Fate of Fusarium mycotoxins during food production. Cases of beer and cereal–based infant food

Xenia Pascari

Universitat de Lleida

TESI DOCTORAL

Fate of *Fusarium* mycotoxins during food production. Cases of beer and cereal–based infant food

Xenia Pascari

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida

Programa de Doctorat en Ciència i Tecnologia Agrària i Alimentària

Director/a

Sònia Marín Sillue

Vicente Sanchis Almenar

Tutor/a

Vicente Sanchis Almenar

2020

The present work is part of the following research projects:

- Transfer of aflatoxins, deoxynivalenol and their modified forms from cereals and other raw materials to the final products (MINECO, Project AGL2014-55379-P).
- Cereal sorting and processing techniques, and their impact on deoxynivalenol contamination in baby foods (MINECO, Project AGL2017-87755-R).

Pre-doctoral fellowship:

- 2016-2020 University of Lleida grant JADE Plus 218/2016.

Training and collaborative institutions:

- March-August 2019: German Federal Institute for Risk Assessment (BfR), National Reference Laboratory for Mycotoxins and Plant Toxins. Research internship.

Lleida, 2016 – 2020.

Author
Xenia Pascari

Supervisors
Vicente Sanchis
Sònia Marín

Major contributors
Antonio J. Ramos
Ronald Maul

Technical contributors
Montserrat Prim
Manel Andujar Moreno

Acknowledgement/Agradecimientos/Agraïments

- To Vicente, Sònia and Antonio, thank you for teaching me so much about mycotoxins and finding the very right way in challenging me. It was a great journey.
- To Ronald, for showing me a different and exciting side of this topic.
- To my parents for supporting me all this time. Thank you for always being there for me.
- To my brother, for always having a good motivational speech prepared and for giving me your support. Thank you for the adventure in Berlin.
- To Jonas for always knowing what to tell me and for making this time more than great.
- To "las antioxidadas", Iziar, Neus and Silvia, for such a beautiful friendship.
- To "the Hikers" for showing me how beautiful is the place I came to and for making me feel like at home.
- To everyone else who was there during these years. Without you this would not be possible.

The present thesis is based on the following articles:

1. Pascari, X., Ramos, A. J., Marín, S., & Sanchis, V. (2018). Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. *Food Research International*, *103*, 121 129. https://doi.org/10.1016/j.foodres.2017.07.038.
2. Pascari, X., Ortiz Solá, J., Marín, S., Ramos, A. J., & Sanchis, V. (2018). Survey of mycotoxins in beer and exposure assessment through the consumption of commercially available beer in Lleida, Spain. *LWT-Food Science and Technology*, *92*, 87 91. https://doi.org/10.1016/j.lwt.2018.02.021
3. Wall Martinez, H.A., Pascari, X., Ramos, A.J., Marin, S., Sanchis, V. (2019). Frequency levels of mycotoxins in beer from the Mexican market and exposure estimate for deoxynivalenol mycotoxins, *Mycotoxin Research*, *35*, 207 216. https://doi.org/10.1007/s12550-019-00347-x
4. Pascari, X., Gil Samarra, S., Marín, S.,Ramos, A. J., & Sanchís, V. (2019). Fate of zearalenone, deoxynivalenol and deoxynivalenol-3-glucoside during malting process. *LWT–Food Science and Technology, 99*, 540 546. https://doi.org/10.1016/j.lwt.2018.10.030
5. Pascari, X., Rodríguez Carrasco, Y., Juan, C., Mañez, J., Marín, S., Ramos, A. J., & Sanchis, V. (2019). Transfer of Fusarium mycotoxins from malt to boiled wort. *Food Chemistry, 278,* 25, 700 710. https://doi.org/10.1016/j.foodchem.2018.11.111
6. Wall-Martinez, H.A., Pascari, X., Bigordà, A., Ramos, A.J., Marin, S., Sanchis, V. (2019). The fate of *Fusarium* mycotoxins (deoxynivalenol and zearalenone) through wort fermenting by *Saccharomyces* yeast (*S. cerevisiae* and *S. pastorianus*). *Food Research International, 126*, 108587. https://doi.org/10.1016/j.foodres.2019.108587
7. Pascari, X., Marín, S., Ramos, A. J., Molino, F. & Sanchis, V. (2018). Deoxynivalenol in cereal-based baby food production process. A review, *Food Control, 99,* 11 20. https://doi.org/10.1016/j.foodcont.2018.12.014

8. Pascari X., Marin, S., Maul, R., Sanchis, V. (2020). Fate of several trichothecenes and zearalenone during cereal-based baby food production process. *Prepared to be submitted to Food Control Journal.*
9. Pascari, X., Marin, S., Sanchis, V., Maul, R. (2020). Detection and quantification of zearalenone, zearalenone-14-sulfate, α- and β-zearalenol, and α- and β- zearalenol-14-sulfate in cereal flour. *Manuscript in preparation.*

Table of content

List of Figures

Figure 11: *Evolution of deoxynivalenol (DON), 3 and 15-acethyl-deoxynivalenol (3-Ac-DON and 15-Ac-DON, respectively), α- and β-zearalenol (α-ZEL and β-ZEL, respectively) and zearalenone (ZEN) concentrations originated from the F. graminearum contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (μg/kg) and wort (μg/L). LOQ was attributed to the values <LOQ.*

Figure 12: *Evolution of fumonisins B_1 (FB1) and fumonisins B_2 (FB2) concentration originated from F. proliferatum contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (μg/kg) and wort (μg/L). LOQ was attributed to the values <LOQ. Spent grains: 45 μg/kg for FB1 and FB2. Wort: 15 μg/L for FB1 and FB2.*

Figure 13: *Alcohol production during fermentation of the contaminated and control wort*

Figure 14: *Concentrations of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) (±SD) in barley (a), wheat (b) and oat (c) during roasting at 105 and 120ºC for 30 and 40 min.*

Figure 15: *Changes in 3-acetyl-deoxynivalenol (3-Ac-DON) concentration (±SD) during barley roasting at 105 and 120ºC for 30 and 40 min.*

Figure 16: *Concentrations (±SD) of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) in barley (a), wheat (b) and oat (c) during enzymatic treatment (α-amylase and glucoamylase) at a dose of 2.1 and 4.0 g/kg during 10, 50 and 90 min.*

Figure 17: *Chemical structure of zearalenone and its modified forms*

Figure 18: *Concentration of zearalenone (ZEN) and its modified forms α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-sulfate (ZENS) and β-zearalenol-14-sulfate (β-ZELS) in a) wheat and b) oat flour during enzymatic treatment at a dose of 2.1 and 4.0 g of enzyme/kg of flour during 10, 50 and 90 min. The error bars represent the standard deviation of the mean concentration*

List of Tables

List of abbreviations

15-Ac-DON: 15-acetyl-deoxynivalenol

3-Ac-DON: 3-acetyl-deoxynivalenol

α-ZEL: α-zearalenol

β-ZEL: β-zearalenol

BEA: beauvericin

DAD: Diode Array Detector

DON-3-Glc: deoxynivalenol-3-glucoside

DON: deoxynivalenol

EC: European Commission

EFSA: European Food Safety Authority

FAO: Food and Agricultural Organization

FB1: fumonisin B_1

FB2: fumonisin B_2

FBs: total fumonisins

FD: fluorescence detector

HPLC: High Performance Liquid Chromatography

IARC: International Agency for Research in Cancer

IUPAC: International Union of Pure and Applied Chemistry

JECFA: Joint WHO/FAO Expert Committee on Food Additives

MS: Mass spectrometry

STE: sterygmatocystin

TDI: Tolerable Daily Intake

WHO: World Health Organization

ZEN: zearalenone

Abstract

Mycotoxins are secondary metabolites of a high concern for the food and feed industry. Their transfer from raw materials to the final product was often studied and demonstrated among the recent years. The present work is designed to investigate the fate of *Fusarium* mycotoxins throughout the production process of two food products with specific consumption characteristics: beer, the most consumed alcoholic beverage worldwide, and cereal-based baby food, which represents the main energy source at the very early stage of human life.

For practical reasons, experimental designs were built for several specific stages of both production processes, focusing on the stages that had been previously reported as able or possibly able of mitigating mycotoxin contamination. Thus, malting, mashing, boiling and alcoholic fermentation were selected for beer making technology; and flour roasting, followed by the pre-gelatinization and enzymatic digestion were chosen for the baby-food production process. All the processes were reproduced at laboratory scale production and samples were taken. Various sample preparation and analytical techniques for *Fusarium* toxins were chosen to achieve the proposed objectives. Sample preparation was mostly performed by immunoaffinity columns and QuEChERS with or without a clean-up. The detection of the mycotoxins was achieved by HPLC coupled to either fluorescence, diode array or triple quadrupole mass spectrometer detectors.

The study of the mycotoxins in beer started by identifying the occurrence of multiple mycotoxins in commercially available beers from Lleida (64 different beers) and Veracruz (61 beers), additionally estimating the possible deoxynivalenol exposure scenarios related to beer consumption in the respective geographical areas. The results showed a total of 20.3 and 26.2% of contaminated samples in Lleida and Veracruz, respectively. Some of the samples were co-contaminated with two or more mycotoxins produced by different *Fusarium* species.

DON, ZEN and DON-3-Glc changes were studied during malting. Barley batches contaminated in two different ways and at different mycotoxin levels were used for malting. The study of the effect of mashing and boiling similarly involved various contamination sources and levels of mycotoxins, and also included other analytes such as

fumonisins and the modified forms of DON and ZEN. The results identified a reduction of DON levels during steeping and its glycosylation during germination. ZEN was unchanged during steeping, but a peaked after 48h of germination. Kilning did not lead to any significant change in the levels of the studied mycotoxins.

The high solubility in water allowed the transfer of DON, DON-3-Glc and FBs from malt to wort during mashing, the first stage of mashing (15 min at 45°C) being the most significant. ZEN also was partly transferred, but more gradually, probably due to the high temperature and relatively long time of contact with water. Boiling led to a significant reduction in all studied mycotoxins after the first 30 min of the treatment. Although almost 99% of ZEN was reduced, final DON concentration represented 30 to 60% of the level initially contained in the contaminated malt.

Fifteen different *Saccharomyces* species were studied for their ability to adsorb DON and ZEN. Malt contaminated with a *Fusarium graminearum* strain was used for the study. The results showed that 11 to 17% for DON and up to 72% of ZEN were adsorbed by the yeast cell wall. The presence of mycotoxins did not affect alcohol production during the process.

Three different cereal flours were used to study the effect of cereal-based baby food production process on mycotoxin contamination, namely barley, wheat and oat. The roasting was performed by choosing two temperature levels (105°C and 120°C) and two treatment times (30 and 40 min). The study did not show a significant change in the initial level of the mycotoxins, similar to the data already reported in the literature.

For the enzymatic treatment two different doses of α-amylase and glucoamylase were chosen, with three different incubation times. The results showed different enzymatic activity levels for the different flours, probably explained by the different structure and composition for each cereal. No significant changes in the mycotoxin levels was identified, however a slight increasing trend in ZEN and its modified forms was found. Also, no relationship of transformation of the parental forms into modified mycotoxins could be proven. Thus, the present thesis proves a partial or complete reduction of *Fusarium* mycotoxins during beer production process at different stages of it. Nonetheless, no significant changes in the mycotoxin levels were registered in the case of infant food.

Resum

Les micotoxines són metabòlits fúngics secundaris que representen un risc important per a la salut pública a causa de la seva freqüent incidència en aliments i pinsos, sent els cereals utilitzats en l'elaboració d'aquests els principals productes que es contaminen. La seva transferència de les matèries primeres al producte final ha estat demostrada però no prou estudiada durant els últims anys. El present treball estudia la transferència de les micotoxines de *Fusarium* durant el procés de producció de dos aliments d'un alt consum, encara que destinat a diferents grups de la població: la cervesa, la beguda alcohòlica més consumida a nivell mundial, i les farinetes, que representen la principal font energètica en l'alimentació infantil.

Per motius pràctics, el disseny experimental per l'estudi de les dues tecnologies de producció es va dividir en estudis específics enfocats a les etapes de producció que han estat prèviament identificades com a possibles determinants en la reducció de la càrrega en micotoxines. Així, el maltatge de l'ordi, la maceració, l'ebullició i la fermentació alcohòlica del most de malt es van triar en l'estudi de la cervesa; el torrat, seguit per la pre-gelatinització i la digestió enzimàtica es van seleccionar per a l'estudi de les farinetes. Tots els processos van ser preparats per a la producció i el mostreig a escala de laboratori.

Es van triar diverses tècniques de preparació i extracció de les micotoxines de les matrius, com la utilització de columnes de immunoafinitat i QuEChERS amb o sense clean-up. La detecció de les micotoxines es va aconseguir mitjançant HPLC acoblat a detectors de fluorescència, de díodes, o espectròmetres de masses de triple quadrupol, depenent de les micotoxines que es pretenia quantificar.

L'estudi de la incidència de les micotoxines de *Fusarium* en cerveses comercials va començar amb l'anàlisi d'aquestes micotoxines en mostres adquirides en diversos punts de venda a Lleida (64 cerveses diferents) i a Veracruz (61 cerveses diferents). A més, es va realitzar, en el marc del present estudi, una avaluació de diversos escenaris d'exposició a deoxinivalenol (DON), que va ser la micotoxina més abundant, en aquestes dues zones geogràfiques. Els resultats obtinguts van mostrar un percentatge de mostres contaminades del 20,3% i 26,2% a Lleida i Veracruz, respectivament. Destaquem que algunes mostres presentaven contaminació per més d'una de les micotoxines analitzades.

S'han estudiat els canvis en els nivells de DON, deoxinivalenol-3-glucòsid (DON-3-Glc) i zearalenona (ZEN) durant el maltatge de l'ordi. Es van assajar dues formes de contaminació de l'ordi i diferents nivells inicials d'aquestes micotoxines. L'estudi sobre l'efecte de la maceració i ebullició del most cerveser sobre el nivell d'aquestes micotoxines es va dissenyar, no sols contemplant diverses fonts de contaminació i nivells d'aquestes toxines, sinó que també va incloure altres micotoxines com les fumonisines i les formes modificades del DON i de la ZEN. Els resultats van mostrar una reducció del nivell de DON durant el remull de l'ordi i la seva glucosilació durant el procés de germinació. Els nivells de ZEN van ser constants durant el remull, però es va observar un augment important a les 48h de germinació. L'assecat del malt no va tenir cap efecte sobre els nivells de les micotoxines analitzades.

La solubilitat elevada en aigua del DON, DON-3-Glc i de les FBs va permetre la seva transferència del malt al most cerveser durant la fase inicial del procés de maceració (15 minuts a 45 °C). La ZEN també es va transferir en part, però de forma més gradual, probablement a causa de les altes temperatures i al llarg temps de contacte del malt amb l'aigua durant aquesta etapa. L'ebullició del most va conduir a una reducció significativa en totes les micotoxines estudiades després dels primers 30 minuts del tractament. Les reduccions obtingudes en el procés van mostrar una gran variabilitat. Així, la ZEN es va reduir un 99%, mentre que el DON va registrar unes reduccions entre el 30 i 60%.

Es va estudiar la capacitat de reduir la contaminació de DON i ZEN per part de quinze soques comercials de *Saccharomyces* utilitzades en la fermentació del most per a l'obtenció de cervesa. En aquest estudi es va utilitzar malt contaminat per DON i ZEN mitjançant la inoculació i incubació del mateix amb una soca micotoxigènica de *Fusarium graminearum*. Els resultats van mostrar una reducció del 11 al 17% per al DON i fins a un 72% en el cas de la ZEN a causa de la seva adsorció a la paret cel·lular del llevat durant el procés de fermentació. El nivell inicial de contaminació amb micotoxines no va afectar el rendiment fermentatiu del llevat.

Es van utilitzar farines d'ordi, blat i civada contaminades per micotoxines de *Fusarium* per a l'estudi de l'impacte del procés de fabricació de farinetes a base d'aquests cereals en els nivells d'aquestes micotoxines. El torrat es va realitzar sotmetent aquestes farines a un tractament tèrmic en sec de 105 °C i 120 °C durant 30 i 40 minuts. L'estudi no va identificar

cap efecte significatiu sobre el nivell inicial de cap de les micotoxines de *Fusarium* analitzades. Aquests resultats corroboren els trobats per altres autors.

El tractament enzimàtic es va realitzar afegint dues dosis de α-amilasa i glucoamilasa amb tres temps d'incubació. Es va observar una activitat enzimàtica diferent en els tres substrats, degut probablement a les diferents propietats d'aquestes matrius. No hi va haver efecte significatiu del tractament sobre el nivell final de les micotoxines. No obstant això, es va observar un lleuger augment en els nivells de ZEN i les seves formes modificades, però sense trobar una relació clara de transformació de les formes parentals a les seves micotoxines modificades.

Per tant, la present tesi demostra un reducció parcial o total del nivell de les micotoxines de *Fusarium* durant el procés de producció de la cervesa. No obstant això, no es van registrar canvis significatius durant el procés de producció de les farinetes infantils.

Resumen

Las micotoxinas son metabolitos fúngicos secundarios que representan un riesgo importante para la salud pública debido a su frecuente presencia en alimentos y piensos, siendo los cereales utilizados en la elaboración de éstos los principales productos que se contaminan. Su transferencia de las materias primas al producto final ha sido demostrada pero no suficientemente estudiada durante los últimos años. El presente trabajo estudia la transferencia de las micotoxinas de *Fusarium* durante el proceso de producción de dos alimentos de un alto consumo, aunque destinados a diferentes grupos de población: la cerveza, la bebida alcohólica más consumida a nivel mundial, y las papillas infantiles a base de cereales, que representan la principal fuente energética en la alimentación infantil.

Por motivos prácticos, el diseño experimental para el estudio las dos tecnologías de producción se dividieron en estudios específicos enfocados a las etapas de producción que habían sido previamente identificadas como posibles determinantes en la reducción de la carga de micotoxinas. Así, el malteado de la cebada, la maceración, la ebullición y la fermentación alcohólica del mosto se eligieron en el estudio de la cerveza; el tostado, seguido por la pre-gelatinización y la digestión enzimática se seleccionaron para el estudio de las papillas infantiles. Todos los procesos fueron preparados para la producción y el muestreo a escala de laboratorio.

Se eligieron varias técnicas de preparación y extracción de las micotoxinas de las matrices, como la utilización de columnas de inmunoafinidad y QuEChERS con o sin clean-up. La detección de las micotoxinas se logró mediante HPLC acoplado a detectores de fluorescencia o de diodos, o a espectrómetros de masas triple cuadrupolo, dependiendo de las micotoxinas que se pretendía cuantificar.

El estudio de la incidencia de las micotoxinas de *Fusarium* en cervezas comerciales empezó con el análisis de estas micotoxinas en muestras adquiridas en varios puntos de venta en Lleida (64 cervezas diferentes) y en Veracruz (61 cervezas diferentes). Además, se realizó, en el marco del presente estudio, una evaluación de varios escenarios de exposición a deoxinivalenol (DON), que fue la micotoxina más abundante, en estas dos zonas geográficas. Los resultados obtenidos mostraron un porcentaje de muestras contaminadas del 20,3 y 26,2% en Lleida y Veracruz, respectivamente. Destacamos que algunas muestras

presentaban contaminación por más de una de las micotoxinas analizadas.

Se han estudiado los cambios en los niveles de DON, deoxinivalenol-3 glucósido (DON-3-Glc) y zearalenona (ZEN) durante el malteado de la cebada. Se prepararon dos formas de contaminación de la cebada y diferentes niveles iniciales de estas micotoxinas. El estudio sobre el efecto de la maceración y ebullición del mosto cervecero sobre el nivel de estas micotoxinas se diseñó, no solo contemplando varias fuentes de contaminación y niveles de estas toxinas, sino que también incluyó otras micotoxinas como las FBs y las formas modificadas del DON y de la ZEN. Los resultados mostraron una reducción del nivel de DON durante el remojo de la cebada y su glucosilación durante el proceso de germinación. Los niveles de ZEN fueron constantes durante el remojo, pero se observó un aumento importante a las 48h de germinación. El secado de la malta no tuvo ningún efecto sobre los niveles de las micotoxinas analizadas.

La solubilidad elevada en agua del DON, DON-3-Glc y de las FBs permitió su transferencia de la malta al mosto cervecero durante la fase inicial del proceso de maceración (15 minutos a 45ºC). La ZEN también se transfirió en parte, pero de forma más gradual, probablemente debido a las altas temperaturas y al largo tiempo de contacto de la malta con el agua durante esta etapa. La ebullición del mosto condujo a una reducción significativa en todas las micotoxinas estudiadas después de los primeros 30 minutos del tratamiento. Las reducciones encontradas en el proceso mostraron una gran variabilidad. Así, la ZEN se redujo un 99%, mientras que el DON registró unas reducciones entre el 30 y 60%.

Se estudió la capacidad de reducir la contaminación de DON y ZEN por parte de quince cepas comerciales de *Saccharomyces* utilizadas en la fermentación del mosto para la obtención de cerveza. En este estudio se utilizó malta contaminada por DON y ZEN mediante la inoculación e incubación de la misma con una cepa micotoxigénica de *Fusarium graminearum*. Los resultados mostraron una reducción del 11 al 17% para el DON y hasta un 72% en el caso de la ZEN debido a su adsorción en la pared celular de la levadura durante el proceso de fermentación. El nivel inicial de contaminación con micotoxinas no afectó el rendimiento fermentativo de la levadura.

Se utilizaron harinas de cebada, trigo y avena contaminadas por micotoxinas de *Fusarium* para el estudio del impacto del proceso de fabricación de papillas infantiles a base de estos

cereales en los niveles de estas micotoxinas. El tostado se realizó sometiendo estas harinas a un tratamiento térmico en seco de 105ºC y 120ºC durante 30 y 40 minutos. El estudio no identificó ningún efecto significativo sobre el nivel inicial de ninguna de las micotoxinas de *Fusarium* analizadas. Estos resultados corroboran los encontrados por otros autores.

El tratamiento enzimático se realizó añadiendo dos dosis de α-amilasa y glucoamilasa con tres tiempos de incubación. Se observó una actividad enzimática diferente en los tres sustratos, debido probablemente a las diferentes propiedades de estas matrices. No hubo efecto significativo del tratamiento sobre el nivel final de las micotoxinas. No obstante, se observó un ligero aumento en los niveles de ZEN y sus formas modificadas, pero sin encontrar una relación clara de transformación de las formas parentales a sus micotoxinas modificadas.

Por lo tanto, la presente tesis demuestra un reducción parcial o total del nivel de las micotoxinas de *Fusarium* durante el proceso de producción de la cerveza. Sin embargo, no se registraron cambios significativos durante el proceso de producción de las papillas infantiles.

Chapter 1: Introduction

1.1 *Fusarium* mycotoxins and their producing species

Fusarium genus includes a high variety of species pathogenic to agriculturally important crops and also is able to cause diseases in humans and animals (Moretti, 2009). From the perspective of host association and produced metabolites, *Fusarium* species represent the highest diversity compared to other foodborne filamentous fungi (Glenn, 2007). Being widely distributed in soils, *Fusarium* plant pathogenicity is manifested in a great range of diseases such as pre- and post-emergence blight, root and stem rots, vascular wilts and others (Pitt & Hocking, 1997). *Fusarium* is one of the three major mycotoxin producing *fungi*. The main species involved in mycotoxin production are *F. graminearum, F. culmorum, F. verticillioides, F. avenaceum, F. proliferatum* and others (Edwards, 2004). Under favourable conditions, cereal infection with *Fusarium* species is often followed by mycotoxins accumulation, which besides a quality alteration of the kernels (Glenn, 2007) represent a threat to the public health due to their toxicity (Lee and Ryu, 2017). The most common groups of mycotoxins produced by *Fusarium* are trichothecenes, zearalenone, fumonisins, enniatins and beauvericin (Asam, Habler, and Rychlik, 2017). The present introduction will focus on the first three groups of compounds.

1.1.1 Trichothecenes

Trichothecenes are a family of secondary metabolites (almost 200 toxins are known nowadays) part of sesquiterpenoid epoxides chemical group, produced by various fungal genera, one of the most frequently reported being *Fusarium* (Yazar and Omurtag, 2008). Their chemical properties and producing fungi determined the classification of trichothecenes into four groups (from group A to D), types A and B being important due to their high occurrence in the commonly cultivated crops (Rocha, Ansari, and Doohan, 2005). Their chemical structure allows them to rapidly proliferate cell tissue, inhibit mitochondrial protein synthesis and interact with protein sulfhydryl groups, which translates into creating free radicals and induce oxidative stress (McCormick et al., 2011). The trichothecenes effect on protein synthesis inhibition can take place in two different ways: initiation type, by postponing the start of the new protein synthesis (effect of the type A group) and elongation- termination type (type B group), by preventing amino acids linkage and their release into the cell (Asam, Habler, and Rychlik, 2017).

1.1.1.1 Type A trichothecenes

Type A trichothecenes includes those trichothecenes that have a hydroxyl group, an ester function or no oxygen substitution at the C-8 (McCormick et al., 2011). They are mainly represented by T-2 and HT-2 toxins, diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (Asam, Habler, and Rychlik, 2017), the first two being most frequently reported in cereals (Eskola et al., 2019). Occurrence studies in Europe indicate the high contamination of oats with T-2 and HT-2 toxins, maize, wheat and barley being also affected but on a lower concentration levels (Van Der Fels-Klerx and Stratakou, 2010). The fungal strains responsible for their production are *F. sporotrichioides, F. langsethiae, F. poae* and *F. kyushuense,* invading the crops under humid and cold conditions (Glenn, 2007). All of them except *F. kyushuense*, which is mainly found in Japan, have a worldwide distribution and were identified as minor contributors to the head blight and maize ear rot diseases (Hellin et al., 2016).

From the toxicological perspective, type A trichothecenes are associated with immunotoxic, cytotoxic and haematotoxic effects. Their carcinogenicity was not proven, however in studies performed in pregnant mice they showed a pronounced faetus and mother toxicity (Van Der Fels-Klerx and Stratakou, 2010). In terms of risk assessment, only T-2 and HT-2 have been evaluated from the perspective of human exposure, with a tolerable daily intake of 0.1μg/kg*bw established by the EFSA (European Food Safety Authority) Panel on Contaminants in the Food Chain (EFSA, 2011a). The legislation in food does not set maximum limits for T-2 and HT-2 toxins, only indicative levels between 50 and 200 μg/kg for cereals intended for direct human consumption have been set (European Commission, 2013).

1.1.1.2 Type B trichothecenes

Type B trichothecenes are tricyclic sesquiterpenes bearing a keto group at the C-8. They are mainly represented by deoxynivalenol (DON), nivalenol (NIV) and their modified forms (Asam, Habler, and Rychlik, 2017). This group of toxins is often produced in cereals by *F. graminearum* and *pseudograminearum, F. culmorum* and *F. cerealis,* also frequently occurring as part of the head blight disease (Glenn, 2007). *Fusarium graminearum* is the most commonly encountered species of the Fusarium Head Blight disease (FHB) affecting

wheat, barley and other small grain crops across the world, being able to destroy a high yield crop in a matter of weeks (Goswami and Kistler, 2004).

Type B trichothecenes are considered less toxic than type A trichothecenes, however their higher occurrence in cereals and resistance to food processing conditions makes them a health threat to consider. DON is one of the most abundant toxin of the group. Its toxicity is manifested by food refusal, nausea, which is later translated into decrease of body weight in animals and humans (Sobrova et al., 2010). Sub-chronic and chronic exposure to DON leads to anorexia, immunosuppressive effects and an impact on the neuronal activity in mice models (Bonnet et al., 2012). NIV also causes vomiting and high doses exposure can lead to histopathologic effects in rats (similar to DON) and even death (Pestka, 2010).

Considering the abovementioned DON toxicity, the concern regarding this toxin has increased with the discovery of its major modified forms, namely deoxynivalenol-3-glucoside (DON-3-Glc) and 3- and 15-acetyldeoxynivalenol (3 and 15-Ac-DON) (Rychlik et al., 2014). Each of these metabolites has its source of formation: DON-3-Glc is a result of plant detoxification strategy (Lemmens et al., 2005), whereas 3- and 15-Ac-DON are synthetized by the *fungus* to protect itself from its own toxins (Berthiller et al., 2013). Figure 1 represents the major mechanisms that cereal plants use to protect themselves from the pathogen. The toxicity of the modified forms is lower compared to the parental form, however human and animal digestion may lead to their conversion back into DON or to the formation of other metabolized forms which proves their contribution to the exposure (Berthiller et al., 2011). A co-occurrence of DON and its metabolites was repeatedly identified in cereal commodities (Bryła et al., 2016; De Boevre et al., 2012; Uhlig et al., 2013), however the existing legislation has not yet defined maximum acceptable levels for those mycotoxins in food products. The available cytotoxicity studies prove that in order of decreasing toxicity DON is followed by 15-Ac-DON, 3-Ac-DON and de-epoxy-deoxynivalenol (DOM-1), nonetheless more investigations are needed to better evaluate the consequences of their metabolism (Dänicke and Brezina, 2013). FAO/WHO Joint Expert Committee on Food Additives (JECFA) extended the provisional maximum tolerable daily intake (PMTDI) from DON to a group of DON, 3-Ac-DON and 15- Ac-DON, the information regarding DON-3-Glc being insufficient to consider its inclusion in the group (JECFA/FAO, 2011).

In terms of regulation of the maximum limits, only DON is being set in cereals and other food products with values between 200 and 1750 µg/kg in food products and raw materials (European Commission, 2006b).

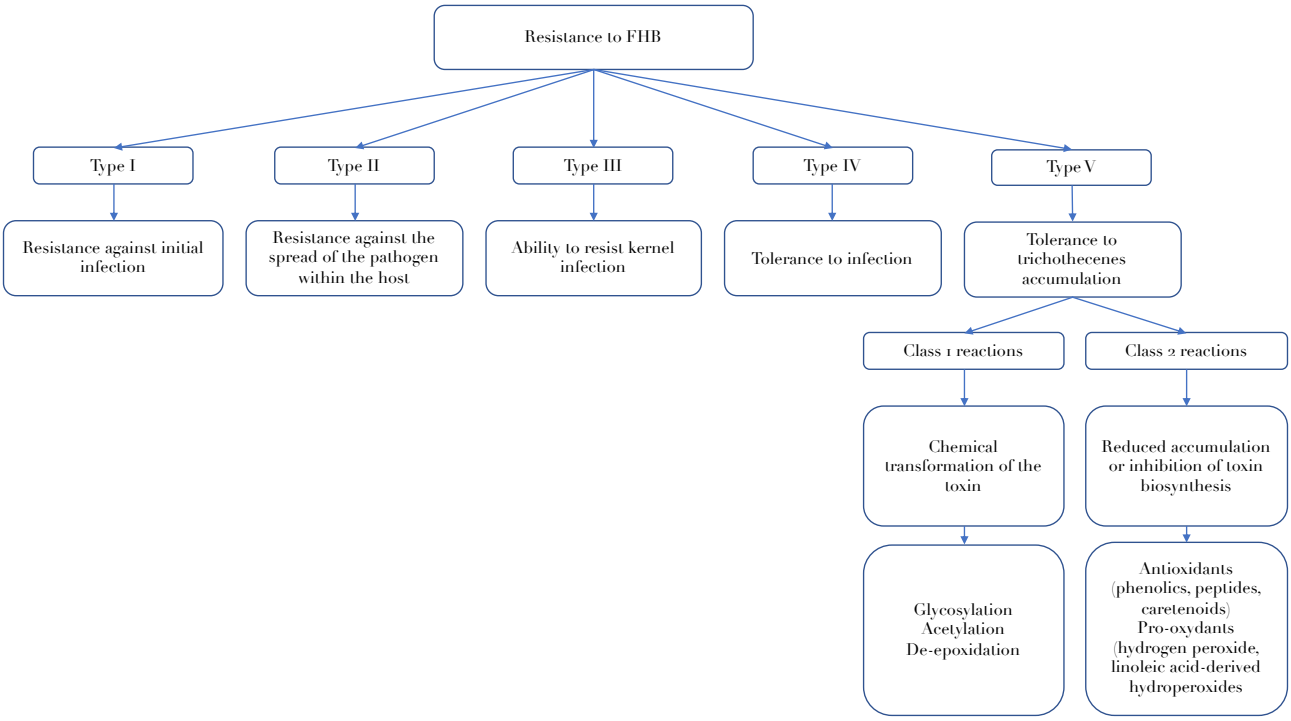

Figure 1: Natural mechanisms for cereal resistance to Fusarium Head Blight (FHB) and trichothecenes accumulation (adapted from Boutigny, Richard-Forget, & Barreau, 2008)

1.1.2 Zearalenone

Zearalenone (ZEN) is a resorcyclic lactone with a chemical structure very similar to 17β-estradiol (estrogen) and is mainly produced by *F. graminearum*, *F. culmorum*, *F. crookwellense, F. cerealis, F. semitectum* and *F. equiseti,* which are common contaminants of cereal crops worldwide (Ismaiel and Papenbrock, 2015). ZEN is usually reported in maize, wheat, barley and sorghum, representing an important health risk due to its estrogenic activity (EFSA, 2011b).

Available studies identified ZEN as having a relatively low acute and sub-chronic toxicities. Nonetheless, a prolonged exposure to the toxin can lead to reproductive disorders (endocrine disruptor agent), immunosuppressing effect (through binding to the estrogen receptors of the immune system and activating immune response related genes), vulvovaginitis (pigs), feminization and depression of serum testosterone and other histopathological changes of the internal organs (Wang et al., 2019; Zinedine et al., 2007). Just like the trichothecenes group of mycotoxins, ZEN can be converted into modified

forms, which have a different relative toxicity compared to the parent form. The most commonly detected ZEN modified forms are α- and β-zearalenone (α-ZEL and β-ZEL) and zearalenone-14-sulfate (ZEN-14-S), all found to be also natural fungal metabolites (De Boevre et al., 2013; Kovalsky et al., 2016). Other modified forms of ZEN were reported by Brodehl et al. (2014), while studying the biotransformation of ZEN by the *Rhizopus* and *Aspergillus,* namely α-zearalenol-14-sulfate (α-ZEL-14-S). The fact that animal metabolism is able to convert the modified forms back to their parental compounds suggests the need in deeper research to assess the exposure to ZEN throughout the consumption of cereal-based products (De Boevre et al., 2012).

