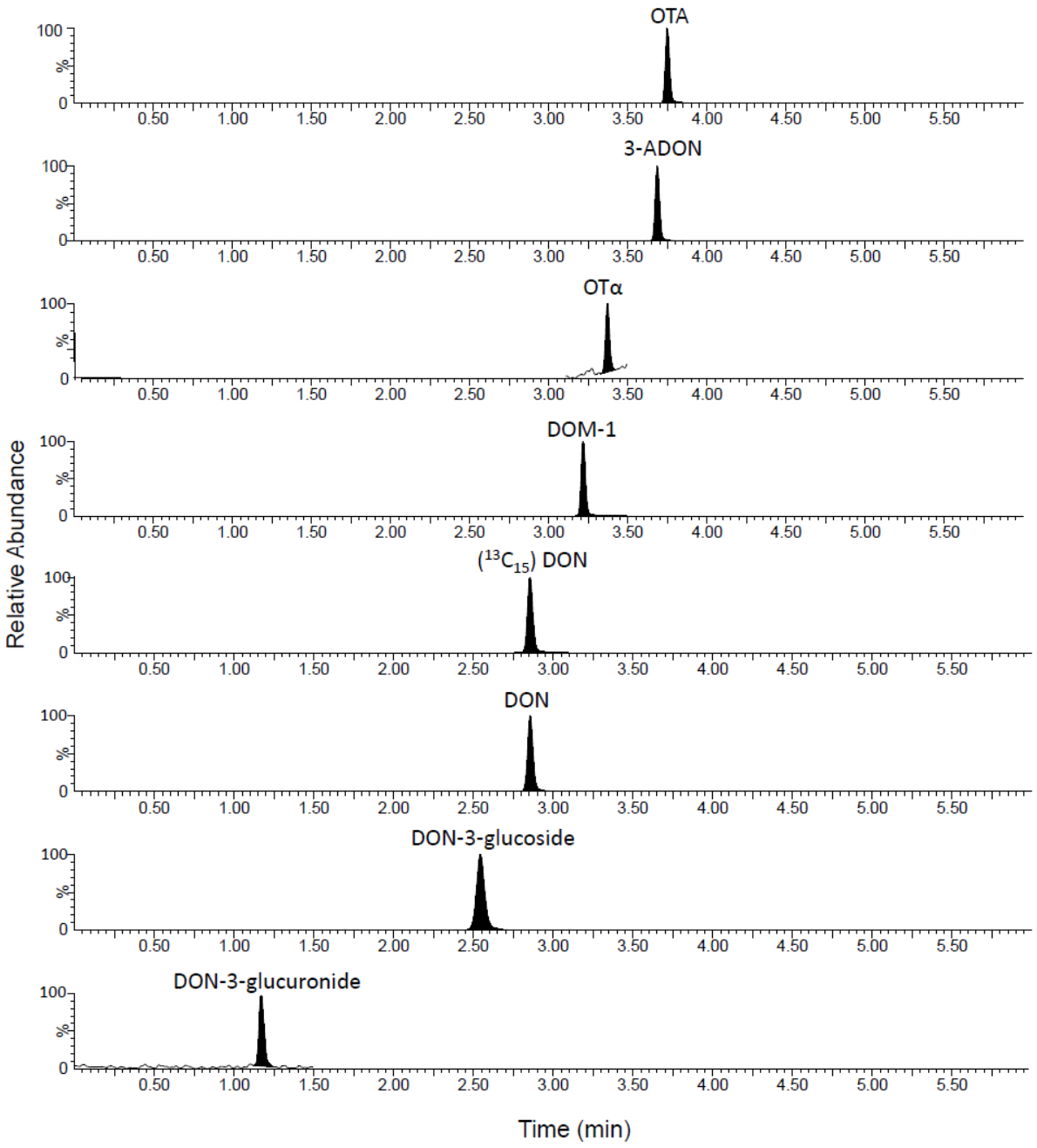


Figure 16.2. Multiple reaction monitoring chromatograms of spiked urine samples under the chromatographic conditions described in the text. Spiking levels for deoxynivalenol (DON), (13C15) DON, DON-3-glucoside, 3-actetyldeoxynivalenol (3-ADON) and deepoxydeoxynivalenol (DOM-1) were 15 µg/kg for OTA and OTα were 0.25 µg/kg.

Table 16.5. Background levels of OTA, DON and its metabolites in urine, without creatinine correction (NC) (ng/mL) or with creatinine correction (YC) (ng/mg), and in plasma (ng/mL) from the volunteers before and after the restriction period.

		Mycotoxin form	Creatinine	% positive samples	Mean (ng/mL or ng/mg)	SD (ng/mL or ng/mg)	Median (ng/mL or ng/mg)	Max (ng/mL or ng/mg)
URINE	Before restriction	DON	NC	91	2.9	3.1	2.1	13.5
			YC	91	1.1	0.8	1	4.1
		DON-3-glucoside	NC	74	1.8	0.8	1.6	3.6
			YC	74	0.8	0.5	0.6	2.4
		DON-3-glucuronide	NC	74	57.0	61.4	36.0	219.5
			YC	74	20.9	15.9	13.8	62.0
		3-ADON	NC	96	1.5	0.5	1.5	2.6
			YC	96	0.7	0.4	0.6	1.9
		DOM-1	NC	96	23.0	51.4	4.9	233.9
			YC	96	8.9	18.9	2.3	87.1
		OTA	NC	100	0.042	0.004	0.041	0.056
			YC	100	0.019	0.008	0.017	0.041
	After restriction	DON	NC	43	2.9	3.6	0.9	9.5
			YC	43	1.4	1.1	1.0	2.9
		DON-3-glucoside	NC	56	1.5	0.7	1.3	3.6
			YC	56	0.7	0.4	0.6	1.3
		DON-3-glucuronide	NC	61	37.5	26.3	26.2	95.4
			YC	61	15.0	10.7	15.8	40.3
		3-ADON	NC	100	1.8	0.5	1.8	3.2
			YC	100	0.7	0.5	0.5	2.7
		DOM-1	NC	78	12.9	15.7	8.8	64.2
			YC	78	5.3	8.3	2.9	35.7
		OTA	NC	96	0.04	0.003	0.04	0.051
			YC	96	0.01	0.008	0.01	0.04
PLASMA	Before restriction	OTA	-	100	0.09	0.08	0.06	0.32
		OTα	-	100	0.28	0.29	0.17	1.11
	After restriction	OTA	-	100	0.05	0.03	0.05	0.13
		OTα	-	100	0.19	0.09	0.19	0.31

Table 16.6. Levels of DON and its metabolites in urine from other biomonitoring studies carried out in Europe.

Mycotoxin form	Country	Extraction[a]	Analytical method[b]	LOD[c] (ng/mL)	% Positive	Mean (ng/mL)	Range (ng/mL)[d]	Reference
DON total	Austria	D&S	LC-MS/MS	6	63	20.4 ± 2.4	nd-63	Warth, 2012
DON	Austria	D&S	LC-MS/MS	4	-	-	nd	Warth, 2012
DON-3-glucoronide	Austria	D&S	LC-MS/MS	6	3.7	13	nd-13	Warth, 2012
DON-15-glucuronide	Austria	D&S	LC-MS/MS	6	63	18.8± 10.6	nd-43	Warth, 2012
DON total	Sweden	IAC (ENZ Tx)	LC-MS	0.5	90	3.5	nd-178	Wallin, 2013
DON total	Sweden	IAC and SPE (ENZ Tx)	LC-MS/MS	1.5	63	7.02 ± 24.4	-	Wallin, 2015
DON total	Italy	IAC and SPE (ENZ Tx)	LC-MS/MS	0.8	70	3.67±1.60	1.1- 14.2	Solfrizzo, 2011
DON total	Italy	IAC and SPE (ENZ Tx)	LC-MS/MS	1.5	96	11.89±10.05	nd-67.36	Solfrizzo, 2014
DON	Germany	D&S	LC-MS/MS	0.05	30	3.38	2.48-17.34	Gerding, 2014
DON-3-glucuronide	Germany	D&S	LC-MS/MS	0.1	84	12.21	4.37-92.95	Gerding, 2014
DON total	UK	IAC	LC-MS	0.6	98.7	9.42	0.6–65.97	Turner, 2008
DON total	UK	IAC (ENZ Tx)	LC-MS	0.6	99.6	12.0	nd-66	Turner, 2009
DON total	UK	IAC (ENZ Tx)	LC-MS	0.6	94.28	8.6	nd-70.7	Turner, 2010a
DON total	UK	IAC and SPE (ENZ Tx)	LC-MS	0.5	100	17.8	5.0–78.2	Turner, 2011
DOM-1	UK	IAC (ENZ Tx)	LC-MS	0.06	2	0.65	nd-0.8	Turner, 2011
DON total	UK	SPE (ENZ Tx)	LC-MS	0.5	72	1.3	nd - 6.5	Turner, 2012
DON total	UK	IAC (ENZ Tx)	LC-MS	0.5	100	10.3	nd-116.7	Hepworth, 2012
DON total	France	IAC and SPE (ENZ Tx)	LC-MS	0.5	98.68	6.8	0.5-28.8	Turner, 2010b
DOM-1	France	IAC (ENZ Tx)	LC-MS	0.2	34	0.2	0.2-2.8	Turner, 2010b
DON total	Spain	SPE	GC-MS/MS	0.12	68	22.78	nd-84.5	Rodriguez-Carrasco, 2014a
DON total	Spain	SPE	GC-MS/MS	0.12	30	7.4	nd-21.1	Rodriguez-Carrasco, 2014b
DON total	Spain	SPE	GC-MS/MS	0.10		35.2 ± 4.3		Rodriguez-Carrasco, 2015
DON	Croatia	D&S	LC-MS/MS	9	76	18.3	nd-275	Sarkanj, 2013
DON-3-glucuronide	Croatia	D&S	LC-MS/MS	7	83	28.8	nd-298	Sarkanj, 2013
DON-15-glucuronide	Croatia	D&S	LC-MS/MS	1	98	120.4	nd-1237.7	Sarkanj, 2013
DON total	Portugal	SPE (ENZ Tx)	GC-MS	0.3	69.2	16.2±10.3	nd-26.2	Cunha, 2012
DON	Portugal	SPE (ENZ Tx)	GC-MS	0.08	15.4	5.3±4.9	nd-8.8	Cunha, 2012
DOM-1	Portugal	SPE (ENZ Tx)	GC-MS	0.15	0	-	nd	Cunha, 2012
DON	Belgium	D&S	LC-MS/MS	0.2	37	6.1	nd-327.0	Heyndrickx, 2015
DON-3-glucuronide	Belgium	D&S	LC-MS/MS	0.2	77	6.7	nd-88.9	Heyndrickx, 2015
DON-15-glucuronide	Belgium	D&S	LC-MS/MS	0.2	100	50.1	nd-546.2	Heyndrickx, 2015

[a] D&S: Dilute and shoot. ENZ Tx: Enzymatic treatment. IAC: Immunoaffinity column. SPE: Solid-phase extraction

[b] LC-MS, Liquid chromatography–mass spectrometry. GC-MS, Gas chromatography–mass spectrometry. MS/MS, tandem mass spectrometry

[c] LOD – limit of detection. [d] nd – not detected

the restriction, a marked increase in DON-3-glucuronide concentration to 92.61 ng/mL (higher than the initial level) was detected ($p<0.05$). Before the restriction diet, an average concentration of 23.05 ng DOM-1/mL was detected in the urine samples, then after five days the mean concentration decreased to 12.89 ng/mL. The concentration of 3-ADON did not show relevant variations during the experimental period. Against our expectations, at day 0 some samples presented notorious levels of DON, DON-3-glucuronide and DON-3-glucoside, while DOM-1 and 3-ADON were detected in almost all samples. After confirming the strict adherence of the participants to the restriction diet we may deduce that other alternative sources of DON may contribute to the global exposure to this mycotoxin. Unfortunately the volunteers did not provide a dietary record from the restriction period to perform a deep study of these alternative sources.

16.4.4. Associations of DON and its metabolites in urine

We first attempted to assess the correlations between biomarkers and explanatory variables by means of bivariate correlation analysis, compiling the urinary data from the entire follow-up study (using the paired datasets from the whole period), and compared against the consumption of cereal based food during the day before. Using this approach we found significant correlations between DON and bread ($r=0.362$, $p<0.001$) and pastries ($r=0.239$, $p<0.01$). Furthermore, DON-3-glucuronide was correlated with DON ($r=0.332$, $p<0.001$) and DON-3-glucoside ($r=0.194$, $p<0.04$). In turn, DOM-1 was correlated with 3-ADON throughout the follow-up study ($r=0.336$, $p<0.001$). We further investigated the correlations of these biomarkers at day 1 (first day after restriction) by multivariate lineal regression, accounting for the consumption of the main food items at day 0 (bread, pasta and pastries) and adjusting for anthropometric variables. Due to the fast excretion rates of DON we also hypothesized that the morning urine levels could be mainly affected by the last meal from the day before, for this reason we performed a refined analysis considering only food from the last meal (commonly reported as dinners in the dietary food records). We found a notable increase on the correlations using this refinement, explained by the short elimination half-live of DON. After applying a backward procedure excluding the un-explanatory variables, the best model showed significant associations of DON-3-glucuronide with total cereal based food intake ($p=0.02$), bread ($p=0.05$), pasta ($p=0.0004$) and pastries ($p=0.04$), adjusted for body mass index and body weight. The overall model showed high and significant correlations ($R^2=0.88$, $p=0.003$). The correlations were not significant for the other models tested with DOM-1, DON-3 glucoside, DON and 3-ADON. These results are in agreement with another previous interventional study designed to assess the associations between urinary DON + DON-glucuronides with DON intake after a 4-day restriction period. Interestingly, a duplicate portion of the consumed bread was analysed, and DON exposure from bread intake was correlated with urinary DON+DON-glucuronide. The authors found high correlation coefficients ranged from 0.49 to 0.64, reaching even higher values after adjustment with age, sex and BMI (R^2 0.83, $p<0.001$) (Turner *et al.*,

2010a). We did not detect statistically significant differences ($p > 0.05$) due to sexes, age or different dietary habits, probably explained by to the low statistical potency of our limited population cohort.

16.4.5. Time-course of OTA

The restriction period triggered only a 3.1 % of OTA average reduction in urine, probably due to the large elimination half-life of OTA, estimated between 19-21 days in monkeys and 35 days in humans (EFSA, 2006). After the restriction period (Day 1 and 2), the urinary OTA levels increased but they did not reach the initial level (Figure 16.3) and tend to decrease at the end of the experimental period (Day 6) without statistical differences among days (Figure 16.3). The slow OTA excretion in urine could determine the reduction of OTA in urine found in the last part of the study. Surprisingly largest differences between the restriction and free-diet period were observed for OTA and OTα in plasma. The OTA and OTα concentration in plasma decreased when the diet was restricted (38.2 % reduction), and the mean OTA concentration in the days -3 and 0 were 0.089 and 0.055 ng/mL, respectively, with the levels always over the LOD (Figure 16.4). While a pronounced depletion was observed for most of the volunteers during the restriction period, the concentrations of OTA and OTα in plasma were significantly restored during the free-diet period. Some extreme values were observed during the first days of free-diet period as a result of an increased consumption of restricted food. The day after the end of the restriction diet, the OTA concentration suffered an important increase ($p<0.05$) from 0.055 ng/mL average value to 0.420 ng/mL. The toxicodynamics of plasmatic OTα were similar to OTA throughout the intervention period, supported by the high positive correlation observed between both mycotoxins (r= 0.997-0.919, $p<0.0001$). The OTα/OTA ratio was slightly reduced after the restriction period, but the differences were not significant during the experimental period.

16.4.6. Associations of OTA in urine

Significant correlations were found between urinary OTA and DON-3-glucuronide (r=0.374; $p<0.001$), DON-3-glucoside (r=0.355; $p<0.001$) and 3-ADON (r=0.3507; $p<0.001$). Concerning the food items, OTA and OTα in plasma showed significant correlations with bread (r=0.67; $p<0.001$), pastries (r=0.38; $p<0.01$) and corn-flakes (r=0.33; $p=0.02$), estimated using a pairwise model compiling all data points. The urinary levels of OTA were not significantly correlated with the different food items through bivariate correlation tests, and only one multivariate linear model showed statistical significance, being urinary OTA predicted by consumption of wine ($p=0.002$) and breakfast cereals ($p<0.001$) during the previous day ($R^2=0.16$, $p<0.001$, adjusted for body weight). The urinary levels of OTA were significantly correlated with plasmatic levels of OTA and OTα when we compared a time interval of 3 days but not when we compared with short time frames (24 h). For instance, the urinary levels of

OTA from day 1 was highly correlated with plasmatic levels of OTA and OTα from day 4 (r=0.611-0.629, *p*=0.02) but not correlated with day 2 (r=0.05-0.06, *p*>0.05). The time elapsed on the correlation could be explained by the slow clearance of OTA, which peak levels in plasma have been estimated in a wide and large interval from 10 hours in pigs to 48 h in rats (Benford *et al.*, 2001).

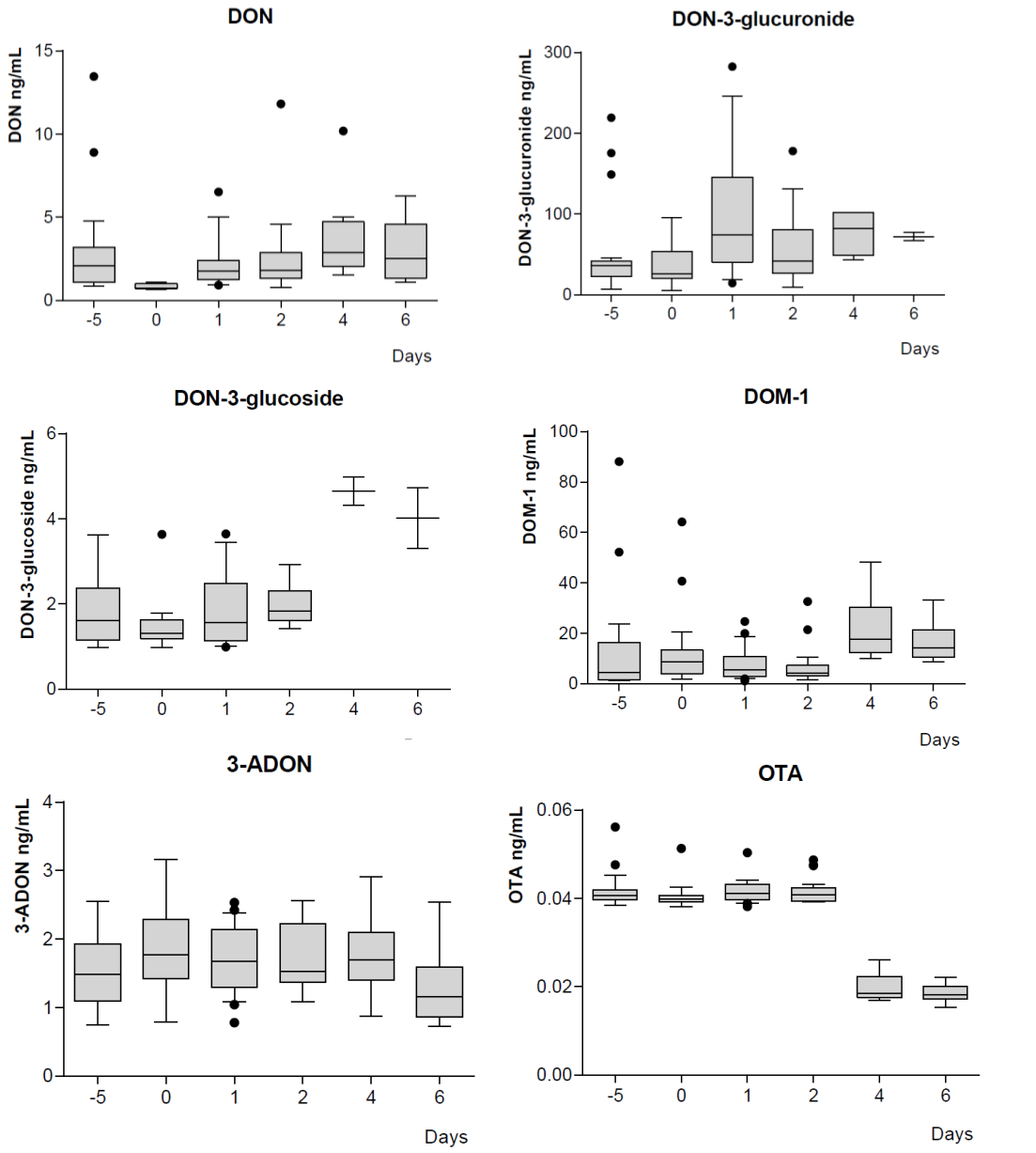


Figure 16.3. Box-plot representing the time-course of deoxynivalenol (DON), DON-3-glucoside, DON-3-glucuronide, 3-acetyldeoxynivalenol (3-ADON), de-epoxydeoxynivalenol (DOM-1) and ochratoxin A (OTA) in urine (units in ng/ml).

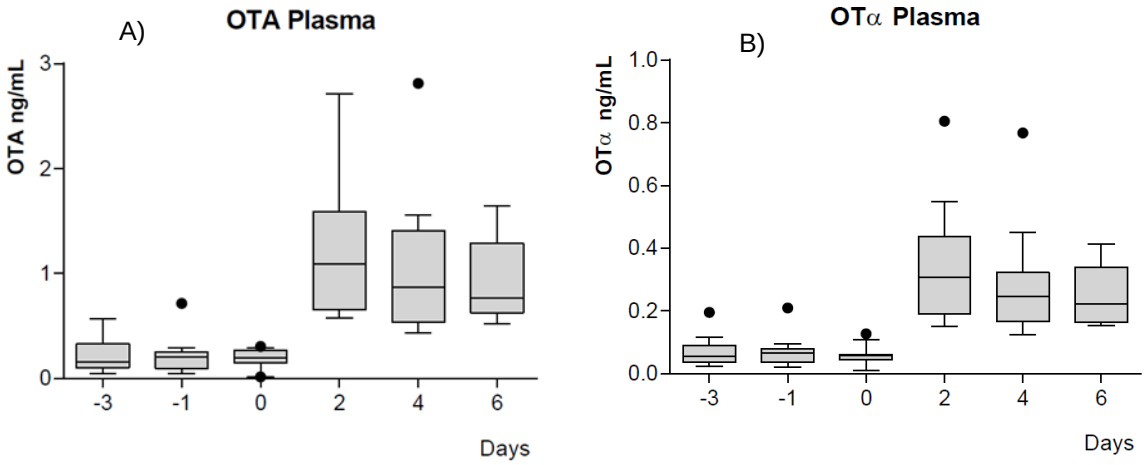


Figure 16.4. Box-plot representing the time-course of ochratoxin A (OTA) and ochratoxin α (OTα) in plasma for the experimental period.

16.4.7. Exposure assessment and health risk characterization

Risk characterization of OTA and DON is routinely addressed by comparing the dietary exposure levels with the corresponding TDIs. For this reason, we derived the external exposure estimates, also known as PDI, throughout the entire experimental period integrating the urinary with the excretion rates, daily urine volume and individual body weight. In case of DON we assumed an excretion rate of 72 % (Turner *et al.*, 2010a). In case of OTA we used the excretion rate of 2.6 % based on a biomonitoring study with pigs using low concentration oral doses of OTA (Gambacorta *et al.* 2013).

The exposure to DON (estimated from the sum of free DON and DON-glucuronide) decreased markedly at day 0, after the 5 days of diet restriction. Afterwards, the levels were similar to the background exposure levels (day -5). The median exposure for the overall period was 0.4 μg/kg bw/day (medians ranging between 0.02 and 1.5 μg/kg bw/day). However, the exposure estimates systematically reached high levels, which often exceeded the TDI of 1 μg/kg bw/day (Figure 16.5). While only 9 % of participants were found to keep the exposure levels below the safety limit during the follow-up period, most of them exhibited repeated journeys exceeding the TDI levels. Even the dietary habits of participants could be slightly modified due to the participation in the interventional trial, the exposure levels after the restriction was normalized in comparison with the background level. Thus, we may suspect that these participants and in extension, the rest of population, could be exposed in normal conditions to moderate and high levels of DON over the time instead of isolated exposure episodes. This pattern was apparently associated to repeated dietary habits of volunteers, which consumed some specific items almost every day instead of following a varied and balanced diet throughout the week. Furthermore, these results are in agreement with our previous estimates for the

Catalonian population obtained from a stochastic model based on a food intake (Cano-Sancho *et al.*, 2009). The median and mean exposure levels were estimated to be 0.32-0.56 and 0.22-0.37 µg/kg bw/day for adult males and females, respectively. Previous exposure studies based on urinary levels of DON also reported high percentages of population exceeding the TDI, such as Austria (33%), Belgium (16-39%), Croatia (48% of women) or Italy (40%) (Heyndrickx *et al.*, 2015; Sarkanj *et al.*, 2013; Solfrizzo *et al.*, 2014; Warth *et al.*, 2012). However, these differences could be caused by the food habits. There is no doubt food habits change among the different countries. Moreover, the mycotoxin exposition could change in function of the collection season; especially the mycotoxin content can change among seasons (Kirinčič, et al., 2012). We would like to emphasize an interesting alternative approach to assess the exposure to mycotoxins based on DON-equivalents, which allows the integration of multiple DON congeners and metabolites using molar mass based conversions (Warth et al., 2012).

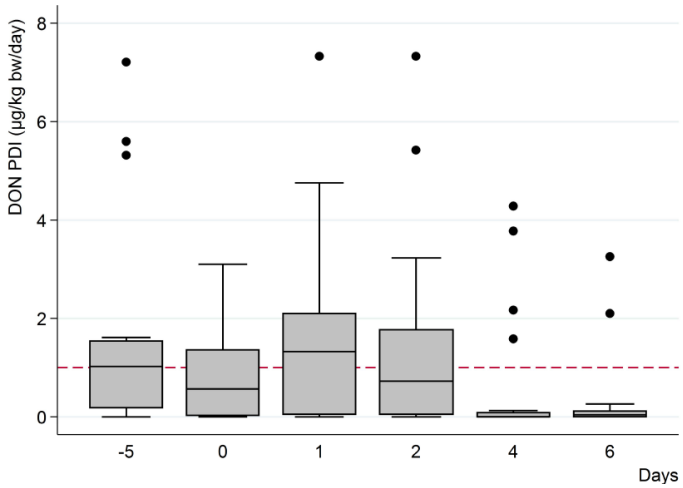


Figure 16.5. Probable daily intake of DON estimated from the sum of urinary levels of DON and DON-3-glucuronide throughout the interventional trial (inter-individual average and standard deviation, units in µg/kg bw/day). The dashed line represents the tolerable daily intake at 1 µg/kg bw/day

The estimated overall median PDI of OTA using the excretion rate 2.6% was 31.5 ng/kg bw/ day (median range, 14.9-48.9 ng/kg bw/ day) throughout the entire experimental period. Considering the TDI of 17 ng/kg bw/ day, most samples would be exceeding the safety values, thus reflecting an underestimation of exposure estimates using the dietary approaches that commonly concluded low risk expected from OTA consumption (Coronel *et al.*, 2012). These values are far of our previous estimates using a food dietary exposure approach for the Catalonian population where we concluded that the median exposure of adults to OTA was in the range of 0.23 and 0.47 ng/kg bw/ day, with highest estimates (99th Percentile) ranging between 1.76 and 2.39 ng/kg bw/ day (Coronel *et al.*, 2012). The exposure of Belgian population to OTA using the urinary biomarkers was estimated in the range between 0.1 and 21 ng/kg bw/ day (Heyndrickx *et al.*, 2015). In agreement, similar high estimates were predicted by Solfrizzo *et al.*, (2015) in Italy, which estimated a mean value of 139 ng/kg bw/day (5.8–127

times higher than the European PDI values estimated with diet approach). The authors attributed this worrying high estimate to the urinary excretion rate (2.6 % in piglets) or a possible underestimation derived from the dietary approach. These results boost an urgent need to refine the implemented approaches by means of more accurate toxicodynamic information with applicability to humans.

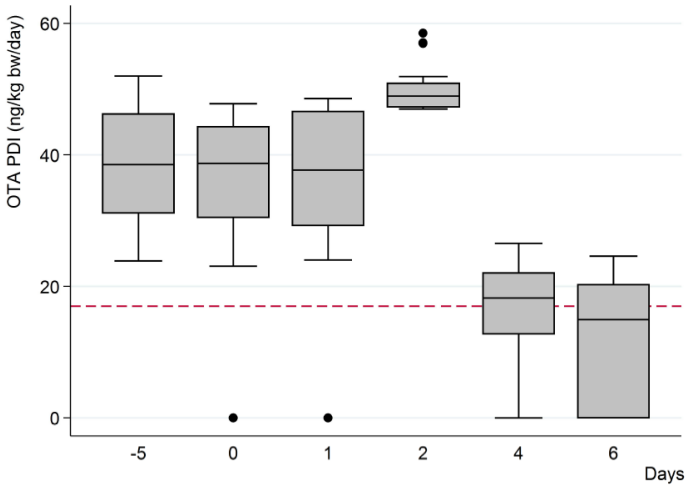


Figure 16.6. Probable daily intake (PDI) of ochratoxin A (OTA) estimated from urinary levels of OTA (inter-individual average and standard deviation, units in ng/kg body weight (bw)/day) considering the urinary excretion rate of 2.6%. The dashed line represents the tolerable daily intake at 17 ng/kg bw/day.

16.5. Conclusions

The proposed multi-detection methodology for urinary OTA, DON and its metabolites was successfully validated, providing suitable recovery, linearity and precision. Subsequently, the analytical methodology was applied in a preliminary pilot trial throughout a follow-up study. To the best of our knowledge, this is the first follow-up study using multi-detection method involving high concerning mycotoxins such us DON and OTA. The results showed that urinary OTA, DON and its metabolites were detected in most of samples, displaying moderate reductions after the restriction period and subsequently recovering the background levels. Despite the restriction period, some DON metabolites, such as 3-ADON or DOM-1, were found in most of urine samples, placing other alternative sources of exposure under suspicion. DON and DON-3-glucuronide were significantly associated with consumption of bread, pasta and pastries, while OTA was only associated with consumption of wine and breakfast cereals. The urinary levels of OTA were significantly correlated with plasmatic levels of OTA and OTα, supporting the results from this convenient biological matrix that allows the simultaneous determination of OTA and DON forms, and also it is more accessible to enrol donors in large scale studies. The results also showed that the high exposure to DON could be held throughout the time by the same person, exceeding the TDIs systematically instead of eventually. The

estimates of OTA exposure through urine are largely higher than those obtained with the dietary approach. The background levels found in urine revealed that the exposure to DON and OTA could be of concern for the Catalonian population, thus, further studies applying this biomonitoring methodology in a large sample of Catalonian population are needed to accurately characterize the human health risks at population level.

16.6. Acknowledgements

The authors are grateful to the Spanish government (projects AGL2010-22182-C04-04 and AGL2011-24862) for the financial support. A. Vidal thanks the Spanish Government (Ministry of Education) for the pre-doctoral grant. The authors are grateful to Dr. Francisca Vilaró for the technical support. The authors thank all the volunteers who participated in the study.

16.7. References

Bader, M., Wrbitzky, R., Blaszkewicz, M. and Van Thriel, C., 2007. Human experimental exposure study on the uptake and urinary elimination of N -methyl-2-pyrrolidone (NMP) during simulated workplace conditions. Archives Toxicology 81: 335-346.

Benford, D., Boyle, C., Dekant, W., Fuchs, R., Gaylor, D.W., Sephard, G., McGregor, D.B., Pitt, J.I., Plestina, R., Shephard, G., Solfrizzo, M., Verger, P.J.P. and Walker, R., 2001. Ochratoxin A. In: Safety evaluations of certain mycotoxins in food, WHO Food Additives Series 47, FAO Food and Nutrition Paper 74, IPCS International Programme on Chemical Safety, WHO, Geneva, 281–387

Cano-Sancho, G., Gauchi, J.P., Sanchis, V., Marín, S. and Ramos, A.J., 2011. Quantitative dietary exposure assessment of the Catalonian population (Spain) to the mycotoxin deoxynivalenol. Food Additives and Contaminants: Part A Chemistry Analysis Control Exposure Risk Assessment 28: 1098-109.

Clewell, H.J., Tan, Y.M., Campbell, J.L. and Andersen, M.E., 2008. Quantitative interpretation of human biomonitoring data. Toxicology and Applied Pharmacology 231: 122-133.

Coronel, M.B., Marin, S., Tarragó, M., Cano-Sancho, G., Ramos, A.J. and Sanchis, V., 2011. Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain. Food and Chemical Toxicology 49: 1436-1442.

Coronel, M.B., Marín, S., Cano-Sancho, G., Ramos. A.J. and Sanchis, V., 2012. Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. Food Additives and Contaminants: Part A Chemistry Analysis Control Exposure Risk Assessment 29: 979-993.

Cunha, S.C. and Fernandes, J.O., 2012. Development and validation of a gas chromatography-mass spectrometry method for determination of deoxynivalenol and its metabolites in human urine. Food and Chemical Toxicology 50: 1019-1026.

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

Edwards, S.G., 2009. *Fusarium* mycotoxin content of UK organic and conventional wheat. Food Additives and Contaminants: Part A Chemistry Analysis Control Exposure Risk Assessment 26: 496-506.

European Commission, 2006. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal European Union L364: 5–24.

European Food Safety Authority (EFSA), 2006. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to ochratoxin A in food. EFSA Journal 365: 1-56.

Ezekiel, C.N., Warth, B., Ogara, I.M., Abia, W.A., Ezekiel, V.C., Atehnkeng, J., Sulyok, M., Turner, P.C., Tayo, G.O., Krska, R. and Bandyopadhyay, R., 2014. Mycotoxin exposure in rural residents in northern Nigeria: A pilot study using multi-urinary biomarkers. Environment International 66: 138-145.

FAO (Food Agriculture and Nutrition), 1991. Food for the future, 1.FAO. Rome: FAO. Global environment Monitoring System/Food World Health Organization (GEMs/Food-WHO). May 1995. Reliable evaluation of low-level contamination of food report of a workshop in the framework of GEMS/Food-EURO.Kulmbach, Germany. Available at: http://www.who.int/foodsafety/publications/chem/lowlevel_may1995/en/index.html.