EFSA Panel on Contaminants in the Food Chain estimated the TDI for ZEN at 0.25μg/kg bw and set the maximum limits for it in food between 20 μg/kg in cereal-based baby food and 350 μg/kg in unprocessed cereals (EFSA, 2011b; European Commission, 2006c). ZEN is always produced together with other trichothecenes, creating a combined effect of exposure, which can be safe as long as the individual recommendations for the co-occurring mycotoxins are followed (EFSA, 2005).

1.1.3 Fumonisins

Fumonisins (FBs) are mycotoxins produced by *F. proliferatum, F. verticillioides* and other *Fusarium* species, which have a chemical similarity with sphinganine and sphingosine (critical compounds in the sphingolipid metabolism) (Voss, Smith, and Haschek, 2007). The prevalent toxin of the group is fumonisin B_1 (FB1), followed by fumonisins B_2 (FB2), B_3 (FB3) and B_4 (FB4). Occurrence studies show their frequent presence in maize and maize based products (Cirillo et al., 2003; Manova and Mladenova, 2009; Yazar and Omurtag, 2008).

The disruption of the sphingolipid metabolism after the exposure to FBs is accounted for an impairment of the cell growth, followed by cellular oxidative stress, apoptosis and necrosis (EFSA, 2005). FB1 is classified as possible human carcinogen by IARC (International Agency for Research on Cancer) of the group 2B (IARC, 2002). Toxicological studies reveal the effect of FBs as a risk factor inducing oesophageal and liver cancer, cardiovascular diseases and neurotube defects (Voss et al., 2007). Besides, animal studies proved it expressing reproductive, developmental toxicity, nonetheless it was not proven

genotoxic (European Commission, 2000).

From the perspective of the European Union (EU) regulations and the available occurrence data, the sum of FB1 and FB2 has a maximum limit set only in maize and maize based products (European Commission, 2006b).

1.2 Examples of transfer of mycotoxins from raw materials to the final product

Mycotoxins are fungal secondary metabolites proven with a high physical and chemical stability (Kabak, 2009; Ryu, Bianchini, and Bullerman, 2008). Cereals are the most susceptible to mycotoxin contamination due to their nutritional composition which stimulates fungal infection and, as a result, toxin accumulation (Marroquín-Cardona et al., 2014). The cereals which are most often contaminated with mycotoxins are maize, barley, wheat, oat, rye, triticale, sorghum etc. (Tangni et al., 2013). The nature of the toxin spreading into the kernel due to their chemical properties (*e.g.* tendency of the toxins to bind to the cereal hulls and external layers), leads to their elimination from the production process by dehulling, grains selection and washing (Kostelanska et al., 2011; Vaclavikova et al., 2013), i.e. before any food process operation is applied. Nonetheless, studies investigating the occurrence of different mycotoxins in the commercially available food products identified not only their presence in the respective foodstuffs but also could assess the risk to the consumer related to the consumption of contaminated foods (Raiola et al., 2015; Rodríguez-Carrasco et al., 2015)

The following part of the present chapter will present the available information regarding the fate of the main *Fusarium* mycotoxins during the production of two cereal-based products characterized by a high consumption and frequent consumer choice, namely beer and cereal-based infant food. Also, the principles of each production process, differences in the levels of mycotoxins found in raw materials and final product surveys and impact of mycotoxin contamination on the quality of the two products will be reviewed.

1.2.1 Mycotoxins in beer. Impact of beer production process on mycotoxin contamination

Beer is identified as the most consumed alcoholic beverage in the world. Its production increases every year in Europe, with Germany leading the list with 21% of the total EU production in 2018, followed by United Kingdom, Poland and Spain (EUROSTAT, 2019). Barley represents one of the main ingredients in beer production together with water, hops and yeast. Its quality is decisive for the quality and acceptance of the beer on the market. Beer also can be subjected to mycotoxin contamination coming from infected raw materials: barley, malt, hops or adjuncts. Figure 2 is a schematic representation of a malting and brewing process.

Many studies have been published concerning the fate of mycotoxins in beer production, analyzing the overall production process or only a part of it and highlighting the physical parameters leading to the variation in mycotoxins' concentration (Inoue et al., 2013; Malachova et al., 2010; Pietri et al., 2010; Vaclavikova et al., 2013). A review on the evolution of mycotoxin during brewing is available, considering also the existing physical, chemical and biological decontamination methods that could be applied (Wolf-Hall, 2007).

1.2.1.1 Incidence of mycotoxins in malting barley

The main mycotoxins present in malting barley are the ones produced by *Fusarium* species. The plant disease Fusarium head blight (FHB) or scab is of a high concern in the production of malting barley (Wolf-Hall, 2007). The major species involved in FHB disease in Europe are *F. graminearum, F. avenaceum* and *F. culmorum* and others of the same genera but in a smaller rate (Nielsen et al., 2014). The major impact for the brewing industry caused by this disease is the negative effect on germination rates which results in worse malting quality and yield reduction (Piacentini et al., 2015). There is a clear relationship between FHB and mycotoxin contamination as the main fungal species responsible for the disease are mycotoxigenic. The predominant mycotoxin present in malting barley is DON, besides ZEN, NIV, T2 and HT-2 toxins (Pestka, 2007).

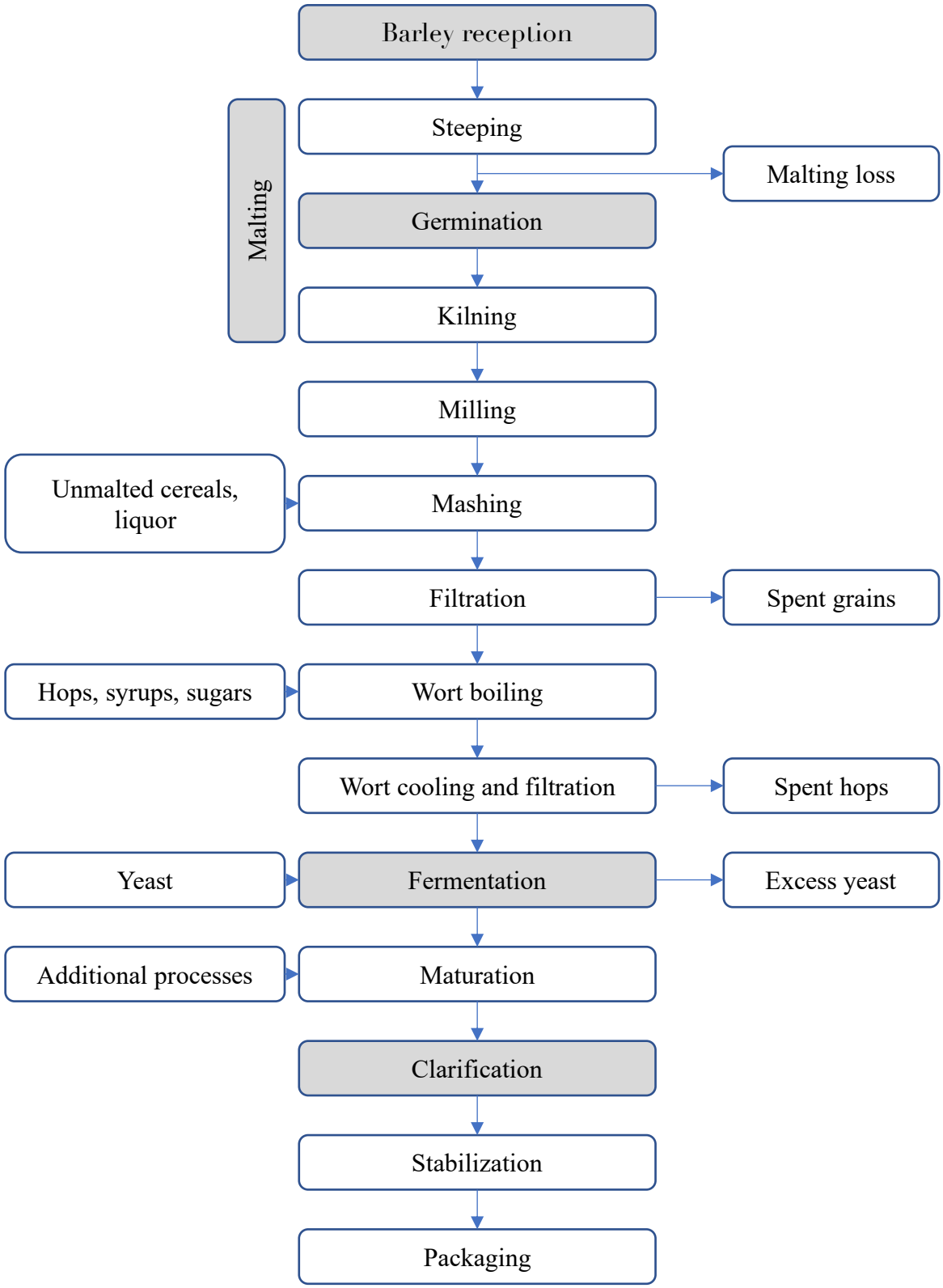

Figure 2: Beer production scheme and steps where possible decontamination strategies could be applied (blocks in grey) (modified from Lewis & Young, 1995)

Various studies have proved the existing positive correlation between FHB intensity and DON accumulation level (Paul et al, 2005; Urrea, et al., 2002). However, a meta analysis performed by Paul et al. (2005), evaluating Pearson's correlation between FHB disease parameters and DON accumulation performed on wheat, ranged from -0.43 to 0.94 for the correlation between disease severity and DON concentration and from -0.47 to 0.98 for the correlation between *Fusarium* damaged kernels rate and DON concentration. This result shows that besides meteorological conditions, defining mycotoxin accumulation, barley variety and its resistance to FHB is an important barrier to mycotoxin synthesis and a factor to consider while evaluating the correlation coefficient (Urrea et al., 2002). Table 1 regroups several surveys concerning the main mycotoxins encountered in malting barley. The natural occurrence of DON is the most studied over years in different regions of the world, as being the mycotoxin reaching the highest concentration levels on barley matrix as compared to AF, ZEN, OTA etc. (Malachova et al., 2010). Considering that the most important part of the contamination takes place on the field, the level of mycotoxins looks to be highly dependent on weather conditions (humidity and temperature) (Ghali et al., 2008; Manova & Mladenova, 2009; Pietri et al., 2010). Particularly important are climate conditions during critical phases of barley plant growth: more fungal and mycotoxin diversity was observed in warm and humid regions and less in countries with a noticeable difference between seasons (Manova & Mladenova, 2009; Tabuc et al., 2009; Piacentini et al., 2015). In cold regions like Romania, the longer period of cold weather barrier the accumulation of OTA and FUM but not of ZEN and AFs, although on comparatively low concentrations (Tabuc et al., 2009). This, together with applied agricultural practices, explains the fungal populations invading the crops on the field (Piacentini et al., 2015). Mbugua & Gathumbi (2004) calculated Pearson's correlation coefficient for the co-occurrence of DON and other mycotoxins and have found a positive association between DON and FB1 and DON and ZEN (if DON is present it is more likely that other mycotoxins are also present in the product), which is explained by the fact that these are all *Fusarium* toxins. Taking into account that different strains are responsible for FB1 and ZEN production, their co-occurrence would mean the presence of more than one fungal strain within the same batch or sample. Legal limits are also set for cereals or barley specifically, namely: 2 µg/kg for AFB1 and 4 µg/kg for total AFs; 100 µg/kg for ZEN; 1250 µg/kg for

Table 1: Incidence of mycotoxins in malting barley

Mycotoxin	Positive samples, % (n)	Concentration, µg/kg		References
		Range	mean	
DON	NA	300 50 800	NA	Ruan et al., 2002
	50 (10)	5.0-80.0	31.0	Olsson et al., 2002
	100 (37)	500-10 000	4098	Pan et al., 2007
	NA	NA	12.0	Lancova et al., 2008
	61.9 (21)	0.0-4000.0	3923.8	Tabuc et al., 2009
	53 (36)	NA	17.0	Malachova et al., 2010
	72 (29)	3.9-112.3	34.4	Gil-Serna et al., 2013
	77.5 (80)	0.0-985.9	48.4	Běláková et al., 2014
	18 (15)	200.0-15 100.0	3400	Piacentini et al., 2015
DON-3-Glc	20 (36)	NA	2.0	Malachova et al., 2010
AcDONs	2.8 (36)	NA	1.0	Malachova et al., 2010
NIV	20 (36)	NA	4.0	Malachova et al., 2010
	17.2 (29)	3.5-5.8	4.6	Gil-Serna et al., 2013
ZEN	8 (25)	0.7-21.5	15	Ghali et al., 2008
	11.1 (18)	0.0-36.3	29.0	Manova & Mladenova, 2009
	71.4 (21)	86.0-202.0	132.7	Tabuc et al., 2009
	39 (123)	0.0-18.5	1.89	Ibáñez-Vea et al., 2012
	37.9 (29)	10.4-34.1	18.5	Gil-Serna et al., 2013
	33.8 (80)	0.0-47.9	3.7	Běláková et al., 2014
OTA	79 (40)	6.7-57.0	25.7	Czerwiecki et al., 2002
	89.6 (295)	0.53-12.0	4.93	Gumus et al., 2004
	52 (25)	0.6-3.4	1.9	Ghali et al., 2008
	58 (123)	0.0-3.53	0.1	Ibáñez-Vea et al., 2012
AFB1	4.8 (21)	0.0-7.2	2.0	Tabuc et al., 2009
AFs	44 (25)	3.5-11.7	7.5	Ghali et al., 2008
	100 (123)	0.0-0.75	0.14	Ibáñez-Vea et al., 2012
FUMs	10 (15)	10.0-13.0	6.0	Piacentini et al., 2015
	34.5 (29)	186.5-347.5	249.1	Gil-Serna et al., 2013
T-2+HT-2	10.3 (29)	14.4-22.7	17.8	Gil-Serna et al., 2013
	40 (80)	0.0-53.4	5.2	Běláková et al., 2014
T-2	86 (36)	NA	34.0	Malachova et al., 2010
HT-2	100 (36)	NA	262.0	Malachova et al., 2010

Note: NA=not available; n=number of samples

DON and 2000 µg/kg for the sum of FB1 and FB2. It is important to notice that a high ratio of malting barley samples from the presented studies were contaminated with mycotoxins at levels that overcome maximum allowed concentrations (especially researches between 2002 and 2010). However, the more recent studies do show a tendency of decreasing contamination levels. Co-occurrence of DON and its derivatives with other *Fusarium* mycotoxins, such as FBs, NIV, ZEN, etc., is very frequent in cereals (Ruprich and Ostrý, 2008) and can result in the contamination of the processed cereal based products such as beer (Cole et al., 1983; Medina et al., 2005; Kostelanska et al., 2011). Although

mycotoxins produced by *Fusarium* are quite similar, their derivatives differ from the parental compounds in both physico-chemical properties and incorporation and distribution into the grain body. This explains the different and sometimes contradictory results obtained concerning mycotoxin concentration in barley samples.

1.2.1.2 Incidence of mycotoxins in beer

Many studies in beer have focused their investigation on DON, which is the most abundant mycotoxin and which represents the highest public health concern related to the consumption of beer (Yoshizawa & Morooka, 1973; Tanaka et al., 1988; Lancova et al., 2008; Kuzdraliński et al., 2013; Piacentini et al., 2015).There are thousands of beer brands in the world and, in order to find a place on the market, each producer aims to obtain its own one according to the demand of the consumer. However, two beer styles are defined worldwide with respect to the fermentation style: top-fermented beer or ale and bottom fermented beer, known as lager. Apart from different yeast strains used in the two aforementioned beer styles, other fermentative characteristics, such as secondary products formed and fermented sugars, define the particularities of these beers (Kunze, 2006).

Current European regulations on mycotoxin sets maximum levels in foodstuff for 13 compounds (EC 1881/2006; Commission Recommendation 2013/165/EU). However, beer is only subjected to maximum limits of FBs and only in the case maize based ingredients are used for its production (maximum 400 μg/kg for the sum of FB1 and FB2). Due to its high worldwide acceptance, beer may contribute to mycotoxins intake, particularly in the case of heavy consumers. Mycotoxin contamination may occur at different stages of brewing. Some mycotoxins can be transferred from cereals to malt and then to beer due to their high thermal stability (AFs, ZEN and DON) and water solubility of mycotoxins (DON and FBs) (Rodriguez-Carrasco et al., 2015). Whatever the origin, numerous surveys on the occurrence of mycotoxins in beer were conducted worldwide up to nowadays analyzing different styles of beer making (Table 2). Many surveys performed on beer are mycotoxin specific, searching for the occurrence and people's exposure to different *Fusarium* mycotoxins found in beer (Shim et al., 1997; Torres et al.,1998; Molto et al., 2000; Papadopoulou-Bouraoui et al., 2004; Bertuzzi et al., 2011; Rubert et al., 2013; Piacentini et al., 2015; Rodríguez-Carrasco et al., 2015; Piacentini et al., 2017).

Table 2: Occurrence of mycotoxins in beers of different commercial styles

Beer type	Toxin	Positive samples, % (n)	Mycotoxin concentration, μg/L		References
			Range	Mean*	
Non-alcoholic (<1% vol. alc.)	DON-3-Glc	NA[b]	0-3.1[f]	2.3	[a] Niessen et al., 1993 [b] Shim et al., 1997 [c] Mbugua & Gathumbi, 2004 [d] Zachariasova et al., 2008 [e] Roger, 2011 [f] Malachova et al., 2012 [g] Kuzdraliński et al., 2013 [h] Rubert et al., 2013 [i] Varga et al., 2013 [j] Piacentini et al., 2015 [k] Rodríguez-Carrasco et al., 2015 [l] Habler & Rychlik, 2016 [m] Piacentini et al., 2017
		47.4 (19)	2.0-6.6[i]	3.0	
		66.7 (3)	18.0-23.0[b]	20.5	
	DON	NA	0.0-3.7[f]	3.7	
		26.3 (19)	3.2-26.1[i]	8.7	
		NA	NA[k]	19.1	
Radler	DON-3-Glc	NA	0.0-5.5[f]	3.5	
		80 (25)	1.8-7.9[i]	3.8	
	DON	NA	0.0-6.4[f]	6.4	
		52 (25)	4.2-12.7[i]	6.9	
		NA	NA[k]	9.4	
Light beer (1.0 to 3.5% vol.)	NIV	100 (6)	3.3-38.0[b]	17.97	
	DON	NA	NA[k]	20.6	
	AcDONs	85.7 (7)	<5-27.6[d]	11.65	
Lager beer (Bottom-fermented)	FB1	72 (75)	0.0-0.78[c]	0.3	
		50 (14)	201.7-1568.62[m]	367.47	
	ZEN	100 (75)	4.3-107.0[e]	8.16	
	NIV	97 (36)	1.0-20.0[b]	4.05	
	DON-3-Glc	85.7 (7)	<2.5-25.8[d]	9.7	
	DON	28.5 (123)	0.0-478[a]	148	
		55.5 (18)	1.0-10.0[b]	3.1	
		100 (75)	1.56-6.4[c]	3.42	
		71.4 (7)	<5-35.9[d]	21.3	
		100 (46)	6.0-70.2[g]	20.01	
		72.4 (58)	<LOQ-42.0[k]	22.9	
		28.6 (14)	4.3-10.1[l]	7.1	
Ale beer (top-fermented)	OTA	10 (10)	3.2[h]	NA	
	DON	100 (17)	8.6-43.3[g]	25.21	
Craft beer	FB1	100 (8)	29.0-285.0[j]	105.0	
	DON	100 (17)	127-501[j]	221.0	
Sorghum beer	FB1	87.5 (120)	0.0-340.0[e]	180.0	
	DON	89.2 (120)	0.0-730.0[e]	485.0	
Pale beer	T-2	15.8 (19)	4.0-12.1[h]	NA	
	HT-2	15.8 (19)	15.1-20.0[h]	NA	
	FB2	36.8 (19)	71.0-87.0[h]	NA	
	FB1	36.8 (19)	71.2-118[h]	NA	
	OTA	21 (19)	2.7-6.9[h]	NA	
	DON-3-Glc	NA	0.0-19.0[f]	8.3	
		65.4 (217)	3.6-81.3[i]	9.3	
	DON	NA	0.0-30[f]	13	
		100 (55)	6.0-70.2[g]	18.3	
		54.4 (217)	5.4-89.3[i]	12.0	

Beer type	Toxin	Positive samples, % (n)	Mycotoxin concentration, µg/L		References
			Range	Mean*	
Dark beer	DON-3-Glc	NA	0.0-16.0[f]	9.6	[i] Varga et al., 2013
		59.6 (47)	4.2-26.2[i]	10.7	[j] Piacentini et al., 2015
	DON	NA	0.0-11.0[f]	11	[k] Rodríguez-Carrasco et al., 2015
		100 (12)	14.3-52.9[g]	28.3	
		29.8 (47)	11.1-45.0[i]	22.4	
		NA	<LOQ-32.8[k]	23.6	
Wheat beer	ENNB	60 (5)	0.01-0.24[l]	0.16	[l] Habler & Rychlik, 2016
	HT-2	56 (25)	<LOQ-38.2[k]	30.9	
	AcDONs	50 (6)	5.1-22.8[d]	11.7	
	DON-3-Glc	100 (6)	6.3-21.0[d]	13.1	
		NA	0.0-15.0[f]	8.6	
		69.6 (46)	13.5-28.4[i]	11.5	
	DON	74.6 (67)	0.0-569.0[a]	245	
		100 (6)	9.0-31.4[d]	24.8	
		NA	0.0-27[f]	14	
		78.3 (46)	5.2-49.6[i]	18.4	
		76 (25)	<LOQ-47.7[k]	34.0	
		40 (5)	3.6-5.8[l]	4.7	

Notes: ENN B: enniantin B; AcDONs: 3- and 15-acetyl-deoxynivalenol; DON-3-G: deoxynivalenol-3-glycoside; * mean from contaminated samples; NA=not available; LOQ= limit of quantification; n= number of samples

Others are beer style specific, regrouping the beer samples according to the malting barley style. Niessen et al. (1993) have found wheat beer containing higher levels of DON and its derivatives compared to barley beer. This can be explained by existing matrix differences between wheat and barley which determine crops mycobiota. Taking into account that different beer styles imply a slightly different physical treatment and substrate composition, Malachova et al. (2012) developed a matrix specific LC-MS/MS method (mycotoxin extraction protocol adjusted to the type of beer) to evaluate the levels of DON and its conjugates in beer samples purchased in Austria, reporting an average concentration of 6.6 µg/L for DON and DON-3-Glc, which does not overcome regulated limits. Varga et al. (2013), focusing their research on different beer styles (374 samples) from 38 countries, have identified that the lowest contamination level of DON and DON-3-Glc was observed in non-alcoholic (2.7 and 1.5 µg/L respectively) and in radler beers (4.4 and 3.2 µg/L respectively), reaching the same conclusion as Kostelanska et al. (2009), but could not prove it as the information concerning raw materials was not available. From the data presented in Table 2, it can be seen that T-2 and HT-2 toxins concentration in pale and wheat beers are near or overcoming the limits recommended by the European

Commission (Commission Recommendation 2013/165/EU) (Rodríguez-Carrasco et al., 2015; Rubert et al., 2013). One of the recent studies performed by Piacentini et al. (2017) have identified very high levels of FB1 in lager beer (four times overpassing the maximum allowed concentration).

DON was firstly isolated in Japan (1972) from moldy barley. In 1973, Yoshizawa and Morooka published an article about the finding of a new mycotoxin, deoxynivalenol monoacetate, found in barley contaminated with *Fusarium roseum*. Consequently, considering a possible carry over of the toxin and its conjugate, many surveys on the occurrence of DON and its derivatives in beer were performed in different countries such as Germany, with detected levels between 172 and 569 μg/L (Niessen et al., 1993); Canada, where more than 50% of beer samples contained up to 50 μg/L of DON (Scott, 1996); Argentina, with a range of 5 to 221 μg/L (Molto et al., 2000); Czech Republic, with 10.9 μg/L and 9.2 μg/L of DON and DON-3-Glc, respectively found (Kostelanska et al., 2009; Benešová et al., 2012); Poland where DON and ZEN concentrations were about 7.5 70.2 μg/L and <0.26 0.36 μg/L, respectively (A. Kuzdraliński, Solarska, and Muszyńska, 2013); Brazil, with levels from 127 to 501 μg/L of DON and from 29 to 285 μg/L of ZEN were found (Piacentini et al., 2015). On a larger regional scale, regrouping several European countries, a contamination range between 4 and 56.7 μg/L of DON was found (Papadopoulou-Bouraoui, et al., 2004).

Bertuzzi et al. (2011) have studied the occurrence of OTA, trichothecenes, FBs and AFs in beer produced in several European countries. In this study aflatoxins were not found in any of the analyzed samples which was confirmed by another study analyzing 117 beer samples (no information was given concerning beer production style) one year later performed by Benešová et al. (2012). However, detectable amounts of other mycotoxins were identified in the majority of the samples (mean levels of 2.1 μg/L for DON, 5.8 μg/L and 0.6 μg/L for FB1 and FB2 respectively and 0.019 μg/L for ochratoxin A) with small differences observed between the countries concerned with the study.

Considering the aforementioned information, researchers are continuously working on the elaboration of fast and reliable methods for mycotoxin identification in both raw materials (such as cereals) and final products as well as preventive and corrective measures in the food and feed chain, but the best measure to avoid mycotoxin accumulation is still

prevention of molds growth in raw materials.

1.2.1.3 Malting

The aim of obtaining a homogeneous quality of the final product within different batches and different harvesting years makes the production process quite challenging. Barley reception and malting are the first decisive steps in beer fabrication. The use of barley in beer production is explained by its high starch content and the good adherence of the husks to the grain body even after malting and milling. Various parameters of barley and malt are to be considered. At the arrival of barley to the brewery, it is first of all submitted to a process of cleaning (to eliminate the present physical contaminants) and sorting (to ensure a maximum of grains size and shape homogeneity) (Kunze, 2006).

Malting is a controlled germination process to produce the malt. It consists of three stages: steeping, germination and kilning. Steeping is a process initiated under specific conditions of temperature and humidity (controlled cycles of water spraying or immersion and aeration until grain water content reaches 42 – 48%). The humidity of the barley after steeping is determined by the type of malt that is aimed to be obtained (42 – 44% for Pilsner and 47 – 48% for dark beers). Steeping purpose is to create favorable humidity conditions inside the grain and activate the enzymes involved in germination. It generally takes place at 10 – 15 °C where, after approximately 30 – 50 hours, the water enters the kernel and first signs of germination appear (Kunze, 2006). Water flow during respective treatment cycle may lead to a spread of fungal contamination into the batch by 15 – 90% (Vegi et al., 2011). Also, as barley steeping implies a treatment of the grain with a quite high amount of water, this process may have an impact on the level of water soluble mycotoxins, such as DON and FBs, by eluting them from the matrix (Schwarz et al., 1995). Lancova et al. (2008) proved a decrease of DON concentration up to 10% compared to the initial content. In addition to that, Maul et al. (2012) found that thanks to the plant cell structure, first 17h of steeping and germination later induced the glycosylation of DON to DON-3-Glc, which explained the decrease in DON concentration (in the favor of its glycosylated metabolite). Oliveira et al. (2012) proved an augmentation in fungal infestation after 48h of steeping (76%) and a 75% increase in DON concentration, which was not detectable after kilning. Vaclavikova et al. (2013), on the contrary, found that after two days of steeping the decrease

in DON concentration occurred, showing up to 30% decline in the final malt.

Germination implies the activation of all enzymatic equipment for the breakdown of reserves of starch and proteins. It starts few hours after water penetration into the grain during steeping and begins with the transport of gibberellic acid (growth promoter) to the aleurone layer where enzyme production and activation take place (Oliveira et al., 2012). The following enzymes are synthetized: amylases and dextrinases, cytolytic enzymes, proteolytic enzymes, lipases, lipoxygenases and phosphatases (Kunze, 2006). The germination temperature, likewise steeping final humidity, is determined by the type of malt that the brewer wants to obtain (17 18°C for Pilsner malt and 23 25°C in the case of dark malt). The main role of malting is expressed in enriching the malt with enzymes and the formation of flavor and aroma compounds. The germination is stopped by kilning (drying) and/or roasting in order to prevent future structural changes of the barley. Fungal biomass may be growing during barley germination (increase of fungal infection up to 39.3% at the end of germination) probably as a result of cross contamination from the residual steeping water or because of a latent barley grain infection, which may be activated with the increase of humidity during this production step (Vegi, Schwarz, and Wolf-Hall, 2011). Fungal infection of barley drastically influence its germinative energy (decrease of germinative energy up to 45%), increase grain water sensitivity and promote DON accumulation up to 199 µg/kg (Oliveira et al., 2012). According to Maul et al. (2012), the possible reason to DON glycosylation during germination is related to the increase in glucose content which might activate the enzyme responsible for the respective reaction and DON transformation into DON-3-Glc (approximately 50% of DON is converted after 5 days of germination).

The kilning prepares the malt for storage and transportation, if needed. It usually takes place at several temperature scales: <50°C until the water humidity of grains reaches 10 12% and then the temperature is gradually increased until 80 90°C. The temperatures chosen are aiming to reduce at a minimum level the degradation of the enzymes (Kunze, 2006). The intensity of kilning and roasting (if applied) is crucial in malt flavor and color formation (Pires & Branyik, 2015). However, the early stages of kilning may promote fungal growth and mycotoxin accumulation by some *Fusarium* strains (Wolf-Hall, 2007). Kostelanska et al. (2011) found that DON may be degraded during malt roasting at 150°C

leading to the formation of de-epoxidized compounds. Also, the enzymatic hydrolytic activity is leading to the increase of DON concentration in the product due to its release from the matrix (Vegi, Schwarz, and Wolf-Hall, 2011).

An increased attention is paid to the transfer of trichothecenes (HT-2 toxin, T-2 toxin, DON and its derivatives) from barley to malt but quite little information is available concerning the fate of AFs and ZEN during malting process which is probably due to their lower occurrence in barley and probably a higher weather dependence (Rodríguez-Carrasco et al., 2015). Weather, fungicide treatment and barley variety look to be the main factors influencing fungal invasion and mycotoxin synthesis in malting barley. Fungicide treated barley showed a 80% decrease in DON concentration after malting (Malachova et al., 2012). Barley rootlets removed at the end of malting were found to contain from 564 to 1383 µg/kg of FB1 (highest rate compare to other brewing intermediates) (Cavaglieri et al., 2009).

1.2.1.4 Milling

Milling of the malt and other grain aims to increase the contact surface between the brewing liquor and malt. Usually, roller and hammer mills are used to obtain the best results because this way the husks are almost intact, which barrier the extraction of tannins and other undesirable compounds (Lewis & Young, 1995). The finer the particles, better the breakdown of malt into fermentable materials such as sugars and assimilable nitrogen compounds while mashing. However, too small particle size may have a negative impact by decreasing filtration yields and increasing wort turbidity (Kunze, 2006). Some researchers have found that the efficiency of milling is not only expressed in the size of final granules, but it should be evaluated together with mashing temperature levels, because starch α-amylase activity depends on both granules size and treatment temperature (Mousia et al., 2004). No direct impact on mycotoxin levels at this stage occurs but probably milling would promote mycotoxin homogeneous spreading into all malt batch and its later solubilization into mashing water.

1.2.1.5 Mashing

Mashing is the mixing of milled malt and a large amount of water (approximately 17 kg of malt are needed for 1 hL of beer) under specific temperatures to activate all the enzymatic

equipment present (inactivated during kilning) and to allow the conversion of starches into fermentable sugars. Two types of enzymes are mainly present: ones acting on sugars and others acting on proteins. The physical conditions applied during mashing are aiming to maximize the efficiency of the enzymes according to their different optimal temperatures (Tse et al., 2003). Four temperature scales are, usually, hold for some time in order to allow the following changes: 45 to 50°C for β-glucans and protein hydrolyzation, 62 to 65°C for maltose production, 70 to 75°C for saccharification and 75 to 78°C for α-amylases activation and finishing of mashing (Briggs et al., 2004; Kunze, 2006). An alternative mashing process exists called "decoction mashing", where different temperatures are achieved by removing repetitively a part of the mash, boiling it and mixing it back (Pires & Branyik, 2015). During this step, it is important to control all possible parameters, starting with temperature and heating time and following with pH (optimal being pH=5.2), oxygenation level and stirring speed.

An infection of the malting barley with *Fusarium* genera (Fusarium Head Blight disease) may lead to a further protein digestion realized by fungal proteases which will affect beer color, flavor, texture and foaming characteristics (e. g. beer gushing related to DON presence) (Wolf-Hall, 2007; Inoue et al., 2013). Concerning the influence on mycotoxin levels, at this production step, there is a possible release of DON or its conjugates from protein structures (due to physico-chemical and biochemical conditions: T=40°C and/or enzymatic changes) and, as a result, increase in total DON concentration (Wolf-Hall, 2007), but according to another study performed later by Kostelanska et al. (2011) the key factor in DON levels in the final product still remain the initial barley contamination as, probably, these conjugated toxins are present in malt but are not extractable using common procedures used for their analysis. Inoue et al. (2013) have identified an almost 20% reduction in all analyzed mycotoxin levels (14 analyzed mycotoxins) which was mainly due to their elimination with the spent grains. Similar is the case of enniatins (Vaclavikova et al., 2013), where 64 to 91% of the initial charge was removed with spent grains, and of ZEN (more than 60% was quantified in the spent grains) (Wolf-Hall, 2007). A particular attention has to be given to unmalted ingredients added at this step, especially the ones coming from maize, which is known to be an important source of AFs, FBs and DON (Torres et al., 1998; Benešová et al., 2012). However, no other references were found

concerning this aspect of mashing.

1.2.1.6 Wort separation and boiling

Wort separation and boiling is performed after the separation of the solid particles, and at this step hops are added. The wort can also be enriched by adding sugars and syrups but also "seasoning" with coriander seeds, orange peel etc. (Pires & Branyik, 2015). The hop boiling typically lasts for 45 – 60 min or more. The process varies as a function of the hops used, hoping rate, boiling time, the moment the hopes are introduced (at the beginning, in the middle or at the end of the boiling process) (Briggs et al., 2004). The main processes taking place during wort boiling are: enzyme inactivation, evaporation of water and volatile compounds (mainly represented by dimethyl sulfides, undesirable in the final product), proteins precipitation, sterilization, isomerization of hop α-acids, Maillard reactions and thus flavor modulation (Briggs, et al., 2004). The wort after all is cooled down, filtered and loaded into fermentation tanks.

Concerning the impact of wort boiling on mycotoxin content, the ingredients added might represent a source of mycotoxins if not controlled (Inoue et al., 2013) but the study performed by Kostelanska et al. (2009) did not identify them to be of a significant importance. Hops are reported to be susceptible to fungal contamination but as the amount added to the wort is low, their impact may be discarded (Vaclavikova et al., 2013). The effect of temperature and beer filtration on mycotoxin levels probably has not to be totally discarded. Taking into account that wort boiling temperature is above 100°C and that the average boiling time is about one hour, a decrease of mycotoxins concentration may occur. Also, filtration residues may contain a certain amount of mycotoxins. However, no studies exist on this particular aspect.

1.2.1.7 Alcoholic fermentation

Fermentation of wort is a process initiated by yeasts of *Saccharomyces* genus. Different yeast strains are used according to the type of beer. Two most common technologies are known: ale or top fermentation performed by *Saccharomyces cerevisiae,* and lager or bottom fermentation leaded by *Saccharomyces pastorianus*. The yeasts will transform sugars into alcohol and carbon dioxide but also a range of secondary compounds such as esters, higher alcohols, volatile compounds etc. The initial yeast concentration at inoculation must be

10^7 cells/mL. The metabolic activity of yeasts is possible at a temperature range of 2 to 30°C. Usually, the fermentation temperature is 18 – 25°C for ale beers and 7 – 15°C for lager beers during 7 – 9 days (Lewis & Young, 1995).

A recently performed study has investigated the adsorption of mycotoxins on beer fermentation residue (BFR), very high ratio of adsorption being observed in the case of ZEN (75.1%) but also for AFB1 (48.1%) and OTA (59.4%) (Campagnollo et al., 2015). The reduction of DON did attain only 11.6%. According to the authors and other previously published researches (Jouany et al., 2005), the adsorption is due to the binding of the toxins (especially ZEN) to β-glucans from yeast cell wall (hydrogen and Van der Waals bonds being involved together with the proportion of β-1,3-D-glucans and β-1,6-D-glucans in the product). Barley is also known as containing a quite high β-glucan content (2.5 to 3.5%). However, by the fifth day of germination, almost 95% of it is broken down losing its binding properties (Agu and Palmer, 2001).

The effect of mycotoxin contaminated raw material on alcoholic fermentation volatile by-products was studied (Kłosowski et al., 2010). It was found that some of the mycotoxins (mainly AFB1 and DON) may inhibit the activity of alcohol dehydrogenase, which is in accordance with Reiss (1973), and results in the decrease in carbon dioxide production. It also implies an increase in acetaldehyde concentration and other undesirable volatile compounds synthetized during alcoholic fermentation but no effect on total ester content was identified (Kłosowski & Mikulski, 2010). However, in a recently performed study by Nathanail et al. (2016), the presence of mycotoxins in wort, even at high concentration (10,000 µg/L), did not influence fermentation parameters, such as alcohol production, pH, sugar utilization and cell viability. The study did not find any impact of different mycotoxin combinations on yeasts' activity and a concentration of 10,000 µg/L of DON was needed to obtain a significant reduction of cell viability. The differences between the two studies concerned with the impact of mycotoxins on yeast activity may be explained by different yeast strains used which can possess a different resistance to mycotoxin action.

1.2.1.8 Maturation and stabilization

Maturation and conditioning are aiming to improve and stabilize the beer taste after fermentation (CO_2 elimination and removal of some undesirable volatile compounds).

During this step, other processes take place such as beer clarification, yeasts sedimentation and flavor formation of the final product. The process of maturation usually takes from 1 to 3 months and involves lowering of the temperature (cold break) to around 0°C. A secondary fermentation is often practiced ($2{\cdot}10^6$ cells/ml) and addition of priming sugars is acceptable.

During conditioning stage, protein and tannins combination takes place (sedimentation of high mass molecules) resulting in beer clarification. Proteins may be also removed by adding enzymes, introducing additional tannins or adsorption on surface (nylon membranes, silica gels etc.) (Lewis & Young, 1995). The clarification process can be accelerated by filtration or centrifugation (also at low temperatures, 0 to -1°C). Yeasts are removed from the beer volume by filtration and the product is transferred to aging tanks for more prolonged storage. The next steps in beer production are aiming to stabilize physically (colloidal stabilization) and microbiologically (filtration and pasteurization) the product before its packaging.

Related to the impact of the stabilization on mycotoxin levels in beer, the use of inorganic adsorbents for clarification is doubted to be involved in mycotoxin removal from the product. The adsorption on the surface is a function of mycotoxin polarity, water solubility, molecule's size, etc. Belajová et al. (2007) have found that -Cl and -CN modified silica gel were very effective in bounding the OTA and the FBs. A dosage of 2.5 6.5 g/L has a good adsorption potential and does not alter beer organoleptic properties. However, there are no studies regarding the impact of inorganic adsorbents in the case of multi-mycotoxin contamination of beer which would take into account possible interferences.

1.2.2 Deoxynivalenol in cereal-based baby food production process

Infants and young children´s diet is mainly restricted to cereal-based food. European Commission defines processed cereal baby-food and baby foods as food intended for use by infants (<12 months) when they are weaned and young children (from 1 to 3 years) as a supplement to their diet and/or for their progressive adaptation to ordinary food (European Commission, 2006a). Several recent surveys prove that the mycotoxin problem is also an issue in this product category (Juan et al., 2014; Pereira, Fernandes & Cunha, 2015; Ul Hassan et al., 2018). Considering the increased vulnerability to contaminants of

this group of population, the European Commission establishes the strictest maximum levels of DON and other mycotoxins allowed in food intended for infants and young children (200 µg/kg), which is 2.5 fold lower than the maximum level in other cereal-based foodstuff and 8.75 times less than the maximum level of DON allowed in unprocessed oats, wheat and maize intended for human consumption (European Commission, 2006b). Although DON is one of the least lethal trichothecenes, its effects in children in the case of an acute exposure (abdominal distress, diarrhea, increase salivation, general malaise) occur within hours after the intake (Raiola et al., 2015).

The production of cereal-based baby foods and food for young children implies the following main process operations: flour roasting, pre-gelatinization, enzymatic hydrolysis, enzymes inactivation, drying and packaging (Figure 3). The objective is to obtain a food product adapted to the particularities of the digestive system of an infant or young child, which has a limited ability to digest starch. The most frequently used cereals are oats, barley, wheat, rice, rye, maize and, on a lower extent, millet, sorghum, triticale and spelt. Also, adjuncts might be added, namely barley or rye malt extracts, maize extracts, quinoa flour, fructo-oligosaccharides (FOS), vitamins and minerals etc., in order to complete or enrich product's nutritional value.

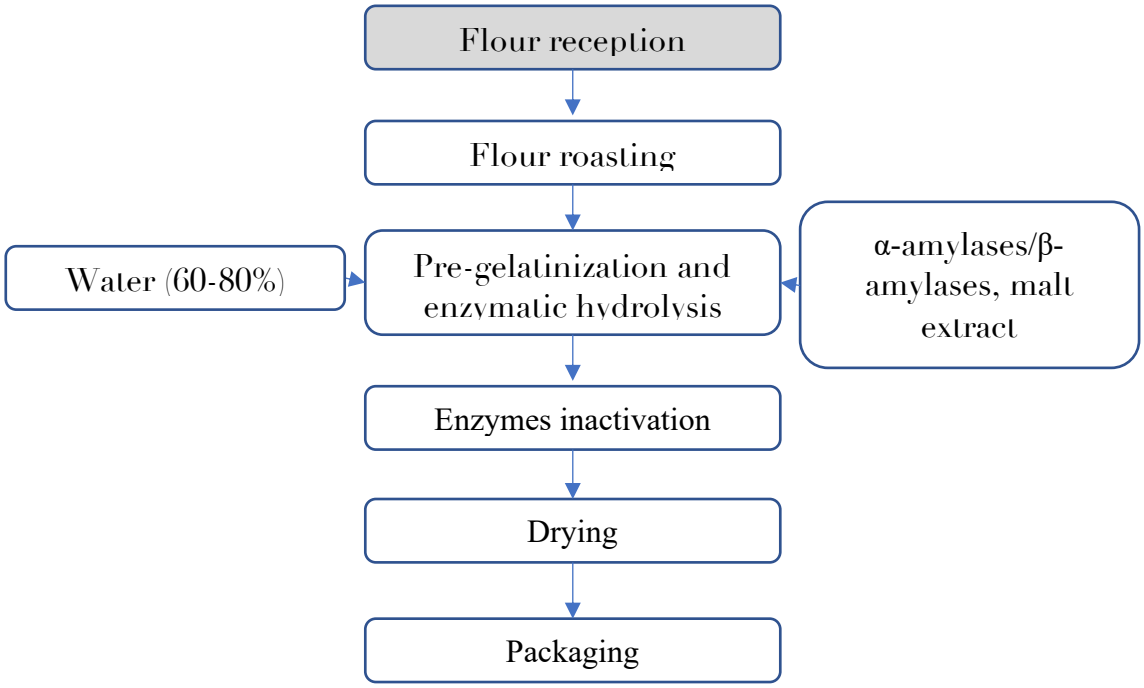

Figure 3: Cereal-based baby food production process

1.2.2.1 DON occurrence in cereals intended for food production

A market research on cereal-based baby foods available in the EU showed that the mainly used commodities in this sector are oats, wheat and barley followed by maize, rye and triticale and, on a lower extent, millet, sorghum and spelt. Very often, these come in formulation of three and more cereals (up to eight cereals), in order to provide a more complex spectrum of nutrients needed to ensure a correct infant growth. Table 3 regroups the available information on the occurrence of DON in cereals intended for food production worldwide.

Usually the studies on mycotoxins incidence in commodities are not only focused on a group of cereals from a country or region but also on more than one mycotoxin, yet their co-occurrence was frequently proved (De Boevre et al., 2012; Bryła et al., 2016; Pleadin et al., 2017; Vanheule et al., 2014). In this section, the occurrence of DON and its metabolites, over the last 25 years, was reviewed in the cereal species which are mainly used in baby food industry. There are very few studies searching for DON metabolites in cereals. The studied metabolites are the ones which are either produced by the mold itself (3-Ac-DON and 15-Ac-DON) or the ones which are a result from plants activity against the contamination (DON-3-Glc) (De Boevre et al., 2012; Bryła et al., 2016; Rasmussen et al., 2012; Uhlig et al., 2013; Vanheule et al., 2014).

One of the largest review on DON occurrence in cereals, after Tanaka et al. (1988b), was performed by Placinta et al. (1999), regrouping the available information on DON contamination in oats, wheat, barley, maize and rye from 15 countries over a decade. Its concentration ranged from 4 to 62050 µg/kg, the highest levels being reported in oats from Norway in 1996's harvest year.

According to a report published by Eurostat (2017), France is the main producer of cereals in the EU, accounting for almost a fifth of total EU production in 2016 (18%), followed by Germany (15.1%), Poland (9.9%) and Spain (8%). However, very limited studies are available on DON occurrence in France (Tangni et al., 2013), wheat presenting the highest DON incidence (145 positive samples from 169 analyzed samples) with a maximum of 3280 µg/kg.

Table 3: Occurrence of deoxynivalenol intended for food production in cereals worldwide

Cereal	Country/region	Positive/total samples analyzed	Concentration (µg/kg)		Reference
			Mean	Range	
Oats	Belgium	1/6	46	-	(De Boevre et al., 2012)
		9/11	810.1	62-2216	(Rasmussen et al., 2012)
	Canada	34/73	330	<100-1200	(Campbell et al., 2000)
		63/82	190	-	(Clear, Patrick & Gaba, 2000)
		6/10	42.5	22-71	(Martos, Thompson & Diaz, 2010)
		4/4	700	200-1200	(Tamburic-Ilincic, 2010)
	Croatia	7/33	145	34-201	(Pleadin et al., 2013)
		8/13	O: 119	O: 32-377	(Pleadin et al., 2017)
		8/14	C: 207	C: 33-546	
	Denmark	-/22	44	-	(Nielsen et al., 2011)
	Finland	ND	-	1300-2600	(Placinta, D'Mello & MacDonald, 1999)
		35/51	154.8	25-896	(Hietaniemi et al., 2004)
		-/470	237.4	Max:8800	(Lindblad et al., 2012)
		129/137	618	0-9298	(Hietaniemi et al., 2016)
	Hungary	8/29	272	222-359	(Tima et al., 2016)
		ND	-	7200-62500	(Placinta, D'Mello & MacDonald, 1999)
	Norway	-/101	O: 111	O: <20-447	(Bernhoft et al., 2010)
			C: 404	C:<20-2056	
		-/171	476.4	Max: 22000	(Lindblad et al., 2012)
		28/28	M: 2070	Max: 7230	(Uhlig et al., 2013)
		260/289	-	50-30000	(Hofgaard et al., 2016)
	Poland	6/6	28	1-48	(Krysinska-Traczyk, Perkowski, and Dutkiewicz, 2007)
		34/35	O: 636	O:250-1700	(Adam Kuzdraliński, Solarska & Mazurkiewicz, 2013)
		14/18	C: 697	C:220-2150	
		7/34	O: 33.1	O: 5-107	(Twaruzek et al., 2013)
		20/24	C: 28.7	C: 5-189	
	Russia	4/4	113	67-149	(Bryła et al., 2016)
		2/8	92	90-94	(Tutelyan et al., 2013)
	Scandinavia	4/4	628	-	(Scudamore et al., 2007)
	Sweden	-/33	199.5	Max: 2340	(Lindblad et al., 2012)
		93/93	M: 172	Max: 5544	(Lindblad et al., 2013)
	UK and Ireland	8/8	13	-	(Scudamore et al., 2007)
	UK	147/458	11	0-282	(Edwards, 2009)

Cereal	Country/region	Positive/total samples analyzed	Concentration (μg/kg)		Reference
			Mean	Range	
Wheat	Argentina	ND	-	100-9250	(Placinta et al., 1999)
	Belgium	4/6	58.5	16-150	(De Boevre et al., 2012)
		6/6	910.5	46-2638	(Rasmussen et al., 2012)
		81/93	1053	-	(Vanheule et al., 2014)
	Brazil	ND	-	470-590	(Placinta, D'Mello & MacDonald, 1999)
	Bulgaria	ND	-	<1800	
	Canada	ND	-	10-10500	(Placinta, D'Mello & MacDonald, 1999)
		40/40	2732	54-8792	(Martos, Thompson & Diaz, 2010)
	Croatia	O: 14/25 C: 16/27	O: 256 C: 252	O: 62.4-678 C: 27-1220	(Pleadin et al., 2017)
	Finland	28/61	420	Max: 2224	(Hietaniemi et al., 2016)
	Germany	ND	-	4-20500	(Placinta, D'Mello & MacDonald, 1999)
	Hungary	2/2	383	<1400	(Uhlig et al., 2013)
		21/29	478	230-1880	(Tima et al., 2016)
	Japan	ND	-	30-1280	(Placinta, D'Mello & MacDonald, 1999)
	Netherlands	ND	-	20-231	(Placinta, D'Mello & MacDonald, 1999)
	Norway	ND	-	450-4300	(Placinta, D'Mello & MacDonald, 1999)
		92/92	O: 91.7 C: 180	O: <20-358 C: <20-797	(Bernhoft et al., 2010)
		163/178	-	Max: 16000	(Hofgaard et al., 2016)
	Poland	ND	-	2000-40000	(Placinta, D'Mello & MacDonald, 1999)
		45/45	770.7	82-2975	(Bryla et al., 2016)
	USA	ND	-	<9300	(Placinta, D'Mello & MacDonald, 1999)
Barley	Belgium	64/65	2029	-	(Vanheule et al., 2014)
	Canada	84/116	1370	Max: 9110	(Campbell et al., 2000)
		20/20	816.4	78-2449	(Martos, Thompson & Diaz, 2010)
	Croatia	18/34	342	74-228	(Pleadin et al., 2013)
		O: 6/11 C: 8/13	O: 71.8 C: 140	O: 32.3-157 C: 42.6-389	(Pleadin et al., 2017)
	Finland	59/86	308.8	Max: 4752	(Hietaniemi et al., 2016)
	Hungary	14/29	339	240-429	(Tima et al., 2016)
	Korea	ND	-	5-361	(Placinta, D'Mello & MacDonald, 1999)

Cereal	Country/region	Positive/total samples analyzed	Concentration (µg/kg)		Reference
			Mean	Range	
Oat	Netherlands	ND	-	4-152	(Placinta, D'Mello & MacDonald, 1999)
	Norway	ND	-	2200-13300	(Placinta, D'Mello, and MacDonald, 1999)
		108	O: 43.7 C: 44	O: <20-154 C: <20-207	(Bernhoft et al., 2010)
		20/20	M: 150	Max: 636	(Uhlig et al., 2013)
	Poland	5/5	22	Max: 40	(Krysinska-Traczyk et al., 2007)
		20/24	370	54-1602	(Bryła et al., 2016)
	Russia	10/214	370.2	60-1280	(Tutelyan et al., 2013)
	USA	ND	-	<500-26000	(Placinta, D'Mello & MacDonald, 1999)
Maize	Belgium	6/6	2036	411-5245	(De Boevre et al., 2012)
	Canada	ND	-	20-4090	(Placinta et al., 1999)
		14/15	1513.5	574-4865	(Martos et al., 2010)
	China	ND	-	490-3100	(Placinta et al., 1999)
	Croatia	45/63	1565	215-1942	(Pleadin et al., 2013)
		O: 31/33 C: 30/37	O: 564 C: 350	O: 35-2260 C: 28-1430	(Pleadin et al., 2017)
	Hungary	25/29	1872	225-2963	(Tima et al., 2016)
	New Zeeland	ND	-	3400-8500	(Placinta, D'Mello & MacDonald, 1999)
	Poland	1/2	180	180	(Krysinska-Traczyk et al., 2007)
	Russia	1/53	70	-	(Tutelyan et al., 2013)
	South Africa	ND	-	Max: 1830	(Placinta, D'Mello & MacDonald, 1999)
Rye	Canada	15/15	269.8	87-500	(Martos, Thompson & Diaz, 2010)
	Croatia	O: 4/7 C: 2/9	O: 68.6 C: 35.4	O: 34-113 C: 31-40.2	(Pleadin et al., 2017)
	Denmark	17/17	56	-	(Nielsen et al., 2011)
	Finland	38/43	-	5-100	(Eskola, Parikka & Rizzo, 2001)
		2/13	25	12-40	(Hietaniemi et al., 2016)
	Netherlands	ND	-	8-384	(Placinta, D'Mello & MacDonald, 1999)
	Poland	5/5	19	-	(Krysinska-Traczyk et al., 2007)
	Russia	1/63	60	-	(Tutelyan et al., 2013)
Triticale	Denmark	4/5	306	43-737	(Rasmussen et al., 2012)
	Poland	15/20	573	196-1326	(Bryła et al., 2016)

O=organic; C=conventional, M=median; ND= not defined

FHB infection and DON contamination is a function of several factors such as climatic factors, crop rotation, fungicide application, debris management and tillage practices applied, variety resistance to FHB, etc. (Blandino et al., 2012). All the above mentioned explains the differences in DON contamination among countries, different cereal varieties and among years. In Norway, Finland and Sweden, for example, important spring rainfalls and relatively warm temperatures during this season would promote the propagation of both *F. graminearum* and *F. culmorum* and mycotoxin formation on cereal crops (Hope, Aldred & Magan, 2005). This explains the high DON accumulation showed in the published studies: maximum 9298 µg/kg of DON in oats in Finland (Hietaniemi et al., 2016), up to 2340 and 22000 µg/kg in oats from Norway and Sweden, respectively (Lindblad, Börjesson, Hietaniemi & Elen, 2012); 2240 and 16000 µg/kg of DON in wheat in Finland and Norway, respectively (Hietaniemi et al., 2016; Hofgaard et al., 2016); 636 and 4752 µg/kg of DON in barley from Norway and Finland, respectively (Hietaniemi et al., 2016; Uhlig et al., 2013). Nonetheless, no such important DON accumulation was found in other crops such as maize, rye and triticale (Table 3), probably because of the differences in fungal ecology characteristics of these crops (Doohan, Brennan, and Cooke, 2003).

Moreover, differences in DON occurrence and accumulation were observed between organic and conventional agricultural systems. Nevertheless, from the available literature, it results that DON accumulation is crop dependent too: in oats, maize and rye from organic agriculture DON concentration is higher compared to the conventional system but it is the opposite in the case of wheat and barley (Bernhoft et al., 2010; Pleadin et al., 2017).

Many of the works listed in the Table 3 were focused on more than one mycotoxin, however all of them found DON as the most prevalent product of fungal activity (in almost all the studies over various years DON positive samples were found in more than 50% of the total analyzed cereal samples). Studies on DON-3-Glc occurrence in cereals and its fate during food and feed processing are quite recent (Berthiller et al., 2007). Moreover, the concern about DON-3-Glc toxic effects on human and animal health is increasing continuously, which made researchers worldwide to study its occurrence (De Boevre, Di Mavungu, Landschoot, et al., 2012; Bryła et al., 2016; Rasmussen et al., 2012; Uhlig et al., 2013; Vanheule et al., 2014) and synthesis pathways in cereals (Berthiller et al., 2007). The levels of DON-3-Glc found are usually lower or similar to DON: a mean of 18 and 51.2

µg/kg of DON-3-Glc was found in wheat and oats from Belgium, respectively (De Boevre, Mavungu, et al., 2012); 252, 56 and 68 µg/kg in oats, wheat and barley, respectively, from Hungary (Uhlig et al., 2013); 99, 102 and 138 µg/kg of DON-3-Glc in wheat, barley and triticale, respectively, from Poland (Bryła et al., 2016). Two years after the study performed by De Boevre et al. (2012), 250 and 390 µg/kg of DON-3-Glc in wheat and barley from the same region in Belgium (Flanders) were found (Vanheule et al., 2014). Interestingly, during these two years a considerable increase in both average rainfall and average temperature was registered (Statistica, 2018), which would explain the higher amount of both DON and DON-3-Glc accumulated (Table 3).

Considering that oats and wheat are the primarily used cereals in baby food production and that these two commodities, together with barley, are the most susceptible for DON contamination, a transfer to the final product may take place, especially in the case of a high contamination level, which would expose these consumers to an important health risk.

1.2.2.2 DON occurrence in cereal-based infant food and food for young children

In Europe, DON maximum allowed levels were applied in baby food, for the first time, in Soviet Ukraine in 1984 (FAO, 2004; Ministry of Health of the Union of Soviet Socialist Republics, 1989). The first EU harmonized regulation on mycotoxins in foodstuffs came in 1998, which included several limits for aflatoxins, also containing sampling and analytical methods procedures (European Commission, 1998). Only with the European Regulation 1881/2006/EC maximum allowed limits for *Fusarium* toxins in foods were legislated (European Commission, 2006b), with its updates and amendments on the following years.

There is a limited number of studies available on the occurrence of DON in cereal-based baby food, even considering that the EU regulation of DON in this product category is particularly strict (maximum allowed DON level is 200 µg/kg). Nowadays, available reliable methods for DON analysis using liquid chromatography coupled with diode array detectors (DAD) have relatively high limits of detection and quantification (usually around 100 µg/L) (Sugita-Konishi et al., 2006), and more sensitive techniques are necessary such as mass spectrometry to increase the accuracy of the analysis, particularly for this product

category (Habler and Rychlik, 2016; Lindblad et al., 2013; Malachova et al., 2012). Table 4 regroups the available published surveys on DON occurrence in infant foodstuffs. Maize, wheat, barley and oats are the cereals most susceptible for *Fusarium* infestation and DON accumulation in the commodity and the final product (EFSA, 2013).

Table 4: Occurrence of deoxynivalenol in cereal-based infant food and food for young children

Food for babies and young children	**Country**	**DON, µg/kg Mean (+ve/total samples)**	**Range**	**Reference**
Cereal-based food	Germany	61(15/25)	15-314	(Schollenberger et al., 1999)
Biscuits and pasta	Italy	35 (7/12)	7-166	(Cirillo et al., 2003)
Oat based cereals		52 (33/53)	Max: 90	
Barley based cereals		260 (29/50)	Max: 980	
Soy based cereals	Canada	116 (8/8)	Max: 240	(Lombaert et al., 2003)
Rice based		(0/9)	-	
Multigrain cereals		116 (62/86)	Max: 400	
Cereal-based food	Italy	(16/44)	ND	(Romagnoli, Ferrari & Bergamini, 2010)
Barley based cereals		63.5 (10/11)		
Oat based cereals	USA	13 (8/18)	ND	(Dombrink-Kurtzman, Poling & Kendra, 2010)
Mixed cereals		35.1 (15/23)		
Cereal-based food	Spain	131 (12/30)	Max: 286	(Cano-Sancho et al., 2011)
Wheat-based cereals		(11/11)	Max: 245	
3 cereals (rice >70 %)		(1/2)	Max: 40.2	
3 cereals (maize > 65%)	Italy	(2/2)	Max: 103.8	(Juan et al., 2014)
4-5 cereals (wheat >50%)		(3/7)	Max: 268	
5 cereals (barley >50%)		(2/3)	Max: 108	
Rice/corn-based meals	ND	(4/7)	46-877	(Zhang et al. 2014)
Cereal-based food	Portugal	160.6(4/9)	29-271	(Pereira, Fernandes & Cunha, 2015)
Breakfast cereals		13 (5/10)	5-47	
Infant cereals	Tunisia	46 (6/6)	10-110	(Oueslati et al., 2018)
Baby mix		61 (7/9)	12-109	

ND=not defined

In the present review, it was observed that maize and wheat-based cereals show not only higher contamination incidence but also present higher levels of mycotoxins, and in the case of DON incidence, often being close to or overcoming legal limits: mean DON of 260 µg/kg in barley based cereals from Canada (Lombaert et al., 2003), mean DON of 103 µg/kg in wheat based cereals from Italy (Juan et al., 2014); mean DON of 131 µg/kg in cereal-based baby food from Spain (Cano-Sancho et al., 2011).

Even considering the abovementioned strict legislation implemented in 2006, there are studies performed on the following years which found DON contaminated commercial products overcoming the stipulated limits (Cano-Sancho et al., 2011; Juan et al., 2014; Pereira, Fernandes & Cunha, 2015; Zhang et al., 2014). Interestingly, multi cereal infant and young children foods are the ones most frequently encountered as contaminated above the regulation, moreover in the ones where barley, wheat or maize were the predominant cereal in the formulation, the highest maximum levels were observed (Juan et al., 2014; Lombaert et al., 2003). On the contrary, Lu, Ruiz Leal, Míguez, & Fernández-Franzón (2013) did not find any DON contamination in the 57 analyzed baby food samples from the Spanish market. Also, very few surveys encountered or analyzed DON-3-Glc, 3- or 15-Ac-DON (Juan et al., 2014; Oueslati et al., 2018). These studies prove again the heterogeneity in mycotoxins contamination in cereals over different harvest seasons (Doohan, Brennan & Cooke, 2003), and justify the need for a deeper investigation on the effect of processing on the evolution of grains contamination with DON and its metabolites.

1.2.2.3 Flour roasting

Roasting consists in increasing flour temperature to the range of 105 – 120°C and maintaining it from 20 to 40 min. This stage is important in the modulation of organoleptic characteristics of the flour, leading to caramelization and Maillard reactions. Also, it helps improving flour dispersibility in water during the next stage (Fernández-Artigas, Guerra-Hernández & García-Villanova, 1999). An important aspect to consider is the change in color of the flour under this production step, browning being an undesirable result for cereal-based baby food products. It is also crucial not to overcome these physical production conditions, yet that a longer time above 120°C might lead to the formation of

a carcinogenic compound, acrylamide, which is proven to be particularly dangerous for children under 2 years old (Erkekoğlu and Baydar, 2010).

Only a few studies are available on the occurrence of DON in cereal flour, mainly being focused on wheat or rye flours used in bakery products industry (Malachova et al., 2011; Rasmussen, Ghorbani, & Berg, 2003; Schollenberger, Jara, Suchy, Drochner, & Muller, 2002; Škrbić, Živančev, Durišić-Mladenović, & Godula, 2012). Moreover, those studies are limited in terms of geographical origin of the analyzed samples and present a high variability of the results. One of the largest occurrence study in cereal flour was performed by Rasmussen et al. (2003). They collected samples of wheat and rye flour from Denmark over 4 years (1998-2001), finding a high incidence of DON contamination with an average level of 114 (85% positive samples) and 42.5 µg/kg (59% positive samples) in wheat and rye flour, respectively. A more recent study in Czech Republic, identified a range of 13 to 320 µg/kg of DON and 11 to 94 µg/kg of DON-3-Glc in wheat flour (Malachova et al., 2011). In the abovementioned studies, very few samples were found exceeding the maximum allowed level of DON in cereal flour (750 µg/kg). Thus, considering the specificity of the contamination with DON mainly in the outer layers of the kernel and the trend in using whole-grain flours for its better nutritional properties compared to the white flour, requires more investigation on mycotoxin occurrence, especially for the products in which flour is used as raw material.

Studies showed a very low DON reduction in roasting cereal flour at temperatures below 180°C. Yumbe-Guevara, Imoto & Yoshizawa (2003) investigated the impact of various temperature levels between 140 and 220°C on DON in standard solution and contaminated wheat and barley (whole grains and powder). They found that 8 and 11 min at 220°C were necessary to reduce 50 and 90% of DON in barley kernels and powder, respectively. The first significant reduction was observed at 180°C. Also, toxin reduction in these conditions was higher in grain powder than in kernels, which led them to the conclusion that heat penetration is an important factor in the effectiveness of thermal treatment. A similar study performed later reached quite controversary results, with only 1.7 to 7.6% reduction after applying a 230°C treatment to wheat flour (Israel-Roming & Avram 2010). Nonetheless, the authors of the mentioned study did not specify the duration of the treatment. Considering the parameters for flour roasting, there is a chance that although

lower temperatures are applied, the longer treatment time could compensate for it, at least, slightly decreasing the level of DON, however there are no available studies that could prove this statement.

Taking into account the risk of acrylamide formation during flour roasting, this stage may not be considered as crucial in the case of adoption of a mitigation strategy against DON contamination, however its possible impact, although small, need to be considered in the evaluation of DON transfer to the final product.

1.2.2.4 Pre-gelatinization and enzymatic hydrolysis

1.2.2.4.1 Process description

One of the most important stages in cereal-based baby food production process is enzymatic hydrolysis. It allows a partial break down of the starch contained in the cereals and facilitates its assimilation by infants digestion system (Fernández-Artigas, Guerra-Hernández & García-Villanova 1999). Starch is the most abundant carbohydrate found in plants, used as source of carbon and energy (Alcázar-Alay & Meireles, 2015). It is formed by two types of polymers: (i) amylose, composed of a linear chain of a-1,4-glucose units representing from 15 to 30 % of the total starch and (ii) amylopectin, composed of α-1,4-glycosidic bonds intensively branched in α-1,6 positions with glucose units (70 to 85% of the starch structure) (Figure 4) (Martinez & Gómez, 2016).

The arrangement of these two polymers form a matrix of starch granules alternating crystalline (amylopectin) and amorphous (amylose) phases. Cereal starches may have associated other molecules to their structure, such as phospholipids and free fatty acids (0.15 to 0.55%) and proteins (up to 0.6%), which increase the nutritional value of the starch (Alcázar-Alay & Meireles 2015). Starch granules have microscopic sizes and a great shape variability depending on their biological origin (Tester & Sommerville, 2001). Cereal starches are mainly characterized by the A-type structure (polymerization degree <15), and B1-type structure (polymerization degree between 15 and 25) (Tester, Karkalas, & Qi, 2004).

Starch is not available to the enzymes without undergoing a pre-gelatinization process (increase of swelling and crystallinity phase which results in the formation of a gel) (Tako et al., 2014). The pre-gelatinization would serve two purposes: (i) increase in starch

swelling capacity, solubility and cold water dispersion (Alcázar-Alay & Meireles, 2015) and (ii) facilitate the access of α-and β-amylases to the polymer chains (Martinez & Gómez, 2016).

The process is determined by transition temperatures (the higher the amount of crystalline structures, the higher the temperatures required for the gelatinization to take place) and gelatinization enthalpies, specific for each botanical source (Tester & Morrison, 1990). Knowing those characteristics together with the temperature for the optimum enzymatic activity allow the producer to identify the best setup of technological conditions in order to minimize energy losses and ensure a more sustainable production. Table 5 regroups the morphology and transition temperatures for native starches from different common cereal sources.

In baby food production, pre-gelatinization consists of mixing the roasted cereal flour with a high amount of water (between 60 and 80%, w/w) and heating the mix from 40 to 70°C.

When gelatinization is achieved a mix of α- and β-amylases is added, followed by a continuous stirring from 10 to 90 min. Amylases are enzymes hydrolysing starch into smaller molecules to produce dextrins and other small polymers composed of glucose units. They can be synthetized from plants, animals or microorganisms. The last source has completely replaced chemical starch hydrolysis in the starch processing industry due to its cost effectiveness, higher stability, great production yields and ease of process modifications (Gupta et al., 2003). Microbial enzymes include fungal and bacterial amylase.