Fruhmann, P., Warth, B., Hametner, C., Berthiller, F., Horkel, E., Adam, G., Sulyok, M., Krska, R. and Fröhlich, J., 2012. Synthesis of deoxynivalenol-3-β-D-O-glucuronide for its use as biomarker for dietary deoxynivalenol exposure. World Mycotoxin Journal 5: 127-132.

Gambacorta, L., Solfrizzo, M., Visconti, A., Powers, S., Cossalter, A.M., Pinton, P., Oswald, I.P. 2013. Validation study on urinary biomarkers of exposure for aflatoxin B1, ochratoxin A, fumonisin B1, deoxynivalenol and zearalenone in piglet. World Mycotoxin Journal 6:299–308.

Gerding, J., Cramer, B. and Humpf, H.-U., 2014. Determination of mycotoxin exposure in Germany using an LC-MS/MS multibiomarker approach. Molecular Nutrition and Food Research 58: 2358-2368.

Hepworth, S.J., Hardie, L.J., Fraser, L.K., Burley, V.J., Mijal, R.S., Wild, C.P., Azad, R., Mckinney, P.A. and Turner, P.C., 2012. Deoxynivalenol exposure assessment in a cohort of pregnant women from Bradford, UK. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 29: 269-276.

Heyndrickx, E., Sioen, I., Huybrechts, B., Callebaut, A., De Henauw, S. and De Saeger, S., 2015. Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study. Environment International 84: 82-89.

IARC (International Agency for Research on Cancer), 2002. Monograph on the evaluation of carcinogenic risk to humans. Some traditional herbal medecines, some mycotoxins, naphthalene and styrene. Vol. 82. Lyon, France.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2011. Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Evaluation of certain contaminants in food. Available at http://apps.who.int/iris/bitstream/10665/44514/1/WHO_TRS_959_eng.pdf

Juan, C., Ritieni, A. and Mañes, J., 2012. Determination of trichothecenes and zearalenones in grain cereal, flour and bread by liquid chromatography tandem mass spectrometry. Food Chemistry 134: 2389-2397.

Kirincic, S., Skrjanc, B., Kos, N., Kozolc, B., Pirnat, N. and Tavcar-Kalcher, G., 2015. Mycotoxins in cereals and cereal products in Slovenia e official control of foods in the years 2008-2012. Food Control 50: 157-165.

Manique, R., Pena, A., Lino, C.M., Moltó, J.C. and Mañes, J., 2008. Ochratoxin A in the morning and afternoon portions of urine from Coimbra and Valencian populations. Toxicon 51: 1281-1287.

Maresca, M., 2013. From the gut to the brain: Journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins 5: 784-820.

Marin, S., Ramos, A.J., Cano-Sancho, G. and Sanchis, V., 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chemical Toxicology 60: 218-237.

Pascale, M. and Visconti, A., 2001. Rapid method for the determination of ochratoxin A in urine by immunoaffinity column clean-up and high-performance liquid chromatography. Mycopathologia 152: 91-95.

Pestka, J.J., 2010a. Toxicological mechanisms and potencial health effects of deoxynivalenol and nivalenol. World Mycotoxin Journal 3: 323–347.

Pestka, J.J., 2010b. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives Toxicology 84: 663–679.

Pitt, J. and Hocking, A., 2009. Fungi and Food Spoilage, Springer, Berlin, Germany, 3rd edition, 2009.

Rodríguez-Carrasco, Y., Moltó, J.C., Mañes, J. and Berrada, H., 2014a. Exposure assessment approach through mycotoxin/creatinine ratio evaluation in urine by GC–MS/MS. Food Chemical and Toxicology 72: 69-75.

Rodríguez-Carrasco, Y., Moltó, J.C., Mañes, J. and Berrada, H., 2014b. Development of a GC-MS/MS strategy to determine 15 mycotoxins and metabolites in human urine. Talanta 128: 125-131.

Rodríguez-Carrasco, Y., Mañes, J., Berrada, H. and Font, G., 2015. Preliminary estimation of deoxynivalenol excretion through a 24 h pilot study. Toxins 7: 705-718.

Šarkanj, B., Warth, B., Uhlig, S., Abia, W.A., Sulyok, M., Klapec, T., Krska, R. and Banjari, I., 2013. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. Food Chemical and Toxicology 62: 231-237.

Scientific Committee on Food (SCF), 1998. Opinion of the Scientific Committee on Food on ochratoxin A. Available at: http://ec.europa.eu/food/fs/sc/scf/out14_en.html.

Shephard, G.S., Burger, H.M., Gambacorta, L., Gong, Y.Y., Krska, R., Rheeder, J.P., Solfrizzo, M., Srey, C., Sulyok, M., Visconti, A., Warth, B. and Van der Westhuizen, L., 2013. Multiple mycotoxin exposure determined by urinary biomarkers in rural subsistence farmers in the former Transkei, South Africa. Food Chemical and Toxicology 62: 217-225.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y. and Qian, S.Y., 2012. Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. Food Control 26: 287-292.

Solfrizzo, M., Gambacorta, L., Lattanzio, V.M.T., Powers, S. and Visconti, A., 2011. Simultaneous LC-MS/MS determination of aflatoxin M1, ochratoxin A, deoxynivalenol, de-epoxydeoxynivalenol, α and β-zearalenols and fumonisin B1 in urine as a multi-biomarker method to assess exposure to mycotoxins. Analytical and Bioanalytical Chemistry 401: 2831-2841.

Solfrizzo, M., Gambacorta, L. and Visconti, A., 2014. Assessment of multi-mycotoxin exposure in southern Italy by urinary multi-biomarker determination. Toxins 6: 523-538.

Turner, P.C., Taylor, E.F., White, K.L.M., Cade, J.E. and Wild, C.P., 2009. A comparison of 24h urinary deoxynivalenol with recent v. average cereal consumption for UK adults. British Journal of Nutrition 102: 1276-1279.

Turner, P.C., White, K.L.M., Burley, V.J., Hopton, R.P., Rajendram, A., Fisher, J.C., Cade, J.E. and Wild, C.P., 2010a. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK adults. Biomarkers 15: 553-562.

Turner, P.C., Hopton, R.P., Lecluse, Y., White, K.L.M., Fisher, J. and Lebailly, P., 2010b. Determinants of urinary deoxynivalenol and de-epoxy deoxynivalenol in male farmers from normandy, France. Journal of Agricultural and Food Chemistry 58: 5206-5212.

Turner, P.C., Hopton, R.P., White, K.L.M., Fisher, J., Cade, J.E. and Wild, C.P., 2011. Assessment of deoxynivalenol metabolite profiles in UK adults. Food Chemical and Toxicology 49: 132-135.

U.S. Department of Health and Human Services Food and Drug Administration, 2001. Guidance for Industry Bioanalytical Method Validation. Available at: http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf

Wallin, S., Gambacorta, L., Kotova, N., Warensjö Lemming, E., Nälsén, C., Solfrizzo, M. and Olsen, M., 2015. Biomonitoring of concurrent mycotoxin exposure among adults in Sweden through urinary multi-biomarker analysis. Food Chemical and Toxicology 83: 133-139.

Wallin, S., Hardie, L.J., Kotova, N., Warensjö Lemming, E., Nälsén, C., Ridefelt, P., Turner, P.C., White, K.L.M. and Olsen, M., 2013. Biomonitoring study of deoxynivalenol exposure and association with typical cereal consumption in Swedish adults. World Mycotoxin Journal 6: 439-448.

Warth, B., Sulyok, M., Berthiller, F., Schuhmacher, R., Fruhmann, P., Hametner, C., Adam, G., Fröhlich, J. and Krska, R., 2011. Direct quantification of deoxynivalenol glucuronide in human urine as biomarker of exposure to the *Fusarium* mycotoxin deoxynivalenol. Analytical and Bioanalytical Chemistry 401: 195-200.

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Fröhlich, J. and Krska, R., 2012. Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins. Rapid Communications in Mass Spectrometry 26: 1533-1540.

Warth, B., Sulyok, M. and Krska, R., 2013. LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxins. Analytical and Bioanalytical Chemistry 405: 5687–5695.

Warth, B., Petchkongkaew, A., Sulyok, M., and Krska, R., 2014. Utilising an LC-MS/MS-based multi-biomarker approach to assess mycotoxin exposure in the Bangkok metropolitan area and surrounding provinces. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 31: 2040-2046.

Yang, D., Geng, Z.M., Yao, J.B., Zhang, X., Zhang, P.P. and Ma, H.X., 2013. Simultaneous determination of deoxynivalenol, and 15- and 3-acetyldeoxynivalenol in cereals by HPLC-UV detection. World Mycotoxin Journal 6: 117-125.

17. Global discussion

From the discovery of Afs at the beginning of the 1960s, a lot of advances have been made to reduce the mycotoxin concentrations in food and feed. Thus, every year a huge amount of new studies appear to enlarge the mycotoxins knowledge. Although large progress has been made in mycotoxin risk assessment and management, a hard work remains to be done before the complete protection of humans and animals against mycotoxins is possible. An overview of the main results presented in this thesis work which enlarge the actual knowledge about mycotoxins is presented and discussed in this section.

This section has been divided into four different parts for a better comprehension:

17.1. Presence of mycotoxins

Although all efforts to control mycotoxins presence through the food chain, according to our results, mycotoxins still have a wide presence in raw cereals and cereal based foods. Thus, more than 74 % of total analysed food samples (bran and bread) from the market contained one or more analysed parent mycotoxins, but the value reached 83 % if conjugated mycotoxins were considered in the total mycotoxin sum (free mycotoxins + conjugated mycotoxins). Trichothecenes are the most common mycotoxins in analysed cereal based food (NIV and DON). Although NIV is not regulated, it was the most common mycotoxin with the highest concentration in the analysed breads. However, recently more studies found high presence of DON in cereal products (Table 17.1).

The highest mycotoxin concentrations in analyzed samples were also found for trichothecenes. Moreover, some cereal based food samples analysed (10.5 %) surpassed the maximum DON concentration set up by the EC (EC 1881/2006). In particular, DON limits were exceeded for pasta, bran, breakfast cereals, bread, bakery products and baby food (Table 17.1). Thus it seems clear that strategies to reduce trichothecene levels in cereal based food are required. The high levels of trichothecenes in cereals cause cereal based food to be the main source of exposure to these mycotoxins (Marín et al., 2013).

Despite the worrying levels of trichothecens, fortunately, Afs, ZEN and OTA are less common in cereals. Afs presence in cereals could be a problem mainly in maize. For instance, our results did not show Afs presence in wheat or oat, and their presence is scarce when wheat or oat based products are analysed, for example, 4.7 % in white bread (Saladino et al., 2017). By contrast, some studies showed higher presence of Afs in maize, as EFSA (2007) found double presence of Afs in maize (14 % of positive samples) than in other cereals (7 %). Although all cereals must be controlled for Afs levels, maize samples should deserve special attention. ZEN has had a moderate presence in our analysed cereal based food samples (19 %). This result agrees with other ZEN presence studies where less than 50 % of ZEN presence in cereal products is reported. Hence, 33 % or 24 % ZEN presence in biscuits and in bakery

products were found, respectively (EFSA, 2011). Despite the common presence of ZEN in cereal based food, ZEN concentrations are customarily below the European legislation limit in these products as our results showed. Cano-Sancho et al. (2013) did not find any ZEN concentration over the limit after they analysed 456 cereal based food samples.

Table 17.1. Number of DON positive samples (%), mean and maximum DON concentration levels found in different studies which analysed cereal based food.

Food Category	N (%)	Mean	Maximum	Reference
Biscuits	23 (24)	50.6	420	SCOOP 2003
Bread	29 (32)	88.9	560*	
Pasta	169 (56)	141.2	3200*	
Breakfast cereals	40 (56)	198.8	426	
Baby food	133 (72)	99.6	1075*	
Baby food	19 (76)	102.6	268*	Juan et al.2014
Bread	24 (96)	316	-	Vanheule et al., 2014
Breakfast cereals	5 (25)	1295	-	
Wheat based products	95 (80)	12.7	83.2	Rodriguez-Carrasco et al., 2014
Pasta	101 (21)	64.8	385.7	Brera et al., 2013
Fibre enriched bread	52 (13)	34	138	De Boevre et al., 2013
Bran enriched bread	36 (15)	25	127	
Breakfast cereal	62 (26)	44	718*	
Wheat flakes	20 (74)	190	437	Cano-Sancho et al., 2013
Pasta	52 (74)	226	946*	
Sliced bread	12 (17)	68	98	
Bread	31 (100)	246	739*	
Bread	5 (17)	491	988*	Savi et al., 2016
Crackers	3 (10)	739	1159*	

* DON concentrations exceed European legislation levels.

OTA presence in the analysed foods during this thesis (25 %) shows the importance to control it in cereal based food as other studies: Assunçao et al. (2016) found 50 % of OTA positive cereal based food samples, Coronel et al. (2012) found 19 % of OTA positive cereal based food samples and Sugita-Konishi et al. (2013) found 63 % positive samples. However, it is difficult to detect high OTA concentrations in cereal based products and samples over the legislation European limit are very scarce. For instance, the cited studies only found one oatmeal sample with more than 3 µg/kg of OTA after analysing more than 1078 samples.

Co-occurrence of mycotoxins in cereal based products is an important research subject because the effects of co-exposure on human and animal health remain unclear. Most of mycotoxin mixtures lead to additive or synergistic effects, which can cause even more health-related issues for humans and animals upon consumption of mycotoxin-contaminated food or

feed (Smith et al., 2016). The obtained results clearly demonstrate co-occurrence of parent mycotoxins as a real threat in cereal-based products and high co-occurrence is found in other studies (Juan et al., 2016; Smith et al., 2016).

So, presence of mycotoxins Afs, DON, NIV, ZEN and OTA in cereals is very common, especially trichothecens, and it is important to find solutions to reduce their presence. The high presence of mycotoxins in cereal based products confirms cereals are the main source of exposure to mycotoxins.

17.1.1. Presence of conjugated mycotoxins

Recent studies show that parent mycotoxins are not the only hazard in mycotoxin exposure, because different researches have detected the common presence of conjugated mycotoxins. While analytical methods are improving, new data about presence of conjugated mycotoxins are appearing every year, showing that it is not uncommon to find this type of toxin when there is presence of parent mycotoxins. Cereals, as one of the main products culprit of high exposure to mycotoxins, have been studied for presence of conjugated mycotoxins.

Up to now, *Fusarium* conjugated mycotoxins are the most common found in cereal based products, as showed in our results. Regarding DON conjugates (3-ADON, 15-ADON and DON-3-glucoside), their high presence, especially of DON-3-glucoside, has been detected in cereal food samples. 3-ADON and 15-ADON concentrations are usually very low (< 50 µg/kg), because they have been shown to be easily converted to DON during food processing (Wu & Wang, 2015). They have been detected in bread during this thesis as in other studies (De Boevre et al., 2013). DON-3-glucoside is of concern, because its presence and concentration is larger in cereal based foods. Concentration of DON-3-glucoside in bread is sometimes similar to that of DON concentration due to a possible increase of DON-3-glucoside during food processing, because while the ratio DON-3-glucoside/DON in raw cereals is from 10 to 30 % (Berthiller et al., 2009; Dall'Asta et al., 2013), much higher ratios are found in bread: De Boevre et al. (2013) found the same concentration of DON-3-glucoside than DON, Vanheule et al. (2014) found a ratio of 1.55 DON-3-glucoside/DON and De Boevre et al. (2012) found a ratio of 0.91.

Although the level of acetylated DON is low in cereal based products, it is important to control their levels because their toxicity is equivalent or stronger than that of DON (Eriksen & Pettersson, 2004). DON-3-glucoside toxicity is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003). Moreover Berthiller et al. (2011) have shown that DON modified forms can be hydrolysed to DON by several intestinal lactic acid bacteria. Due to their level of toxicity and large presence, the FAO/WHO Expert Committee (JECFA) considered acetylated forms, as DON-3-glucoside, to be an additional contributing factor to the total dietary exposure to DON (JECFA, 2011).

Unlike DON conjugates, ZEN conjugates presence has been less studied. However, they could have a wide presence in cereal based products, especially α-ZEL and β-ZEL. Moreover, high concentrations were detected in recent studies (De Boevre et al., 2013; Juan et al., 2014; Nathanail et al., 2015), even higher concentration of α-ZEL and β-ZEL than ZEN have been detected (De Boevre et al., 2013) as it was also found in this thesis. ZEN glucoside conjugates (ZEN-14-glucoside) are less common than DON glucoside conjugates (DON-3-glucoside) and their presence has always been less than 20 % of total analysed cereal based foods (De Boevre et al., 2013; Nathanail et al., 2015).

Although α-ZEL and β-ZEL are not regulated, the high levels detected are of concern, especially for α-ZEL. β-ZEL has a 2.5 times lower affinity for the estrogen receptor than ZEN, while α-ZEL has a 92 times higher affinity in comparison to ZEN. So, more control of these mycotoxins is required. Even if the toxicity of ZEN-14-glucoside is not known, the transformation from ZEN-14-glucoside to ZEN during digestion is probable.