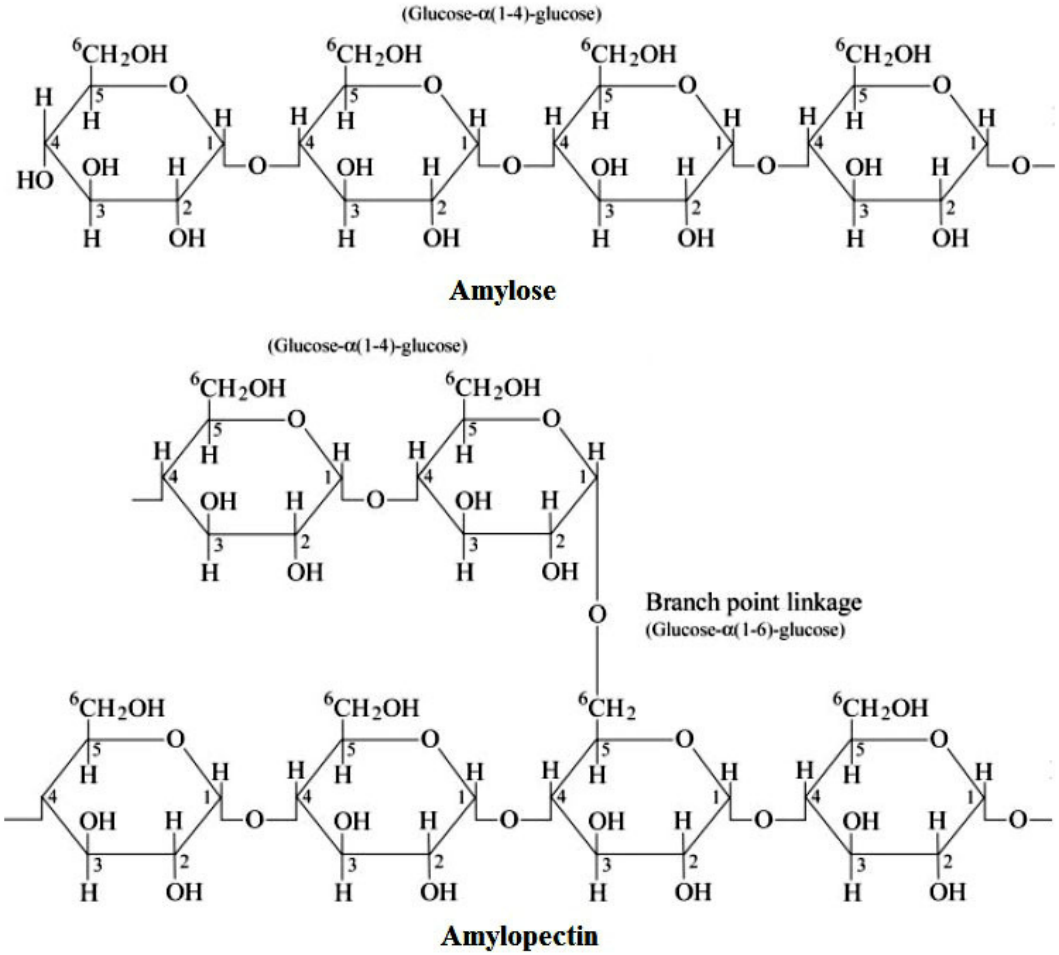

Figure 4: Chemical structure of amylose and amylopectin (Ghanbarzadeh and Almasi, 2013)

Table 5: Morphology and transition temperatures for native starches from different cereal sources

Species	Granule shape	Diameter (μm)	Amylose (%, w/w)	Crystalline type	Gelatinization temperatures[1]			Reference
					T_o (°C)	T_p (°C)	T_c (°C)	
Wheat	Spherical and lenticular*	<30*	25.6±1*	A*	52**	57**	63**	*Alcázar-Alay & Meireles, 2015 **Sasaki & Matsuki, 1998
Barley	Lenticular (A-type), spherical (B-type)*	15-25, 2-5*	19-22.1*	A, B*	46.7**	56.5**	73.7**	*Tester et al., 2004 **Tester & Morrison, 1990
Oats	Polyhedral*	3-10 (single) 80 (compound)*	28.4±0.8**	Do not fall into discrete size distribution***	45**	57**	72**	*Tester et al., 2004 **Tester & Karkalas, 1996 ***Zhou, Robards, Glennie-Holmes, & Helliwell, 1998
Maize	Angular*	11.5±0.3*	23-28*	A*	67.3**	72.9**	82.7**	*Alcázar-Alay & Meireles, 2015 **Chung, Liu, & Hoover, 2009
Rye	Lenticular (A-type), spherical (B-type)*	10-40 5-10*	29.7**	A**	51**	56**	62**	*Tester et al., 2004 **Verwimp, Vandeputte, Marrant, & Delcour, 2004
Sorghum	Polygonal, dented, round*	0.8*	23.7-27.6*	A*	67.9**	70.7**	75.7**	*Alcázar-Alay & Meireles, 2015 **Sang, Bean, Seib, Pedersen, & Shi, 2008
Triticale	Disc-shaped, Spherical*	1-30*	26.9**	A, B**	43.8**	53.6**	60.1**	*Tester et al., 2004 **Ao & Jane, 2007
Rice	Angular, polygonal*	<20*	21-25*		72**	76.6**	89.2**	*Alcázar-Alay & Meireles, 2015 **Lii, Shao, & Tseng, 1995

[1] T_o, T_p and T_c are onset, peak and conclusion temperatures, respectively; the stars in a line correspond to the references that the data was cited from.

There are two groups of amylases: endoamylases (α-amylase) and exoamylases (β-hydrolases). The α-amylase can be obtained from several bacteria or fungi, bacterial enzyme being preferred due to a larger spectrum of optimum conditions of temperature (30-50°C) and pH (2 to 12, most of the α-amylases being active within the neutral range) (Martínez & Gómez, 2016). The mechanism of their action allows to rapidly fragment the poly-glucoside chain and decrease the viscosity of the paste. The products of their activity (mono- and oligo-saccharides) are very numerous, depending on the nature of the α-amylase active site and on the biological origin of the starch (Martínez & Gómez, 2016). There are two types of exo-acting hydrolases: β-amylases and glucoamylases. β-amylase activity leads to the formation of β-maltose and β-limit dextrins of high molecular weight (the distribution of hydrolysation products of amylopectin is half maltose and half limit dextrins). Glucoamylases are able to hydrolyse both α-1,4 and α-1,6 bonds of the starch molecule into D-glucose. β-amylases can be obtained from microbial origins, however the most cost-effective are β-amylases from plant sources (sweet potatoes, soybean, barley and wheat). Glucoamylases can be also derived from animal sources besides plants and microorganisms. Their optimum temperature and pH for activity are 40 60°C and 4.9 6.6, respectively (Martínez & Gómez, 2016).

Considering the high diversity and complexity of the product, each producer would establish the technological parameters in accordance to the cereal species used and the desired degree of hydrolysis.

1.2.2.4.2 Impact on DON

There are no available studies on the impact of enzymatic hydrolysis on DON during infant food production process, however the addition of enzymes is common in breadmaking and brewing processes. Garda-Buffon, Baraj, & Badiale-Furlong (2010) studied the effect of DON on malt amylase activity (both α- and β-amylases). They found not only an increase in DON content during mashing but also an impact of its concentration by inhibiting the enzymatic activity. While observing those effects, DON concentration varied between 146 and 854 ng/g. Moreover, an increase in the enzymatic activity outside of this range was registered. Simsek, Burgess, Whitney, Gu, & Qian (2012) studied the evolution of DON concentration in wheat flour, dough and bread, with the addition of α-amylase, cellulase,

protease and xylanase during breadmaking. The increase of DON level in bread compared to the wheat flour was registered together with the decrease of DON-3-Glc level after baking. Enzymatic activity was found to be the main cause of the increase, especially the protease and xylanase activity, suggesting the release of DON from the grain wall matrix. α-amylase was not found to have a significant impact on DON, probably because of the higher exposure to the toxin of the outer layers of the seed compared to the endosperm, where starch is stored. This result is not confirmed by the work performed by Vidal, Ambrosio, Sanchis, Ramos, & Marín (2016), where they identified a significant increase in DON concentration under amylases activity (10%), nevertheless confirming that xylanase activity had a slightly higher impact on the increase in DON concentrations during the process (15% increase). Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova (2012) also suggested a release of DON and its conjugates from starch and dextrins due to hydrolytic enzymatic activity, however the extent of this release depended on wheat variety and the mechanism of *Fusarium* infection.

In conclusion, previous studies show an increase of DON and DON-3-Glc levels due to amylase activity, however these studies are limited to certain cereal species (mainly wheat and barley). Considering the complexity of food matrices and the differences in their composition, which result in differences in *Fusarium* infection and, as a result, DON contamination, more studies are needed in order to complete the knowledge regarding the evolution of DON levels under hydrolases activity, especially taking into account that infant food production usually implies the use of a mix of cereals. Also, an interesting object for study would be identifying if there is a correlation between the progress of starch hydrolysis by amylases and the change of mycotoxins level.

1.2.2.5 Enzymes inactivation

Enzymes inactivation is performed by heating the mass until 105 – 135°C, from 2 to 120 s. The applied temperature will depend on the biological origin of the enzymes: bacterial are the most thermostable, then fungal ones and then the enzymes coming from plant sources (Martinez & Gómez 2016). The heating would take place in a recirculation circuit in order to ensure a minimal exposure of the mass to the high temperature (browning reactions are undesirable at this stage) and to the environmental factors.

According to the studies on the impact of heat operations on DON, this stage might not be of a special concern for toxin reduction, especially because of the time of action: research works suggest the need of at least 180°C temperature to be applied in order to observe significant modifications in DON levels (Yumbe-Guevara, Imoto & Yoshizawa, 2003). Nevertheless, considering the efficient temperature penetration, it has to be considered for further studies.

1.2.2.6 Drying and packaging

Drying is performed using a drum drying system which consists of applying the paste as a thin layer (approximately 1.5 – 2 mm) on the outer face of an internally steam-heated revolving drum. The applied internal steam temperature is up to 200°C. The product becomes dry after three quarters of a drum revolution, with a residence time ranging from few seconds to dozens of seconds, being then removed with a static scraper with a final humidity <5%, on a wet basis. The obtained dry material is then ground into flakes or powder and packaged for delivering (Heldman, 2003). Similar to the roasting stage, an important safety factor to control is browning reactions (Maillard or caramelization reactions), which, as mentioned above, are highly undesirable, and thus the duration of the treatment has to be strictly controlled.

Regarding DON contamination and the impact of drum drying process, there are no studies available in baby-food or flour processing. Nevertheless, a study on cereal kernels drum drying indicates this process as highly efficient, leading to a 25 and 50% reduction of DON at 160 and 185°C, respectively (initial concentration of DON was 15.8±1.5 µg/g (Pronyk, Cenkowski & Abramson, 2006). Thus, considering the better potential for heat penetrability in the case of a flour material obtained during cereal-based infant food production process, a greater decrease in DON and its conjugates might be expected, nonetheless experimental studies have to be performed in order to confirm that.

1.3 References

Agu, R. C., and G.H. Palmer. (2001). The Effect of Nitrogen Level on the Performance of Malting Barley Varieties during Germination. *Journal of the Institute of Brewing* 107: 93 98.

Alcázar-Alay, S.C., and Almeida Meireles., M.A., (2015). Physicochemical properties, modifications and applications of starches from different botanical sources. *Food Science and Technology (Campinas), 35*, 215 36.

Ao, Z., and Jay lin J. (2007). Characterization and modeling of the A- and B-granule starches of wheat, triticale, and barley. *Carbohydrate Polymers,* 67, 46 55.

Asam, S., Habler, K., and Rychlik, M. (2017). *Fusarium* mycotoxins in food. *Chemical Contaminants and Residues in Food: Second Edition*, 295 336.

Belajová, Elena, Drahomíra Rauová, and Lubomír Daško. (2007). Retention of Ochratoxin A and Fumonisin B1 and B2 from Beer on Solid Surfaces: Comparison of Efficiency of Adsorbents with Different Origin. *European Food Research and Technology, 224*, 301 308.

Běláková, S., et al. (2014). The Occurrence of the Selected Fusarium Mycotoxins in Czech Malting Barley. *Food Control, 37*, 93 98.

Bellver Soto, J., Fernández-Franzón, M., Ruiz, M.J., and Juan-García, A. (2014). Presence of ochratoxin a (OTA) mycotoxin in alcoholic drinks from Southern European countries: wine and beer. *Journal of Agricultural and Food Chemistry, 62*, 7643 51.

Benešová, K., Běláková, S., Mikulíková, R. and Svoboda, Z. (2012). Monitoring of selected aflatoxins in brewing materials and beer by liquid chromatography/mass spectrometry." *Food Control, 25*, 626 30.

Bernhoft, A., P. E. Clasen, A. B. Kristoffersen, and M. Torp. (2010). Less *Fusarium* infestation and mycotoxin contamination in organic than in conventional cereals. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 27*, 842 52.

Berthiller, F. et al. (2007). Short Review: Metabolism of the *Fusarium* Mycotoxins Deoxynivalenol and Zearalenone in Plants. *Mycotoxin Research, 23*, 68 72.

Berthiller, F. et al. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters, 206*, 264 67.

Berthiller, F. et al. (2013). Masked mycotoxins: A review *Molecular Nutrition and Food Research, 57*, 165-186.

Bertuzzi, T. et al. (2011). Mycotoxin Occurrence in Beer Produced in Several European Countries." *Food Control, 22*, 2059 64.

Blandino, M. et al. (2012). Integrated strategies for the control of Fusarium Head Blight and deoxynivalenol contamination in winter wheat. *Field Crops Research, 133*, 139 49.

De Boevre, M., J. D. Di Mavungu, P. Maene, et al. (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 35.

De Boevre, M., Di Mavungu, J. D., S. Landschoot, et al. (2012). Natural occurrence of mycotoxins and their masked forms in food and feed products. *World Mycotoxin Journal, 5*, 207 19.

De Boevre, M. et al. (2013). Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281 92.

Bonnet, M. S. et al. (2012). Advances in deoxynivalenol toxicity mechanisms: The brain as a target. *Toxins, 4*, 1120 38.

Boutigny, A.L., Richard-Forget, F., and Barreau, C. (2008). Natural mechanisms for cereal resistance to the accumulation of *Fusarium* trichothecenes. *European Journal of Plant Pathology, 121*, 411 23.

Brodehl, A. et al. (2014). Biotransformation of the mycotoxin zearalenone by fungi of the genera *Rhizopus* and *Aspergillus*. *FEMS Microbiology Letters, 359*, 124 30.

Bryła, M. et al. (2016). Occurrence of 26 mycotoxins in the grain of cereals cultivated in Poland. *Toxins, 8*, pages 20.

Campagnollo, F.B. et al. (2015). In vitro evaluation of the ability of beer fermentation residue containing *Saccharomyces cerevisiae* to bind mycotoxins. *Food Research*

International, 77, 643–48.

Campbell, H., T. M. Choo, B. Vigier, and L. Underhill. (2000). Mycotoxins in barley and oat samples from Eastern Canada. *Canadian Journal of Plant Science, 80*, 977–80.

Cano-Sancho, G. et al. (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.

Cavaglieri, L. R. et al. (2009). Fungi and natural incidence of selected mycotoxins in barley rootlets. *Journal of Stored Products Research, 45*, 147–50.

Chung, H.J., Qiang, L., and Ratnajothi, H. (2009). Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches. *Carbohydrate Polymers, 75*, 436–47.

Cirillo, T., A. Ritieni, F. Galvano, and R. Amodio Cocchieri. (2003). Natural co-occurrence of deoxynivalenol and fumonisins B_1 and B_2 in Italian marketed foodstuffs. *Food Additives and Contaminants*, *20*, 566–71.

Clear, R. M., S. K. Patrick, and D. Gaba. (2000). Prevalence of fungi and fusariotoxins on oat seed from Western Canada, 1995-1997. *Canadian Journal of Plant Pathology, 22*, 310–14.

Cole, R J, J W Dorner, R H Cox, and L W Raymond. (1983). Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* isolated from contaminated beer. *Journal of Agricultural and Food Chemistry, 31*, 655–57.

Czerwiecki, L, D Czajkowska, and A Witkowska-Gwiazdowska. (2002). On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms - Part 1: occurrence of ochratoxin A and fungi in cereals in 1997. *Food Additives & Contaminants, 19*, 470–77.

Dänicke, S., and Ulrike, B. (2013). Kinetics and metabolism of the *Fusarium* toxin deoxynivalenol in farm animals: consequences for diagnosis of exposure and intoxication and carry over. *Food and Chemical Toxicology, 60*, 58–75.

Dombrink-Kurtzman, M.A., Stephen, T., Poling, M., and Kendra, D.F. (2010). Determination of deoxynivalenol in infant cereal by immunoaffinity column Cleanup

and High-Pressure Liquid Chromatography UV detection. *Journal of Food Protection, 73*, 1073 76.

Doohan, F.M., Brennan, J. and Cooke, B.M. (2003). Influence of climatic factors on *Fusarium* species pathogenic to cereals. *European Journal of Plant Pathology, 109*, 755 68.

Edwards, S G. (2009). *Fusarium* mycotoxin content of UK organic and conventional oats. *Food Additives and Contaminants, 26*, 1063 69.

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

EFSA. (2005). Opinion of the Scientific Panel on Contaminants in the Food Chain related to fumonisins as undesirable substances in animal feed. *EFSA Journal* 235

EFSA. (2011a). Scientific opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA Journal, 9*, 2481.

EFSA. (2011b). Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal* : 2197 2321.

Erkekoğlu, P., and Terken, B. (2010). Toxicity of acrylamide and evaluation of its exposure in baby foods. *Nutrition Research Reviews, 23*, 323 33.

Eskola, M. et al. (2019). Worldwide contamination of food-crops with mycotoxins: validity of the widely cited 'FAO estimate' of 25%. *Critical Reviews in Food Science and Nutrition*, 1-17.

Eskola, M., Päivi P., and Aldo R. (2001). Trichothecenes, ochratoxin A and zearalenone contamination and *Fusarium* infection in Finnish cereal samples in 1998. *Food Additives and Contaminants, 18,* 707 18.

European Commission. (1998). Commission Regulation EC No. 1525/98 Amending Regulation EC No 194/97 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Official Journal of the European Communities, 201,* 43 46.

European Commission. (2000). Opinion of the Scientific Committee on Food on *Fusarium* toxins part 3: fumonisin B_1 (FB1). *EFSA Journal.*

European Commission. (2006a). Commission Directive 2006/125/EC on Processed Cereal-Based Foods and Baby Foods for Infants and Young Children. *Official Journal of the European Union*, *396*, 16–35.

European Commission. (2006b). Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Official Journal of the European Union*, 5–24.

European Commission. (2013). Commission Recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxins in cereals and cereal products. *Official Journal of the European Union*.

EFSA. (2013). Deoxynivalenol in food and feed: occurrence and exposure. *EFSA Journal 11*.

EUROSTAT. (19 de 11 de 2019). *Happy International Beer Day*. Obtenido de Eurostat website : https://ec.europa.eu/eurostat/web/products-eurostat-news/-/EDN-20190802-1

Fernández-Artigas, P., Guerra-Hernández, E., and García-Villanova, B. (1999). Browning indicators in model systems and baby cereals. *Journal of Agricultural and Food Chemistry*, *47*, 2872–78.

Garda-Buffon, J., Edlira, B., and Badiale-Furlong, E. (2010). Effect of deoxynivalenol and T-2 toxin in malt amylase activity. *Brazilian Archives of Biology and Technology*, *53*, 505–11.

Ghali, R. et al. (2008). Incidence of aflatoxins, ochratoxin A and zearalenone in Tunisian foods. *Food Control, 19*, 921–24.

Ghanbarzadeh, B., and Almasi, H. (2013). Biodegradable Polymers. Biodregradation-Life of Sciences. *IntechOpen,* pages 141-185.

Gil-Serna, J. et al. (2013). Contamination of barley seeds with *Fusarium* species and their toxins in Spain: an integrated approach. *Food Additives & Contaminants: Part A, 30*, 372–80.

Glenn, A. E. (2007). Mycotoxigenic *Fusarium* species in animal feed. *Animal Feed Science and Technology*, *137*, 213–40.

Goswami, R.S., and Corby Kistler, H. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology, 5,* 515–25.

Gumus, T., Arici, M., and Demirci, M. (2004). A survey of barley, malt and beer contamination with ochratoxin A in Turkey. *Journal of the Institute of Brewing, 110*, 146–49.

Gupta, R. et al. (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry, 38*, 1599–1616.

Habler, K., and Rychlik, M. (2016). Multi-mycotoxin stable isotope dilution LC-MS/MS method for *Fusarium* toxins in beer. *Analytical and Bioanalytical Chemistry, 408*, 307–17.

Heldman, D. R. (2003). *Encyclopedia of Agricultural, Food and Biological Engineering.* New York: Marcel Dekker, Inc.

Hellin, Pierre et al. (2016). Relationship between *Fusarium* spp. diversity and mycotoxin contents of mature grains in Southern Belgium. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 33*, 1228–40.

Hietaniemi, V. et al. (2004). Contents of trichothecenes in oats during official variety, organic cultivation and nitrogen fertilization trials in Finland. *Agricultural and Food Science, 13*, 54–67.

Hietaniemi, V. et al.. (2016). Updated survey of *Fusarium* species and toxins in Finnish cereal grains. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 33*, 831–48.

Hofgaard, I.S. et al. (2016). Associations between *Fusarium* species and mycotoxins in oats and spring wheat from farmers' fields in Norway over a six-year period. *World Mycotoxin Journal, 9,* 365–78.

Hope, R., Aldred, D., and Magan, N. (2005). Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F. graminearum* on wheat grain. *Letters in Applied Microbiology, 40*, 295–300.

IARC. (2002). International Agency for Research on Cancer Iarc Monographs on the Evaluation of Carcinogenic Risks To Humans. Monograph 96.

Ibáñez-Vea, M., González-Peñas, E., Lizarraga, E., and López de Cerain, A. (2012). Co-occurrence of aflatoxins, ochratoxin A and zearalenone in barley from a northern region of Spain. *Food Chemistry, 132*, 35–42.

Inoue, T., Nagatomi, Y., Uyama, A., and Mochizuki, N.. (2013). Fate of mycotoxins during beer brewing and fermentation. *Bioscience, Biotechnology, and Biochemistry, 77*, 1410–15.

Ismaiel, A., and Papenbrock, J. (2015). Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture, 5*, 493–537.

Israel-Roming, F., and Avram, M. (2010). Deoxynivalenol stability during wheat processing. *Romanian Biotechnological Letters, 15*, 47–50.

JECFA/FAO. (2011). Deoxynivalenol. *Safety Evaluation of Certain Contaminants in Food*, 317–85.

Jouany, J.P., Yiannikouris, A., and Bertin, G. (2005). The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. *Archiva Zootechnica*: 26–50.

Juan, C., Raiola, A., Mañes, J., and Ritieni, A. (2014). Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control, 39*, 227–36.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture, 89*, 549–54.

Kłosowski, G., and Mikulski, D. (2010). The effect of raw material contamination with mycotoxins on the composition of alcoholic fermentation volatile by-products in raw Spirits. *Bioresource Technology, 101*, 9723–27.

Kostelanska, M. et al. (2009). Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3-glucoside, in beer and some brewing intermediates. *Journal of Agricultural and Food Chemistry, 57*, 3187–94.

Kostelanska, M. et al.. (2011). The study of deoxynivalenol and its masked metabolites fate during the brewing process realised by UPLC-TOFMS method. *Food Chemistry, 126,* 1870–76.

Kovalsky, P. et al. (2016). Co-occurrence of regulated, masked and emerging mycotoxins

and secondary metabolites in finished feed and maize an extensive survey. *Toxins, 8,* 1 29.

Krysinska-Traczyk, E., Perkowski, J., and Dutkiewicz, J. (2007). Levels of fungi and mycotoxins in the samples of grain and grain dust. *Annals of Agricultural and Environmental Medicine, 14,* 159 67.

Kunze, W. (2006). *Tecnologia para cerveceros y malteros* (1 ed.). Berlin: Westkreuz-Duckerei Ahrens KG.

Kuzdraliński, A., Solarska, E., and Muszyńska, M. (2013). Deoxynivalenol and zearalenone occurence in beers analyzed by an enzyme-linked immunosorbent assay method. *Food Control, 29,* 22 24.

Kuzdraliński, A., Solarska, E., and Mazurkiewicz, J. (2013). Mycotoxin content of organic and conventional oats from Southeastern Poland. *Food Control, 33*, 68 72.

Lancova, K et al. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food Additives & Contaminants: Part A, 25,* 732 44.

Lee, H.J., and Ryu, D. (2017). Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: public health perspectives of their co-occurrence. *Journal of Agricultural and Food Chemistry, 65,* 7034 51.

Lemmens, M. et al. (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 24.

Lewis, M.J. & Young, T.W. (1995). *Brewing (1st ed.)*. Norwich: Page Bros.

Lii, Cheng Yi, Yi Yuan Shao, and Kuo Hsuen Tseng. (1995). Gelation mechanism and rheological properties of rice starch. *Cereal Chemistry, 72*, 393 400.

Lindblad, M., T. Börjesson, V. Hietaniemi, and O. Elen. (2012). Statistical analysis of agronomical factors and weather conditions influencing deoxynivalenol levels in oats in Scandinavia. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 29*, 1566 71.

Lindblad, M. et al. (2013). Deoxynivalenol and other selected *Fusarium* toxins in Swedish

wheat - occurrence and correlation to specific *Fusarium* species. *International Journal of Food Microbiology, 167*, 284–91.

Lombaert, G. A. et al. (2003). Mycotoxins in infant cereal foods from the Canadian retail market. *Food Additives and Contaminants, 20,* 494–504.

Lu, H., Leal Ruiz, M.J., Míguez, M.P., and Fernández-Franzón, M. (2013). Detection of seven trichothecene mycotoxins in infant cereal foods by QuEChERS extraction and liquid chromatography coupled to tandem mass spectrometry. *Revista de Toxicologia*, *30*, 203–8.

Malachova, A. et al. (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,* 261–70.

Malachova, A. et al. (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. et al. (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,* 12990–97.

Manova, R., and Mladenova, R. (2009). Incidence of zearalenone and fumonisins in Bulgarian cereal production. *Food Control, 20*, 362–65.

Marroquín-Cardona, A.G., Johnson, N.M., Phillips, T.D., and Hayes, A.W. (2014). Mycotoxins in a changing global environment - a Review. *Food and Chemical Toxicology, 69,* 220–30.

Martinez, M.M., and Gómez, M. (2016). Insight of the α-amylase family of enzymes. In *Microbial Enzyme Technology in Food Applications*, 21–45.

Martos, P., Thompson, W., and Diaz, G. (2010). Multiresidue mycotoxin analysis in wheat, barley, oats, rye and maize grain by high-performance liquid chromatography-tandem mass spectrometry. *World Mycotoxin Journal, 3,* 205–23.

Maul, R. et al. (2012). Germination induces the glucosylation of the *Fusarium* mycotoxin deoxynivalenol in various grains. *Food Chemistry, 131*, 274–79.

Mbugua, S.K., and Gathumbi, J.K. (2004). The contamination of Kenyan lager beers with

Fusarium mycotoxins. *Journal of the Institute of Brewing, 110*, 227–29.

McCormick, S.P., Stanley, A.M., Stover, N.A., and Alexander, N.J. (2011). Trichothecenes: from simple to complex mycotoxins. *Toxins, 3,* 802–14.

Medina, Á. et al. (2005). Determination of ochratoxin A in beer marketed in Spain by liquid chromatography with fluorescence detection using lead hydroxyacetate as a clean-up agent. *Journal of Chromatography A, 1083*, 7–13.

Molto, G et al. (2000). Occurrence of trichothecenes in Argentinean beer: a preliminary exposure assessment. *Food additives and contaminants, 17,* 809–13.

Moretti, A. (2009). Taxonomy of *Fusarium* genus: a continuous fight between lumpers and splitters. *Zbornik Matice srpske za prirodne nauke, 117,* 7–13.

Nathanail, A.V. et al. (2016). The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. *Food Chemistry, 203*, 448–55.

Nielsen, L. K., Cook, D.J., Edwards, S.G., and Ray, R.V. (2014). The prevalence and impact of Fusarium Head Blight pathogens and mycotoxins on malting barley quality in UK. *International Journal of Food Microbiology, 179,* 38–49.

Nielsen, L.K. et al. (2011). Fusarium Head Blight of cereals in Denmark: species complex and related mycotoxins. *Phytopathology, 101*, 960–69.

Niessen, L., Böhm-Schrami, M., Vogel, H., and Donhauser, S. (1993). Deoxynivalenol in commercial beer - screening for the toxin with an indirect competitive ELISA. *Mycotoxin Research, 9,* 99–109.

Novo, P. et al. (2013). Detection of ochratoxin A in wine and beer by chemiluminescence-based ELISA in microfluidics with integrated photodiodes. *Sensors and Actuators, B: Chemical, 176*, 232–40.

Oliveira, P.M. et al. (2012). Fundamental study on the influence of *Fusarium* infection on quality and ultrastructure of barley malt. *International Journal of Food Microbiology, 156,* 32–43.

Olsson, J., Börjesson, T., Lundstedt, T., and Schnürer, J. (2002). Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and

electronic nose. *International Journal of Food Microbiology, 72*, 203–14.

Oueslati, S., Berrada, H., Mañes, J., and Juan, C. (2018). Presence of mycotoxins in Tunisian infant foods samples and subsequent risk assessment. *Food Control, 84,* 362–69.

Pan, D. et al. (2007). Deoxynivalenol in barley samples from Uruguay. *International Journal of Food Microbiology, 114,* 149–52.

Papadopoulou-Bouraoui, A. et al. (2004). Screening survey of deoxynivalenol in beer from the European market by an enzyme-linked immunosorbent assay. *Food Additives & Contaminants, 21*, 607–17.

Paul, P. A., Lipps, P. E., and Madden, L. V. (2005). Relationship between visual estimates of Fusarium Head Blight intensity and deoxynivalenol accumulation in harvested wheat grain: a meta-analysis. *Phytopathology, 95,* 1225–36.

Pereira, V. L., Fernandes, J. O., and Cunha, S.C. (2015). Comparative assessment of three cleanup procedures after QuEChERS extraction for determination of trichothecenes (type A and type B) in processed cereal-based baby foods by GC–MS. *Food Chemistry, 182,* 143–49.

Pestka, J.J. (2010). Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal, 3,* 323–47.

Pestka, J. J. (2007). Deoxynivalenol: toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology, 137,* 283–98.

Piacentini, K.C. et al. (2017). Mycotoxin analysis of industrial beers from Brazil: the influence of fumonisin B_1 and deoxynivalenol in beer quality. *Food Chemistry, 218,* 64–69.

Piacentini, K. C., Savi, G. D., Pereira, M.E.V., and Scussel, V.M. (2015). Fungi and the natural occurrence of deoxynivalenol and fumonisins in malting barley (*Hordeum Vulgare L.*). *Food Chemistry, 187,* 204–9.

Piacentini, K.C., Savi, G.D., Olivo, G., and Scussel, V.M. (2015). Quality and occurrence of deoxynivalenol and fumonisins in craft beer. *Food Control, 50,* 925–29.

Pietri, A., Bertuzzi, T., Agosti, B., and Donadini, G. (2010). Transfer of aflatoxin B_1 and

fumonisin B_1 from naturally contaminated raw materials to beer during an industrial brewing process. *Food additives & contaminants. Part A, 27*, 1431–39.

Pires, E., and Branyik, T. (2015). Biochemistry of beer fermentation.. *Springerbriefs in biochemistry and molecular biology*,

Pitt, J. I., and Hocking, A. D. (1997). *Fungi and food spoilage.* London: Blackie Academic and Professional.

Placinta, C. M., D'Mello, J. P.F., and MacDonald, A. M.C.. (1999). A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology, 78,* 21–37.

Pleadin, J. et al. (2013). *Fusarium* mycotoxins' occurrence in cereals harvested from Croatian fields. *Food Control, 32*, 49–54.

Pleadin, J. et al. (2017). Mycotoxins in organic and conventional cereals and cereal products grown and marketed in Croatia. *Mycotoxin Research, 33*, 219–27.

Pronyk, C., Cenkowski, S. and Abramson, D. (2006). Superheated steam reduction of deoxynivalenol in naturally contaminated wheat kernels. *Food Control, 17*, 789–96.

Raiola, A. et al. (2015). Risk analysis of main mycotoxins occurring in food for children: an overview. *Food and Chemical Toxicology, 84*, 169–80.

Rasmussen, P. H. et al. (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*, 181–90.

Rasmussen, P. H., Ghorbani, F. and Berg, T. (2003). Deoxynivalenol and other *Fusarium* toxins in wheat and rye flours on the Danish market. *Food Additives and Contaminants, 20*, 396–404.

Reiss, J. (1973). Influence of the mycotoxins aflatoxin B_1, rubratoxin B,, patulin and diacetoxyscirpenol on the fermentation activity of baker's yeasts. *Mycopathologia et Mycologia applicata, 51*, 337–45.

Rocha, O., Ansari, K., and Doohan, F.M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Additives and Contaminants, 22*, 369–78.

Rodríguez-Carrasco, Y. et al. (2015). Occurrence of *Fusarium* mycotoxins and their dietary

intake through beer consumption by the European population. *Food Chemistry, 178*, 149–55.

Roger, D.D. (2011). Deoxynivalenol (DON) and fumonisins B_1 (FB1) in artisanal sorghum opaque beer brewed in North Cameroon. *African Journal of Microbiology Research, 5*, 1565–67.

Romagnoli, B., Ferrari, M. and Bergamini, C. (2010). Simultaneous determination of deoxynivalenol, zearalenone, T-2 and HT-2 toxins in breakfast cereals and baby food by high-performance liquid chromatography and tandem mass spectrometry. *Journal of Mass Spectrometry, 45*, 1075–80.

Ruan, R., Li, Y., Lin, X. and Chen, P. (2002). Non-destructive determination of deoxynivalenol in barley using near-infrared spectroscopy. *Applied Engineering in Agriculture, 18*, 549–53.

Rubert, J. et al. (2013). Mass spectrometry strategies for mycotoxins analysis in European beers. *Food Control, 30*, 122–28.

Ruprich, J., and Ostrý, V. (2008). Immunochemical methods in health risk assessment: cross reactivity of antibodies against mycotoxin deoxynivalenol with deoxynivalenol-3-glucoside. *Central European Journal of Public Health, 16*, 34–37.

Rychlik, M. et al. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including 'masked' mycotoxins. *Mycotoxin Research, 30*, 197–205.

Ryu, D., Bianchini, A. and Bullerman, L.B. (2008). Effects of processing on mycotoxins. *Stewart Postharvest Review, 4*, 1–7.

Sang, Y. et al. (2008). Structure and functional properties of sorghum starches differing in amylose content structure and functional properties of sorghum starches differing in amylose content. *Journal of Agricultural and Food Chemistry, 56*, 6680–85.

Sasaki, T., and Matsuki, J. (1998). Effect of wheat starch structure on swelling power. *Cereal Chemistry, 75*, 525–29.

Schollenberger, M. et al. (1999). A survey of *Fusarium* toxins in cereal-based foods marketed in an area of Southwest Germany. *Mycopathologia, 147*, 49–57.

Schollenberger, M. et al. (2002). *Fusarium* toxins in wheat flour collected in an area in

Southwest Germany. *International Journal of Food Microbiology, 72*, 85 89.

Scott, P.M. (1996). Mycotoxins transmitted into beer from contaminated grains during brewing. *Journal of AOAC International, 79*, 875 82.