Apart from conjugated mycotoxins, some mycotoxins could be embedded in the cereal matrix and they could be ignored in routinary analysis. Thus, they could be only detected after enzymatic release. Fbs, DON and Afs are some mycotoxins that could be embedded in the matrix. Embedded Fbs were the first mycotoxins to be detected thanks to alkali hydrolisation (Dall'Asta et al., 2009). These could represent more than 25 % of total fumonisins. Hidden DON could also exist because enzymatic activity caused an increase of DON in wheat grain (39 %) (Simsek et al., 2012) and their increase was not caused by the reduction of conjugated mycotoxins as showed for Afs in this thesis.

The presence of embedded mycotoxins enlarges the list of modified mycotoxins, which are different to parent compounds. For this reason a recent classification of mycotoxins that are different to parent mycotoxins was made by Rychlik et al. (2014) (Table 17.2).

To sum up, not only parent mycotoxins should be analysed because modified mycotoxins have shown a high presence in food. However, analyses of conjugated mycotoxins are still difficult and easier analytical methods are required. Thus more research is required to find all conjugated mycotoxins and develop suitable analytical methods for their easy detection. Moreover, there is not legislation about them, however, it should exist to protect consumers because they can have a higher toxicological power than parent mycotoxins or they can be transformed to parent mycotoxins due to digestion.

Table 17.2. Classification of mycotoxins different to parent mycotoxins.

First level	Second level	Third level	Fourth level	Examples
Free or parent mycotoxins				DON, AFB1, ZEN, OTA
Matrix-associated mycotoxins	Complexes physically dissolved or trapped.			Fumonisins bound to starch. DON and Afs released for enzymes
Modified mycotoxins	Biologically modified	Functionalised (phase I metabolites)		Aflatoxin B1-epoxide
		Conjugated (phase II metabolites)	Conjugated by plants	DON-3-glucoside
			Conjugated by animals	DON-3/15-glucuronide, HT2-3/4-glucuronide
			Conjugated by fungi	3-ADON and 15-ADON
	Chemically modified	Thermally formed		norDON A-C, N-carboxy-methyl-Fumonisin B1, 14-(R)-ochratoxin A
		Non-thermally formed		norDON A-C (under alkaline conditions)

17.2. Food processing

In the present work a great number of studies have been made about the stability of OTA, DON and its conjugates during fermentation, baking and boiling. Specifically, studies about breadmaking, baking and pasta making process have been developed. Due to the high presence of these mycotoxins in cereals, methods to reduce them are urgently needed and food processing is an important part of the food chain to reduce mycotoxin concentration. However, mycotoxins are very stable compounds and it is extremely difficult to decrease their concentration, especially after cleaning and milling steps. Cleaning and sorting reduce mycotoxins concentrations during this first step because dust, broken grains and other foreign material, where mould is present, are removed. Milling causes a reduction of mycotoxins in the internal fractions of the grain because usually there is a larger mycotoxin concentration in the external layers. For this reason external layers are usually destined to animal feed. However, bran is sometimes consumed by people with the aim to increase fiber consumption for healthier diets.

17.2.1. Fermentation

Fermentation is a common process to produce a lot of different products and it is able to produce changes in mycotoxin stability. Beer or wine fermentations have been widely studied with huge reductions of mycotoxin presence. Thus, OTA has been removed from wine (more than 80 %) after fermentation (Piotrowska et al., 2013). But bread fermentation has been less studied and lower reductions exist during its fermentation. Firstly, shorter fermentation times compared to beverage fermentations could be one reason of this low level of reduction. Our results showed the importance of time for DON reduction during bread fermentation. Longer times caused larger DON reductions. However more factors are essential for the stability of this toxin. While reduction of DON occurs with the absence of added enzymes, Suman et al. (2012) detected an increase of up to 14 % using non-specific enzymes; studies in which either malt flour or other enzymes were added reported increases in DON levels (Simsek et al., 2012; Suman et al., 2012) as we found in Study IV and V (Table 17.3). When we used flour improvers with non-specific enzymes, increase of DON up to 30 % was detected. We observed that some enzymes can have a lot of importance in DON stability. Thus, the use of α-amylase during the fermentation at 30 ºC led to an increase of 10 % in the DON level. Moreover, xylanase, cellulase, protease and glucose-oxidase use resulted in increases of DON of 15, 63, 75 and 78 %, respectively, in the fermented dough compared with the fermented dough without enzymes when fermentation was at 45 ºC. This could be associated with the release of DON from the wheat matrix through enzyme catalysis. Temperature, apart from modifying enzyme activity, may directly affect DON stability; Samar et al. (2001) assayed fermentation temperatures from 30 to 50 ºC, and found higher DON reduction as the temperature increased (from 0 to 56 %). The highest reduction in DON levels was observed at the highest temperature (50 ºC) and the

longest time tested (60 min). This result is consistent with our results where larger DON reductions were achieved during fermentation at 45 ºC than at 30 ºC. Similarly, Generotti et al. (2015) assayed different fermentation temperatures (from 26 to 46 ºC), and they found reduced DON stability with higher fermentation temperatures. Thus, fermenting at high temperatures may be a feasible alternative to reduce DON content in bread, as long as bread quality is not affected.

On the other hand, Generotti et al. (2015) pointed out that DON is more affected by initial yeast amount in the recipe than by fermentation time and temperature; a possible explanation for the observed DON reduction is DON adsorption on the yeast wall (De Souza et al. 2015), however more studies to confirm this point are necessary. By contrast, we observed that sourdough use led to increases in DON content during fermentation; it was suggested that sourdough bacteria could be able to either transform DON precursors into DON or to release bound DON, increasing DON content at the end of fermentation. For example, increase of DON can also be favoured by the conversion of 3-ADON and 15-ADON forms to DON. Wu and Wang (2015) showed that almost all 3-ADON and 15-ADON were converted to DON during kneading and fermentation.

In conclusion, DON can be either reduced or increased in dough fermentation. The use of high fermentation temperatures and avoiding enzyme use should produce DON reductions at the end of fermentation, while use of enzymes (especially xylanase and α-amylase) can cause important increases of DON due to enzymatic release.

Due to the high presence of DON-3-glucoside, its stability during bread fermentation has been studied in this thesis, however it is not widely studied yet. DON-3-glucoside concentration suffered a high variation at the end of this step. Thus, when enzymes were added to the dough, a clear decrease in DON-3-glucoside during fermentation was observed, regardless of the temperature. Despite a reduction in the DON-3-glucoside concentration at the end of fermentation, xylanase and protease at 30 ºC and cellulase and lipase at 45 ºC led to less pronounced reductions in DON-3-glucoside concentrations than fermented doughs with no added enzymes. Conversely, the exception to DON-3-glucoside reduction at the end of fermentation was for glucose oxidase activity, which led to a significant increase in DON-3-glucoside. The presence of glucose oxidase generated an important increase in DON-3-glucoside in the fermented dough compared to the initial mix.

The use of enzymes as improvers in past studies caused increases in DON-3-glucoside during fermentation; however, the exact enzymes responsible for this increase were not described (Kostelanska et al. 2011). Simsek et al. (2012) used only α-amylase and detected a reduction in DON-3-glucoside at the end of fermentation at 30 ºC (5 %) similar to this thesis.

OTA is a very common mycotoxin in food products and several studies about its stability during food processing exist. Thus, high reductions of OTA during wine fermentation have been observed (Piotrowska et al., 2013). However OTA seems to be more stable during bread

fermentation and its concentration is not affected by it. Similarly, some studies also concluded that OTA is stable during bread fermentation. For instance, Scudamore et al. (2003) did not find any reduction at 43 ºC for 35 min. On the other hand, Valle-Algarra et al. (2009) found a reduction from 29.8 to 33.5 % (30 ºC, 1 h), but they used spiked OTA contamination which is likely easily reduced. The huge difference in OTA behaviour among food processes could be caused by the short time of fermentation in bread compared for instance to wine fermentation.

To sum up, bread fermentation can cause variations in some mycotoxin's stability and it could be a key step to reduce mycotoxin presence in food. Time, temperature, enzyme presence, yeast and sourdough are factors which cause variations in mycotoxins stability.

17.2.2. Baking

Mycotoxins are thermostable compounds, however, some reductions can be achieved after a thermal treatment. Several cereal based food processes involve a baking step.

Studies that have examined DON concentrations after baking are contradictory: some studies have observed reductions (Bergamini et al., 2010; Neira et al., 1997), while other studies have reported no changes or even increases (Simsek et al., 2012; Zachariasova et al., 2012). Firstly, the inconsistencies may exist because these studies have been conducted on different scales: some studies have been conducted in laboratories, while other studies have been conducted at the industrial level (Bergamini et al., 2010). On the other hand, when we used small items, DON levels were widely reduced. Thus, size of baked items may also provide an explanation for inconsistent results reported in baking studies. Moreover, we also showed DON is rather more affected by the time of baking than the used temperature in bread, even Generotti et al. (2015) considered the used temperature as a factor with low importance for reduction of DON (vip score = 0.15). It is because temperature inside the loaves is always below 100 ºC regardless the baking temperature. The use of enzymes could also affect DON stability in the baking step. Hence, we showed xylanase and α-amylase caused an increase of DON after baking, however the increase of DON for each enzyme depended on the fermentation temperature, due to the different optimum temperature of each enzyme. The initial DON concentration affects the reduction level, when the initial concentration is higher, higher reduction is got (Bergamini et al., 2010).

Reduction of DON could be due to DON binding to matrix compounds or it might be transformed to other toxins as the detected DON degradation products norDON A, B and C (Bretz et al., 2006).

The behaviour of DON-3-glucoside is extremely worrying because incredible increases of it have been observed (> 100 %) at the end of baking and this causes the high ratio DON-3-glucoside/DON found in some breads which is in some cases 1 (De Boevre et al., 2013), much

higher than the ratio found in cereals which is around 0.1-0.3 (Berthiller et al., 2011). Other studies have shown a reduction in DON-3-glucoside after baking (Kostelanksa et al., 2011; Simsek et al., 2012). But several factors, as for DON, produce differences in DON-3-glucoside behaviour during baking. Thus, size of the product, temperature and time have a lot of importance. The mild baking conditions caused mainly by slow heat transmission, which is present in normal size bread products, induce an increase in DON-3-glucoside during baking. While harsher treatments would have led to a reduction in DON-3-glucoside, but they are difficult to obtain in normal breads; harsh conditions can be only obtained in cookies or small bakery products. Our results showed the large effect that enzymes have in the release of DON-3-glucoside during baking in breads with added xylanase, α-amylase, cellulase, protease and lipase. DON-3- glucoside may be bound to flour components and released during baking. In general, no significant correlation was found between increases in DON-3-glucoside and decreases in DON or vice versa. Similarly, Kostelanska et al. (2011) concluded that the behaviour of the two mycotoxins was not linked because, in their study, the concentration of DON did not change. These authors suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may occur.

Few works exist on OTA stability in the baking process and they show conflicting results. On the one hand, some studies concluded that OTA reduction is produced during baking process (Boudra et al., 1995; Valle-Algarra et al., 2009). On the other hand, high stability has been also observed in other studies (Scudamore et al., 2003), as we observed in our studies. The different results found by past studies showed OTA reduction is affected by different factors. Firstly, the heat penetration is an essential factor to get OTA reduction, because Boudra et al. (1995) and us got important reductions, working with small pieces of dough (3 g) or cookies. In the same way, Valle-Algarra et al. (2009) reported more OTA reduction in the crust than in the inner part of the bread ($p<0.05$) showing that heat transmission is an important factor in OTA reduction. Moreover, time is an important factor in OTA reduction during baking, so when temperature increases a lower time is needed to get the same OTA reduction obtained in lower temperatures and longer times.

No studies about OTA degradation products resulting from baking exist, but comparing with the roasting process, in it, OTA is converted to 14-(*R*)-Ochratoxin A and 14-decarboxy-ochratoxin A, which are less toxic than OTA (Cramer et al., 2008). Specific studies on OTA degradation due to baking are necessary to know the true degradation products.

To conclude, time, temperature, enzymes, initial mycotoxin concentration and chiefly the size of the product are the factors which affect the mycotoxin stability during baking. However the stability changes among mycotoxins and for instance, OTA is more stable than DON.

Table 17.3. Effect of bread making process in DON stability in recent studies.

Reference	DON level in flour (µg/kg)	Loaf size	Fermentation + proofing conditions	Use of improvers	Baking conditions	%DON reduction referred to flour	Results referred to equal basis (in general dw basis)
Bergamini et al. (2010)	100/160/967 (natural)	140 g (pilot scale)	45-85 min, 30-40 ºC	Malt flour, other	8-18min 180-210 ºC	-77-5% (fermented dough) -88-3% (baked product)	Yes
	100/160/967 (natural)	140 g (industrial scale)	45-85 min, 30-40 ºC	Malt flour, other	8-18min 180-210 ºC	-39% (fermented dough) -61% (baked product)	Yes
Pacín et al. (2010)	72 (natural)	35-65 g (industrial)	-	-	-	42-58% (baked product)	No
Kostelanska et al. (2011)	48-1049 (natural)	55g	95 min, 30 ºC	No	14 min 240 ºC	0% (fermented dough) 3% (baked product) Referred to kneaded dough	No
Simsek et al. (2012)	48-182 (natural)	>100 g	180min, 30 ºC	α-amylase	25 min 220 ºC	-99% (fermented dough) -69% (baked product) Referred to kneaded dough	Yes
Zachariasova et al. (2012)	729 (natural)	500 g	95 min, 30 ºC	Yes	14 min 240 ºC	28% (fermented dough) -4% (baked product)	No
Suman et al. (2012)	238-633 (natural, from bran)	Biscuits	2-4 min, 20-30 ºC	Flour malt, enzymes	1.5-5 min 225-270 ºC	-14-10% (fermented dough) -17-42% (baked product) Referred to kneaded dough	Yes
De Angelis et al. (2013)	816/954/1824 (natural)	500 g	60 min, ambient temp.	No	55 min, 200ºC	-18% (baked product)	Yes
Generotti et al. (2016)	1500/1050/600 (natural, from bran)	800 g	40-60 min, 26-46 ºC	Enzymes	12-30 min, 180-210 ºC	-12 – 14 % (fermented dough) -16 – 37 % (baked product) Referred to kneaded dough	Yes
Wu et al., (2015)	100-500 (spiked)	239 g	60 min, 30 ºC	No	20 min, 225 ºC	-2 % (fermented dough) 10 % (baked product)	Yes
Wu et al., (2016)	300/500/1000 (spiked)	161 g	60 min, 38 ºC	No	20 min, 100 ºC (steamed)	2 % (fermented dough) 8 % (baked product)	Yes
Zhang et al., (2014)	5970-940 (natural)	149 g	60 min, 38 ºC	No	20 min, 100 ºC (steamed)	-2.8 (fermented dough) -210 % (baked product)	Yes
Study IV	1012/1459/2090 (natural)	260 g	75 min, 30 ºC	Other	45-135 min, 170-210 ºC.	-18 % (fermented dough) -45 – 61 % (baked product) Referred to kneaded dough	Yes
Study V	1197/565 (natural, from bran)	260 g	75 min, 30 ºC	Other and sourdough	105 min, 180 ºC.	6 (without sourdough) -27 % (with sourdough) (fermented dough) -4 % (baked product) Referred to kneaded dough	Yes
Study VI	1042/550 (natural)	3 g	-	Other	0-40 min, 160-200 ºC	-30 – 90 %	Yes
Study VII	651 (natural, from bran)	257 g	60 min, 30-45 ºC	Xylanase, α-amylase, cellulase, protease, lipase and glucose-oxidase.	75 min, 180 ºC	23 (without enzymes) – > 30 %(with some enzymes) -35 – 49 % (baked product) Referred to kneaded dough	Yes
Sutdy VIII	651 (natural, from bran)	257 g	60 min, 30-45 ºC	Xylanase and α-amylase	75 min, 180 ºC	25 (without enzymes) – > 10 %(with some enzymes)	Yes

17.2.3. Boiling

Boiling is the process of cooking by the application of heat at the water boiling point of temperature, and it is usually used to cook pasta. It is a wet heat treatment and it is important because DON is one of the more polar trichothecenes with a solubility of 11 g/L at 25 °C in water (Chemicaldictionary, 2009). A few studies about DON stability have been made in the past about the fate of DON during the cooking of durum wheat pasta, but significant DON reductions have been reported: Nowicki et al. (1988) with a DON reduction approximately of 50 % after 12 and 22 min, Visconti et al. (2004) with a DON reduction over 50 % after 7 min, Sugita-Konishi et al. (2006) with a DON reduction of 69 % after 10 min and Cano-Sancho et al. (2013) with a DON reduction of 75 % after 10 min. All of these studies agreed DON was leached to water in a big percentage because all of them found high amounts of toxin in the final broth, as we detected in the analysed broth. However, great variability of results exists among them showing some other factors may affect the level of reduction. Firstly, Visconti et al. (2004) pointed out that the ratio pasta:water affects the leaching of DON, with higher reduction with increasing water. Our results showed that boiling time is also important, thus longer times cause larger DON reductions. Also Cano-Sancho et al. (2013) observed the importance of time for DON reduction during boiling, when they analysed pasta samples after 2, 6 and 10 min of boiling and they observed reduction of this secondary metabolite in pasta through the time. We observed reduction of DON in pasta stopped after a given time, this means that this mycotoxin is leached from pasta to water till an equilibrium is reached between toxin in pasta and in broth. Moreover, doing a mass balance final concentration of DON in pasta in equilibrium can be estimated. It is only necessary to know DON content in uncooked pasta, humidity of uncooked pasta, volume of broth and final humidity of cooked pasta. The use of a mass balance worked out correctly final concentration of DON in pasta from other studies if all data is described, as in Visconti et al. (2004). As DON leaches from pasta to broth, it was experimentally confirmed that the sum of DON in pasta and in broth was constant, therefore DON thermal stability was confirmed and DON is only leached to the broth. In fact, boiling conditions (100 °C) are mild and boiling time is short, thus this result was expectable. The high stability of DON in broth agrees with Mishra et al. (2014), who observed that this trichothecene was only unstable at 125-250 °C, showing 16-100 % degradation.

In spite of the importance to study DON-3-glucoside stability in food processing, few studies exist about it. However our results, unlike DON, showed that the concentration of DON-3-glucoside in pasta seems to be stable through the time. So, kneading, drying and boiling pasta do not change the concentration of DON-3-glucoside. On the other hand, Zhang and Wang (2015), who studied DON-3-glucoside stability in noodle production detected a significant increase of DON-3-glucoside (69%) in uncooked pasta. However, they used fermentation (30 min at room temperature) after mixing of the ingredients and as we observed in this thesis fermentation can cause an increase in DON-3-glucoside content. Zhang and Wang (2015) agreed that DON-3-glucoside concentration is not reduced in pasta during boiling after boiling

noodles for 5 minutes. On the other hand, despite the absence of reduction of DON-3-glucoside in pasta, increases of DON-3-glucoside were detected in broth (Zhang & Wang, 2015) as in our case. This increase suggests that an increase in the total amount of DON-3-glucoside occurred during boiling. The mild conditions involved in boiling (100 °C and short times) may lead to DON-3-glucoside release instead of thermal degradation. DON-3-glucoside found in broth was not linked to DON presence, because no change in the total amount of DON was detected. Other baking studies did not find any relation between both toxins (Kostelanska et al., 2011); they pointed out to a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides. DON-3-glucoside presence in the broth confirms that leaching from pasta took place. The high solubility of DON-3-glucoside and other DON conjugates has been observed in malting and brewing processes (Lancova et al., 2008).

As in baking, OTA seems again to be more stable than DON during boiling. Thus, OTA concentration did not change during pasta making process. Contrary to DON, OTA is poorly hydro soluble (1.31 mg/L at 25 °C) (SRC, 2010), and this helps to be very stable during boiling. However, slight increases of OTA through time have been detected in broth. The few existing information about OTA stability in boiling cereals showed a 35% of OTA reduction after boiling 10 g during 6 min in 400 mL of water (Sakuma et al., 2013). However they worked with OTA spiked spaghettis, which may easily loose the toxin. Reduction of OTA during boiling in other food products has been more successful, for instance, Milanez et al. (1996) observed an important reduction of OTA (up to 64 %) by soaking and boiling beans. The different results of reduction may be produced by the high time (12 hours) or by the use of ethanol/water for washing beans before soaking used by Milanez et al. (1996). Long boiling times, not used during boiling of food, could increase the reduction of OTA. Thus, 47% of OTA transfer was reached after boiling for 3 h in decoctions of herbal medicines (Shim et al., 2014) and 1% of OTA transfer occurred after 5 min of boiling an infusion tea (Ariño et al., 2007).

All in all, pasta making process reduced DON concentration in pasta, but DON-3-glucoside and OTA concentrations remained without variations. However, reduction of DON is due to a transfer of DON in pasta to water due to his high hydrosolubility. The ratio pasta:water and boiling time seem the most important factors in mycotoxin reduction from pasta, however there is not a mycotoxin degradation during this step, there is only a leaching process of mycotoxins from the pasta to the broth.

17.2.4. Kinetics

Knowing the inactivation kinetics of a mycotoxin under a given food process would allow the estimation of the inactivation constants, as the base for developing secondary models of prediction; such models would link process parameters and time and enable the prediction of the expected reduction. Such parameters and predictions would enable to obtain the PC and

PcC for the food safety management in the food industries. At the moment, mycotoxin inactivation kinetics of most food production processes remain unknown. Some examples exist in the literature, for example, Ferraz et al. (2010) studied OTA inactivation kinetics during roasting of coffee at different temperatures (180, 200, 220 and 240 ºC) and time periods (5, 8 and 12 minutes); they concluded that OTA reduction followed a first order reaction kinetics in coffee. The first work on inactivation kinetics of mycotoxins belongs to Dupuy et al. (1993), who reported that the decomposition of fumonisins in dry corn followed a first order reaction kinetics. Later, Jackson et al. (1996) worked with aqueous solutions of FB1 and FB2, and confirmed the first order reaction kinetics. Many factors may affect destruction of mycotoxins, which make establishment of inactivation kinetics quite complex.

The developed kinetics for DON and OTA reduction during baking have followed a fist-order equation as the other kinetics developed in past studies (Castell et al., 2006; Numanoglu et al., 2012, Ferraz et al. 2010; Dupuy et al., 1993 and Jackson et al., 1996) regardless mycotoxin studied. The Arrhenius equation showed a good fit for both high concentration (r^2 = 0.94) and low concentration (r^2 = 0.79) of DON. The highest degradation of DON occurred when initial concentration was higher. The development of DON reduction kinetics can be important control tools for bakery industry, through the prediction of expected final levels once the mycotoxin concentrations in the raw materials are known. DON-3-glucoside and DOM-1 kinetics were also calculated with a good fit (r^2 > 0.83). Kinetics of DON-3-glucoside formation during baking are very interesting due to its high increase during the thermal process, which could result in an unfit product, despite using raw materials with lower mycotoxin levels than established by the legislation

Also DON kinetics of reduction have been developed for the boiling process, and a pseudo-second-order reaction model was used because reduction of DON from pasta is rather milder. Moreover, leaching equation found can be used for any initial concentration and showed a very good fit (r^2 = 0.99).

It must be pointed out that modelling of mycotoxins behaviour during food processes is essential to provide an applied knowledge about mycotoxins intake by the population, but nowadays scarce works exist about this (Castells, Pardo, Ramos, Sanchis, & Marín, 2006; Ferraz et al., 2010; Numanoglu, Gökmen, Uygun, & Koksel, 2012). In particular, exposure assessment studies could benefit from correction of the initial mycotoxin concentration in raw cereals or food industry could benefit from the establishment of PO.

17.3. Exposure

High concentrations of mycotoxins are found in cereal products thus human exposure may be high. So, quantify mycotoxin exposure is important to see if the mycotoxin intake is over the adviced levels. Exposure assessment is a wide procedure and it is one of the four steps included in the risk assessment process. Although many mycotoxin exposure assessments have been published, there are different methodologies for exposure assessment and to date harmonisation is far from being achieved.

To calculate exposure to DON due to bran consumption we used the common approach widely used which is the combination of contamination data with consumption data. Contamination data were obtained from the obtained DON results. We took from 1 to 30 g/day of bran consumption per people. Results showed bran consumption as a great contribution to the global exposure of Catalonian adults to DON. These results reinforce that cereals are the main source of exposure to DON.

Exposure assessments are approaches that merge dietary contamination and consumption data from a subs-sample to extrapolate a distribution of exposure estimates on the whole population and obviously some assumptions and missing data are produced in these processes. Biomarker quantification is an objective assessment, which avoids the biased answers provided by interviewed humans, because the biomonitoring approach provides an internal measure of the individual exposure to pollutants which accounts for all intake routes and inter-individual variability (Clewell et al., 2008). For these reasons developing accurate and reliable biomarkers for human studies is so important. However, up to now, several studies have reported data on absorption, toxicokinetics, toxicodynamics and metabolism in animals, but few studies have been conducted in human, and for example there is a lack of information in excretion mycotoxin rate. Therefore, excretion rate of 2.6 % based on a biomonitoring study with pigs using low concentration oral doses of OTA is commonly used (Gambacorta et al., 2013). Afs were the first mycotoxins in which biomarker studies were made in the late 1980s (Gan et al., 1988; Zhu et al., 1987). They were able to relate the AFB1 intake with the urinary excretion of AFM1. If possible urine analysis is always better as a biomarker than plasma because its higher accessibility. Direct measurement of OTA or OTα in plasma or urine has been shown to be a suitable indicator of exposure to this mycotoxin (Coronel et al., 2010), as it was shown in this thesis where we got a significant correlation between urinary and plasmatic levels of OTA and OTα when we compared a time interval of 3 days but not when we compared in short time frames (24 h). Moreover, OTA and OTα in urine showed significant correlation with bread, pastries and corn-flakes consumption. In the case of DON and considering its short excretion half-life, only the urinary levels of DON or its glucuronide forms have been proposed as reliable biomarkers (Turner et al., 2010) showing successful results, with positive correlations between estimated dietary intake of toxin and urinary levels. Besides, we found significant correlations between urinary DON and bread ($r=0.362$, $P<0.001$) and pastries ($r=0.239$, $P<0.01$)

consumption. The new analytical methods developed recently which allow to detect conjugates should led to make important advances in biomonitoring mycotoxin exposure. Thus, biomarkers will have an important role on mycotoxin risk assessment in the future.

Large exposure exists among European population to DON and OTA and several studies in this thesis showed it. Different studies to calculate the exposure to OTA have been made through the consumption of coffee, wine and cereal based food and different biomarker studies also have been developed to study OTA exposure. But important differences in OTA exposure levels exist between consumption data and biomarkers. Biomarkers studies found higher OTA exposure than estimated exposures from food intake data. Thus levels of OTA found in urine during this thesis pointed out that the population is regularly exceeding the PDI with an average PDI level of 31.5 µg/kg. Other European studies using the same method also found that population is exceeding the PDI level: Belgian population exposure to OTA was in the range between 0.1 and 21 ng/kg bw/day (Heyndrickx et al., 2015) and Solfrizzo et al. (2014) in Italy, estimated a mean value of 139 ng/kg bw/day. On the other hand, estimated exposures through food intake data were lower and they hardly ever exceed the PDI levels for OTA. For instance, Coronel et al. (2012) used food intake data and they found OTA exposure levels from 0.23 to 0.47 ng/kg bw/day. The important difference could be caused by estimated excretion OTA rate (2.6 % in pigs) that it is widely used in biomarker studies.

European population could be excessively exposed to DON. Thus, biomarker results showed only 9 % of population were not exceeding the PDI DON level (1 µg/kg bw/day). Similar studies found a high percentage of population exceeding the PDI such as Austria (33%), Belgium (16-39%), Croatia (48% of women) or Italy (40%) (Heyndrickx et al., 2015; Šarkanj et al., 2013; Solfrizzo et al., 2014; Warth et al., 2012a). Food habits could be the main reason of differences among European conturies.

17.4. New analytical methods

Every year a great number of new analytical methods to quantify mycotoxins appear, especially to determine conjugated forms. Thereby new tendencies in analytical methods try to determine with LC-MS/MS a great number of mycotoxins in one run like 35 (Han et al., 2012) and 33 (Heyndrickx et al., 2015). But the quantification of an exceptional number of mycotoxins is not an advantage because a loss of sensitivity is usually observed. Limits of detection are really important in terms of conjugated mycotoxins because their concentrations are usually very low. So, before to decide a methodology is important to weight up all factors.

DON and its conjugates are often analysed in cereal products and concentration of 3-ADON, 15-ADON and DOM-1 are usually below 20 µg/kg. LC-MS/MS is almost the only method

able to determine all of them in so low concentrations. Although a new method with HPLC-UV to determine DON and 3-ADON and 15-ADON in cereals was developed (Yang et al., 2013), the quantification limits were near to 60 µg/kg. For this reason, LC–MS is usually applied for simultaneous detection of DON and its conjugates (Vendl et al, 2009), specially DON-3-glucoside (Dall'asta, et al., 2009) and it has demonstrated to be highly effective for quantification of DON conjugates in cereal products, such as malt and beer (Zachariasova et al., 2012). Our developed method to determine DON and its conjugates in bakery products was the first for simultaneous analysis of DON, DON-3-glucoside, 3-ADON, 15-ADON, and DOM-1 in bakery products. Though DON, DON-3-glucoside, 3-ADON and 15-ADON are usually analysed together (De Boevre et al., 2013), DOM-1 is always forgotten. But we showed it can be present in raw cereals and even to increase during baking. Thus it is important to analyse it also in cereals.

To avoid immunology-based clean-up steps is required for analysis of conjugates, because conjugates are almost always lost during the clean-up step. Therefore, extracting the target compounds from the matrix with weak interference and high recovery is a critical point. Due to the different polarity and solubility of the compounds to be extracted, mixtures consisting of different acetonitrile/water/acetic acid ratios are used. For instance: acetonitrile (84%)/water (16%) (Malachova et al., 2011), acetonitrile (79%)/water (19%)/ acetic acid (1%) (De Boevre et al., 2012) and the developed method in this thesis for bakery products water (54%)/acetonitrile (45%)/acetic acid (1%). We detected that with our extraction solution DON-3-glucoside recovery improved.

Urine analysis to determine DON required enzymatic treatment (*β-glucuronidase*) to detect total DON (sum of free DON and DON released during enzymatic treatment) in urine until recent years, because glucuronide forms (DON-3-glucuronide and DON-15-glucuronide) are the majoritary compounds of DON in urine (Warth et al., 2012b). However, the new analytical methods allow directly quantifying glucuronide forms and enzyme treatment is not necessary. Since, a direct analytical method has been described (Warth et al., 2012b) without enzymatic treatment called "dilute and shoot" which is faster and glucuronide forms are quantified. However, DON glucuronide commercial standards do not exist yet notwithstanding a method to synthesise DON-3-glucuronide has been published (Fruhmann et al., 2012). Limit of detection is of special importance in analytical methods for mycotoxins because their levels in urine are generally extremely low (< 10 µg/kg or lower) as we found in our results. All new analytical methods developed in recent years tried to get the lowest possible limit of detection as we achieved in our developed method where all LOD were lower than 3 µg/kg. A low urine dilution is important to get a low limit of detection; some urine analysis used a dilution of 1:9 (Warth et al., 2012), however we applied 1:4 which improved the limit of detection.

17.5. References

Ariño, A., Herrera, M., Estopañan, G., & Juan, T. 2007. High levels of ochratoxin A in licorice and derived products. *International Journal of Food Microbiology, 114*, 366-369.

Assunção, R., Martins, C., Dupont, D., & Alvito, P. 2016. Patulin and ochratoxin A co-occurrence and their bioaccessibility in processed cereal-based foods: A contribution for Portuguese children risk assessment. *Food and Chemical Toxicology, 96*, 205-214.

Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. 2010. Fate of *Fusarium* mycotoxins in the cereal product supply chain: the deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 677-687.

Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. 2009. Occurrence of deoxynivalenol and its 3-b-D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 507-511.

Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. 2011. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264-267.

Boudra, H., Le Bars, P., & Le Bars, J. 1995. Thermostability of ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology, 61*, 1156-1158.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.-U. 2006. Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry, 54*, 6445-6451.

Cano-Sancho, G., Sanchis, V., Ramos, A.J., & Marín, S. 2013. Effect of food processing on exposure assessment studies with mycotoxins. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 867-875.

Castells, M., Pardo, E., Ramos, A.J., Sanchis, V., & Marín, S. 2006. Reduction of ochratoxin A in extruded barley meal. *Journal of Food Protection, 69*, 1139-1143.

Chemicaldictionary. 2009. http://www.chemicaldictionary.org/dic/D/Deoxynivalenol_842.html (2009) (accessed: 07.01.2015).

Clewell, H.J., Tan, Y.M., Campbell, J.L., & Andersen, M.E. 2008. Quantitative interpretation of human biomonitoring data. *Toxicology and Applied Pharmacology, 231*, 122-133.

Coronel, M.B., Sanchis, V., Ramos, A.J., & Marin, S. 2010. Review. ochratoxin a: Presence in human plasma and intake estimation. *Food Science and Technology International, 16*, 5-18.

Coronel, M.B., Marín, S., Cano-Sancho, G., Ramos, A.J., & Sanchis, V. 2012. Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 979-993.

Cramer, B., Königs, M., & Humpf, H.-U. 2008. Identification and *in vitro* cytotoxicity of ochratoxin A degradation products formed during coffee roasting. *Journal of Agricultural and Food Chemistry, 56*, 5673-5681.

Dall'Asta, C., Mangia, M., Berthiller, F., Molinelli, A., Sulyok, M., Schuhmacher, R., Krska, R., Galaverna, G., Dossena, A., & Marchelli, R. 2009. Difficulties in fumonisin determination: The issue of hidden fumonisins. *Analytical and Bioanalytical Chemistry, 395*, 1335-1345.

Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. 2013. Occurrence of deoxynivalenol and deoxynivalenol-3- glucoside in durum wheat. *World Mycotoxin Journal, 6*, 83-91.

De Boevre, M., Di Mavungu, J.D., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut, A., Berthiller, F., Van Peteghem, C., & De Saeger, S. 2012. Development and validation of an LC–MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 819-835.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Di Mavungu, J.D., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B., & De Saeger, S. 2013. Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281-292.