Scudamore, K. A., Baillie, H., Patel, S. and Edwards, S.G. (2007). Occurrence and fate of *Fusarium* mycotoxins during commercial processing of oats in the UK. *Food Additives and Contaminants, 24,* 1374 85.

Shim, W-B., Kim, J-C., Seo, J-A., and Lee, Y-W. (1997). Natural occurrence of trichothecenes and zearalenone in Korean and imported beers. *Food Additives and Contaminants, 14,* 1 5.

Simsek, S. et al. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control, 26*, 287 92.

Škrbić, B., Živančev, J., Durišić-Mladenović, N. and Godula, M. (2012). Principal mycotoxins in wheat flour from the Serbian market: levels and assessment of the exposure by wheat-based products. *Food Control, 25*, 389 96.

Sobrova, P. et al. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology, 3*, 94 99.

Statistica. (2018). *Statistic Portal*. Obtenido de www.statistica.com: https://www.statista.com/statistics/526023/annual-rainfall-in-belgium/

Sugita-Konishi, Y. et al. (2006). Effect of cooking process on the deoxynivalenol content and its subsequent cytotoxicity in wheat products. *Bioscience, Biotechnology, and Biochemistry, 70*, 1764 68.

Tabuc, C et al. (2009). Molds and mycotoxin content of cereals in Southeastern Romania. *Journal of food protection, 72*, 662 65.

Tako, M., Tamaki, Y., Teruya, T., and Takeda, Y. (2014). The principles of starch gelatinization and retrogradation. *Food and Nutrition Sciences, 5*, 280 91.

Tamburic-Ilincic, L. (2010). *Fusarium* species and mycotoxins associated with oat in Southwestern Ontario , Canada. *Canadian Journal of Plant Science, 90*, 211 16.

Tanaka, T.. et al. (1988). Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. Survey of 19 countries. *Journal of*

Agricultural and Food Chemistry, 36, 979–83.

Tangni, E.K., Pussemier, L., Schneider, Y-J., and Larondelle, Y. (2013). Mycotoxines dans les céréales et produits dérivés : revue de la littérature sur les filières biologiques et conventionnelles en Europe. *Cahiers Agriculture, 22*, 152–64.

Tester, R. F., and Sommerville, M. D. (2001). Swelling and enzymatic hydrolysis of starch in low water systems. *Journal of Cereal Science, 33*, 193–203.

Tester, R.F., and Morrison, W.R. (1990). Swelling and gelatinization of cereal starches. I. Effects of amylopectin, amylose, and lipids. *Cereal Chemistry, 67*, 551–57.

Tester, R.F., and Karkalas, J. (1996). Swelling and gelatinization of oat starches. *Cereal Chemistry, 73*, 271–77.

Tester, R.F., Karkalas, J., and Qi, X. (2004). Starch composition, fine structure and architecture. *Journal of Cereal Science* 39: 151–65.

Tima, H., Brückner, A., Mohácsi-Farkas, C., and Kiskó, G. (2016). *Fusarium* mycotoxins in cereals harvested from Hungarian fields. *Food Additives and Contaminants: Part B Surveillance, 9,* 127–31.

Torres, M. R., Sanchis, V., and Ramos, A.J. (1998). Occurrence of fumonisins in Spanish beers analyzed by an enzyme-linked immunosorbent assay method. *International Journal of Food Microbiology, 39*, 139–43.

Tutelyan, V.A. et al. (2013). Fusariotoxins in Russian Federation 2005-2010 grain harvests. *Food Additives and Contaminants: Part B Surveillance, 6,* 139–45.

Twaruzek, M. et al. (2013). Statistical comparison of *Fusarium* mycotoxins content in oat grain and related products from two agricultural systems. *Food Control, 34*, 291–95.

Uhlig, S. et al. (2013). Faces of a changing climate: semi-quantitative multi-mycotoxin analysis of grain grown in exceptional climatic conditions in Norway. *Toxins, 5*, 1682–97.

Ul Hassan, Z. et al. (2018). Co-occurrence of mycotoxins in commercial formula milk and cereal-based baby food on the Qatar market. *Food Additives and Contaminants: Part B Surveillance*: 1–7.

Urrea, C.A., Horsley, R.D., Steffenson, B.J. and Schwarz, P. (2002). Heritability of

Fusarium Head Blight resistance and deoxynivalenol accumulation from barley accession CIho 4196. *Crop Science, 42*, 1404–8.

Vaclavikova, M. et al. (2013). 'Emerging' mycotoxins in cereals processing chains: changes of enniatins during beer and bread making. *Food Chemistry, 136*, 750–57.

Van Der Fels-Klerx, H., and Stratakou, I. (2010). T-2 toxin and HT-2 toxin in grain and grain-based commodities in Europe: occurrence, factors affecting occurrence, co-occurrence and toxicological effects. *World Mycotoxin Journal, 3*, 349–67.

Vanheule, A. et al. (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.

Varga, E. et al. (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*, 137–46.

Vegi, A., Schwarz, P. and Wolf-Hall, C.E. (2011). Quantification of Tri5 gene, expression, and deoxynivalenol production during the malting of barley. *International Journal of Food Microbiology, 150*, 150–56.

Verwimp, T., Vandeputte, G.E., Marrant, K. and Delcour, J.A. (2004). Isolation and characterisation of rye starch. *Journal of Cereal Science, 39*, 85–90.

Vidal, A. et al. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry, 208*, 288–96.

Voss, K.A., Smith, G.W. and Haschek, W.M. (2007). Fumonisins: toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137*, 299–325.

Wang, N., Wu, W., Pan, J. and Long, M. (2019). Detoxification strategies for zearalenone using microorganisms: A review. *Microorganisms,* 7, 1–14.

Wolf-Hall, C.E. (2007). Mold and mycotoxin problems encountered during malting and brewing. *International Journal of Food Microbiology, 119*, 89–94.

Yazar, S., and Omurtag, G.Z. (2008). Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences, 9,* 2062–90.

Yoshizawa, T., and Morooka, N. (1973). Deoxynivalenol and its monoacetate: new mycotoxins from *Fusarium roseum* and moldy barley. *Agricultural and Biological Chemistry, 37,* 2933 34.

Yumbe-Guevara, B. E., Imoto, T. and Yoshizawa, T. (2003). Effects of heating procedures on deoxynivalenol, nivalenol and zearalenone levels in naturally contaminated barley and wheat. *Food Additives and Contaminants, 20*, 1132 40.

Zachariasova, M. et al. (2008). Deoxynivalenol and its conjugates in beer: a critical assessment of data obtained by enzyme-linked immunosorbent assay and liquid chromatography coupled to tandem mass spectrometry. *Analytica Chimica Acta, 625,* 77 86.

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 91.

Zhang, K., Wong, J.W., Krynitsky, A.J. and Trucksess, M.W. (2014). Determining mycotoxins in baby foods and animal feeds using stable isotope dilution and liquid chromatography tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 62*, 8935 43.

Zhou, M., Robards, K., Glennie-Holmes, M. and Helliwell, S. (1998). Structure and pasting properties of oat starch. *Cereal Chemistry, 75,* 273 81.

Zinedine, A., Soriano, J.M., Moltó, J.C. and 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.

Chapter 2: Objectives

Chapter 2. Objectives

The general objective of the present thesis was to assess the changes of *Fusarium* mycotoxins during food production processes. Two food products were chosen for that purpose: beer and cereal-based infant food.

In order to achieve the proposed objective, the work was divided into several sub-objectives:

- Determine the level of mycotoxins in commercially available beer samples from Lleida and Mexico, assessing a possible exposure scenario (Chapters 4 and 5);
- Study the changes in the concentration of *Fusarium* mycotoxins in naturally contaminated barley during malting process (Chapter 6);
- Evaluate the changes of *Fusarium* mycotoxins during brewing (mashing, boiling and fermentation processes), developing different contamination scenarios and mycotoxin sources (Chapters 7 and 8);
- Study the changes of mycotoxin levels in *Fusarium graminearum* contaminated flours during roasting and enzymatic hydrolysis applied for the production of cereal-based infant foods (Chapters 9 and 10).

Chapter 3: Global methodology

3.1 Experimental design

The global design of the study was divided into two main parts: (i) identifying the technological parameters to be applied during each laboratory scale production process and set the sampling stages; (ii) developing the analysis methods for the selected mycotoxins in each sample type (Figure 5). In the case of beer, an updated survey on mycotoxins occurrence in beer from Lleida (Spain) and Veracruz (Mexico) was performed, which, besides the evaluation of a possible exposure scenario, helped define the mycotoxin selection for the following work related to the technological side of the study (i.e. ingredients, mycotoxins, contamination ways etc.).

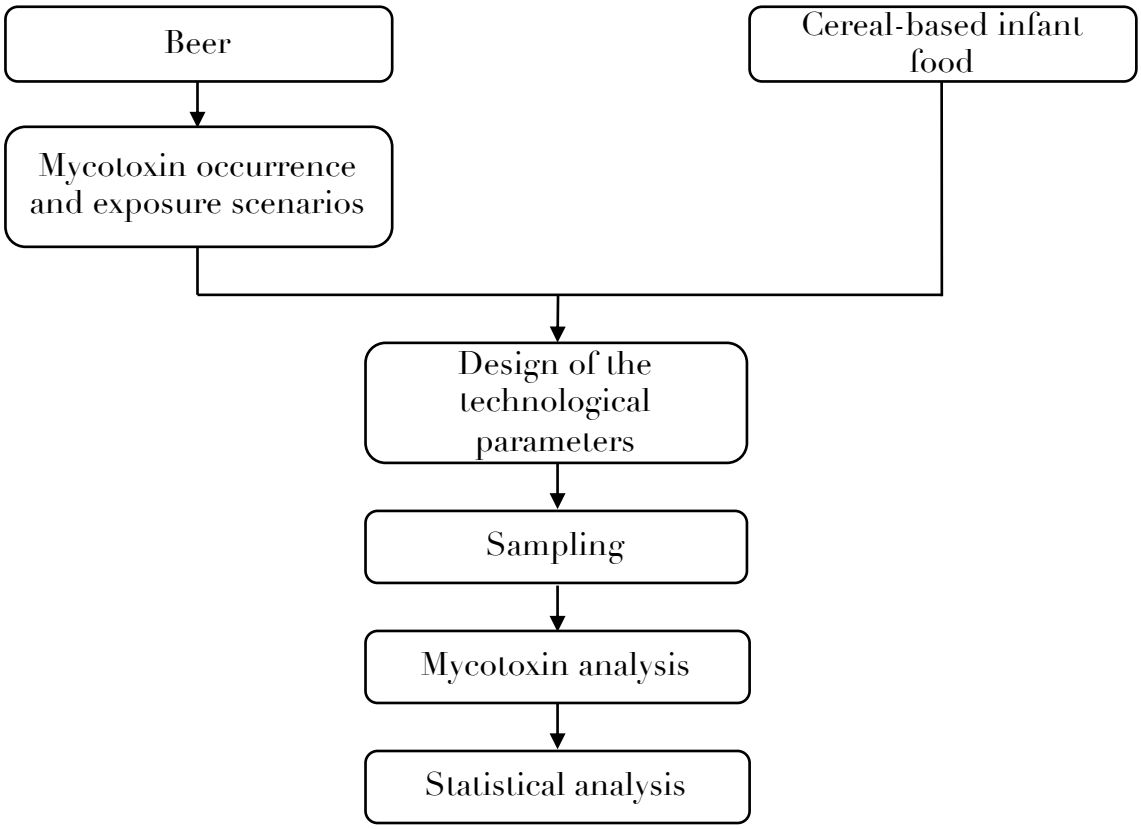

Figure 5: Global study design

3.2 Laboratory scale production processes

3.2.1 Beer

Beer production process was divided into three important stages: (i) malting, (ii) mashing and boiling and (iii) fermentation. For an easier sample manipulation, the stages were performed independently using *Fusarium* contaminated barley and malt.

Malting was composed of three steps: steeping, germination and kilning. The first two steps were performed in the Applied Mycology Unit of the Food Technology Department of the University of Lleida, whereas kilning was performed at the experimental pilot plant of the Malting Plant "La Moravia" (Bell·lloc d'Urgell, Spain). Briefly, steeping consisted of alternated phases of water and air applied to the prepared contaminated samples during 26h, at 10ºC. This helped kernels humidification and the activation of the enzymes involved in the germination process which followed afterwards (duration 96h at 15ºC). Kilning was performed by applying cycles of increasing temperature (maximum 80ºC, during 20h), which aimed stopping the germination process and further changes in the grain.

Mashing consisted of mixing the coarse ground malt with water, heating the obtained mix in a step-wise manner and maintaining the reached temperature right enough for different hydrolysing enzymes to be activated, namely 15 min at 45ºC (protein hydrolysis), 1h at 65ºC (maltose production) and 15 min at 75ºC (α-amylase activation) (Kunze, 2006). The obtained wort was then separated from the spent grains and submitted to boiling for 1.5h. The latter helps protein sedimentation and flavor modulation also due to hops addition.

Fermentation was performed at 20ºC for 96h (250 mL wort samples), using the wort obtained from contaminated malt. Fifteen different *Saccharomyces* strains (10 strains of *S. cerevisiae* and 5 strains of *S. pastorianus*) were analyzed from the perspective of their ability to mitigate mycotoxin contamination in barley wort. During the process, several parameters were monitored daily, such as physico-chemical characteristics of the obtained wort (e.g. relative density, pH, alcohol production) and yeast cell count.

3.2.2 Cereal-based baby food

Two stages of the cereal-based baby food production process were studied in the present work, namely flour roasting and amylolytic treatment. For that, three different cereal flours were used: barley, wheat and oat. Before the treatment they were contaminated with a strain of *F. graminearum* able to produce DON and ZEN. The combinations of time and temperature were considered for roasting by performing it at 105 and 120ºC for 30 and 40 min. Each treatment was triplicated.

Amylolytic treatment was performed by adding α-amylase and glucoamylases to the

roasted flours at two concentration levels (2.1 and 4.0 g of enzyme/kg flour). Then warm water was added to the mix (30% flour, w/w) and the obtained slur was maintained at 53ºC for 10, 50 and 90 min in a water bath. After the incubation time expired, the samples were rapidly cooled on an ice bath and freeze-dried before the analysis of mycotoxins.

3.3 Analytical methods

This section refers to the analytical techniques used in the present study for mycotoxin analysis in the obtained samples. The validation of the analysis methods was performed according to Commission Regulation No 401/2006 laying down the methods of sampling and analysis for the official control of the level of mycotoxins in foodstuffs (European Commission, 2006). The present study was focused on the following Fusarium mycotoxins: DON, DON-3-Glc, 3-AcDON, 15-AcDON, ZEN, α-ZEL, β-ZEL, ZEN-14-S, α-ZEL-14-S, β-ZEL-14-S, FB1 and FB2. In each of the following chapters the analytical methods used for each study are described in-depth.

Figure 6 shows a schematic representation of the mycotoxins and analytical methods used for their identification and quantification during the study of their fate throughout beer production process. Briefly, the analysis of the mycotoxins in the samples obtained from laboratory scale malting and fermentation processes were extracted using immunoaffinity columns (IAC) and solid-liquid (S/L) extraction (yeast). They were analyzed using an Agilent Technology 1260 Infinity HPLC system (California, USA) coupled with a fluorescent detector (FLD) for ZEN analysis and a diode array detector (DAD) for the analysis of DON and DON-3-Glc. The samples obtained after mashing, boiling and the commercial beer samples were purified by QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique and submitted to the analysis by HPLC-MS/MS. This difference in the sample preparation and analysis techniques is explained by the expansion of the focus of the study to the modified forms of DON and ZEN (i.e. less specific sample preparation procedure is required for a multi-mycotoxin analysis method).

Oat, wheat and barley flours were used for the laboratory scale cereal-based baby food production process. Before applying the treatments, the flours were infected with a mycotoxin producing *F. graminearum* strain.

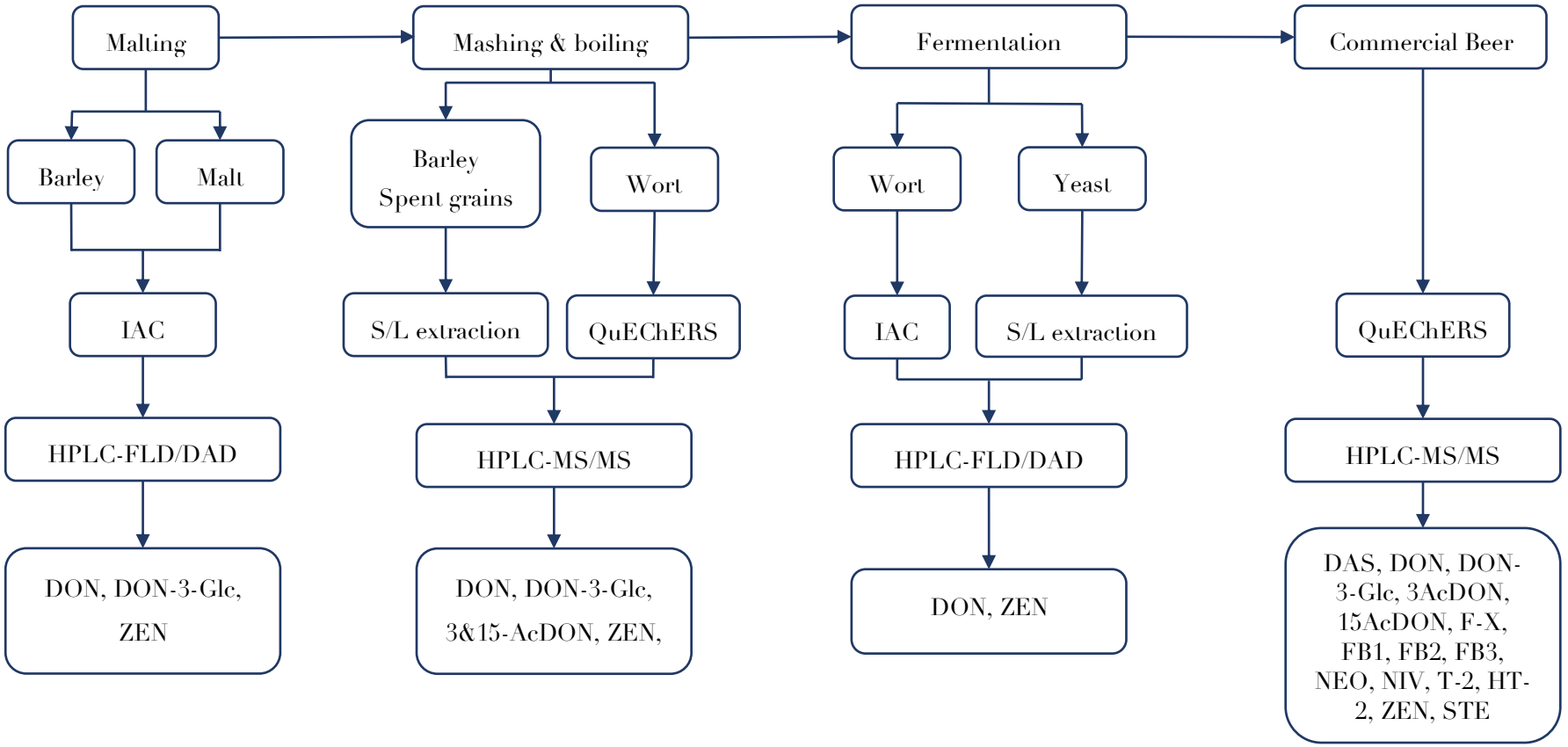

Figure 6: Analytical methods applied for the assessment of the fate of mycotoxins during beer production process

Two treatments (roasting and treatment with amylolytic enzymes) were tested separately for their ability to impact the initial mycotoxin level identified in the raw materials. Mycotoxin extraction from the three matrices was performed using the QuEChERS technique without a following clean-up to reduce the losses during sample preparation. Separation, identification and quantification of the mycotoxins was also achieved using an HPLC system coupled to a triple quadrupole mass spectrometer. Two aspects were studied in this part of the present work: (i) the fate of *Fusarium* mycotoxins under the treatments applied and (ii) chromatographic separation and identification of the newly found modified forms of ZEN, α- and β-ZEL: ZEN-sulfate and α- and β- ZEL-sulfates.

3.4 Statistical analysis

The obtained results were represented in international system units. The software that allowed data handling and evaluation was JMP Pro 13, Microsoft Office Excel 2016, @risk 7.5 and Minitab 18. The statistical analysis applied was one-way analysis of variance (ANOVA) and Tukey-HSD test ($p=0.05$) to identify significant changes of the mycotoxins during between different production stages. LOQ*1/2 was attributed to the non-quantitated data. For the assessment of the exposure scenario, deterministic and probabilistic approaches were used.

Chapter 4: Survey of mycotoxins in beer and exposure assessment through the consumption of commercially available beers from Lleida, Spain

Pascari, X., Ortiz – Solá, J., Marín, S., Ramos, A. J., & Sanchis, V.

LWT – Food Science and Technology 92 (2018) 87-91

4.1 Abstract

A multianalyte method, using a MS/MS detector, was applied for a simultaneous determination of 23 mycotoxins in 64 beer products purchased from the supermarket in Lleida, Spain. The samples varied by their origin, brewing technology, alcohol content, etc. The results showed that 20.3% of the tested samples were mycotoxin contaminated overpassing the limit of detection (LOD). None of the alcohol-free samples (17%) were contaminated with mycotoxins. The most frequently occurring toxin was zearalenone (ZEN), being quantified in 65% of the positive samples, with levels ranging from 8.24 to 62.96 μg/L. Regarding the co-occurrence of mycotoxins, three samples were found to contain two or more mycotoxins simultaneously. A deterministic approach was used to evaluate the contribution of beer consumption to daily intake and the proportion of the established tolerable daily intake (TDI) for ZEN and deoxynivalenol (DON) and its metabolite deoxynivalenol-3-glucoside.

Keywords

Mycotoxins, beer, LC-MS/MS, tolerable daily intake.

4.2 Introduction

Mycotoxins are natural compounds with a low molecular weight produced by filamentous fungi as secondary metabolites with no biochemical significance for fungal development. When exposed to optimal mycotoxin synthesis conditions, they create a toxic substrate which if ingested is able to cause diseases in animals and human beings (Benett & Klich, 2003).

Beer is one of the products that is susceptible to mycotoxin contamination. Spain is the fourth beer producing country in the European Union. Beer production in the country represents the major economic impact compared to other agrifood sectors (1.4% of GDP) (Cerveceros de España, 2016). As the main ingredient in brewing is barley, *Fusarium, Aspergillus, Penicillium* and *Alternaria* mycotoxins are highly probable to be present, if barley contamination in the field has occurred or good storage practices have not been applied (Medina et al., 2006). The most abundant mycotoxin in beer is found to be deoxynivalenol (DON) (Lancova et al., 2008; Piacentini, Savi, Olivo, & Scussel, 2015). However, other studies did prove the presence of toxins such as zearalenone (ZEN), fumonisins B_1 (FB1), B_2 (FB2) and B_3 (FB3), ochratoxin A (OTA) together with their modified forms (Bauer, Gross, Gottschalk, & Usleber, 2016; Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2011; Medina, Jiménez, Gimeno-Adelantado, Valle-Algarra, & Mateo, 2005; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015; Rubert, Soler, Marín, James, & Mañes, 2013; Zachariasova et al., 2008). The EU Regulation EC 1881/2006 establishes maximum allowed levels for 13 mycotoxins; however, the modified forms are not yet included. Beer is subjected to EU regulation on mycotoxins only in the case of containing maize adjuncts, FBs having set their maximum limits at 400 µg/kg for the sum of FB1 and FB2 (EC 1881/2006). The majority of modified mycotoxins are less toxic than their parent forms, nevertheless enzymes present in the digestive system may be able to transform the modified forms into parent forms and may have consequences on human health that are difficult to predict (Berthiller et al., 2013).

Considering the existing studies on the carryover of mycotoxins from barley to beer (Inoue et al., 2013; Kostelanska et al., 2011; Lancova et al., 2008) and the few mycotoxin survey studies in Spain, the aim of this work was to study the occurrence of mycotoxins in 64

different beer products, varying by their origin and brewing technology, purchased in the area of Lleida, Spain. In the present research it was aimed to use an analytical LC-MS/MS method for the simultaneous determination of diacetoxyscirpenol (DAS), DON, deoxynivalenol-3-glucoside (DON-3-G), 3- and 15-acetyl-deoxynivalenol (3-Ac-DON and 15-Ac-DON), fusarenon-X (F-X), the three main fumonisins (FB1, FB2, FB3), neosolaniol (NEO), nivalenol (NIV), T-2 and HT-2 toxins, zearalenone (ZEN), four aflatoxins (AFB1, AFB2, AFG1, AFG2), sterigmatocystin (STE), ochratoxin A (OTA), roquefortine-C (ROQ-C), alternariol (AOH) and alternariol-methyl-ether (AME). Also, an assessment of population exposure to mycotoxins through beer consumption was performed.

4.3 Materials and methods

4.3.1 Chemicals and reagents

The standards of DAS, DON, DON-3-Glc, 3-Ac-DON, 15-Ac-DON, F-X, FB1, FB2, FB3, NEO, NIV, T-2, HT-2, ZEN, AFB1, AFB2, AFG1, AFG2, STE, OTA, ROQ-C, AOH and AME were obtained from Sigma Aldrich (Bornem, Belgium). Internal standard deepoxy-deoxynivalenol (DOM-1) was obtained from Romer Lab (Getzersdorf, Austria). All mycotoxin solid standards were dissolved in methanol (1 mg/mL) and stored at -18°C.

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), was obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany). Magnesium sulphate and sodium chloride were purchased from Fischer Scientific (New Jersey, USA).

4.3.2 Samples

Various bottled and canned beers (*n=64*) were bought from supermarkets of the area of Lleida between May and July 2017. Every product was purchased in a duplicate or triplicate (2 or 3 different lots of each beer) according to their availability at the time of buying (n=165 samples). Fourteen different brands originating from nine countries, namely Spain (5), Germany (2), France (1), Belgium (1), Netherland (1), Scotland (1), Czech

Republic (1), Argentina (1), Mexico (1) were chosen for the analysis according to their availability. The samples were bought considering the differences in consumer preferences, *i.e.* according to their fermentation style, ale (9.4%) and lager (90.6%); their alcohol content, alcohol free (17.2%), between 4 and 5 % vol. (60.9%) and >5.5% vol. (21.9%); their color, yellow (75%), amber (15.6%) and dark colored (9.4%). Table 6 regroups the description of the samples that were found to be contaminated with mycotoxins.

4.3.3 Sample preparation

Beer samples purification was carried out following a protocol validated by the Laboratory of Food Analysis from Ghent University, Belgium. Briefly, from each bottle (or can) 100 mL of sample was fractioned and degassed by sonication during 15 min (Branson 2800, Newtown, USA). Then, 18 mL of extraction solvent composed by acetonitrile: water: acetic acid (59:40:1, v/v/v) was added to 2 mL of degassed beer sample containing the internal standard (DOM-1) at a concentration of 10 µg/L. The mixture was vigorously shaken for 30 s prior to the addition of premixed 4 g of MgSO4 and 1 g of NaCl. Afterwards, it was again intensively shaken for 60 s and agitated during 30 min at 200 rpm (Infors AG CH-4103, Bottmingen, Switzerland). The mixture was then centrifuged at 4500 rpm during 10 min with Hettich Universal 320R centrifuge (Tuttlingen, Germany) and 7 mL of supernatant were collected and evaporated to dryness under a low nitrogen stream (40°C).

4.3.4 LC-MS/MS analysis

A Waters Acquity UPLC system coupled to a Quattro XEVO TQ mass spectrometer (Waters, Milford, MA, USA) was used to analyse the samples. Data acquisition and processing was performed with MassLynx™ version 4.1 and QuanLynx® version 4.1 software (Waters, Manchester, UK). A Waters Acquity UPLC ® HSS T3 2.1 x 100 mm, 1.8 µm column was applied (Milford, Massachusetts, US).

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.

Table 6: Description of the contaminated beers purchased in Lleida, Spain

Sample*	Country of origin	Alc. content, % vol.	Color	Malt type	Fermentation style	Unmalted adjuncts
01	Spain	7.5	Ambar with orange reflections	Barley	Lager	None
02	France	5.0	Golden yellow	Barley	Lager	None
03	Spain	7.2	Golden yellow	Barley	Lager	Maize, rice
04	Spain	4.8	Cloudy golden	Barley and Wheat	Lager	None
05	Spain	7.5	Golden	Barley	Lager	Maize
06	Spain	6.8	Ambar	Barley	Lager	Maize
07	Belgium	6.0	Blond	Barley and wheat	Lager	None
08	Spain	5.4	Golden bright	Barley	Lager	Rice
09	Spain	5.2	Blond	Barley and wheat	Lager	None
10	Germany	5.0	Blond	Barley and wheat	Lager	None
11	Spain	4.8	Golden	Barley	Lager	Maize
12	Czech Republic	4.4	Pale to golden yellow	Barley	Lager	None
13	Spain	4.0	Bright yellow	Barley	Ale	None

The flow rate was set at 0.3 mL/min. Initially, the mobile phase gradient was set at 5% of the solvent B. Then, it was changed linearly to 65% B in 7 min and to 75% B in the next 4 min. Following that, the proportion dropped to 1% B within 2 min and increased to 99% B in the next minute. Afterward, the proportion of the solvent B came back to 5% within 0.1 min, increased to 65% B and 75% B in the next 3.5 min and 1 min, respectively. The next 1.2 min was characterized by a drop to 1% of solvent B and its increase to 5% in the following minute. Then, the solvent B proportion was linearly increased to 65% in 3.5 min, to 75% in 1 min and to 99% in the next 1.6 min. The last 2 min of the chromatogram, solvents proportion was kept at 5% B until the next injection.

The mass spectrometer was operated in the positive electrospray ionization mode (ESI+). The capillary voltage was 30 kV, and nitrogen was applied as spray gas. Source and dissolution temperatures were set at 150°C and 200°C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 L/h and the dissolution gas flow 4 m^3/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.

4.3.5 MS/MS method validation

The LC-MS/MS method was successfully validated based on European Commission Decision 401/2006 laying down the rules for the analytical methods to be used in the testing of official samples. Matrix-matched calibration plots were constructed for the determination of the analytes. DOM-1 was used as internal standard in the multi-mycotoxin analysis. Evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. The linearity was interpreted graphically using a scatter plot. The precision was calculated in terms of the relative standard deviation (RSD) and the bias of the method (uncertainty related to the reference standard, the accuracy of the bias and the root mean square (RMS_{bias})), represented by measurement uncertainty (MU). The MU evaluation was performed according to European Union Decision 2002/657/EC, which corresponds to a confidence interval of 95%. Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; the 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 (IUPAC, 1995). The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in European Commission Decision 401/2006. Table 7 describes the abovementioned parameters.

4.3.6 Risk assessment and mycotoxin daily intake

A deterministic approach was used in order to evaluate the probable daily intake of mycotoxins throughout beer consumption based on the obtained mycotoxin levels and beer consumption data available, considering an average body weight of 70 kg (Juan et al., 2017). Taking into account that more than 80% of the samples were found to be below the detection limit (left-censored data), the recommendations of EFSA applying the substitution method (best case scenario the <LOD values were considered equal to zero, worst case scenario the <LOD values were equaled to LOD) were followed (European Food Safety Authority, 2010). Afterwards, the following equation was used to calculate the PDI (1):

$$PDI = (C_m * K)/bw \ , \quad (1)$$

- PDI: probable daily intake for each mycotoxin (ng/kg bw/day);
- C_m: mean of mycotoxins in the analyzed samples (ng/L);
- K: average beer consumption (L/day);
- bw: body weight (kg).

Table 7: MS/MS parameters for the analysis of the target analytes by MRM ESI (+) positive mode ionization

Mycotoxin	Precursor ion (m/z)	Product ions (m/z)	CE[a] (eV)	CV[b] (v)	Retention time (min)	LOD[c] (µg/L)	LOQ[d] (µg/L)	CCα[e], µg/L	CCβ[f], µg/L	MU[g] (2x), %
NIV	313.1	175.0/177.0	21/16	35	3.02	31.75	63.50	18.26	23.07	4.51
DON-3-G	476.1	249.0/297.0	18/12	15	3.75	22.36	44.71	12.21	12.28	0.16
DON	297.0	249.0/231.0	15/10	26	4.43	51.76	103.53	27.16	32.44	4.58
3-AcDON	356.1	203.1/339.2	16/15	25	5.53	4.97	9.95	2.82	3.34	4.95
15-AcDON	356.1	339.2/137.4	25/8	18	5.47	2.65	5.29	1.52	1.84	1.88
F-X	355.0	137.1/247.1	21/12	16	5.37	20.68	41.35	11.25	11.47	0.32
ZEN	319.2	187.2/203.0	19/20	27	11.54	14.12	28.23	7.42	9.24	3.88
STE	325.0	281/310.0	24/30	40	11.25	5.27	10.54	2.70	3.51	0.46
DAS	384.1	247.1/307.1	12/9	35	7.59	0.52	1.03	0.29	0.55	3.74
AOH	258.9	185.1/213.1	30/26	40	9.56	7.78	15.57	3.71	6.18	2.45
AME	272.9	199.3/258.2	30/26	57	12.11	24.73	49.47	12.23	22.60	7.96
AFB1	313.0	241.1/270.1/	32/35	65	8.21	3.22	6.43	1.77	2.39	3.48
AFB2	315.0	259.0/286.9	28/40	25	7.95	2.29	4.57	0.97	2.35	5.71
AFG1	329.0	243.0/311.0	24/20	50	7.20	2.10	4.20	1.10	1.46	2.68
AFG2	331.0	285.0/313.0	28/24	40	6.84	1.16	2.33	0.69	1.42	2.48
NEO	400.0	215.0/305.0	12/9	30	5.34	9.58	19.16	4.57	8.68	4.73
OTA	403.9	239.0/358.0	22/12	40	11.36	4.04	8.08	2.25	2.46	15.46
HT2	442.3	215.7/263.2	12/12	40	9.13	6.39	12.79	3.47	4.25	1.35
T2	484.3	215.9/305.2	18/12	40	9.74	8.23	16.46	5.03	7.45	3.83
FB1	706.2	354.3/530.2	30/28	70	10.51	42.77	85.54	19.87	59.66	7.70
FB2	706.1	336.2/354.2	36/30	70	11.15	172.91	345.82	102.48	159.31	31.40
FB3	722.1	334.2/352.1	36/32	40	9.65	23.20	46.40	11.76	25.02	6.20

[a] CE=collision energy; [b] CV=cone voltage; [c] LOD= limit of detection; [d] LOQ= limit of quantification; [e]CCα=decision limit; [f] CCβ=detection capability; [g]MU=measurement uncertainty.