De Souza, A.F., Borsato, D., Lofrano, A.D., De Oliveira, A.S., Ono, M.A., Bordini, J.G., Hirozawa, M.T., Yabe, M.J.S., & Ono, E.Y.S. 2015. *In vitro* removal of deoxynivalenol by a mixture of organic and inorganic adsorbents. *World Mycotoxin Journal, 8*, 113-119.

Dupuy, J., Le Bars, P., Boudra, H., & Le Bars, J. 1993. Thermostability of fumonisin B1, a mycotoxin from *Fusarium moniliforme*, in corn. *Applied and Environmental Microbiology, 59*, 2864-2867.

Eriksen, G.S., & Pettersson, H. 2004. Toxicological evaluation of trichothecenes in animal feed. *Animal Feed Science and Technology, 114*, 205-239.

European Commission (EC). 2006. European Commission N° 1881/2006 of December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L 364 (2006)*, 5-24.

European Food Safety Authority (EFSA). 2011. Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal, 9*, 2197.

Ferraz, M.B.M., Farah, A., Iamanaka, B.T., Perrone, D., Copetti, M.V., Marques, V.X., Vitali, A.A., & Taniwaki, M.H. 2010. Kinetics of ochratoxin A destruction during coffee roasting. *Food Control, 21*, 872-877.

Fruhmann, P., Warth, B., Hametner, C., Berthiller, F., Horkel, E., Adam, G., Sulyok, M., Krska, R., & Fröhlich, J. 2012. Synthesis of deoxynivalenol-3-β-D-O-glucuronide for its use as biomarker for dietary deoxynivalenol exposure. *World Mycotoxin Journal, 5*, 127-132.

Gambacorta, L., Solfrizzo, M., Visconti, A., Powers, S., Cossalter, A.M., Pinton, P., Oswald, I.P. 2013. Validation study on urinary biomarkers of exposure for aflatoxin B1, ochratoxin A, fumonisin B1, deoxynivalenol and zearalenone in piglet. *World Mycotoxin Journal, 6,* 299–308.

Gan, L.S., Skipper, P.L., Peng, X., Groopman, J.D., Chen, J.S., Wogan, G.N., & Tannenbaum, S.R. 1988. Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: Correlation with aflatoxin B1 intake and urinary excretion of aflatoxin m1. *Carcinogenesis, 9*, 1323-1325.

Generotti, S., Cirlini, M., Malachova, A., Sulyok, M., Berthiller, F., Dall'Asta, C., & Suman, M. 2015. Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins, 7*, 2773-2790.

Han, Z., Ren, Y., Zhu, J., Cai, Z., Chen, Y., Luan, L., & Wu, Y. 2012. Multianalysis of 35 mycotoxins in traditional Chinese medicines by ultra-high-performance liquid chromatography-tandem mass spectrometry coupled with accelerated solvent extraction. *Journal of Agricultural and Food Chemistry, 60*, 8233-8247.

Heyndrickx, E., Sioen, I., Huybrechts, B., Callebaut, A., De Henauw, S. & De Saeger, S., 2015. Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study. *Environment International, 84*, 82-89.

Jackson, L.S., Hlywka, J.J., Senthil, K.R., Bullerman, L.B., & Musser, S.M. 1996. Effects of time, temperature, and pH on the stability of fumonisin B1 in an aqueous model system. *Journal of Agricultural and Food Chemistry, 44*, 906-912.

JEFCA: WHO Technical Report Series 959: JEFCA. 2010: Seventy second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food. WHO Technical Report Series; 2010, 959.

Juan, C., Raiola, A., Mañes, J., & Ritieni, A. 2014. Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control, 39*, 227-236.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. 2011. Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I.,Prokinova, E.,Skerikova, A., & Hajslova, J. 2011. Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry, 59*, 9303-9312.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H., & Vanova, M. 2008. Fate of trichothecene mycotoxins during the processing: milling and baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25*, 650-659.

Malachova, A., Dzuman, Z., Veprikova, Z., Vaclavikova, M., Zachariasova, M., & Hajslova, J. 2011. Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *Journal of Agricultural and Food Chemistry, 59*, 12990-12997.

Marin, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology, 60*, 218-237.

Milanez, T.V., & Leitão, M.F.F. 1996. The effect of cooking on ochratoxin A content of beans, variety 'Carioca'. *Food Additives and Contaminats, 13*, 89-93.

Mishra, S., Dixit, S., Dwivedi, P.D., Pandey, H.P., & Das, M. 2014. Influence of temperature and pH on the degradation of deoxynivalenol (DON) in aqueous medium: Comparative cytotoxicity of DON and degraded product. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 31*, 121-131.

Nathanail, A.V., Syvähuoko, J., Malachová, A., Jestoi, M., Varga, E., Michlmayr, H., Adam, G., Sieviläinen, E., Berthiller, F., & Peltonen, K. 2015. Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. *Analytical and Bioanalytical Chemistry, 407*, 4745-4755.

Neira M.S, Pacin, A.M., Martinez, E.J., Molto, G., & Resnik, S.L. 1997. The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology, 37*, 21-25.

Nowicki, T.W., Gaba, D.G., Dexter, J.E., Matsuo, R.R., & Clear, R.M. 1988. Retention of the *Fusarium* mycotoxin deoxynivalenol in wheat during processing and cooking of spaghetti and noodles. *Journal of Cereal Chemistry, 8*, 189-202.

Numanoglu, E., Gökmen, V., Uygun, U., & Koksel, H. 2012. Thermal degradation of deoxynivalenol during maize bread baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 423-430.

Piotrowska M., Nowak, A., & Czyzowska, A. 2013. Removal of ochratoxin A by wine *Saccharomyces cerevisiae* strains. *European Food Research and Technology, 236*, 441-447.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. 2003. Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278*, 47905-47914.

Rychlik, M., Humpf, H.-U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., & Lorenz, N. 2014. Proposal of a comprehensive definition of modified and other forms of mycotoxins including “masked” mycotoxins. *Mycotoxin Research, 30*, 197-205.

Sakuma, H., Watanabe, Y., Furusawa, H., Yoshinari, T., Akashi, H., Kawakami, H., Saito, S., & Sugita-Konishi, Y. 2013. Estimated dietary exposure to mycotoxins after taking into account the cooking of staple foods in Japan. *Toxins, 5*, 1032-1042.

Saladino, F., Quiles, J.M., Mañes, J., Fernández-Franzón, M., Luciano, F.B., & Meca, G. 2017. Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain. *LWT - Food Science and Technology, 75*, 697-701.

Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. 2001. Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants,18*, 1004-1010.

Šarkanj, B., Warth, B., Uhlig, S., Abia, W.A., Sulyok, M., Klapec, T., Krska, R., & Banjari, I. 2013. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. *Food and Chemical Toxicology, 62*, 231-237.

Scudamore, K.A., Banks, J., & MacDonald, S.J. 2003. Fate of ochratoxin A in the processing of whole wheat grains during milling and bread production. *Food Additives and Contaminants, 20*, 1153-1163.

Shim, W.-B., Ha, K.-S., Kim, M.-G., Kim, J.-S., & Chung, D.-H. 2014. Evaluation of the transfer rate of ochratoxin a to decoctions of herbal medicines. *Food Science and Biotechnology, 23*, 2103-2108.

Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. 2012. Analysis of Deoxynivalenol and Deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287-292.

Smith, M.-C., Madec, S., Coton, E., & Hymery, N. 2016. Natural Co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. *Toxins, 8*, Article number 94.

Solfrizzo, M., Gambacorta, L., & Visconti, A. 2014. Assessment of multi-mycotoxin exposure in southern Italy by urinary multi-biomarker determination. *Toxins, 6*, 523-538.

SRC. 2010. Interactive PhysProp Database Demo Syracuse Research Corporation. (https://esc.syrres.com/fatepointer/webprop.asp?CAS=303479 (accessed: 07.01.2017))

Sugita-Konishi, Y., Kamata, Y., Sato, T., Yoshinari, T., & Saito, S. 2013. Exposure and risk assessment for ochratoxin A and fumonisins in Japan. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*, 1392-1401.

Sugita-Konishi, Y., Park, B.J., Kobayashi-Hattori, K., Tanaka, T., Chonan, T., Yoshikawa, K., & Kumagai, S. 2006. Effect of cooking process on the deoxynivalenol content and its subsequent cytotoxicity in wheat products. *Bioscience, Biotechnology and Biochemistry, 70*, 1764-1768.

Suman, M., Manzitti, A., & Catellani, D. 2012. A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC–MS/MS techniques. *World Mycotoxin Journal, 5*, 241-249.

Turner, P.C., White, K.L.M., Burley, V.J., Hopton, R.P., Rajendram, A., Fisher, J.C., Cade, J.E. & Wild, C.P. 2010. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK adults. *Biomarkers,* 15, 553-562.

Valle-Algarra, F.M., Mateo, E.M., Medina, Á., Mateo, F., Gimeno-Adelantado, J.V., & Jiménez, M. 2009. Changes in ochratoxin A and type B trichothecenes contained in wheat flour during dough fermentation and bread-baking. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 26*, 896-906.

Vanheule, A., Audenaert, K., De Boevre, M., Landschoot, S., Bekaert, B., Munaut, F., Eeckhout, M., Hofte, M., De Saeger, S., & Haesaert, G. 2014. The compositional mosaic of *Fusarium* species and their mycotoxins in unprocessed cereals, food and feed products in Belgium. *International Journal of Food Microbiology, 181*, 28-36.

Vendl, O., Berthiller, F., Crews, C., & Krska, R. 2009. Simultaneous determination of deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food by LC-MS-MS. *Analytical and Bioanalytical Chemistry, 395*, 1347-1354.

Visconti, A., Haidukowski, E.M., Pascale, M., & Silvestri, M. 2004. Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters, 153*, 181-189.

Warth, B., Sulyok, M., Fruhmann, P., Berthiller, F., Schuhmacher, R., Hametner, C., Adam, G., Fröhlich, J., & Krska, R. 2012a. Assessment of human deoxynivalenol exposure using an LC-MS/MS based biomarker method. *Toxicology Letters, 211*, 85-90.

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Fröhlich, J., & Krska, R. 2012b. Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins. *Rapid Communications in Mass Spectrometry, 26*, 1533-1540.

Wu, L., & Wang, B. 2015. Evaluation on levels and conversion profiles of DON, 3-ADON, and 15-ADON during bread making process. *Food Chemistry,185*, 509-516.

Yang, C., Lates, V., Prieto-Simón, B., Marty, J.-L., & Yang, X. 2013. Rapid high-throughput analysis of ochratoxin A by the self-assembly of DNAzyme-aptamer conjugates in wine. *Talanta, 116*, 520-526.

Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. 2012. Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry, 60*, 9280-9291.

Zhang, H., & Wang, B. 2015. Fates of deoxynivalenol and deoxynivalenol-3-glucoside during bread and noodle processing. *Food Control, 50*, 754-757.

Zhu, J.-Q., Xiao, Y., & Chen, J.-S. 1987. Correlation of Dietary Aflatoxin B1 Levels with Excretion of Aflatoxin M1 in Human Urine. *Cancer Research, 47*, 1848-1852.

18. Conclusions

This Thesis resulted in the following conclusions:

1. Large presence of mycotoxins in cereals and cereal based products.

- Mycotoxins and conjugated mycotoxins presence in cereals are greatly common. Trichothecenes are the most usual mycotoxins found and their presence can be over the legislation European limit. The other analysed mycotoxins also showed a large presence in cereals, but concentration and incidence found were under trichothecens levels. Moreover, the conjugated mycotoxins concentrations can sometimes surpass parent mycotoxin level. On the other hand, some mycotoxins can be embedded to the matrix and they are not detected in routinary analysis.

2. Mycotoxins can be reduced during food processing.

- DON and its conjugates can be reduced during bread fermentation correctly using temperature, time, enzymes and sourdough presence. On the other hand, increase of their concentration can occur at the end of the process, under certain conditions. OTA reminds without variation during the bread fermentation.

- Baking is able to reduce OTA, DON and its conjugates concentration. Long times, high temperatures, fast heat transmission and enzyme absence are described as important factors to get large reductions. DON and DON-3-glucoside can increase during baking due to the possible presence of embedded mycotoxins in cereals.

- Boiling pasta reduces widely DON concentration in pasta, because it is highly soluble in water and it is transferred to broth. But there is not thermodegradation process during boiling. Other studied mycotoxins (OTA and DON-3-glucoside) do not suffer important variations during this themo step.

- The developed kinetics about mycotoxins fate during food processing can be an interesting tool for the food industry to prevent mycotoxin presence in food.

3. Population are highly exposed to DON and OTA.

- OTA urinary levels could be used as OTA biormarkers because they were correlated successfully with plasmatic levels of OTA and OTα. Most of DON found in urine is in glucuronide forms, and their detection is essential to study biomarkers from DON in urine. So, developing new analytical methods to study biomarkers is an important issue because it is a good way to analyse mycotoxin exposure.

- Catalonia population has a high exposure to DON and OTA, the exposure level to these mycotoxins is of concern. Consumption of fibre, bread, pasta and pastries cause a high exposure to DON, while wine and breakfast cereals lead to high OTA exposure.

Aquesta tesis ha obtingut les següents conclusions:

1. Gran presència de micotoxines en els cereals in els productes a base de cereals.

- La presència de micotoxines i micotoxines conjugades en els cereals és molt freqüent. Els tricotecens són les micotoxines analitzades més usuals i la seva presència pot ser per sobre del límit legislatiu establert per la Unió Europea. La resta de micotoxines analitzades també han presentat una elevada presència en els cereals, però la concentració i la incidència trobades han estat inferiors als nivells presents en els tricotecens. A més, les concentracions de les micotoxines conjugades poden ser algunes vegades superiors a la concentració de les micotoxines lliures. Per altra banda, algunes micotoxines poden estar unides a la matriu i no són detectades en els anàlisis rutinaris.

2. Les micotoxines poden ser reduïdes durant el processat dels aliments.

- El DON i els seus conjugats poden ser reduïts duran la fermentació del pa utilitzant correctament la temperatura, el temps, els enzims i la presència de massa mare. Per altra banda, increments de les seves concentracions poden ser assolides en la massa fermentada. La OTA es manté sense variacions durant la fermentació del pa.

- El fornejat del pa és capaç de reduir la concentració de la OTA, del DON i dels seus conjugats. Temps llargs, altes temperatures, ràpida transmissió del calor i presència d'enzims són els factors trobats com a importants per assolir grans reduccions. Encara que el DON i el DON-3-glucòsid poden incrementar les seves concentracions durant el fornejat a causa de la presència de micotoxines unides a la matriu.

- L'ebullició de pasta redueix la concentració de DON a la pasta, perquè és un compost altament hidrosoluble i la micotoxina és transferida a l'aigua. Però, no existeix una degradació tèrmica durant aquesta etapa. Les altres micotoxines estudiades (la OTA i el DON-3-glucòsid) no presenten variacions importants durant aquest procés tèrmic.

- Les cinètiques desenvolupades sobre l'estabilitat de les micotoxines durant els processos d'elaboració dels aliments poden ser una eina molt interessant per la indústria alimentària per tal de prevenir la presència de micotoxines en els aliments.

3. La població està altament exposada el DON i a la OTA.

- Els nivells d'OTA en urina poden ser utilitzats com a biomarcadors d'OTA perquè han estat satisfactòriament correlacionats amb els nivells plasmàtics d'OTA i OTα. La gran part del DON trobat a la orina està en forma de glucuronids, i les deteccions d'aquestes formes conjugades són essencials per l'estudi dels biomarcadors de DON en orina. Per tant, desenvolupar nous mètodes analítics per estudiar els biomarcadors és un punt important perquè és una bona manera d'analitzar l'exposició a les micotoxines

- La població catalana presenta un elevat nivell d'exposició al DON i a la OTA, l'elevada exposició a aquestes micotoxines suposa un problema. El consum de fibra, de pa, de pasta i de pastes causa una elevada exposició el DON, mentre que el vi i els cereals d'esmorzar provoquen una elevada exposició a la OTA.

Esta tesis ha obtenido las siguientes conclusiones:

1. Elevada presencia de micotoxinas en los cereales en los productos a base de cereales.

- La presencia de micotoxinas y de micotoxinas conjugadas en los cereales estudiados es muy frecuente. Los tricotecenos son las micotoxinas analizadas más comúnmente detectadas y las concentraciónes encontradas pueden ser en algunas muestras superiores al límite legislativo establecido por la Unión Europea. El resto de micotoxinas analizadas también han presentado una elevada presencia en las muestras de cereales estudiadas, pero la concentración y la incidencia encontradas han sido inferiores a los niveles presentes en los tricotecenos. Además, las concentraciones de las micotoxinas conjugadas pueden ser algunes veces superiores a la concentración de las micotoxinas libres a pesar de que algunas de estas no son detectadas en los análisis rutinarios por estar unidas a la matriz. .

2. Las micotoxinas pueden ser reducidas durante el procesado de los alimentos.

- El DON y sus conjugados pueden ser reducidos durante la fermentación del pan utilizando correctamente la temperatura, las enzimas y la adición de masa madre, mientras que se pueden incrementar durante la fermentación de la masa. La OTA se mantiene sin variaciones durante la fermentación del pan.

- El horneado del pan es capaz de reducir la concentración de la OTA, DON y de sus conjugados. Así, tiempos largos y temperaturas elevadas de cocción, así como la rápida transmisión del calor y la presencia de enzimas son los factores encontrados como importantes para alcanzar grandes reducciones. Aunque el DON y el DON-3-glucósido pueden incrementar sus concentraciones durante el horneado debido a la presencia de micotoxinas ligadas a la matriz.

- La ebullición de pasta reduce la concentración de DON, porque es un compuesto altamente hidrosoluble y es transferido al agua de ebullición. No obstante, no existe una degradación térmica durante esta etapa. Las otras micotoxinas estudiadas (OTA y DON-3-glucosido) no presentan variaciones importantes durante este proceso térmico.

- Las cinéticas desarrolladas sobre la estabilidad de las micotoxinas durante los procesos de elaboración de los alimentos pueden ser una herramienta muy interesante para la industria alimentaria con el objetivo de prevenir la presencia de micotoxinas en los alimentos.

3. La población está altamente expuesta a DON y a OTA.

- Los niveles de OTA en orina pueden ser utilizados como biomarcadores de OTA porque han estado satisfactoriamente correlacionados con los niveles plásmaticos de la OTA y la OTα. Gran parte del DON encontrado en la orina está en forma de glucurónidos, y la detección de estas formas conjugadas es esencial para el estudio de los biomarcadores de DON en orina. Por lo que desarrollar nuevos métodos analíticos para estudiar los biomarcadores es muy importante porque es una buena manera de analizar la exposición de los consumidores a las micotoxinas.

- La población catalana tiene un elevado nivel de exposición al DON y a la OTA,y esta exposición puede suponer un problema para su salud. El consumo de fibra, de pan, de pasta y de pastas causan una elevada exposición al DON, mientras que el vino y los cereales de desayuno provocan una elevada exposición a la OTA.

19. Annex I. The fate of deoxynivalenol through wheat processing to food products.

Current Opinion in Food Science (2016) 11, 34-39.

Authors: Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S.

19. The fate of deoxynivalenol through wheat processing to food products

Arnau Vidal, Asier Ambrosio, Vicente Sanchis, Antonio J. Ramos, Sonia Marín*.

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

* Corresponding author. Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Rovira Roure 191, 25198 Lleida, Spain. Tel.: +34 973 702542; fax: +34 973 702596. smarin@udl.cat (S. Marín).

19.1. Abstract

Deoxynivalenol (DON) is one of the most frequently occurring mycotoxin in wheat crops worldwide and it poses a risk to human and animal health due to its wide range of adverse effects. As its accumulation at field seems to be unavoidable, it is very important to investigate its stability during food processing. Recent outcomes of DON stability during milling, fermentation, and baking show some opportunities to reduce DON. In-depth knowledge of such processes is required. Moreover, DON-3-glucoside seems to increase during the breadmaking process, thus actions to prevent this to occur are required. Finally, recent studies have pointed out that ozonation may help reducing DON content in wheat, which provides a new alternative to the food industry.

19.2. Introduction

Fusarium species produce a heterogeneous blend of mycotoxins known as trichothecenes. The most abundant trichothecene is deoxynivalenol (DON), also known as vomitoxin. It is mainly produced by *Fusarium graminearum* and *Fusarium culmorum* [1] and it is not classified as to its carcinogenicity to human by IARC (International Agency for Research on Cancer) [2]. However, DON can inhibit the synthesis of proteins when humans or animals ingest contaminated food, causing immune dysregulation, chronic autoimmune diseases and aberrant intercellular signalling [3]. Recent exposition studies showed the high exposure of human to DON, with high percentages of population exceeding the tolerable daily intake [4,5]. The high presence of DON in human urine is mostly attributed to consumption of contaminated wheat derived products [6].

Wheat is highly susceptible to DON accumulation in field, and a high percentage of analysed wheat samples, often above 85 %, contain DON [7-9]. Due to the high presence of DON in raw wheat, studying the stability of DON during food processing is critical. In addition, some studies showed that despite cereal processing, wheat based products also contain relatively high DON concentrations such as 246 ng/g [6] and 437 ng/g [10] in bread, 137.1 ng/g in pasta [11] and 14.6 ng/mL in beer [12]. In recent years research has been mainly focussed in DON stability during milling and breadmaking (Figure 19.1). Moreover, wheat treatment with ozone has been proposed as a new alternative in the past few years [13].

The co-occurrence of conjugated DON forms has been documented in raw wheat, especially deoxynivalenol-3-glucoside (DON-3-glucoside) [14,15] which is a plant metabolite of DON [16]. Despite the high presence of DON-3-glucoside in wheat, few studies exist on its occurrence.

The present review summarizes the latest outcomes in DON and DON-3-glucoside stability during different food processing, in particular milling, fermentation and baking, as well as ozonation.

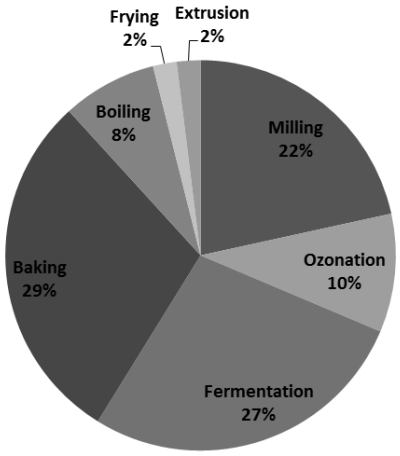


Figure 19.1. Quantity of studies about DON stability for each type of food process since 2010.

19.2. Wheat processing and DON

19.2.1. Milling

Most of the wheat harvested in the world is subjected to milling. A large amount of studies have been made about how wheat milling can affect DON reduction [17-20]. DON is not eliminated during this step but it is only redistributed and concentrated in certain milling fractions. Milling of DON contaminated wheat results in less contaminated fractions intended for human consumption (flour or semolina); while concentration is observed in the animal feed fractions (bran, shorts and middlings). The significantly lower DON levels in finished flour may be attributed to the potential of the bran layer to behave as a physical barrier preventing the mycelia from penetrating further into the kernel structure [21]. The major DON concentration in the external parts is of concern because although these parts are almost always used for animal feed, sometimes they are used for direct human consumption due to their benefits (improved large bowel function to slowed digestion and absorption of carbohydrate and fat and reduced risk for certain diseases). As Vidal et al. [22] showed wheat bran consumption can be an important source of DON intake. For instance, DON was found in 19 % of wheat bran samples, at concentration above the EU legal limit (750 ng/g).

Recent studies were performed using various kinds of wheat, natural and spiked contaminated, with different DON levels, from a low (0.001 mg/kg) to a high level (36.72 mg/kg) and different milling methods. This results in certain variability in the reported distribution factors. The levels of DON reduction in flour are extremely different, from a high reduction (> 80 %) [23**] to a very low one (4 %) [24]. A factor of reduction variability is the initial DON concentration, the higher the initial DON concentration the higher is the reduction in the final flour [23**]. Another possible explanation to the big variability of DON reduction during milling is the extent of mould penetration in the grain. Although there is usually more DON in the external grain layers mould can penetrate inside the grain which would end in a higher DON concentration in the inner part and a lower DON reduction in the final flour than others grains which mould could not penetrate. But DON concentration will be always higher in the external part than internal part, even in the grains which mould can penetrate in the internal parts. Almost all the studies used laboratory mills (mainly the Bühler laboratory mill MLU-202) [10,23**,24,25,26**,27,28] and few of them used industrial equipment [29,30]. Anyway, few differences exist between the results of the different methods. In addition, wheat moisture is usually increased to 14-17 % to get a good split of the grain; although Zhang et al. [23**] got the highest DON reductions (> 80 %) in the final flour with the highest moisture (16.5 %), Schwake-Anduschus et al. [26**] using the same moisture (16.5 %) only got a 15 % of DON reduction in the flour. Relative distribution is the percentage of DON content in each milling fraction relative to the initial DON content in the initial grain, thus it is necessary to know the weight of each fraction to calculate it. Unlucky, few studies include this information and the absence of DON

degradation cannot be confirmed. For instance, Zhang et al. [23**] recovered the 97 % of the initial DON content in the different milling parts (shorts, bran and flour).

Regarding durum wheat, the milling process has the same impact in DON content, and different types of cereal did not cause different levels of reduction [21,29,31]. All in all, milling causes a reduction of the DON concentration in the final flour or semolina, while a DON increase in the external parts of wheat grains (shorts and bran) is detected. More studies using industrial conditions are needed.

19.2.2. Fermentation

Most studies in the fate of DON during fermentation deal with breadmaking. Although a huge amount of studies have been made in the past, they showed contradictory results and while some of them have suggested that DON concentrations are reduced during fermentation [32], others have shown that DON concentration significantly increases after fermentation [33,34]. Firstly, the presence of enzymes can have an important role. Suman et al. [35] detected an increase of up to 14 % in DON using non-specific enzymes. Moreover, Vidal et al. [36], using flour improvers with non-specific enzymes, detected a 30 % increase in DON during fermentation. Studies in which either malt flour or other enzymes were added reported increases in DON levels [27,35,36], while in the absence of added enzymes, reductions in DON content often occur [32]. The importance of enzymes in detected DON was showed by Vidal et al. [37*]. They tested different enzymes presence compared to fermented doughs without enzymes. Without the use of enzymes DON was reduced, 5 % when the fermentation was at 30 ºC and 23 % when it was at 45 ºC. On the other hand, the use of α-amylase during the fermentation at 30 ºC led to an increase of 10 % in the DON level. Moreover, xylanase, cellulase, protease and glucose-oxidase use resulted in an increase of DON of 15, 63, 75 and 78 %, respectively, in the fermented dough compared with the fermented dough without enzymes when the fermentation was at 45 ºC. This could be associated with the release of DON from the wheat matrix through enzyme catalysis. Recently, Wu and Wang [38*] and Wu and Wang [39] working at 38 ºC for 1 hour without neither improvers nor enzymes, showed no significant change in DON. Temperature, apart from modifying enzyme activity, may directly affect to DON stability; Samar et al. [40] assayed fermentation temperatures from 30 to 50 ºC, and found higher DON reduction as the temperature increased (from 0 to 56 %). The highest reduction in DON levels was observed at the highest temperature (50 ºC) and the longest time tested (60 min). This result is consistent with the results by Vidal et al. [37*] who observed only a 5 % of DON reduction when fermentation was at 30 ºC, and 23 % when it was at 45 ºC. Similarly, Generotti et al. [41**] assayed different fermentation temperatures (from 26 to 46 ºC), and they found reduced DON stability with higher fermentation temperatures. Thus, fermenting at high temperatures may be a feasible alternative to reduce DON content in bread, as long as bread quality is not affected.

On the other hand, Generotti et al. [41**] pointed out that DON is more affected by initial yeast amount in the recipe than by fermentation time and temperature; a possible explanation for the observed DON reduction is DON adsorption on the yeast wall [42], however more studies to confirm this point are necessary. By contrast, sourdough use led to increases in DON content during fermentation [43]; it was suggested that sourdough bacteria could be able to either transform DON precursors into DON or to release bound DON, increasing the DON content at the end of the fermentation. For example, the increase of DON can also be favoured by the conversion of acetyldeoxynivalenol (3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol) forms to DON. Wu and Wang [38] showed that almost all 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol were converted to DON during kneading and fermentation.

In conclusion, DON can be either reduced or increased in dough fermentation. The use of high fermentation temperatures and avoiding enzyme use should produce DON reductions at the end of the fermentation, while use of enzymes (especially xylanase and α-amylase) can cause important increases of DON due to enzymatic release.

19.2.3. Baking

Mycotoxins are considered thermostable compounds; however, baking affects the stability of DON to a certain extent. Studies are contradictory: some studies have observed reductions in DON levels [32,33], while other studies have reported no changes or even increases in DON levels [36,44]. Firstly, the inconsistencies may exist because these studies have been conducted on different scales: some studies have been conducted in laboratories, while other studies have been conducted at the industrial level [20,32]. Moreover, Vidal et al. [45**], using small items, demonstrated that DON levels may be widely reduced only in the external part of the loaves due to the reduced heat transmission. Thus, the size of the baked items may also provide an explanation for the inconsistent results reported for baking studies. Moreover, DON is rather more affected by the time of baking than the baking temperature [20,36]. Generotti et al. [41**] even considered that baking temperature had negligible importance, for instance they found that the fermentation temperature is a more important factor to reduce DON than baking temperature. The reason is that temperature inside the bread loaves is always below 100 ºC regardless the baking temperature [36]. The use of enzymes could modify the potential DON reducing effect of baking. Hence, xylanase and α-amylase use caused an increase of DON after baking [37*], however, the increase of DON for each enzyme depended on the fermentation temperature, as these enzymes have different optimum temperatures. The initial DON concentration affects the level of DON reduction, when the initial DON concentration is higher more reduction is got [33,45**].

Kinetics for DON degradation during baking have been studied [45**], it followed a first-order equation and the Arrhenius model showed a good fit.

19.2.4. Ozonation

The use of ozone to reduce DON concentration has been tested recently with important outcomes [13*,46,47,48**]. Although ozone has not been widely tested in DON in the past, previous studies have proven that ozone can detoxify mycotoxins, especially aflatoxins, effectively in food, such as corn [49] and wheat [50]. Ozone gas is a strong oxidizing reagent that can destroy the double bonds in organic compounds, and further produce simple products with low molecular weight [51,52]. In addition, ozone has favourable penetration, and can decompose to oxygen with no toxic residual [53]. Another positive point of ozone is that it does not cause alterations to the physical and biochemical characteristics of the whole wheat grains showing that the ozone treatment would not decrease the quality of wheat [46,47].

Some factors can affect the level of DON reduction during ozone treatment. The higher the contact time the higher the DON reduction [46–48**], even Savi et al. [47] achieved a total DON reduction after 2 h of ozone treatment at 60 mg/L of ozone. However, they used spiked wheat samples and DON artificial contamination could be easier to reduce, because lower reductions were observed in naturally contaminated samples with longer treatments. Some of the studies [46–48**] followed the evolution of DON during ozone treatment time with decreasing DON concentration through the time. Moreover, DON is more reduced with higher wheat moisture [46,48**]. Ozone can quickly be decomposed into atomic oxygen with strong oxidation and hydroxyl ions in water, which are effective in oxidative degradation of DON. Under higher water contents more reactive ions can be generated because ozone is soluble in water, so it has a stronger effect on the degradation of DON. Another important factor is ozone concentration; some studies showed highly variable results with higher ozone concentration [46,48**]. For instance, Li et al. [46] only obtained a reduction of 3.5 % of DON with an ozone concentration of 20 mg/L, while they got a reduction of 26 % using an ozone concentration of 80 mg/L, and similar tendency has been observed in Wang et al. [48**] who tested 25, 50, 75 and 100 mg/L of ozone. Few studies checked the impact of initial DON concentration in wheat. Wang et al. [48**] assured that ozone effects had no correlation with initial DON concentration, but their tested DON range was very narrow. On the other hand, Li et al. [46] who worked in aqueous solutions (1 and 5 µg DON/mL) observed higher reduction in lower DON concentrations when the remaining factors (moisture, ozone concentration and time) were constant. It can be hypothesized that ozone generated reactive ions in solution was roughly the same under the same ozone concentration, therefore the lower the concentration of DON, the higher chance to react with reactive ions in solution, so the lower concentration of DON in the same concentration of ozone had higher rate of decline. Finally, when ozone treatment is made in whole wheat grain the reduction of DON in ozone treatment is mainly produced in the external layers of wheat grain and a small reduction is detected in the internal layers of wheat grain [46]. In the same way, Savi et al. [47] showed that the reduction is higher in whole wheat flour than in wheat kernels, so an ozone treatment after milling could be theoretically implemented as a critical control point.

For ozone degradation in wheat also a first order reaction was followed [48**]. According the $t_{1/2}$ values, ozone treatment is slower than baking for DON reduction. To sum up, long time, high ozone concentration, high percentage of moisture and low initial DON concentration increase the effectivity of ozone treatments. Up to now, ozone studies have been only made in raw material.

19.3. Increasing interest in DON-3-glucoside

The availability of DON-3-glucoside standards has triggered the number of studies. Results show that DON-3-glucoside and DON in milling fractions is similar and the bran fractions were clearly higher contaminated with the DON-3-glucoside [26**,28,54]. Recently, Vidal et al. [45**] showed that DON-3-glucoside may be released under mild baking conditions of temperature and time (for instance, 140 ºC for 35 min or 200 ºC for less than 10 min) but reduced under harsher baking conditions (i.e., longer periods of time and higher temperatures). On the other hand, the enzyme use can also produce an increase of DON-3-glucoside concentration during the breadmaking process, in particular with the use of xylanase, α-amylase, cellulase and lipase (> 100 % of increase of DON-3-glucoside) [37*]. Although DON-3-glucoside is a DON conjugate, authors pointed out the DON-3-glucoside behaviour is not linked to DON [28,37*] and Kostelanska et al. [28] suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may cause the release.