4.4 Results and discussion

4.4.1 Mycotoxin contamination

From 64 analyzed beers, thirteen (20.3%) were found to be contaminated with mycotoxins (Table 8). However, none of the contaminated samples overpassed the maximum allowed limits. From the thirteen positive samples, only in one sample (01) the three batches were mycotoxin contaminated and in four samples (03, 05, 06 and 10) 2/3 batches were contaminated. None of the eleven analyzed alcohol free samples (17%) contained mycotoxins, which is in accordance with previously published researches (Kostelanska et al., 2009; Varga et al., 2013). However, the lack of knowledge concerning the raw materials does not let us explain the different incidence of mycotoxin in alcohol free and the beer containing alcohol.

According to previously reported studies, the unmalted adjuncts (e.g. maize) are susceptible to be contaminated with mycotoxins and to transfer them to the beer (Torres et al., 1998). Nevertheless, no significant correlation between mycotoxin contamination and the presence of maize adjuncts was found in this particular case.

DON, DON-3G, ZEN, FB1 and HT-2 toxin were found in the mycotoxin positive samples with and average concentration in the positive samples of 31.28, 13.19, 15.06, 32.78 and 23.72 μg/L, respectively (Table 8). The most frequently encountered mycotoxin resulted to be ZEN (in 65% of positive samples) which concentration ranging between 8.24 and 62.96 μg/L (legal limit for ZEN in beer is 75 μg/L) (Regulation EC 1881/2006). However, none of other monitored toxins were found in the tested beer samples (AOH, AME, NIV, AFs, STE, 3-Ac-DON, 15-Ac-DON, F-X, DAS, ROQ-C, NEO, OTA, T-2 toxin, FB2 and FB3). In one of the samples (021) the co-occurrence of three mycotoxins was identified, namely DON, DON-3G and FB1 with a concentration of 20.97, 13.05 and 32.78 μg/L, respectively. In two samples the co-occurrence of DON and DON-3-G was observed which can be explained by a possible conversion from one form to another during the stages of brewing (Kostelanska et al., 2011).

Table 8: Mycotoxin levels (μg/L) in contaminated beer samples purchased from Lleida, Spain

Sample ID*		DON	DON-3-Glc	ZEN	HT-2	FB1
01						
	011	<LOD	<LOD	**8.77**	<LOD	<LOD
	012	<LOD	<LOD	**8.95**	<LOD	<LOD
	013	<LOD	<LOD	**8.24**	<LOD	<LOD
02						
	021	**20.97**	**13.05**	<LOD	<LOD	**32.78**
03						
	032	<LOD	<LOD	**9.98**	<LOD	<LOD
	033	**46.74**	<LOD	<LOD	<LOD	<LOD
04						
	041	<LOD	<LOD	**10.98**	<LOD	<LOD
05						
	051	<LOD	<LOD	**11.66**	<LOD	<LOD
	052	<LOD	<LOD	**10.72**	<LOD	<LOD
06						
	061	<LOD	<LOD	**8.69**	<LOD	<LOD
	062	<LOD	<LOD	**8.53**	<LOD	<LOD
07						
	072	<LOD	**13.76**	<LOD	<LOD	<LOD
08						
	083	<LOD	<LOD	**10.33**	<LOD	<LOD
09						
	091	<LOD	<LOD	<LOD	**23.72**	<LOD
10						
	101	**26.82**	<LOD	<LOD	<LOD	<LOD
	102	**26.13**	**11.94**	<LOD	<LOD	<LOD
11						
	112	<LOD	**14.00**	<LOD	<LOD	<LOD
12						
	121	<LOD	<LOD	**62.96**	<LOD	<LOD
13						
	131	<LOD	<LOD	**20.97**	<LOD	<LOD

DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside; ZEN=zearalenone; FB1= fumonisins B_1; LOD=limit of detection

However, in this case the ratio DON-3-Glc/DON <1 (0.53), which is in opposition with previously mentioned study. Another study performed by Inoue et al. (2013) on the fate of mycotoxins during brewing showed a reduction of DON levels up to 50% compared to the initial contamination but DON-3-Glc was not an object of the study, thus DON reduction was attributed only to its possible adsorption on spent grains. Also, two samples contained only DON and other two only DON-3-Glc, which prove, in line with other published

researches, that there is not a unique correlation of transformation from one form to another during brewing processes, but more complexed origins of these two toxins are modulating their concentration in the final product (nature of contamination of raw materials, enzymatic activity etc.) (Habler & Rychlik, 2016; Kostelanska et al., 2011; Scott, 1996; Wolf-Hall, 2007).

One of the samples (09) was found to contain HT-2 toxin in a concentration of 23.72 µg/L. HT-2 toxin's main source is wheat and the HT-2 toxin contaminated sample is a wheat beer, which explains that a possible contamination at the level of raw materials occurred (Schothorst & Van Egmond, 2004).

The fact that none of the samples overpassed the legal limits suggests that good reception and storage practices are applied, yet that at the level of reception, the rejection of the contaminated raw materials is an important preventive measure that companies are implementing (Medina et al., 2006).

4.4.2 Exposure assessment

Results allowed the evaluation of the probable daily intake for ZEN and the sum of DON and DON-3-Glc as they were the most frequently and significantly occurring mycotoxins in the tested samples (Table 9). The exposure was assessed using the available national beer consumption data for 2016 provided by two sources: Spanish Brewers Association (Cerveceros de España, 2016) and Spanish Ministry of Agriculture, Fishing, Alimentation and Environment (MAPAMA). The databases showed a slightly different annual per capita consumption, namely 46.4 L/person (Cerveceros de España, 2016) and 40.67 L/person (MAPAMA, 2016). In the light of the knowledge that in alcohol free beers mycotoxins contamination has not occurred (Kostelanska et al., 2009; Varga et al., 2013) and, considering an average proportion of alcohol free beer in the diet of Spanish consumer of 14% (Cerveceros de España, 2016) and 13.3% (MAPAMA, 2016), the annual consumption levels were considered as 39.9 L/person (corresponding to 109 mL/day) and 35.27 L/person (corresponding to 97 mL/day), respectively. The established tolerable daily intake (TDI) for ZEN is 0.25 µg/kg body weight (EFSA, 2014). In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) extended the group of DON and DON-3-Glc by including 3 and 15- Ac-DON as a factor increasing consumers exposure risk to these toxins

and established a TDI of 1 µg/kg body weight for the sum of the four toxins (JECFA/FAO, 2011).

Table 9: Results of the probable daily intake (PDI) assessment for the tested mycotoxins expressed as LB and UB scenarios (ng/kg bw/day).

Mycotoxin	TDI[c], (ng/kg bw/day)	PDI, ng/kg bw/day			
		LB[d]	%TDI	UB[e]	%TDI
ZEN	250	[a]1.71	[a]0.68	[a]12.56	[a]5.02
		[b]1.52	[b]0.61	[b]11.18	[b]4.47
Sum of DON, DON-3-Glc, 3-Ac-DON, 15-Ac-DON	1000	[a]1.64	[a]0.16	[a]61.45	[a]6.14
		[b]1.46	[b]0.15	[b]54.69	[b]5.47

[a] Source: Spanish Brewers Association; [b] Source: MAPAMA; [c]Tolerable Daily Intake; [d]Lower bound; [e]Upper Bound.

The left-censored data approach was used to treat the obtained dataset. Two exposure scenarios, the lower bound (LB) or the best-case scenario and the upper bound (UB) or the worst-case scenario, were defined (Table 9). Regarding the LB scenario, the obtained PDI is less than 1% from the established TDI for both sum of DONs and ZEN (0.15 and 0.65%, respectively). In the case of UB scenario, the PDI for the sum of DONs and for ZEN represent an average of 5.81 and 4.75% from the recommended TDI for these toxins.

In a study performed by Juan et al. (2017) on consumers´ exposure to mycotoxins through the consumption of barley derived products in Tunisia, beer represented the highest contribution to the TDI compared to other analyzed products. Nevertheless, studies evaluating the exposure of the European population to mycotoxins, specially dedicated to DON and its metabolites, found out that beer is not a significant source of exposure, unless it is consumed in high amounts (e.g. more than 0.5 L/day) (Pietri et al., 2010; Varga et al., 2013).

Only several toxicological studies are published investigating the combined toxic effect on health of two or more simultaneously present or ingested mycotoxins (Speijers & Speijers, 2004). This, considering the findings of the present work (several samples were found contaminated with more than one mycotoxin), proves the need for establishing more combined TDI for the mycotoxins that have additive or synergic effects as the effect of multiple mixtures of mycotoxins must be better understood. Also, considering that beer is

only a part of daily diet, studies on the interaction of mycotoxins and other contaminants (e.g. heavy metals) are needed, yet that DON is already known to be decreasing micro-nutrients absorption at intestinal level (Hunder et al., 1991).

4.5 Conclusion

From the 64 tested beer products, 20.3% were found to contain mycotoxins over the limit of detection (LOD) without overcoming the legal limits. Ordered by their prevalence in the tested beer samples, the found mycotoxins were ZEN, DON, DON-3-Glc, FB1 and HT-2 toxin. Three samples were characterized by a co-occurrence of two or more mycotoxins. In none of the cases the contamination exceeded the legally established maximum limits for mycotoxins. Regarding the mycotoxin exposure risk assessment, it was found that, according to the available national data on beer consumption, the consumers are not at risk (<1% from the TDI for LB scenario and about 5% from the TDI for the UB scenario). However, the situation might change in the case of heavy drinkers (>0.5 L/day).

4.6 Acknowledgement

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2014 55379 P) for funding this work.

4.7 References

Bauer, J. I., Gross, M., Gottschalk, C., & Usleber, E. (2016). Investigations on the occurrence of mycotoxins in beer. *Food Control*, *63*, 135 139.

Benett, J. W., & Klich, M. (2003). Mycotoxins. *Clinical Microbiology Review*, 497-516.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. De, Haesaert, G., Karlovsky, P., Stroka, J. (2013). Masked mycotoxins: A review. *Molecular Nutrition and Food Research*, *57*, 165 186.

Bertuzzi, T., Rastelli, S., Mulazzi, A., Donadini, G., & Pietri, A. (2011). Mycotoxin occurrence in beer produced in several European countries. *Food Control*, *22*, 2059 2064.

Cerveceros de España. (2016). *Informe socioeconómico del sector de la cerveza en España 2016*.

Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. Retrieved from Cerveceros de España web site: http://www.cerveceros.org/pdf/CE-informe-economico-2017-FINAL.pdf. Accessed: 03/11/2017

EFSA. (2014). Evaluation of the increase of risk for public health related to a possible temporary derogation from the maximum level of deoxynivalenol, zearalenone and fumonisins for maize and maize products. *EFSA Journal*, *12*, 1–61.

European Food Safety Authority. (2010). Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal*, *8*, 1–96.

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

Hunder, G., Schümann, K., Strugala, G., Gropp, J., Fichtl, B., & Forth, W. (1991). Influence of subchronic exposure to low dietary deoxynivalenol, a trichothecene mycotoxin, on intestinal absorption of nutrients in mice. *Food and Chemical Toxicology*, *29*, 809–814.

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

JECFA/FAO. (2011). Safety evaluation of certain contaminants in food. Monographs 8, *FAO* (Vol. 82), 1-778.

Juan, C., Berrada, H., Manes, J., Oueslaty, S. (2017). Multi-mycotoxin determination in barley and derived products from Tunisia and estimation of their dietary intake. *Food and Chemical Toxicology*, *103*, 148–156.

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, J., Krska, R. (2009). Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3-glucoside, in beer and some brewing intermediates. *Journal of Agricultural and Food Chemistry*, *57*, 3187–3194.

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.

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 & Contaminants: Part A*, *25*, 732 744.

Medina, Á., Jiménez, M., Gimeno-Adelantado, J. V., Valle-Algarra, F. M., & Mateo, R. (2005). Determination of ochratoxin A in beer marketed in Spain by liquid chromatography with fluorescence detection using lead hydroxyacetate as a clean-up agent. *Journal of Chromatography A*, *1083*, 7 13.

Medina, Á., Valle-Algarra, F. M., Mateo, R., Gimeno-Adelantado, J. V., Mateo, F., & Jiménez, M. (2006). Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria*, *Aspergillus* and *Fusarium*. *International Journal of Food Microbiology*, *108*, 196 203.

Ministerio de Agricultura Alimentación y Medio Ambiente. (2016). *Informe del Consumo de Alimentación en España 2014*. Retrieved from MAPAMA web site: http://www.mapama.gob.es/fr/alimentacion/temas/consumo-y-comercializacion-y-distribucion-alimentaria/informe_del_consumo_de_alimentos_en_espana_2016_webvf_tcm12-460602.pdf. Accessed: 03/11/2017

Piacentini, K. C., Savi, G. D., Olivo, G., & Scussel, V. M. (2015). Quality and occurrence of deoxynivalenol and fumonisins in craft beer. *Food Control*, *50*, 925 929.

Pietri, a, Bertuzzi, T., Agosti, B., & Donadini, G. (2010). Transfer of aflatoxin B1 and fumonisin B1 from naturally contaminated raw materials to beer during an industrial brewing process. *Food Additives & Contaminants. Part A, 27*, 1431 1439.

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., & Mañes, J. (2015). Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry*, *178*, 149 155.

Rubert, J., Soler, C., Marín, R., James, K. J., & Mañes, J. (2013). Mass spectrometry strategies for mycotoxins analysis in European beers. *Food Control*, *30*, 122 128.

Schothorst, R. C., & Van Egmond, H. P. (2004). *Report from SCOOP task 3.2.10 "collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states" Subtask: Trichothecenes*. *Toxicology Letters* (Vol. 153).

Scott, P. M. (1996). Mycotoxins transmitted into beer from contaminated grains during brewing. *Journal of AOAC International*, *79*, 875–882.

Speijers, G. J. A., & Speijers, M. H. M. (2004). Combined toxic effects of mycotoxins. *Toxicology Letters*, *153*, 91–98.

Torres, M. R., Sanchis, V., & Ramos, A. J. (1998). Occurrence of fumonisins in Spanish beers analyzed by an enzyme-linked immunosorbent assay method. *International Journal of Food Microbiology*, *39*, 139–143.

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 & Contaminants: Part A*, *30*, 137–146.

Wolf-Hall, C. E. (2007). Mold and mycotoxin problems encountered during malting and brewing. *International Journal of Food Microbiology*, *119*, 89–94.

Zachariasova, M., Hajslova, J., Kostelanska, M., Poustka, J., Krplova, A., Cuhra, P., & Hochel, I. (2008). Deoxynivalenol and its conjugates in beer: A critical assessment of data obtained by enzyme-linked immunosorbent assay and liquid chromatography coupled to tandem mass spectrometry. *Analytica Chimica Acta*, *625*, 77–86.

Chapter 5. Frequency and level of mycotoxins in beer from Mexican market and exposure estimate for deoxynivalenol mycotoxins

Wall – Martinez, H.A., Pascari, X., Ramos, A.J., Marin, S., Sanchis, V.

Mycotoxin Research 35 (2019) 207-216

5.1 Abstract

The aim of the present study was to evaluate the occurrence of 23 mycotoxins in beer purchased in Mexico and to assess two exposure scenarios in the Mexican population through beer consumption. Multi-mycotoxin analysis of a total of 61 different beers (132 samples) was carried out using UHPLC-MS/MS equipment. Probability density functions were used to describe mycotoxin contamination. The daily intake of mycotoxins was estimated using a semi-probabilistic approach, applying the Monte Carlo method. Deoxynivalenol (DON) and its metabolites (deoxynivalenol-3-glucoside (DON-3-Glc) and 3-acetyl-deoxynivalenol (3-Ac-DON)) were the mycotoxins found in higher proportions in the contaminated samples. None of other mycotoxins overpassed the limit of quantification (LOQ) of the method. The combined intake of DON and its analogues ranged from 5.24 to 86.59 ng/kg bw day, which represent from 1.2 to 19.83% of the DON tolerable daily intake (TDI). The results suggest that depending on the individual consumption of beer and depending on the type of beer, the intake of DON via beer consumption could represent a significant percentage of the TDI.

Keywords

Mycotoxins, deoxynivalenol, beer, occurrence and estimated daily intake

5.2 Introduction

Beer is the most consumed alcoholic beverage worldwide, with an annual *per capita* consumption greater than 100 litres in some European countries (Euromonitor International 2014; Kirin 2016). Mexico, with a production of 10.5 billion litres, is the country with the highest export of beer worldwide. In 2016, Mexico beer exports reached 2.814 billion dollars, followed by Holland (1.905 billion), Belgium (1.438 billion) and Germany (1.307 billion) (INEGI, 2017). About 80% of Mexican beer is exported to the United States, the rest being distributed to more than 184 countries (Kantar Worldpanel Mexico, 2015; INEGI, 2017).

Cereals used in brewing are mainly barley, wheat and corn (Shetty and Jespersen, 2006). These cereals can be subjected to contamination by different mycotoxins. Barley and wheat are mainly contaminated by ochratoxin A (OTA), trichothecenes (deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins) and zearalenone (ZEN). Corn is usually infested by fumonisins (FBs) and aflatoxins (AFs) producing *fungi*. All these mycotoxins have been associated with human and animal diseases (Zain 2011). *Alternaria* mycotoxins in cereals have been largely ignored both in Europe and overseas (Müller and Korn, 2013). *Alternaria* species produces several mycotoxins, such as alternariol and alternariol monomethyl ether. Strong evidence suggests that they are genotoxic (Pfeiffer et al., 2007) and mutagenic (Schrader et al., 2001; Brugger. et al., 2006).

The International Agency for Research on Cancer (IARC) classified AFs as a human carcinogen (Class 1), OTA and fumonisin B_1 (FB_1) as a possible human carcinogen (Class 2B), DON, ZEN, NIV and T-2/HT-2 toxins were not classifiable as to their carcinogenicity to humans (Class 3) (IARC, 1993, IARC 2002; FAO/WHO, 2006; EFSA, 2014).

The accumulation of mycotoxins in cereals, or derived foods and feeds, has been sporadically documented in Mexico, reaching concentrations higher than 1000 μg kg^{-1} for ZEN in wheat (Gonzalez-Osnaya and Farres, 2011), 200 μg kg^{-1} for AFs in maize and maize products (Martínez-Flores et al., 2003; Castillo-Urueta et al., 2011), 5.8 μg kg^{-1} for OTA (Reyes-Velázquez et al., 2008) and 5600 μg kg^{-1} for FB_1 (Robledo et al., 2001) in maize silage. In Mexico, there is no comprehensive food mycotoxins monitoring program carried out by the governmental agencies (Guzmán-de-Peña and Peña-Cabriales, 2005).

AFs are the only mycotoxins legislated in Mexico by the standards NOM-184-SSA1/SCF1-2002, NOM-187-SSA1-2002 NOM-247-SSA1-2008. The maximum allowed limit of AFs in cereals for human and animal consumption is 20 μg/kg. In the United States, AFs (20 μg/kg), DON (1000 μg/kg), FBs (2000-4000 μg/kg), and patulin (50 μg/kg) have been regulated (USDA, 2015). European regulations on mycotoxin set maximum levels in foodstuff for 14 compounds (Commission Regulation European (EC) 1881/2006; Commission Recommendation 2013/165/EU). Regulation 1881/2006 establishes a limit for fumonisins content in maize based foods (applicable to beer) intended for human consumption to 1000 μg/kg.

Mycotoxin contamination can occur during cereal growth in the field, during post-harvest storage or during malting (Bertuzzi et al., 2011). Considering mycotoxins thermal stability (AFs, ZEN, and DON) and solubility in water (DON and FBs), they can be partially transferred from cereals to malt and then to beer (Rodríguez-Carrasco et al., 2015). Several authors have studied the occurrence of mycotoxins in industrial and craft beers commercialized in Argentine (Molto et al., 2000), Brazil (Piacentini et al., 2017), Spain (Torres et al., 1998; Pascari et al., 2018b), Poland (Kuzdraliński et al., 2013), Belgium (Tangni et al., 2002), and other European countries (Papadopoulou-Bouraoui et al., 2004; Bertuzzi et al., 2011). There are no studies on the occurrence of mycotoxins in beer consumed in Mexico or in the United States, however, some of the surveys mentioned above included Mexican beers in their study (Table 10).

To estimate the dietary exposure, it is necessary to combine the data on food consumption and the levels of contamination in order to allow conclusions to be drawn about the amount of a substance being consumed by population (FAO/WHO, 2006). Monte Carlo simulation is a statistical method commonly used in the probabilistic approach assessment. Monte Carlo simulation relies on a sequence of random numbers to carry out a simulation. This enables obtaining a probability distribution of study regarding instead of a single value to represent this risk (Landau and Binder, 2015). Among the studies of exposure to mycotoxin through beer intake that have been made so far, none has been conducted exclusively in Mexico. Therefore, the objective of this work was to assess two exposure scenarios to mycotoxins throughout beer consumption, focusing on the data for Mexican population (daily beer consumption, average body weight).

Table 10: Occurrence of mycotoxins in Mexican beers

References	Country	Total beer analyzed	Mexican beer analyzed	Contamination level of Mexican beers Percentage of contaminated samples /range
Hlywka and Bullerman 1999	Unites States	29	3	FBs < 1 ng L^{-1}
Tangni et al. 2002	Belgium	62	2	OTA 100% /12 13 ng L^{-1}
Kuzdraliński et al. 2013	Poland	91	1	DON 100 % /16.7 □g L^{-1} ZEN n.d.[2]
Rodriguez-Carrasco et al. 2015	Spain	60	5	DON 100 % / 25.1 27.3 □g L^{-1} HT2-T2 < LOD[1]
Bauer et al. 2016	Germany	44	1	No results regarding country of origin DON 75 % of the total samples, ZEN and AOH in 100 % of the total samples.
Pascari et al. 2018b	Spain	64	1	23 mycotoxin analyzed / < LOD

Mycotoxins: alternariol (AOH); deoxynivalenol (DON); total fumonisins (FBs); HT-2 toxin (HT2); ochratoxin A (OTA); T-2 toxin (T2); zearalenone (ZEN). [1]LOD: limit of detection; [2]n.d.: not detected

5.3 Materials and methods

5.3.1 Chemicals and reagents

The standards of 15-Ac-DON, 3-Ac-DON, AFB_1, AFB_2, AFG_1, AFG_2, AOH, AME, DON, DON-3-Glc, DAS, FB_1, FB_2, FB_3, F-X, HT-2, NEO, NIV, OTA, ROQ-C, STE, T-2, STE, and ZEN were obtained from Sigma Aldrich (Bornem, Belgium). An internal standard of deepoxy-deoxynivalenol (DOM-1) was obtained from Romer Labs (Getzersdorf, Austria). All mycotoxin solid standards were dissolved in methanol (1 mg/mL) and stored at -18 °C.

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) was obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany). Magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Fischer Scientific (New Jersey, USA).

5.3.2 Samples

Various types of bottled and canned beers (n=61) were bought from supermarkets and beer stores of Veracruz city (Mexico) between July and October 2017. Every product was purchased by duplicate or triplicate (2 or 3 different lots of each beer) according to their availability at the time of buying (total of 132 samples). Twenty-five different beer producing companies, originating from eight countries, Mexico (40), Unites States (10), Belgium (4), Germany (3), Spain (1), Netherland (1), Argentina (1), and Guatemala (1) were chosen for the analysis. To facilitate results interpretation and discussion, all the samples were grouped as follows: according to their fermentation style, ale (31.1%) and lager (68.9%); their alcohol content, alcohol-free (3.3%), between 4 and 5% vol. (80.3%) and > 5.5% vol. (16.4%); their color, golden (62.2%), amber (28.0%) and dark colored (9.8%); and their way of production, industrial (73.8%) and craft (26.2%).

5.3.3 Samples pre-treatment

Beer samples extraction was carried out following a protocol modified of Monbaliu et al. 2009, validated by the Laboratory of Food Analysis from Ghent University, Belgium.

Briefly, from each sample, a 100 mL aliquot was taken, degassed, sonicated for 15 minutes and stored at -18ºC until analysis. Then, 18 mL of extraction solvent composed by acetonitrile:water:acetic acid (59:40:1, v/v/v) was added to 2 mL of degassed beer sample containing the internal standard (DOM-1) at a concentration of 10 µg L^{-1}. The mixture was vigorously shaken for 30 s prior to the addition of premixed 4 g of $MgSO_4$ and 1 g of NaCl. Afterwards, it was again intensively shaken for 60s and agitated during 30 min at 200 rpm in an orbital rotary shaker (Infors AG CH 4103, Bottmingen, Switzerland). The mixture was then centrifuged at 2336 x g during 10 min with a Hettich Universal 320R centrifuge (Tuttlingen, Germany) and 7 mL of supernatant were collected and evaporated to dryness under a low nitrogen stream (40°C). The dry extract was resuspended in 0.5 mL of methanol:water (95:5, *v/v*) and filtered (PTFE syringe filter, 0.22 µm) before injection in HPLC-MS/MS system.

5.3.4 Mycotoxin analysis

A Waters Acquity UHPLC system coupled to a Quattro XEVO TQ mass spectrometer (Waters, Milford, MA, USA) was used to analyse the samples. Data acquisition and processing were performed with MassLynx™ version 4.1 and QuanLynx® version 4.1 software (Waters, Manchester, UK). A Waters Acquity UPLC® HSS T3 2.1 × 100 mm, 1.8 µm column was applied (Milford, Massachusetts, US).

The mobile phase consisted of a gradient with phase A: water:methanol (95:5, *v/v*) and phase B: methanol:water (95: 5, *v/v*), both buffered with 10 mmol L^{-1} ammonium acetate and acidified with 0.3 % of glacial acetic acid.

The phase gradient was adjusted with 5 % of solvent B and the rest of solvent A. After 7 minutes; it was linearly placed at 65% of solvent B, and 4 minutes later it was increased to 75% of B. Following that, the proportion dropped to 1% B within 2 min and increased to 99% B in the next minute. After that, the proportion of solvent B again decreased to 5%, increased to 65% B and 75% B in the next 3.5 min and 1 min, respectively. In the following 1.2 min, the proportion of solvent B decreased to 1%, increasing to 5% after 1 minute. Then, the solvent B proportion was linearly increased to 65% in 3.5 min, to 75% in 1 min and to 99% in the next 1.6 min. The last 2 min of the chromatogram, solvents proportion was kept at 5% B until the next injection. The flow rate was set at 0.3 m*min^{-1} through the

entire analysis process.

The mass spectrometer was operated in the positive electrospray ionization mode (ESI+). The ESI parameters were set up as follows: capillary voltage 30 kV, and nitrogen applied as spray gas; source and dissolution temperatures 150 and 200°C, respectively; argon collision gas pressure 9×10^{-6} bar; cone gas flow 50 L*h^{-1}; dissolution gas flow 4 m^3*h^{-1}. 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.

5.3.5 LC-MS/MS method validation

The LC-MS/MS method for the simultaneous detection of 23 mycotoxins was in-house successfully validated based on European Commission Decision 401/2006. Validation data for each selected compound are presented in Table 11. Matrix matched calibration plots were constructed for the determination of the analytes. Linearity and the homogeneity of variance were checked for each studied mycotoxin. The linearity was interpreted graphically using a scatter plot. The precision was represented in terms of the relative standard deviation (RSD) and the bias of the method represented by measurement uncertainty (MU). The MU evaluation was performed according to EU Decision 2002/657/EC, which corresponded to a confidence interval of 95%. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as three and six times the standard error of the intercept divided by the slope of the calibration curve, respectively. 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 (Curie, 1995). The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in European Commission Decision 401/2006. The resulted detection and quantification limits are higher compared to the ones obtained in similar studies (Bertuzzi et al. 2011; Rodriguez-Carrasco et al. 2015; Bauer et al. 2016; Piacentini et al. 2017), however none of them performed a simultaneous multi-analysis study of 23 mycotoxins with conversion rates close to 100%.

Table 11: Validation parameters for the LC-MS/MS method for mycotoxin analysis in beer

Analyte	LOD (µg L^{-1})	LOQ (µg L^{-1})	Recovery range (%)	Lowest recovery level (µg L^{-1})	Recover Range at lowest level (%)	RSDr, % (n = 10)	CC_α (µg/L)	CC_β (µg/L)	Measurement uncertainty (2x)
15-Ac-DON	2.65	5.29	106.18	12.5	103.97	4.08	1.52	1.84	1.88
3-Ac-DON	4.97	9.95	103.59	25	102.57	3.08	2.82	3.34	4.95
AFB1	3.22	6.43	107. 86	10	103.74	3.86	1.77	2.39	3.48
AFB2	2.29	4.57	106.00	10	104.47	3.38	0.97	2.35	5.71
AFG1	2.10	4.20	105.83	10	107.02	3.16	1.10	1.46	2.68
AFG2	1.16	2.23	103.11	10	103.65	1.60	0.69	1.42	2.48
AME	24.73	49.47	109.06	100	111.12	2.62	12.23	22.60	7.96
AOH	7.78	15.57	104.37	50	104.57	1.14	3.71	6.18	2.45
DAS	0.52	1.03	104.76	5	104.47	1.68	0.29	0.55	3.74
DON	51.76	103.53	107.21	200	108.85	0.84	27.16	32.44	4.58
DON-3-Glc	22.36	44.71	101.32	20	102.59	1.08	12.21	12.28	0.16
F-X	20.68	41.35	104.59	100	106.78	1.53	11.25	11.47	0.32
FB1	42.77	85.54	106.96	200	108.23	3.76	19.87	59.66	7.70
FB2	172.91	345.82	123.51	200	124.23	10.42	102.48	159.31	31.40
FB3	23.20	46.40	105.90	125	105.26	2.06	11.76	25.02	6.20
HT-2 toxin	6.39	12.79	102.61	50	104.82	2.0	3.47	4.25	1.35
NEO	9.58	19.16	104.11	50	103.86	1.65	4.57	8.68	4.73
NIV	31.75	63.50	107.71	100	103.49	3.22	18.26	23.07	4.51
OTA	4.04	8.08	122.08	25	107.50	9.71	2.25	2.46	15.46
ROQ-C	0.67	1.34	105.9	2.5	104.47	1.24	0.35	0.42	2.41
STE	5.27	10.54	104.41	25	107.60	3.65	2.70	3.51	0.46
T-2 toxin	8.23	16.46	105.50	50	105.20	1.19	5.03	7.45	3.83
ZEN	14.12	28.23	103.11	50	108.24	1.60	7.42	9.24	3.88

15-acetyl-deoxynivalenol (15-Ac-DON); 3-acetyl-deoxynivalenol (3-Ac-DON); aflatoxin B_1 (AFB1); aflatoxin B_2 (AFB2); aflatoxin G_1 (AFG1); aflatoxin G_2 (AFG2); alternariol (AOH); alternariol-methyl ether (AME); deoxynivalenol (DON); deoxynivalenol-3-glucoside (DON-3-Glc); diacetoxyscirpenol (DAS); fumonisin B_1 (FB1); fumonisin B_2 (FB2), fumonisin B_3(FB3); fusarenon-X (F-X); HT-2 toxin (HT-2); neosolaniol (NEO); nivalenol (NIV); ochratoxin A (OTA); roquefortin C (ROQ-C); sterigmatocystin (STE); T-2 toxin (T-2); sterigmatocystin (STE) and zearalenone (ZEN), CCα = decision limit., CCβ = detection capability.

5.3.6 Treatment of the left-censored data

Analytical methods are defined by LOD and LOQ, to express quantitatively a result below these limits several techniques can be used. EFSA (2010) has published a scientific report resulted from the estimation of the accuracy of methods currently used and recommendation for more advanced alternative statistical approaches. WHO proposed recommendations for replacing the non-detected samples by LOD/2, or 0 and LOD according to the percentage of non-detects in the samples, similar guidelines were provided in the case of non-quantified values.

In this study, taking into account that more than 60% but less than 80% of the samples were found to be below the detection limit (with <25 results quantified) EFSA's recommendations were applied: Lower bound (LB) or best-case scenario the < LOD values were considered equal to zero and Upper bound (UB) or worst-case scenario the < LOD values were equalled to LOD) (EFSA, 2010).

5.3.7 Theoretical distribution fitting the mycotoxin beer contamination data

Using the software risk @Risk 7.5 (Palisade, Inc.), a comparison of different probability distribution functions were carried out and considering the asymmetry of the histogram of mycotoxin contamination in beer, the data were adjusted to an exponential function. Probability density functions and descriptive statistics (the mean, median, standard deviation and the 95th percentile) of mycotoxin concentration in beer were also determined and analyzed. Monte Carlo method was applied with the iterations number (10,000) recommended by international agencies (US-EPA, 1997).

5.3.8 Body weight population and beer consumption data

The high variability of alcohol consumption makes it one of the most difficult food items for exposure assessment studies. According to the WHO (2014), in Mexico alcohol consumption is six times higher in men (12.4 L of pure alcohol per year) than in women (2.6 L of pure alcohol per year) and 76 % of the alcohol consumed comes from the intake of beer. Because there are no available studies describing the behavior of beer consumption in groups of population, such as age, gender, region or socioeconomic level,

the national average volume of 60 L of beer per year, equivalent to 164.38 mL/day, established by the Mexican Ministry of Economy will be applied in the present publication. (Secretaría de Economía, 2015). To estimate the levels of intake in high drinkers, the beer consumption average of Czech Republic (143.3 L per year), the country with the highest consumption of beer in the world was used.

The reference body weight used was the one established by CANAIVE (2012) for an average Mexican (71.7 kg) (Cámara Nacional de la Industria del Vestido, CANAIVE 2012).

5.3.9 Estimation of mycotoxin daily intake and exposure risk

Daily intake was then calculated under a semi-probabilistic approach by equation (1):

$$EDI = \frac{Mc * Bc}{bw} \ldots (1)$$

Where:

$EDI =$ Probability Density Function of Estimated Daily Intake (ng mycotoxin kg^{-1} bw d^{-1})

$Mc =$ Probability Function Density of mycotoxin concentration in beer (ng L^{-1})

$Bc =$ Beer consumption (L d^{-1})

$bw =$ Body weight (kg)

In case of mycotoxins that are not classified as genotoxic and carcinogenic, the exposure estimates were compared with the guidance values of Tolerable Daily Intake (TDI). Safety levels used in the present study are summarised in Table 12.