The increase of DON-3-glucoside during the breadmaking process agrees with the high ratio DON-3-glucoside/DON found in some analysed breads. While the ratio DON-3-glucoside/DON in raw cereals is from 10 to 30 % [14,15], some studies showed higher ratios in breads: De Boevre et al. [55*] found the same concentration of DON-3-glucoside than DON, Vanheule et al. [7] found a ratio of 1.55 DON-3-glucoside/DON and De Boevre et al. [56] found a ratio of 0.91.

Finally, the increase of DON-3-glucoside is of concern because, although DON-3-glucoside is far less active as protein biosynthesis inhibitor than DON [57], DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, to microbial activity in the intestine as shown *in vivo* in swine and *in vitro* using human intestinal microbiota [58], thus its presence is important for food safety and the Joint European Commission FAO/WHOExpert Committee (JEFCA) considered DON-3-glucoside as an additional contributing factor of the total dietary exposure to DON [59].

19.4. Conclusions

Although difficult to remove, some possibilities for DON reduction through wheat processing exist. In-depth knowledge of the food processes is, however, required to avoid those practices which could lead to increasing DON presence. DON-3-glucoside is of concern during breadmaking process because high increases of concentration are detected at the end of the baking process. There are still many factors to investigate which may affect DON stability. In recent years, ozonation has been proven to be a promising decontamination alternative, thus implementation of an additional step in food processing could be a choice. Food treatments can reduce DON concentration but attention should be paid to degradation products. There is little information on them and their associated toxicity. Degradation products have been only analysed in baking products and norDONs A-F and DON lactones have been described. All of them are less toxic than DON itself. The losses that cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions [60]. On the other hand, two unknown compounds have been described after ozone treatment [61] and they had a lower molecular weight ($C_{11}O_4H_{22}$ and $C_{13}O_2H_{25}$) than DON ($C_{15}O_6H_{20}$) but their toxicology have not been studied.

19.5. Acknowledgements

The authors thank the Spanish Ministry of Economy and Competitiveness (project AGL2014-55379-P) A. Vidal thanks the Spanish Ministry of Economy and Competitiveness for the pre-doctoral grant.

19.6. References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

** of outstanding interest

[1] Richard E, Heutte N, Sage L, Pottier D, Bouchart V, Lebailly P, Garon D: **Toxigenic fungi and mycotoxins in mature corn silage.** *Food Chem Toxicol* 2007, **45**:2420-2425.

[2] IARC (International Agency for Research on Cancer): **Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC monographs on the evaluation of carcinogenic risks to humans.** 1993, 56. Lyon, France.

[3] Pestka JJ: **Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol.** *World Mycotoxin J* 2010, **3**:323-347.

[4] Heyndrickx E, Sioen I, Huybrechts B, Callebaut A, De Henauw S, De Saeger S: **Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study.** *Environ Int* 2015, **84**:82-89.

[5] Vidal A, Cano-Sancho G, Marín S, Ramos AJ, Sanchis V: **Multidetection of urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and risk assessment in Catalonia, Spain.** *World Mycotoxin J* 2016**, In press**.

[6] Cano-Sancho G, Gauchi JP, Sanchis V, Marín S, Ramos AJ: **Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol.** *Food Addit Contam A* 2011*,* **28***:*1098-1109.

[7] Vanheule A, Audenaert K, De Boevre M, Landschoot S, Bekaert B, Munaut F, Eeckhout M, Höfte M, De Saeger S, Haesaert G: **The compositional mosaic of *Fusarium* species and their mycotoxins in unprocessed cereals, food and feed products in Belgium.** *Int J Food Microbiol* 2014, **181**:28-36.

[8] Liu Y, Lu Y, Wang L, Chang F, Yang L: **Occurrence of deoxynivalenol in wheat, Hebei Province, China.** *Food Chem* 2016, **197**:1271-1274.

[9] Tralamazza SM, Bemvenuti RH, Zorzete P, De Souza Garcia F, Corrêa B: **Fungal diversity and natural occurrence of deoxynivalenol and zearalenone in freshly harvested wheat grains from Brazil.** *Food Chem* 2016, **196**:445-456.

[10] Savi GD, Piacentini KC, Tibola CS, Santos K, Sousa Maria G, Scussel VM: **Deoxynivalenol in the wheat milling process and wheat-based products and daily intake estimates for the Southern Brazilian population.** *Food Control* 2016, **62**:231-236.

[11] González-Osnaya L, Cortés C, Soriano JM, Moltó JC, Mañes J: **Occurrence of deoxynivalenol and T-2 toxin in bread and pasta commercialised in Spain.** *Food Chem* 2011, **124**:156-161.

[12] Varga E, Malachova A, Schwartz H, Krska R, Berthiller F: **Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples.** *Food Addit Contam A*, 2013, **30**:137-146.

[13] * Wang L, Shao H, Luo X, Wang R, Li Y, Li Y, Luo Y, Chen Z: **Effect of ozone treatment on deoxynivalenol and wheat quality.** *PLoS ONE* 2016, **11**: e0147613.

The no effect of ozone in wheat quality, or even the improve of flour quality when DON is reduced is showed in this study. They compared different times of ozone treatment and the reduction of ozone for each used time.

[14] Berthiller F, Dall'asta C, Corradini R, Marchelli R, Sulyok M, Krska R, Adam G, Schuhmacher R: **Occurrence of deoxynivalenol and its 3-β-D-glucoside in wheat and maize.** *Food Addit Contam A* 2009, **26**:507-511.

[15] Dall'Asta C, Dall'Erta A, Mantovani P, Massi A, Galaverna G: **Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat.** *World Mycotoxin J* 2013, **6**:83-91.

[16] Berthiller F, Schuhmacher R, Adam G, Krska R: **Formation, determination and significance of masked and other conjugated mycotoxins.** *Anal Bioanal Chem* 2009, **395**:1243-1252.

[17] Cheli F, Pinotti L, Rossi L, Dell'Orto V: **Effect of milling procedures on mycotoxin distribution in wheat fractions: A review.** *LWT-Food Sci Technol* 2013, **54**:307-314.

[18] Kushiro M: **Effects of milling and cooking processes on the deoxynivalenol content in wheat.** *Int J Mol Sci* 2008, **9**:2127-2145.

[19] Ryu D, Bianchini A, Bullerman LB: **Effects of processing on mycotoxins.** *Stewart Postharvest Review* 2008, **4**:1-7.

[20] Suman M, Generotti S: **Transformation of mycotoxins upon food processing: masking, binding and degradation phenomena.** In *Issues in Toxicology*. Edited by Dall'Asta C, Berthiller F. Royal Society of Chemistry Publishing; 2015:73-96.

[21] Rios G, Zakhia-Rozis N, Chaurand M, Richard-Forget F, Samson MF, Abecassis J, Lullien-Pellerin V: **Impact of durum wheat milling on deoxynivalenol distribution in the outcoming fractions.** *Food Addit Contam A* 2008, **26**:487-495.

[22] Vidal A, Marín S, Ramos AJ, Cano-Sancho G, Sanchis V: **Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market.** *Food Chem Toxicol* 2013, **53**:133-138.

[23] ** Zhang H, Wang B: **Fate of deoxynivalenol and deoxynivalenol-3-glucoside during wheat milling and Chinese steamed bread processing.** *Food Control* 2014, **44**:86-91.

This paper is one of the few studies which described clearly the relative distribution of DON in the different milling parts (whole wheat, bran, shorts, flour and lost). Moreover, they also studied the relative distribution of DON-3-glucoside and the fate of DON and DON-3-glucoside during steamed bread.

[24] Zheng Y, Hossen SM, Sago Y, Yoshida M, Nakagawa H, Nagashima H, Okadome H, Nakajima T, Kushiro M: **Effect of milling on the content of deoxynivalenol, nivalenol, and zearalenone in Japanese wheat.** *Food Control* 2014, **40**:193-197.

[25] Tibola CS, Fernandes JMC, Guarienti EM: **Effect of cleaning, sorting and milling processes in wheat mycotoxin content.** *Food Control* 2015, **60**:174-179.

[26] ** Schwake-Anduschus C, Proske M, Sciurba E, Muenzing K, Koch M, Maul R: **Distribution of deoxynivalenol, zearalenone, and their respective modified analogues in milling fractions of naturally contaminated wheat grains.** *World Mycotoxin J* 2015, **8**:433-443.

This study is very interesting because they described with a high level of detail the process of milling. It is very useful to understand deeply the milling process and how to build correctly a milling study. Moreover, they analysed DON in the milling fractions during the process. On the other hand, they also analysed the level of conjugated mycotoxins.

[27] Simsek S, Burgess K, Whitney KL, Gu Y, Qian SY: **Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat.** *Food Control* 2012, **26***:*287–292.

[28] Kostelanska M, Dzuman Z, Malachova A, Capouchova I, Prokinova E, Skerikova A, Hajslova J: **Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside.** *J Agr Food Chem* 2011, **59**:9303-9312.

[29] Stuper-Szablewskaa K, Szablewskib T, Buśkoa M, Perkowskia J: **Changes in contents of trichothecenes during commercial grain milling.** *LWT-Food Sci Technol* 2016, **69**:55-58.

[30] Brera C, Peduto A, Debegnach F, Pannunzi E, Prantera E, Gregori E, De Giacomo M, De Santis B: **Study of the influence of the milling process on the distribution of deoxynivalenol content from the caryopsis to cooked pasta.** *Food Control* 2013, **31**:309-312.

[31] Herrera M, Juan T, Estopañan G, Ariño A: **Comparison of deoxynivalenol, ochratoxin A and aflatoxin B1 levels in conventional and organic durum semolina and the effect of milling.** *J Food Nutr Res* 2009, **48**:92-99.

[32] Neira MS, Pacin AM, Martínez EJ, Moltó G, Resnik SL: **The effects of bakery processing on natural deoxynivalenol contamination.** *Int J Food Microbiol* 1997, **37**:21-25.

[33] Bergamini E, Catellani D, Dall'Asta C, Galaverna G, Dossena A, Marchelli R, Suman M: **Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology**. *Food Addit Contam A* 2010, **27**:677-687.

[34] Lancova K, Hajslova J, Kostelanska M, Kohoutkova J, Nedelnik J, Moravcova H, Vanova M: **Fate of trichothecene mycotoxins during the processing: Milling and baking.** *Food Addit Contam A* 2008, **25**:650-659.

[35] Suman M, Manzitti A, Catellani D: **A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques.** *World Mycotoxin J* 2012, **5**:241-249.

[36] Vidal A, Morales H, Sanchis V, Ramos AJ, Marín S: **Stability of DON and OTA during the breadmaking process and determination of process and performance criteria.** *Food Control* 2014, **40**:234–242.

[37] * Vidal A, Ambrosio A, Sanchis V, Ramos AJ, Marin S: **Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.** *Food Chem* 2016, **208**:288–296.

This study is very interesting to check the importance of enzymes for DON and DON-3-glucoside during breadmaking process, showing the levels of DON and DON-3-glucoside after fermentation and baking. Also the importance of fermentation temperature for DON is studied.

[38] * Wu L, Wang B: **Evaluation on levels and conversion profiles of DON, 3-ADON, and 15-ADON during bread making process.** *Food Chem* 2015, 185:509-516.

It is an interesting study because they observed the stability of DON during breadmaking process and moreover they also checked the transformation of acetyl DON forms to DON during breadmaking process. They also detected degradation products of DON in the crust.

[39] Wu L, Wang B: **Transformation of deoxynivalenol and its acetylated derivatives in Chinese steamed bread making, as affected by pH, yeast, and steaming time.** *Food Chem* 2016, 202:149-155.

[40] Samar MM, Neira MS, Resnik SL, Pacin A: **Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology.** *Food Addit Contam* 2001, **18**:1004-1010.

[41] ** Generotti S, Cirlini M, Malachova A, Sulyok M, Berthiller F, Dall'Asta C, Suman M: **Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology.** *Toxins* 2015, **7**:2773-2790.

This paper is important because they studied a high level of factors to see the importance of them in the stability of DON and DON-3-glucoside during the breadmaking process in an industrial process. The obtained results provided interesting information about the trends and directions useful to be followed exactly in this industrial perspective.

[42] De Souza AF, Borsato D, Lofrano AD, De Oliveira, AS, Ono, MA, Bordini JG, Hirozawa MT, Yabe MJS, Ono EYS: ***In vitro*** **removal of deoxynivalenol by a mixture of organic and inorganic adsorbents.** *World Mycotoxin J* 2015, **8**:113-119.

[43] Vidal A, Marín S, Morales H, Ramos AJ, Sanchis V: **The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content.** *Food Chem Toxicol* 2014, **68**:53-60.

[44] Zachariasova M, Vaclavikova M, Lacina O, Vaclavik L, Hajslova J: **Deoxynivalenol oligoglycosides: New "masked"** ***Fusarium*** **toxins occurring in malt, beer, and breadstuff.** *J Agr Food Chem* 2012, **60**:9280-9291.

[45] ** Vidal A, Sanchis V, Ramos AJ, Marín S: **Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products.** *Food Chem* 2015, **178**:276-286.

The study is important because kinetics of degradation for DON and DON-3-glucoside are built. These results show the evolution of DON and DON-3-glucoside during baking process in different times and temperatures of baking. An increase of DON-3-glucoside in the first stages of baking and a total reduction of it at the end of baking has been observed in this study.

[46] Li MM, Guan EQ, Bian K: **Effect of ozone treatment on deoxynivalenol and quality evaluation of ozonised wheat.** *Food Addit Contam A* 2015, **32**:544-553.

[47] Savi GD, Piacentini KC, Bittencourt KO, Scussel VM: **Ozone treatment efficiency on** ***Fusarium graminearum*** **and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum L.*) quality and germination.** *J Stored Prod Res* 2014, **59**:245-253.

[48] ** Wang L, Luo Y, Luo X, Wang R, Li Y, Li Y, Shao H, Chen Z: **Effect of deoxynivalenol detoxification by ozone treatment in wheat grains.** *Food Control* 2016, **66**:137-144.

This article evaluated the effectiveness of destroying DON in wheat via ozonation under different conditions (moisture content, ozone concentration and exposure time). Moreover they worked with different parts of

wheat grain. Kinetics assaying different factors is built for DON reduction. This kinetics can be very useful for industry.

[49] Luo X, Wang R, Wang L, Li Y, Bian Y, Chen Z: **Effect of ozone treatment on aflatoxin B1 and safety evaluation of ozonized corn.** *Food Control* 2014, **37**:171-176.

[50] Raila A, Lugauskas A, Steponavičius D, Railiene M, Steponavičiene A, Zvicevičius E: **Application of ozone for reduction of mycological infection in wheat grain.** *Ann Agric Environ Med* 2006, **13**:287-294.

[51] Bailey PS: **The reactions of ozone with organic compounds.** *Chem Rev* 1958, **58**:925-1010.

[52] Cataldo S, Iannì A, Loddo V, Mirenda E, Palmisano L, Parrino F, Piazzese D: **Combination of advanced oxidation processes and active carbons adsorption for the treatment of simulated saline wastewater.** *Sep Purif Technol* 2016, **171**:101-111.

[53] McKenzie KS, Sarr AB, Mayura K, Bailey RH, Miller DR, Rogers TD, Norred WP, Voss KA, Plattner RD, Kubena LF, Phillips TD: **Oxidative degradation and detoxification of mycotoxins using a novel source of ozone.** *Food Chem Toxicol* 1997, **35**:807-820.

[54] Zhang H, Wang B: **Fates of deoxynivalenol and deoxynivalenol-3-glucoside during bread and noodle processing.** *Food Control* 2015, **50**:754-757.

[55] * De Boevre M, Jacxsens L, Lachat C, Eeckhout M, Di Mavungu JD, Audenaert K, Maene P, Haesaert G, Kolsteren P, De Meulenaer B, De Saeger, S: **Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium.** *Toxicol Lett* 2013, **218**:281-292.

The study is relevant because high concentration of DON-3-glucoside is detected in commercial breads, showing a higher concentration of DON-3-glucoside in the final product than in initial flour and the importance to deeper study DON-3-glucoside.

[56] De Boevre M, Di Mavungu JD, Maene P, Audenaert K, Deforce D, Haesaert G, Eeckhout M, Callebaut A, Berthiller F, Van Peteghem C, De Saeger S: **Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food.** *Food Addit Contam A* 2012, **29**:819-835.

[57] Poppenberger B, Berthiller F, Lucyshyn D, Siebrer T, Schumacher R, Krska R, Kuchler K, Glössl J, Luschnig C, Adam G: **Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*.** *J Biol Chem* 2003, **278**:47905-47914.

[58] Berthiller F, Krska R, Domig KJ, Kneifel W, Juge N, Schuhmacher R, Adam G: **Hydrolytic fate of deoxynivalenol-3-glucoside during digestion.** *Toxicol Lett* 2011, **206**:264-267.

[59] JEFCA: **WHO Technical Report Series 959: JEFCA. 2010: Seventy-second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food.** *WHO Technical Report Series* 2010, 959

[60] Bretz M, Beyer M, Cramer B, Knecht A, Humpf HU: **Thermal degradation of the Fusarium mycotoxin deoxynivalenol.** *J Agr Food Chem* 2006, **54**:6445–6451.

[61] Sun C, Ji J, Wu S, Sun C, Pi F, Zhang Y, Tang L, Sun X: Saturated aqueous ozone degradation of deoxynivalenol and its application in contaminated grains. Food Control 2016, 69:185-190.