Table 12: Compilation of tolerable daily intake values (TDI) for mycotoxins issued by the EU

Mycotoxins	Safety level (ng kg^{-1} bw day^{-1})	Reference
DON	1000	SCF 2000
FBs	2000	SCF 2003
HT-2	100	EFSA 2011a
NIV	700	SCF 2002
OTA	17	EFSA 2010b
T-2	60	EFSA 2011a
ZEN	250	EFSA 2011b

Deoxynivalenol (DON); fumonisins (FBs); HT2 toxin (HT2); nivalenol (NIV); ochratoxin A (OTA); T2 toxin (T2); zearalenone (ZEN).

5.4 Results and discussion

5.4.1 Occurrence of mycotoxins in beer

Mycotoxins were detected in 16 of the 61 analyzed samples (26.2% positive samples), however, none overpassed the limits of quantification of the methodology used. Only one beer presented contamination in the two analyzed replicates (different production batches).

The samples were purchased in supermarkets and beer stores in Veracruz city, so there is no information available on the traceability of the raw material or of the process, however, all the mycotoxins detected are produced by *Fusarium* fungi, which are characterized by invading cereals in the field (Gimeno and Martins, 2003). Thus, the contamination probably originates from the field, minimizing the option of contamination during storage or processing. From the analyzed samples, nine presented contamination with DON, two with 3-Ac-DON, six with DON-3-Glc and three with FB_1. The characteristics of beers and mycotoxins detected are described in Table 13. Similar results were reported by Pascari et al. (2018b) in beer purchased in Lleida, Spain, with 20.3% of samples contaminated by DON, DON-3-Glc, ZEN, HT2, and FB_1. Kuzdraliński et al. (2013) and Rodríguez-Carrasco et al. (2015) reported contamination by DON in 100% of beers analyzed; however, all samples showed contamination less than 48 μg L^{-1}. This concentration is lower than the LOQ of our methodology, so decreasing the LOQ of our methodology, the proportion of positive samples would probably increase to a large extent.

The most frequent contaminants were DON and its metabolites, detected in 87.5% of the positive samples. In two samples co-occurrence of DON and 3-Ac-DON was detected, which could have been due to their liberation from barley matrix during mashing and transfer to wort and beer because of their relatively high solubility in water (Samar, 2001; Kostelanska et al., 2011). Similarly, the presence of DON-3-Glc in five samples can be attributed to DON conversion during malting due to grain defence mechanisms against the presence of the contaminant (Lancova et al. 2008). ZEN was not detected in any of the samples. It would have been advisable to analyze α-zearalenone (α-ZEL) and β-zearalenone (β-ZEL) to discard contamination by ZEN metabolites (Karlovsky et al., 2016).

Table 13: Beer classification and mycotoxin contamination

Sample	Country of origin	Mycotoxin detected	Process style	Fermentation style	Color	Contamination replicate	Alcohol content	Type of Malt	Unmalted Adjuncts
Leffe blonde	Belgic	DON	Industrial	Ale	Golden	No	> 5.5%	Barley	None
Leffe Radieuse	Bélgica	DON	Industrial	Ale	Amber	No	> 5.5%	Barley	None
Leffe Brune	Bélgica	DON	Industrial	Ale	Dark	No	> 5.5%	Barley	None
Barrilito	Mexico	DON	Industrial	Lager	Amber	Yes	4 to 5%	Barley	None
Budweiser	USA	DON	Industrial	Lager	Golden	No	4 to 5%	Barley	Maize
Cerveza papantla	Mexico	DON, FB1	Craft	Lager	Golden	No	4 to 5%	Barley	Maize
Cucapa Ambar	Mexico	DON, 3AcDON	Craft	Ale	Amber	No	> 5.5%	Barley	None
Minerva Stout	Mexico	DON, 3AcDON	Craft	Ale	Dark	No	> 5.5%	Barley	Coffee and chocolate
Tijuana Xolos	Mexico	DON-3-Glc	Craft	Ale	Amber	No	> 5.5%	Barley	None
Boca Negra Pilsner	Mexico	DON-3-Glc	Craft	Lager	Amber	No	4 to 5%	Barley	Wheat
Tijuana Morena	Mexico	DON-3-Glc	Craft	Lager	Amber	No	4 to 5%	Barley	None
Bacanal	Mexico	DON-3-Glc	Craft	Ale	Amber	No	4 to 5%	Barley	None
Boca Negra Pilsner	Mexico	DON-3-Glc	Craft	Ale	Amber	No	4 to 5%	Barley	None
Tecate titanium	México	DON-3-Glc	Industrial	Lager	Amber	No	4 to 5%	Barley	None
Morelos	México	FB1	Craft	Lager	Golden	No	4 to 5%	Barley	Maize
Gallo	Guatemala	FB1	Industrial	Lager	Amber	No	4 to 5%	Barley	Maize

Deoxynivalenol (DON); 3-acetyl-deoxynivalenol (3AcDON); deoxynivalenol-3-glucoside (DON-3-Glc); fumonisin B_1 (FB1)

FB_1 contamination was found in three analyzed beers; this could be a consequence of the use of corn as an unmalted adjunct, corn grits are commonly used in order to achieve a better degree of lightness in color, clarity, calories, and flavor (Bertuzzi et al., 2011). Corn has been proved sensitive to the infestation by FBs producing *Fusarium*, which would explain the abovementioned finding (Mendoza et al., 2017, Robledo et al., 2001). Malt type and unmalted adjuncts used in beers contaminated with mycotoxins are described in Table 13.

There are limited surveys that classify the samples for data analysis (Rodriguez Carrasco et al., 2015; Peters et al., 2017; Pascari et al., 2018). In our study, beers with an alcohol content greater than 5.5% had mycotoxin contamination in 60% of the samples analyzed, similar to the results reported by Pascari et al (2018). A possible explanation would be the necessity to use more grain in high-density malt wort to reach these alcohol levels, which could contribute to mycotoxin contamination. Light and non-alcoholic beers did not show contamination above LOD.

Craft beer presented a higher percentage of mycotoxin contamination (56.3%) than industrial beers (15.55%). In the same way, Peters et al. (2017) detected more mycotoxins (AFB_1, OTA, ZEN, FBs, DON, T-2, and HT2) in craft beer than in industrial beer from 1,000 beers analyzed. It is recommended for small craft breweries to consider the implementation of rapid analysis techniques for mycotoxins in cereals to control the purchased malts and adjuncts as well as their final products.

The Mexican-brand or Mexican-made beers presented contamination in 27.5%. Although with a non-representative sample size (3 positive samples from a total of 7 analyzed), the results agree with that reported by Bauer et al. (2016), who found a high frequency in mycotoxin contamination (75% for DON) although in low concentrations (2.2 - 20 $\mu g\ L^{-1}$) in European beers. Regarding the color category, similar contamination was found, dark beers presented 33%, amber 26%, and golden 23%. Finally, as for the fermentation type, ale beers had a higher percentage of contamination (42%) than lager (29%), which could be probably explained by different adsorption of the toxins to the yeast cell during fermentation (Lancova et al., 2008), nonetheless more investigation is needed to confirm this statement.

5.4.2 Estimation of DON in various exposure scenarios

Due to the limited number of positive samples contaminated with FB_1 and other mycotoxins, only the assessment of exposure to DON will be performed. The suggestion of EFSA (2010) of using the sum of DON metabolites (DON-3-Glc, 3-Ac-DON, and 15-Ac-DON) to the total DON group was considered for risk assessment.

Table 14 shows the statistical parameters of the probability density function for mycotoxin contamination in beer for the two risk scenarios (LB and UB). It can be seen that even in the 99th percentile the values are below the legal limit of 1000 ng L^{-1} (SCF, 1999). Similar concentrations were presented by Bryla et al. (2018) (9.0 µg L^{-1}), Kuzdraliński et al. (2013) (20.66 µg L^{-1}) and Rodriguez Carrasco et al. (2015) (28.9 µg L^{-1}) in beer from different countries. The data on contamination by DON and its metabolites were adjusted to an exponential function. Figure 7 presents the probability density function of DON contamination in LB scenario.

Table 14: Fitted Exponential probability density function (PDF) parameters for the content of DON mycotoxins (DON+DON-3-Glc+3-Ac-DON+15-Ac-DON) in beer marketed in Veracruz (Mexico)

PDF parameters (**µg L^{-1}**)	**Lower bound** (**LB**)	**Upper bound** (**UB**)
Mean	5.24	86.59
Median	3.64	85.02
Standard Deviation	5.24	4.79
95th Percentile	15.71	96.05
99th Percentile	24.12	103.77

Deoxynivalenol (DON); 3-acetyl-deoxynivalenol (3-Ac-DON); deoxynivalenol-3-glucoside (DON-3-Glc); 15-acetyl-deoxynivalenol (15-Ac-DON).

Probability density function and probability density function parameters of the EDI calculated by Monte Carlo method are shown in Figure 7 and Table 15. EDI average was 12.03 ng kg^{-1} bw day^{-1} (LB) and 198.31 ng kg^{-1} (UB) or 28.69 ng kg^{-1} bw day^{-1} (LB) and 473.64 ng kg^{-1} bw day^{-1} (UB) in high consumers scenario. Those are lower than the recommendation of the JEFCA (2010) of 1000 ng kg^{-1} bw d^{-1}. The percentage of TDI that beer provides for DON mycotoxins as a result of beer consumption in LB is similar that

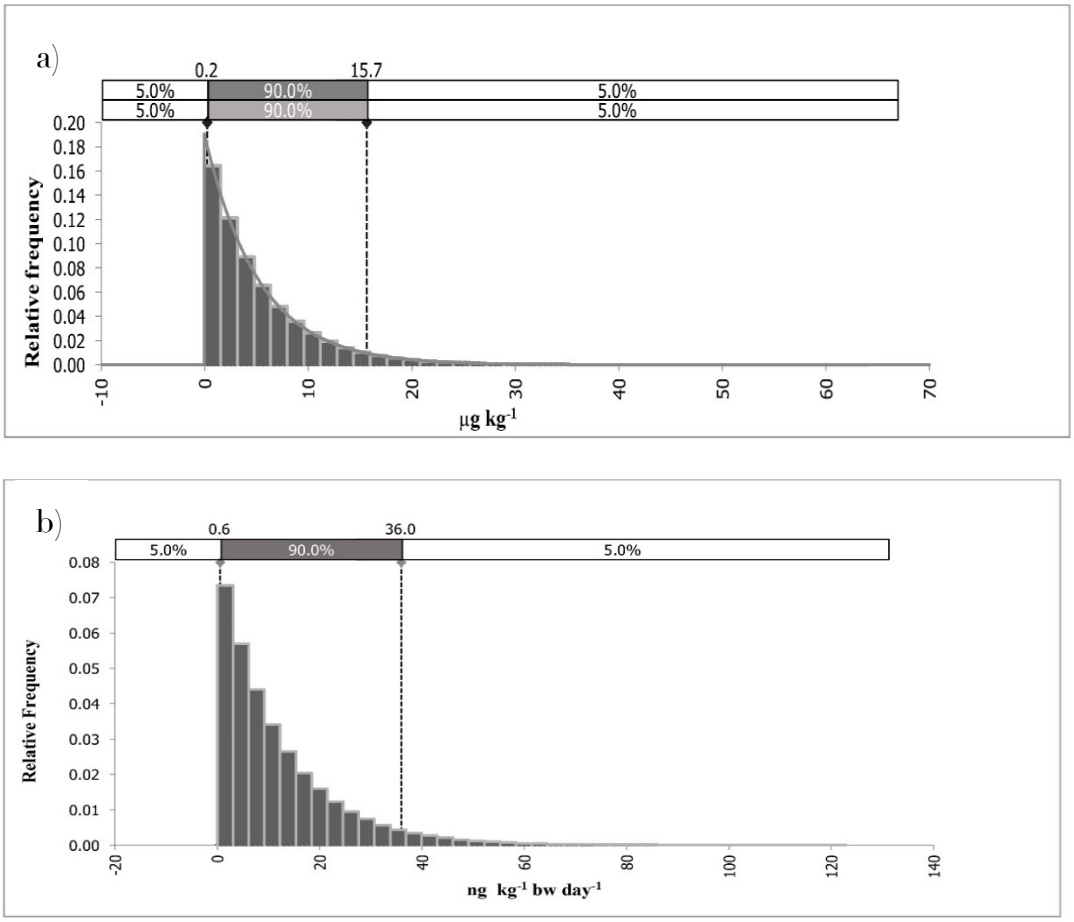

Figure 7: a) Probability density function fitted exponential distribution (solid line) for DON contamination in beer marketed in Mexico (Lower bound values), obtained by the Monte Carlo method, showing contamination in the 5th and 95th percentiles (broken line); b) Probability density function for estimated daily intake of DON (Lower bound values) though beer, obtained by Monte Carlo method, showing exposure in the 5th and 95th percentiles (broken line).

Table 15: Probability density functions (PDF) parameters for estimated daily intake of DON mycotoxins (DON+DON-3-Glc+3-Ac-DON+15-Ac-DON) through beer consumption in Veracruz (México)

PDF parameters	Lower bound (LB)	Upper bound (UB)
Mean (*ng kg^{-1} bw day^{-1}*)	12.03	198.31
Median (*ng kg^{-1} bw day^{-1}*)	8.33	194.93
Standard Deviation (*ng kg^{-1} bw day^{-1}*)	12.04	10.98
95th Percentile (*ng kg^{-1} bw day^{-1}*)	36.00	220.23
99th Percentile (*ng kg^{-1} bw day^{-1}*)	55.35	237.91
DON TDI (*ng kg^{-1} bw day^{-1}*)	1000	1000
Mean for a high consumer (*ng kg^{-1} bw day^{-1}*)	28.69	473.64
% TDI 50th percentile	1.20	19.83
% TDI 50th percentile for a high consumer	2.87	47.36

Deoxynivalenol (DON); 3-acetyl-deoxynivalenol (3-Ac-DON); deoxynivalenol-3-glucoside (DON-3-Glc); 15-acetyl-deoxynivalenol (15-Ac-DON), TDI: Tolerable daily Intake.

reported by Pascari et al (2018) in Spain (1.6%) and lower to the ones obtained by Bauer et al. (2016) (5 10%) and Rodríguez-Carrasco 2015 (10%) in beer consumers from Germany and Ireland respectively. Regarding other products, the TDI in LB scenario that beer provides for exposure to DON is similar to bread (5.3 ng kg^{-1} bw d^{-1}) and cookies (5.7 ng kg^{-1} bw d^{-1}) in the population of Brazil (Savi et al. 2016) and pasta (22 ng kg^{-1} bw d^{-1}) in Spain. It is lower than corn flour (1600 ng kg^{-1} bw d^{-1}) and greater than oat flakes (0.07 ng kg^{-1} bw d^{-1}) in China (Ji et al. 2018).

This is the first study with a large number of mycotoxins analyzed in beer commercialised in Mexico, the country with the largest world export of beer. Mycotoxins were present in a greater proportion in craft beers than in commercial beers. DON and its modified forms (DON-3-Glc, 3-Ac-DON) were most frequently occurring mycotoxins compared to other analized compounds. Although the contamination data obtained in the present study were not above the legal limits, DON intake through beer consumption should not be ignored (contribution to exposure from 1.20 to 19.83% of TDI). An even greater contribution may take place for the population consuming a daily amount of beer above the national average, as Mexican male population (according to WHO reports, men consume six times more alcohol than women).

5.5 Acknoledgement

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), to the Spanish Ministry of Economy, Competitiveness (MINECO, Project AGL2014-55379-P) for funding this work and Consejo Nacional de Ciencia y Tecnología (CONACyT) by the scholarship assigned to Wall-Martínez HA.

5.6 References

Bauer JI, Gross M, Gottschalk C, Usleber E (2016) Investigations on the occurrence of mycotoxins in beer. *Food Control, 63*, 135 139.

Bertuzzi T, Rastelli S, Mulazzi A, et al (2011) Mycotoxin occurrence in beer produced in several European countries. *Food Control, 22*, 2059 2064.

Bryła, M., Ksieniewicz-Woźniak, E., Waśkiewicz, A., et al. (2018) Co-occurrence of nivalenol, deoxynivalenol and deoxynivalenol-3-glucoside in beer samples. *Food Control, 92*, 319 324.

Cámara Nacional de la Industria del Vestido (CANAIVE) (2012) ¿Cuánto mide México? El tamaño sí importa., *Cámara Nacional de la Industria del vestido*. [How much does Mexico measure? size does matter]. [accessed 2018 June 13]. México. www.canaive.com

Castillo-Urueta, P., Carvajal, M., Méndez, I., Meza, F., Gálvez, A. (2011) Survey of aflatoxins in maize tortillas from Mexico City. *Food Additives and Contaminants Part B 4*: 42-51.

Commission Regulation (European Commission) (EC) 401/2006 Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union*:12 34.

Commission Regulation (European Commission) (EC= 1881/2006. Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*: 5-24.

Commission Recommendation (European Commission) (EC) 2013/165 Commission Recommendation of March 27, 2013 on the presence of T-2 and HT-2 toxin in cereal and cereal products (2013/165/EU), *Official Journal of the European Union*: 1-12.

Curie LA (1995) Nomenclature in evaluation of analytical methods including detection and

quantification capabilities (IUPAC Recommendations). Pure and Applied Chemistry 67:1699-1723.

European Food Safety Authority (EFSA) (2010a) Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal, 8*, 1-96.

European Food Safety Authority (EFSA) (2010b) Opinion of the Scientific Panel on Contaminants in the Food Chain on recent scientific information on the toxicity of ochratoxin A. EFSA, Parma, Italy.

European Food Safety Authority (EFSA) (2011a) Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA Journal, 9*, 1 187.

European Food Safety Authority (EFSA) (2011b) Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal*, 1 124.

European Food Safety Authority (EFSA) (2014) Scientific opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal*, 1-61.

Euromonitor International **Beer in México,** (2014) London. Retrieved from Euromonitor Passport database. [accessed 2018 Feb 11]. http://www.euromonitor.com/beer

Food and Agriculture Organization and World Health Organization (FAO/WHO) (2006) The use of microbiological risk assessment outputs to develop practical risk management strategies: Metrics to improve food safety. Report of a Joint FAO/WHO Meeting in collaboration with the German Federal Ministry of Food. Kiel, Germany. [accesed 2018 jun 13].

http://www.who.int/foodsafety/publications/micro/MRA_Outputs.pdf

Food and Agriculture Organization and World Health Organization (FAO/WHO) (2014) Global Alcohol reports: Mexico. *Report of a Joint FAO/WHO* [accesed 2018 jun 14]

http://www.who.int/substance_abuse/publications/global_alcohol_report/profiles/mex.pdf

Gimeno, A., Martins, M. (2003) Micotoxinas y micotoxicosis en animales y humanos. *Talleres gráficos del SRL*, Buenos Aires.

Gonzalez-Osnaya, L., Farres, A. (2011) Deoxynivalenol and zearalenone in Fusarium-contaminated wheat in Mexico City. *Food Additives and Contaminants Part B, 4*, 71 78.

Guzmán-de-Peña, D., Peña-Cabriales, J.J. (2005) Regulatory considerations of aflatoxin contamination of food in Mexico. *Revista Latinoamericana de Microbiologia, 47*, 160 164.

Instituto Nacional de Estadística y Geografía (INEGI) (2017) Estadísticas a propósito de la actividad de elaboración de la cerveza [Statistics on the activity of brewing]. México. [Accessed 2018 [junio 01].

http://cervecerosdemexico.com/wp-content/uploads/2017/08/estadisticas-a-proposito-de-la-elaboracion-de-la-cerveza.pdf

International Agency for Research on Cancer (IARC) (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the evaluation of carcinogenic risk to humans, vol. 56, IARC, WHO, Lyon, France, 245-395.

International Agency for Research on Cancer (IARC) (2002) Aflatoxins Monograph on the evaluation of carcinogenic risks to humans, vol. 82, IARC, World Health Organization, Lyon, France. 171-300.

Ji, X., Yang, H., Wang, J., et al. (2018) Occurrence of deoxynivalenol (DON) in cereal-based food products marketed through e-commerce stores and an assessment of dietary exposure of Chinese consumers to DON. *Food Control, 92*, 391 398.

Joint FAO/WHO Expert Committee on food additives (JECFA) (2010) Summary and conclusions. In: Seventy-Second Meeting, Rome, 16 25 February 2010, FAO/ WHO, Rome, Italy.

Kantar Worldpanel México (2015) "México 2º lugar en consumo de cerveza en Latinoamérica". [Mexico 2nd place in beer consumption in Latin America] [Accessed 2018 June 01]. https://www.kantarworldpanel.com/mx/Noticias-/Mexico-2-lugar-en-consumo-de-Cerveza-en-Latinoamerica.

Karlovsky, P., Suman, M., Berthiller, F., Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T., Dussor, P. (2016) Impact of food

processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Research, 32*, 179-205.

Kirin Holding Company (2016) Global Beer Consumption by Country in 2015. [Accessed 2018 June 01] https://www.kirinholdings.co.jp/english/news/2017/1221_01.html.

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, .J (2009). Occurrence of deoxynivalenol and its major conjugate deoxynivalenol-3-glucoside in beer and brewing intermediates. *Journal of Agricultural and Food Chemistry, 57*, 3187-3194.

Kuzdraliński, A., Solarska, E., Muszyńska, M. (2013) Deoxynivalenol and zearalenone occurrence in beers analyzed by an enzyme-linked immunosorbent assay method. *Food Control, 29*, 22 24.

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, 25*, 732-744.

Landau, D., Binder, K. (2015) A guide to Monte Carlo simulations in statistical physics. *Cambridge university Press*, Cambridge, United Kingdom.

Martínez-Flores, R., García-Aguirre, G., Melgarejo-Hernández, M. (2003) Inspección de aflatoxinas en maíz cultivado, almacenado y transportado en el estado de Tamaulipas, México en 1988. [Inspection of aflatoxins in corn grown, stored and transported in the state of Tamaulipas, Mexico in 1988] Instituto de Biología, UNAM, serie Botánica 74:313 321. http://www.redalyc.org/articulo.oa?id=40074209 , ISSN 0185-254X

Mendoza, J.R., Kok, C.R., Stratton, J., Bianchini, A., Hallen-Adams, H.E. (2017) Understanding the mycobiota of maize from the highlands of Guatemala, and implications for maize quality and safety. *Crop Protection, 101*, 5-11.

Molto, G., Samar, M., Resnik, S., Martínez, E.J., Pacin, A. (2000) Occurrence of trichothecenes in Argentinean beer: A preliminary exposure assessment. *Food Additives and Contaminants, 17*, 809-813.

Monbaliu, S., Van Poucke, C., Van Peteghem, C., Van Poucke, K., Heungens, K., De

Saeger, S. (2009) Development of a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for sweet pepper analysis. *Rapid Communications in Mass Spectrometry, 21*, 3-11.

NOM-188-SSA1-2002. Norma Oficial Mexicana. Productos y Servicios. Control de aflatoxinas en cereales para consumo humano y animal. Especificaciones sanitarias.

NOM-247-SSA1-2008. Productos y servicios. Cereales y sus productos. Cereales, harinas de cereales, sémolas o semolinas. Alimentos a base de: cereales, semillas comestibles, de harinas, sémolas o semolinas o sus mezclas. Productos de panificación. Disposiciones y especificaciones sanitarias y nutrimentales. Métodos de prueba.

Papadopoulou-Bouraoui, A., Vrabcheva, T., Valzacchi, S., Stroka, J., Anklam, E. (2004) Screening survey of deoxynivalenol in beer from the European market by an enzyme-linked immunosorbent assay. *Food Additives and Contaminants, 21*, 607-617.

Pascari, X., Ortiz-Solá, J., Marín, S., Ramos, A.J., Sanchis, V. (2018a) Survey of mycotoxins in beer and exposure assessment through the consumption of commercially available beer in Lleida, Spain. *LWT Food Science and Technology, 92,* 87-91.

Pascari, X., Ramos, A.J., Marín, S., Sanchis, V. (2018b) Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. *Food Research International, 103,* 121-129.

Peters, J., Van Dam, R., Van Doorn, R., Katerere, D., Berthiller, F., Haasnoot, W., Nielen, M.W.F. (2017) Mycotoxin profiling of 1000 beer samples with a special focus on craft beer. *PLoS One 12*: 1-27.

Piacentini, K.C., Rocha, L.O., Fontes, L.C., Carnielli, L., Reis, T.A., Corrêa, B. (2017) Mycotoxin analysis of industrial beers from Brazil: The influence of fumonisin B_1 and deoxynivalenol in beer quality. *Food Chemistry, 218,* 64-69.

Reyes-Velázquez, W.P., Isaías-Espinoza, V.H., Rojo, F., Jiménez-Plascencia, C., De Lucas-Palacios, E., Hernández-Góbora, J., Ramírez-Álvarez, A. (2008) Occurrence of fungi and mycotoxins in corn silage, Jalisco State, México. *Revista Iberoamericana de Micologia, 25,* 182-185.

Robledo, M., Marín, S., Ramos, A.J. (2001) Contaminación natural con micotoxinas en

maíz forrajero y granos de café verde en el Estado de Nayarit (México) [Natural contamination with mycotoxins in fodder corn and green coffee beans in the State of Nayarit (Mexico)]. *Revista Iberoamericana de Micologia, 18,* 141-144.

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., Mañes, J. (2015) Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry, 178,* 149-55.

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.

Savi, G.D., Piacentini, K.C., Tibola, C.S., Santos, K., Maria, G.S., 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.

Scientific Committee for Food (SCF) (2000) Opinion of the scientific committee on food on *Fusarium* toxins. Part 2: Zearalenone (ZEA), *Official Journal of the European Union.*

Scientific Committee for Food (SCF) (2002). Opinion on *Fusarium* toxins, Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol. *Official Journal of the European Union.*

Scientific Committee for Food (SCF) (2003) Updated opinion of the scientific committee on food on fumonisin B_1, B_2 and B_3. *Official Journal of the European Union.*

Secretaría de Economía (SE) (2015) Industria de la Cerveza en México [Beer Industry in Mexico]. Subsecretaría de Industria y Comercio. Catálogo de Publicaciones de la Administración General del Estado: http://publicacionesociales.boe.es/NIPO:280-16-007-6 Deposito legal: M-25254-2016.

Shetty, P.H., Jespersen, L. (2006) *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends Food Science and Technology, 17,* 48-55.

Tangni, E.K., Ponchaut, S., Maudoux, M., Rozenberg, R., Larondelle, Y. (2002) Ochratoxin A in domestic and imported beers in Belgium: occurrence and exposure assessment. *Food Additives and Contaminants, 12,:* 1169-1179.

Torres, M.R., Sanchis, V., Ramos, A.J. (1998) Occurrence of fumonisins in Spanish beers analyzed by an enzyme-linked immunosorbent assay method. *International Journal of Microbiology*, *39*, 139 143.

U.S. Environmental Protection Agency (US EPA) (1997) "Exposure factors handbook. US EPA/600/P-95/002Fb", Volume II, Food ingestion factors, Office of Research and Development, Washington, DC.

Unites States Department of Agriculture (USDA) (2015) Mycotoxin Handbook. Grain Inspection Packers and Stockyards Administration. Washington, D.C. [accessed 2018 June 13].

https://www.gipsa.usda.gov/fgis/handbook/MycotoxinHB/MycotoxinHandbook_2016-07-12.pdf

Zain, M.E. (2011) Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, *15*, 129 144.

Chapter 6. Fate of zearalenone, deoxynivalenol and deoxynivalenol-3-glucoside during malting process

Pascari, X., Gil–Samarra, S., Marín, S., Ramos, A. J., & Sanchis, V.

Mycotoxin Research 99 (2019) 540-546

6.1 Abstract

Malting was simulated using two different batches of barley as raw material: a naturally contaminated batch and laboratory inoculated (with a deoxynivalenol (DON) and zearalenone (ZEN) producing *Fusarium graminearum* strain) one. Up to three contamination levels were prepared, every process being carried out in triplicate.

A significant washout effect on DON was observed by the end of the first water phase (between 22.4 and 34 % reduction) with an even more pronounced reduction (up to 75 % decrease) by the end of the steeping process. ZEN content remained almost unchanged. Germination was characterized by an increase in all the three toxins (ZEN, DON and DON-3-Glc) concentrations, however showing a decreasing trend in the last 24 h of the stage, compared to the first day of germination. Kilning lead to a significant reduction of DON in the naturally contaminated batch, nevertheless an increase in all other toxins and contamination levels was observed.

Keywords

Deoxynivalenol, deoxynivalenol-3-glucoside, zearalenone, HPLC-DAD/FLD, malting

6.2 Introduction

Barley was domesticated, for the first time, in the southern part of Fertile Crescent (Israel-Jordan) about 8000 BC (Badr et al., 2018) and in about 5000 BC the first records exist about malting and brewing practices by early societies (Meussdoerffer, 2009). Malting process is a crucial stage in brewing and barley quality plays the most important role.

Fusarium Head Blight disease (FHB), which is highly occurring on barley crops, is an important issue for farmers and brewers, firstly, because the disease drastically affects grains' quality (*e.g.* germination energy, nutrients content) and secondly, because it is usually accompanied with mycotoxins accumulation. The main species responsible for FHB are *Fusarium graminearum, Fusarium avenaceum* and *Fusarium culmorum*. The predominant mycotoxins contaminating malting barley are deoxynivalenol (DON), zearalenone (ZEN), nivalenol (NIV), T-2 and HT-2 toxins, which may induce neurotoxic, teratogenic, immunosuppressive, oestrogenic and carcinogenic effects in case of ingestion, inhalation or skin contact (Pestka, 2007; Zinedine, Soriano, Moltó, & Mañes, 2007). A previously published review found DON and ZEA as the most commonly occurring mycotoxins on malting barley crops worldwide (Pascari, Ramos, Marín, & Sanchís, 2018).

Besides mycotoxins co-occurrence, a transformation of the original mycotoxins molecule into a different structure is possible which might result unreported because of its undetectability by traditional analytical methods. Deoxynivalenol-3-glucoside (DON-3-Glc) is the most common modified form of DON as a result of plant detoxification mechanism catalyzed by glucosyltransferase enzyme (Freire & Sant'Ana, 2018). However, after ingestion, the digestive tract hydrolases tend to break the glucosyl bound and DON is freed in the body (Berthiller et al., 2013). The abovementioned facts corroborate the need for a deeper study of mycotoxins fate during processing in order to evaluate the possible risk for consumers and to revise the current legal limits for mycotoxins and their derived forms.

There are various researchers dedicated to study malting barley infestation with *Fusarium* spp. and their mycotoxins (Beccari, Caproni, Tini, Uhlig, & Covarelli, 2016; Medina et al., 2006; Piacentini, Savi, Pereira, & Scussel, 2015). After proving fungal activity during

germination process, by one hand, and the discovery of modified mycotoxins, by the other, researchers started to question the fate of mycotoxins during malting (Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Kostelanska et al., 2011; Pietri, Bertuzzi, Agosti, & Donadini, 2010; Vaclavikova et al., 2013).

The aim of the present work is to study the fate of DON, DON-3-Glc and ZEN, during malting process, focusing on naturally contaminated barley. Moreover, a comparison with mycotoxins' fate during malting process of laboratory infested barley was performed. Also, different concentration levels were taken into consideration for the study of the evolution of mycotoxins level throughout the process. The results will also allow to identify the stages at which significant changes in mycotoxins' concentration take place.

6.3 Materials and methods

6.3.1 Chemicals and reagents

Deoxynivalenol, deoxynivalenol-3-glucoside and zearalenone standards were purchased from Romer Labs (Tulln, Austria) and stored at -18°C. Solid standards (deoxynivalenol and zearalenone) were resuspended in methanol before storage.

Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol and acetonitrile (HPLC grade) were purchased from Scharlab (Sentmenat, Spain). Sodium chloride was purchased from Fischer Scientific (New Jersey, USA) whereas ammonium formate from Sigma-Aldrich (St. Louis, MO, USA).

6.3.2 Barley

Uncontaminated malting barley was supplied by the malting plant Malteria la Moravia S.L. (Bell-lloc d'Urgell, Spain). The absence of DON, DON-3-Glc and ZEN in the uncontaminated batch was confirmed by LC-DAD/FD system (see 2.7). Barley grains were stored in a cool and dry place until malting. Naturally contaminated malting barley, already containing DON, DON-3-Glc and ZEN, was taken from a rejected batch in a food company.

6.3.3 DON and ZEN contamination of the inoculated barley

A strain of *F. graminearum* (F.46) (producer of DON and ZEN) from the Food Technology Department's culture collection was used for fungal contamination of the grain and mycotoxin production.

Barley grains were disinfected according to Andrews, Pardoel, Harun, & Treloar (1997). Briefly, 500 g of grains were immerged into 0.4% chlorine solution for 2 minutes and then abundantly rinsed with sterile distilled water.

Then, the grains were placed in a hermetically closed sterile ISO bottle and left overnight with a small amount of water (approximately 340 cm^3/kg) at 4°C to achieve a water activity of 0.99 (Aqualab Series 3 TE, Decagon Devices Inc., Washington, USA). Afterwards, the humidified barley was aseptically transferred to Petri dishes and 1 mL of spore suspension of *F. graminearum* (10^6 spores/mL) was pulverized on each dish. Petri dishes with barley were then incubated at 25°C for 30 days. Afterwards, the contaminated grains were dried at 40°C for 24 hours, homogenized and DON and ZEN levels were determined (see 2.6 and 2.7).

6.3.4 Experimental design

Experiments were carried out using naturally contaminated barley. Moreover, an additional experiment was performed using barley inoculated in the laboratory in order to assess the impact of two sources of contamination on mycotoxins' concentration change during malting. Before malting, contaminated and uncontaminated barley were mixed to achieve two and three concentration levels for naturally contaminated and laboratory inoculated barley batches, respectively. All tested contamination levels were prepared per triplicate. Table 16 shows the data concerning the prepared samples.

Table 16: Rate of mycotoxins contaminated barley grains added to uncontaminated barley in the mixes prepared for micro malting

Barley batches	**Mycotoxins contaminated grains contained in the mix, %**		
	Level 1	***Level 2***	***Level 3***
Naturally contaminated	62	100	NP*
Laboratory infected	5.3	11.36	22.8

*NP=not performed

6.3.5 Laboratory scale malting

Laboratory scale malting was performed following three main malting stages (steeping, germination and kilning). Briefly, 60 g of dry grains were placed in glass recipients of 100 mL and washed under flowing water stream before steeping. The process was repeated per triplicate for every desired mycotoxin level and barley batch. Steeping stage consisted of alternated controlled cycles of water immersion and aeration in order to achieve moisture content of 45-48% by the end of the stage. The following order of cycles was applied: 13 hours of water immersion, 9 hours of aeration (all water from the previous stage was drained and barley grains were exposed to a gentle air flow in the thermoregulated chamber with a periodic mixing to facilitate water evaporation on grain's surface) and then another 4 hours of water immersion. The process was carried out at 10 °C in a thermoregulated chamber Memmert GmbH+ (Schwabach, Germany). At the end of steeping, the excess water was drained, and the samples were placed at 15 °C for germination for 96 hours. During the first 24 hours of germination, the grains were gently mixed, and water was sprayed (40 cm^3/kg) every 4 – 5 hours, in order to avoid grain's surface dehydration. The grains were gently mixed daily to provide a good aeration and to ensure a good germination yield. Once the germination was over, all the samples were located in the drying chamber (Malting Plant "Malteria la Moravia SL", Bell-lloc d'Urgell, Spain), where four temperature levels were applied: 3 hours at 50°C, 16 hours at 60°C, 2 hours at 68°C and 3 hours at 85°C.

Sampling was performed after the following stages of malting process: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, then every 24 h of

germination process (g24h, g48h, g72h and g96h) and finally, at the end of drying process (dry24h). Totally, twenty-seven recipients were prepared for each desired contamination level (9 sampling stages per replicate).

6.3.6 Sample preparation

All samples were dried (40°C) and ground with IKA® A11 Basic (Darmstadt, Germany). For the extraction of DON, DON-3-G and ZEA specific immunoaffinity columns from R-Biopharm (Rhone LTD, Glasgow, Scotland) were used.

6.3.6.1 DON and DON-3-Glc extraction

Five grams of ground sample was mixed with 1 g of sodium chloride and 40 mL of Mili Q water into a 250 mL Erlenmeyer flask, followed by 30 minutes stirring. Then, samples were centrifuged for 10 minutes at 1846 g. Supernatant was filtered through a glass microfiber paper filter (Whatman, Maidstone, UK) and 2 mL of the filtrate was passed through the column. The immunoaffinity column was then washed with 10 mL of bi-distilled water and the toxins were eluted with 3 mL of methanol HPLC grade (1.5 mL performing back-flushing and 1.5 mL for the final elution). Samples were then evaporated under a gentle nitrogen stream at 40 °C. Dry extract was resuspended in 0.5 mL of mobile phase, filtered with nylon filter (0.4 µm) and 50 µL were injected into UHPLC-DAD system.

6.3.6.2 ZEN extraction

Five grams of ground sample was mixed with 25 mL of extraction solvent acetonitrile:water (75:25, v/v) and stirred for 30 min. Samples were centrifuged for 10 min at 1846 g and 10 mL of supernatant was mixed with 40 mL of Phosphate Buffer Saline at pH of 7.4 (PBS: 8 g of sodium chloride, 1.2 g of disodium hydrogen phosphate, 0.2 g of potassium di-hydrogen phosphate, 0.2 g of potassium chloride and 1 L of distilled water). The pH of the obtained extract was adjusted back to 7.4 with 2 M solution (80 g/L) of sodium hydroxide. The obtained 50 mL was passed through the immunoaffinity column which was afterwards washed with 20 mL of PBS. ZEN was eluted with 3 mL of acetonitrile (1.5 mL performing back-flushing and 1.5 mL for the definite elution). Samples were evaporated under a low nitrogen stream at 40°C. The dry extracts were resuspended in 1 mL of acetonitrile:water (50:50, v/v) and 50 µL were injected into UHPLC-FD system.

6.3.7 UHPLC analysis

6.3.7.1 DON and DON-3-Glc analysis

For DON and DON-3-Glc detection, an Agilent Technologies 1260 Infinity UHPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD) and a Gemini® C18 column from Phenomenex (California, USA) with a particle size of 5 µm and a pore size of 110 Å was used as a stationary phase. Absorption wavelength was setup at 220 nm. The mobile phase was composed of methanol:acetonitrile:water (5:5:90, v/v/v) and set at a flow rate of 1 mL/min.

6.3.7.2 ZEN analysis

For ZEN analysis, the same equipment as in 2.7.1 and same column were used but with an Agilent 1260 Infinity Fluorescence detector. The excitation and emission wavelengths were 274 nm and 455 nm, respectively. The mobile phase was acetonitrile:water (60:40, v/v) and a pH adjusted to 3.2 with acetic acid. Flow rate was set at 0.6 mL/min.

6.3.7.3 Validation of the analytical methods

Analytical methods were checked for selectivity, linearity and precision. Selectivity was checked by injecting 50 µL of standard solution for at least three times, comparing retention time and peak resolution between injections. For linearity check, a calibration curve of at least seven concentration levels was prepared and injected into the system, generating a linear regression plotting solutions' concentration versus peak area. Finally, precision was evaluated preparing blank barley samples spiked with DON, DON-3-Glc and ZEN at several concentration levels and percentage recoveries were identified. The limit of detection (LOD) was considered as three times the signal of blank noise (Table 17). Method characteristics of performance are according to Commission Regulation (EC) Nr. 401/2006 (European Commission, 2006).

Table 17: Method performance parameters for deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-G) and zearalenone (ZEA)

Mycotoxin	LOD[1], µg/kg	Spiking levels, µg/kg	Replicates	Recovery±SD[2], %	RSD_r[3], %
DON	20	143.4	5	121.46±12.27	10.11
		750	7	105.6±6.32	5.99
		1075.5	7	74.12±3.36	4.54
		1434.0	5	82.93±0.91	1.10
DON-3-Glc	30	50	3	93.0±6.82	7.33
		250	5	80.76±13.54	16.76
		500	3	77.20±12.36	16.01
ZEN	2.5	35	5	96.07±11.02	11.47
		75	7	98.17±4.88	4.98
		150	5	75.18±4.43	5.90

[1]LOD limit of detection; [2]SD standard deviation; [3]RSD_r relative standard deviation; DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside; ZEN=zearalenone.

6.3.8 Statistical analysis

ANOVA test was applied to assess the effect of the type of contamination and the initial mycotoxins concentration. Ad hoc multiple comparison Tukey HSD's test was also performed to locate more specifically the significant changes during malting process, using JMP Pro® 13 software (SAS Institute, New York, USA).

6.4 Results and discussion

6.4.1 Fate of DON and DON-3-Glc during malting

There are several published studies on *Fusarium* mycotoxins' transfer from barley to malt (Kostelanska et al., 2009; Lancova et al., 2008), but the majority used artificially contaminated grains, either with standard mycotoxin solutions or with a mycotoxin producer *Fusarium* spore suspension.

Firstly, the results on naturally contaminated barley will be presented. Table 18 shows the transfer of DON and DON-3-Glc throughout malting process for naturally contaminated barley. As showed by previous studies, DON was partially washed out from barley grains during steeping due to its water solubility. However, its decrease achieved a lower extent

compared to the results obtained by Lancova et al. (2008) in their work (90% of DON was washed out compared to the initial content): for naturally contaminated barley, in the present work, a significant decrease ($p<0.05$) of 22.4% after 13h of water phase, reaching a 75.52% decrease by the end of the process was recorded .

Table 18: Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-Glc) mean concentration (µg/kg) ±SD[1] at each malting stage in naturally contaminated barley

Malting stage	Level 1, µg/kg		Level 2, µg/kg	
	DON	DON-3-Glc	DON	DON-3-Glc
T=0	407.21±54.1 a	64.16±40.3 a	676.49±304.6 a	170.21±123.5 a
S13h	316.19±118.5 ab	72.23±48.7 a	235.85±20 b	125.10±42.2 a
S22h	216.26±71.3 bc	108.52±75.9 a	172.22±24.9 b	69.60±26.3 a
S26h	209.02±80.3 bc	99.95±70.5 a	165.63±54.3 b	69.82±49.2 a
G24h	166.41±27.4 bc	116.96±43.1 a	195.26±60.6 b	89.43±50 a
G48h	244.98±17.9 abc	194.35±103.1 a	212.44±58.1 b	171.05±50 a
G72h	169.03±37.1 bc	183.08±81 a	139.99±10.4 b	227.80±88.8 a
G96h	130.33±36.2 c	173.25±62.5 a	112.86±19.4 b	70.88±94.6 a
Dry24h	217.3±69.6 bc	242.12±107 a	143.42±26.5 b	127.44±115.9 a

[1] SD=standard deviation; Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different); Samples: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, every 24 h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).

DON-3-Glc behaved significantly different depending on the initial concentration ($p<0.05$): a constant increase in DON-3-Glc concentration for low level of contamination of the naturally contaminated barley (Figure 8a) was observed, but a variable trend in the high contamination level occurred in the steeping process.

Germination temperature and humidity, being close to the optimal for *Fusarium* development (Medina et al., 2006), explain the increase in DON concentration at this stage in all the studied scenarios, a peak concentration being achieved on the second

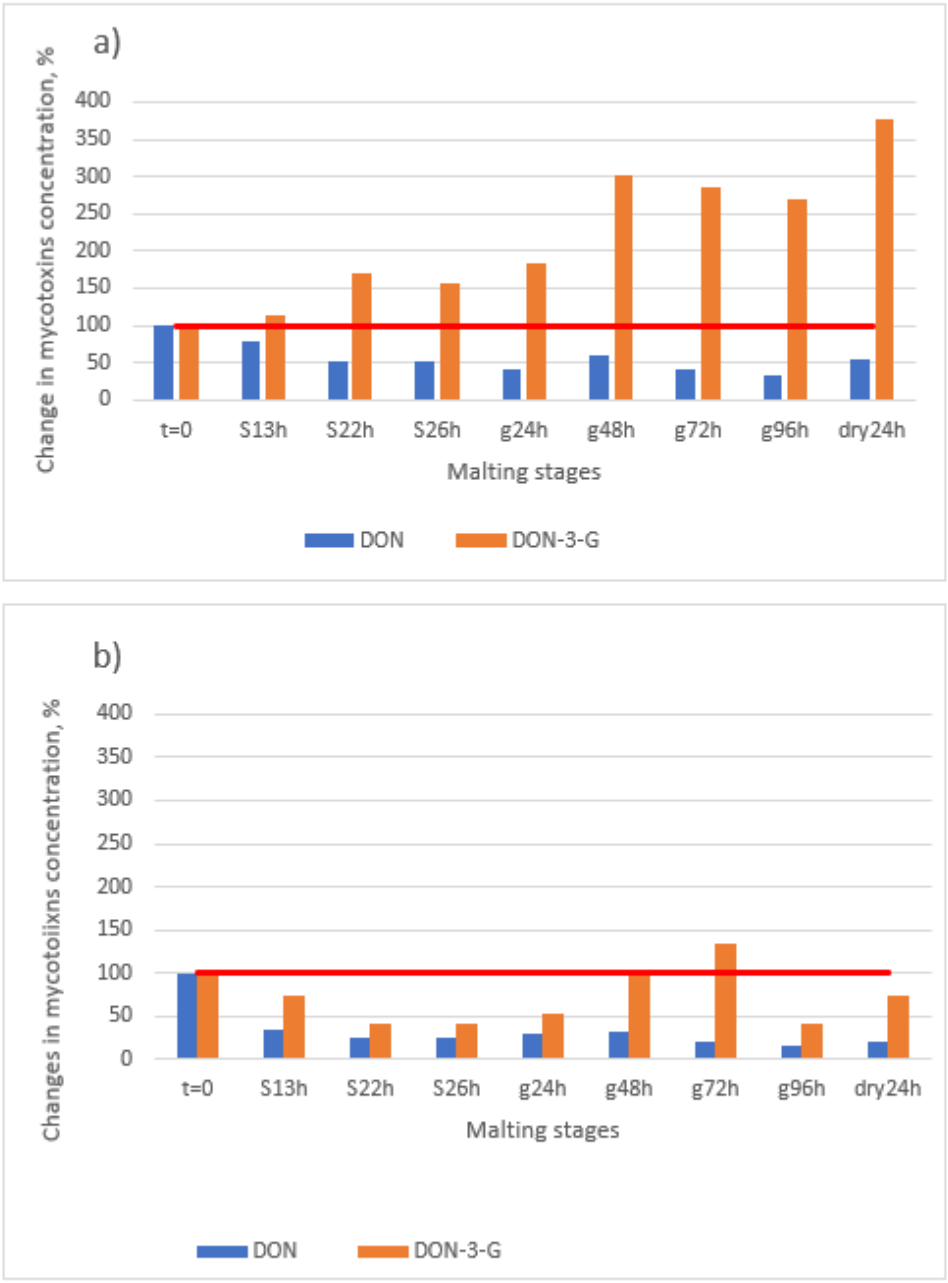

Figure 8: Evolution of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) concentrations in naturally contaminated barley at a) low and b) high contamination levels during malting process. Samples: right after barley mixing (t=0); 13h (s13h), 9h (s22h) and 4h (s26h) of steeping, every 24h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).

day of the process. No fungal growth was observed in naturally contaminated barley batches. Worth noting, DON level was quite similar after 48h of germination whatever the initial contamination level, within the same contaminated batch, nevertheless its level was significantly lower compared to the initial contamination (t=0). DON-3-Glc levels also increased, however its increase was intensified by the end of the germination process, probably due to the continuous accumulation of glucose molecules and intensification of enzymatic DON glycosylation reaction (Maul et al., 2012). Nonetheless, Lemmens et al. (2005), in their work, identified a close relationship between DON-3-Glc formation and the resistance of the grains variety to DON. DON's level decrease on the germination step was accompanied by an important increase in DON-3-Glc concentration at the same malting stage (from 20 to 147% increase after the second day of germination, in the case of low level of contamination in the naturally infected barley and from 11.5 to 92.8% increase in the batch with a higher contamination). The last 24h of germination were characterized by a significant decrease in both DON and DON-3-Glc concentrations in all the samples.

Kilning, as proven by previously reported studies, did not show any destructive effect on either DON nor DON-3-Glc levels in the case of naturally contaminated barley (Lancova et al., 2008). Moreover, an increase of mycotoxin concentration compared to the end of germination process was noticed in this batch, particularly for the low levels of contamination: 21.3 and 4.5% increase in DON for low and high contamination levels, respectively; 107.3 and 33.2% increase in DON-3-Glc concentration for low and high contamination levels, respectively.

The same decreasing trend of DON concentration occurred in the laboratory inoculated barley batch after steeping (approximately 34% decrease), but only for the low contaminated batch and almost no changes were reported in the two other contamination levels (Table 19). This may be partially explained by DON washing from the grains with steeping water (Maul et al., 2012) and partially by the weight difference between the two types of grains used (molded grains are lighter than the

Table 19: Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-Glc) average concentration (µg/kg) ± SD at each malting stage in laboratory contaminated barley

Malting stage	Low concentration, µg/kg		Medium concentration, µg/kg		High concentration, µg/kg	
	DON	DON-3-Glc	DON	DON-3-Glc	DON	DON-3-Glc
T=0	117.33±43 b	<LOD	164.88±52.4 b	<LOD	167.72±88.2 b	<LOD
S13h	70.01±36.5 b	34.8±32.1 b	101.04±7 b	<LOD	86.99±13.4 b	<LOD
S22h	50.40±10.6 b	<LOD	109.71±50.3 b	<LOD	146.34±117.2 b	67.31±44.2 c
S26h	39.65±8.9 b	38.36±38.3 b	126.94±36.7 b	53.05±33.8 a	166.09±11 b	103.4±38.3 c
G24h	150.68±24 b	37.06±36 b	154.78±23.6 b	35.45±33.2 a	167.79±10.8 b	125.46±18.5 bc
G48h	4079.01±2826.6 a	527.26±351.9 ab	5320.28±887.3 a	501.37±590.8 a	4363.42±1167.7 a	1072.73±826.6 ab
G72h	3638.83±1732.1 a	1174.66±580.5 a	4563.66±1578.5 a	1057.21±1154.8 a	4603.73±985.5 a	513.08±235 bc
G96h	143.81±157.1 b	254.68±367.6 b	676.90±554.2 b	1362.21±1259.4 a	1106.44±469.3 b	1620.94±394.7 a
Dry24h	235.15±55.3 b	260.84±85.3 b	844.16±372.2 b	1179.35±550.3 a	351.55±270 b	357.31±340.3 bc

[1] SD=standard deviation; [2] Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different). Samples: right after barley mixing (t=0); 13h (s13h), 9h (s22h) and 4h (s26h) of steeping, every 24h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h)

uncontaminated ones and might be accidentally removed with the drained water). The initial low DON-3-Glc concentration (<LOD) in laboratory inoculated batch and the moderate increment during steeping (which may result from the activation of grains enzymatic equipment), clearly shows that its increase is purely a part of grains' metabolism.

Fungal growth during germination process in laboratory infected barley led to a greater DON concentration rise, especially in the samples with a lower level of contamination (9066, 3496.3 and 2671.6% rise in the contamination levels 1, 2 and 3, respectively). Also, DON-3-G concentration raised following the same pattern as DON (2959.1, 1892.8 and 396.2% for low, medium and high contamination levels). In laboratory inoculated barley, DON and DON-3-Glc concentrations variations behaved significantly different compared to the naturally contaminated batch. Only low levels of contamination showed a very similar behavior to naturally contaminated barley (increase of DON and DON-3-Glc concentrations by 63.5 and 2.1%, respectively). After kilning, almost a 78% decrease of DON-3-Glc was observed. Also, in the medium contamination level, there was a 24.7% increase in DON content and a 13.4% decrease in DON-3-Glc.

6.4.2 ZEN fate during malting

There is scarce information concerning the fate of ZEN during malting (Habschied, Šarkanj, Klapec, & Krstanović, 2011). A very similar trend was observed between the two levels of contamination for naturally contaminated barley (Table 20). More than 40% decrease in ZEN concentration was obtained after removing the first steeping water (t=13h) in naturally contaminated barley. During the following steps of the steeping process, the decrease was not so important (48 and 72% for low and high contamination levels, respectively). No significant differences, however, were observed.

Germination process was characterized by an increase of ZEN's concentration at the beginning of the process, with a peak at 48h, followed by a decrease compared to ZEA's concentration before the beginning of germination. No fungal growth was

noticed in naturally contaminated barley and the increase in ZEN concentration to an almost same level as before malting process could be explained by the high amount of water available for reactions and an elution of ZEN from grain's matrix might take place, however there are no studies investigating ZEN transfer through malting which would confirm this statement.

Except for low contaminated samples (decrease up to 64% in naturally contaminated barley), kilning process led to an increase in ZEN levels present in barley compared to the initial concentration, with 64% in the high level of contamination of naturally contaminated barley, although the changes were not significant. Considering the physical parameters of the kilning process (moderate change in temperature and water evaporation), infected grains could be still subjected to ZEN production by *F. graminearum* (Kostelanska et al., 2011).

The evolution of ZEN concentration in the laboratory inoculated barley showed almost no change up after 48h of germination (Table 20), where ZEN level increased by 350, 134.3 and 40% compared to the initial concentration in contamination levels 1, 2 and 3, respectively, although such increases were not significant.

Table 20: Zearalenone (ZEN) average concentration (µg/kg) ±SD[1] at each malting stage for both naturally contaminated and laboratory inoculated barley batches.

Malting stage	Naturally contaminated barley		Laboratory infected barley		
	Level 1, µg/kg	***Level 2, µg/kg***	***Level 1, µg/kg***	***Level 2, µg/kg***	***Level 3, µg/kg***
T=0	38.42±33.98 a[2]	55±31.26 a	33.97±6.14 a	109.74±47.26 a	194.22±96.69 a
S13h	22.52±6.68 a	17.56±1.66 a	34.6±21.28 a	131.95±92.28 a	272.72±189.85 a
S22h	17.44±2.98 a	14.61±0.97 a	32.82±8.96 a	60.89±14.5 a	200.42±211.22 a
S26h	19.69±3.57 a	15.48±3.03 a	36.19±25.5 a	58.29±20.74 a	239.35±195.25 a
G24h	20.2±4.36 a	31.45±26.77 a	42.83±28.82 a	230.6±257.6 a	248.13±148.9 a
G48h	49.26±44.84 a	30.44±11.49 a	153.06±188.98 a	257.12±109.03 a	271.88±88.75 a
G72h	36.34±17.97 a	21.73±3.4 a	152.55±44.94 a	268.67±138.34 a	314.42±64.61 a
G96h	31.69±5.98 a	17.42±2.16 a	220.2±257.52 a	126.24±36.67 a	229.68±29.24 a
Dry24h	17.73±2.31 a	42.28±31.17 a	27.91±7.44 a	155.77±89.85 a	431.41±166.52a

[1] SD=standard deviation; [2] Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different). Samples: right after barley mixing (t=0); 13h (s13h), 9h (s22h) and 4h (s26h) of steeping, every 24h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).

6.5 Conclusion

The transfer of DON and ZEN through malting process was investigated in naturally contaminated barley. From the three malting stages (steeping, germination and kilning), the effect on the three mycotoxins had a similar tendency. It was confirmed, as in previous studies, that DON is washed out with steeping water (up to 75% of reduction). ZEN concentration remains stable during malting. DON contamination, on the contrary, is significantly reduced during malting process. An increase of DON-3-Glc levels was reported at the end of the process (277% in the low level of contamination), however a decrease was registered for the level 2 of contamination (25%). Considering the variability of the results, the abovementioned changes in DON-3-Glc concentration were not significant.

In the case of laboratory contaminated barley, on the contrary, malting led to a rise in DON and DON-3-Glc concentration but without overpassing the legal limits, except for the medium contamination level. ZEN was subjected to an almost two-fold increase in medium and high contamination levels, compared to its initial concentration, however the performed statistical analysis did not prove this increase significant.

Besides showing the decrease in DON concentration, this study shows that barley analysis and diversion at the entry point and implementing good storage practices is highly important to ensure a safe final product. Also, considering the obtained differences between the naturally contaminated batch and the laboratory infested one, it would be highly recommended that the following studies on mycotoxin transfer to be performed with naturally present mycotoxins to ensure a more realistic situation.

6.6 Acknowledgement

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2014-55379-P) for funding this work. The authors are also grateful to the malting plant La Moravia SL for the supplied material and installations.

6.7 References

Andrews, S., Pardoel, D., Harun, A., & Treloar, T. (1997). Chlorine inactivation of fungal spores on cereal grains. *International Journal of Food Microbiology*, *35*, 153–162.

Badr, A., Mu, K., Scha, R., Rabey, H. El, Effgen, S., Ibrahim, H. H., Pozzi, C., Rohde, W., Salamini, F. (2018). On the Origin and Domestication History of Barley (Hordeum vulgare). *Genetic Engineering and Biotechnology Research Institute*, *17*, 499–510.

Beccari, G., Caproni, L., Tini, F., Uhlig, S., & Covarelli, L. (2016). Presence of Fusarium species and other toxigenic fungi in malting barley and multi-mycotoxin analysis by liquid chromatography-high-resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, *64*, 4390–4399.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. De, Haesaert, G., Karlovsky, P., Oswald, I. P., Seefelder, W., Speijers, G., Stroka, J. (2013). Masked mycotoxins: A review. *Molecular Nutrition and Food Research*, *57*, 165–186.

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. *Official Journal of the European Union*

Commission Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: an updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189–205.

Habschied, K., Šarkanj, B., Klapec, T., & Krstanović, V. (2011). Distribution of zearalenone in malted barley fractions dependent on *Fusarium graminearum* growing conditions. *Food Chemistry*, *129*, 329–332.

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

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, J.,

Fiala, J., Scott, P. M., Berthiller, F., Krska, R. (2009). Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3- glucoside, in beer and some brewing intermediates. *Journal of Agricultural and Food Chemistry*, *57*, 3187 3194.

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.

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 & Contaminants: Part A*, *25*, 732 744.

Lemmens, M., Scholz, U., Berthiller, F., Dall 'asta, C., Koutnik, A., Schuhmacher, R., Adam, G., Buerstmayr, H., Mesterházy, A., 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.

Maul, R., Müller, C., Rieß, S., Koch, M., Methner, F. J., & Irene, N. (2012). Germination induces the glucosylation of the Fusarium mycotoxin deoxynivalenol in various grains. *Food Chemistry*, *131*, 274 279.

Medina, Á., Valle-Algarra, F. M., Mateo, R., Gimeno-Adelantado, J. V., Mateo, F., & Jiménez, M. (2006). Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria, Aspergillus* and *Fusarium*. *International Journal of Food Microbiology*, *108*, 196 203.

Meussdoerffer, F. G. (2009). A comprehensive history of beer brewing, pp. 1 42 in *Handbook of Brewing: Processes, Technology, Markets* edited bby H. M. Weiheim, Germany.

Pascari, X., Ramos, A. J., Marín, S., & Sanchís, V. (2018). Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. *Food Research International*, *103*, 121 129.

Pestka, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal*

Feed Science and Technology, *137*, 283 298.

Piacentini, K. C., Savi, G. D., Pereira, M. E. V, & Scussel, V. M. (2015). Fungi and the natural occurrence of deoxynivalenol and fumonisins in malting barley (Hordeum vulgare L.). *Food Chemistry*, *187*, 204 209.

Pietri, a, Bertuzzi, T., Agosti, B., & Donadini, G. (2010). Transfer of aflatoxin B1 and fumonisin B1 from naturally contaminated raw materials to beer during an industrial brewing process. *Food Additives & Contaminants. Part A*, *27*, 1431 1439.

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.

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.

Chapter 7. Transfer of *Fusarium* mycotoxins from malt to boiled wort

Pascari, X., Rodríguez–Carrasco, Y., Juan, C., Mañez, J., Marín, S., Ramos, A. J., & Sanchis, V.

Food Chemistry 278 (2019) 700–710

7.1 Abstract

The fate of deoxynivalenol, deoxynivalenol-3-glucoside, 3- and 15-acetyl-deoxynivalenol, zearalenone, (α- and β-zearalenol and fumonisins (fumonisin B_1 and fumonisin B_2) through mashing and wort boiling was studied. Three different mycotoxin contamination scenarios were considered. In almost all samples an increase in the level of mycotoxins in wort was observed during mashing followed by a decrease after just 30 min of the process, with levels remaining constant until the end of boiling. Deoxynivalenol and its metabolites were reduced to their initial level contained in the malt before mashing, or even lower, however in none of the samples they were completely eliminated. Zearalenone was not quantitated at the end of boiling, although there was a significant initial level of ZEN. β-zearalenol remained unaltered during the process. Fumonisins were reduced by between 50 and 100 per cent during mashing and boiling.

Keywords

Deoxynivalenol, zearalenone, fumonisins, masked mycotoxins, HPLC-MS/MS

7.2 Introduction

Cereal grains might be contaminated by molds both in the field and during storage. Apart from commodity losses, fungal contamination may represent a safety risk because of the synthesis of mycotoxins by certain strains. Barley, which is the main ingredient in beer production, is predominantly exposed to fungal infestation throughout FHB disease (Fusarium Head Blight) (Baenziger, Fredy, Nopsa, & Bockus, 2015). The main *Fusarium* mycotoxinogenic species involved in FHB in barley are *F. graminearum*, *F. avenaceum*, *F. culmorum* and, to a lesser extent, *F. proliferatum* (Nielsen, Cook, Edwards, & Ray, 2014). They are known as mainly being deoxynivalenol (DON), zearalenone (ZEN) and fumonisins (FBs) producers (Beccari, Senatore, Tini, Sulyok, & Covarelli, 2018). An important aspect that has been investigated during recent years are the modified forms of mycotoxins, which initially might be formed by the plant or the fungus (Berthiller et al., 2013; Rychlik et al., 2014; Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012). Concern was raised because of their proven bioavailability and toxicity in humans and animals (Freire & Sant'Ana, 2018). Considering the abovementioned, researchers began to be concerned with both their co-occurrence with parental forms and their origin in the final product (Kostelanska et al., 2011; Lancova et al., 2008; Zachariasova et al., 2008). Modified forms of mycotoxins are not yet included in the current European Union legislation, however, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) has extended the tolerable daily intake for DON to 3- and 15-acetyldeoxynivalenol (3- and 15-Ac-DON) (JEFCA/FAO, 2011). In a recently published EFSA scientific opinion on the risk of sum exposure of the population to DON and its metabolites, the important contribution of acute exposure was attributed to DON on different age groups (from 50 to 90%) (Knutsen et al., 2017). Nonetheless, in the long run (chronic exposure), the same document concluded the considerable additive health impact of DON metabolites (3- and 15- Ac-DON and DON-3-Glc). ZEN and its metabolites, α- and β-zearalenol, (α- and β-ZEL, respectively) represent a higher risk for public health compared to DON metabolites, especially if their level is closer to the upper bound scenario defined by EFSA (up to 2.2 fold the tolerable daily intake) (European Food Safety Authority, 2014).

Another aspect to be considered with regard to *Fusarium* mycotoxins is their resistance to physical parameters applied in food industry and possible subsequent transfer to the final product (Wolf & Bullerman, 1998). Besides that, other chemical or physical transformation might occur, depending on the applied production operations (Vidal, Morales, Sanchis, Ramos, & Marín, 2014; Zachariasova et al., 2008, 2012). The brewing process includes several main steps: mashing, wort boiling, fermentation, maturation, stabilization and packaging (with or without previous pasteurization), the first three representing a significant impact on the levels of mycotoxins possibly present in malt (Pascari, Ramos, Marín, & Sanchís, 2018). The present study will be focusing on two brewing stages (mashing and boiling processes), giving a detailed vision from the inside of each of them. Mashing consists of mixing coarse ground malt with a large amount of water (approximately 170 g per liter) under specific temperatures to activate all the enzymes present in malt, namely: 45-50°C is set to ensure protein hydrolysis, 60-65°C lead to maltose production, and 75-78°C activate α-amylases (Kunze W. , 2006). Then, malt grist is removed from the wort and after adjusting the relative density of the wort with water (final density should vary between 1005 and 1010 kg/m^3) boiling starts, lasting from 45-60 min up to 3h depending on the beer style to be obtained (Pascari et al., 2018).

Beer production implies the use of five main commodities, namely barley, water, hops, yeast and adjuncts (e.g. maize, sugar syrup, unmalted cereals etc.). Besides barley, mentioned above, beer adjuncts can represent another source of mycotoxins, of particular note being maize (Marin et al., 2013), which is proven to be susceptible mainly for *Aspergillus* section *Flavi* (aflatoxin producers), *F. proliferatum* and *F. verticillioides* (FBs producers) infestation. Hops added during the boiling stage may also be subject to fungal invasion with subsequent mycotoxin accumulation. However, the study performed by Vaclavikova et al. (2013) did not find hops as a significant source of mycotoxins in brewing wort because of the relatively low quantity added to beer.

According to previously published studies, a transfer of mycotoxins from raw materials to beer is possible due to their relatively high resistance to physical treatments applied during brewing (Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Pascari et al., 2018). Nevertheless, several stages of the production scheme are proved to decrease the initial mycotoxin

contamination levels (Lancova et al., 2008). In the EU, maximum allowed mycotoxin levels are regulated by the Regulation EC 1881/2006 with its subsequent updates and amendments. The applicable limits in barley are set as follows: 1250 µg/kg for DON and 100 µg/kg for ZEN. Beer is subjected only to legal limits in FBs content (maximum 400 µg/kg for the sum of fumonisin B_1 and B_2) in the case maize is used as an ingredient, other toxins not being included. This result in the hypothesis that brewing process may lead to a reduction in mycotoxin content.

This study aims to identify the impact of the obtention of brewing wort on mycotoxin contamination, a special focus being given to mashing and boiling parameters. The fate of DON, ZEN and their metabolites was investigated because of their frequent occurrence in malting barley: DON levels range from 69.9 to 602.3 µg/kg, ZEN varies from 181.2 to 204.4 µg/kg (Bolechova et al., 2015), DON-3-Glc and AcDONs have an average occurrence of 2 and 1 µg/kg, respectively (Malachova et al., 2010). Taking into account the lack of available studies on the fate of α- and β-zearalenol in brewing and their possible production by some *Fusarium* spp. (Bottalico et al., 1985), these two metabolites were also included in the study. FBs are less occurring in barley, however their incidence in maize is significantly frequent (94,7%) (Manova & Mladenova, 2009) and in relatively high concentrations in maize grits used in brewing (from 1146 to 3194 µg/kg) (Pietri, Bertuzzi, Agosti, & Donadini, 2010). The different levels of mycotoxin contamination found in barley and in beer (Piacentini, Savi, Pereira, & Scussel, 2015; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015) leads to the conclusion that an efficient strategy is applied in the field to discard heavily contaminated barley, which, in combination with the mitigation potential of the production process, might reduce the health concern related to this product from the perspective of mycotoxin contamination.

Considering the different mycotoxin producing fungal strains, different sources of contamination (from infected malt or added maize) and the possible impact of the contamination level on mycotoxin transfer, three scenarios will be tested: two scenarios of grain inoculation with mycotoxin producing *Fusarium* strains and one of spiking with mycotoxin standard solutions. Results will help understand the interactions between mycotoxins, malt and beer matrices that might take place and possible mitigation strategies

to be considered in the future. One of the strengths of the present study is that it better simulates natural contamination scenarios compared to a study where only spiking with standard solutions of the raw materials is performed.

7.3 Materials and methods

7.3.1 Chemicals and reagents

Mycotoxin standard solutions of DON, DON-3-Glc, 3- and 15-Ac-DON, ZEN, α-ZEL), β-ZEL, FB1 and FB2 were purchased from Romer Lab Diagnostic (Tulln, Austria). Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol and acetonitrile (HPLC grade) were purchased from Scharlab (Sentmenat, Spain), and ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

7.3.2 Preparation of mycotoxin contaminated grains

Three origins of contamination were studied: two sources of contamination with *Fusarium graminearum* (F.46) and *F. proliferatum* (F2.333) (infected malt and maize) and contamination by spiking the malt with mycotoxins standard solutions. Mycotoxin producing fungal strains were taken from the strains collection of the Food Technology Department of the University of Lleida.

Before inoculation with mycotoxin producing strains, malt and maize grains were disinfected according to Andrews, Pardoel, Harun, & Treloar (1997). Briefly, a batch of grains (500 g) was immerged into 0.4% chlorine solution for 2 min and then abundantly rinsed with sterile distilled water. Then, all the grains were placed in an hermetically closed ISO bottle and left overnight with the addition of sterile water for grain hydration (approximately 300 and 340 mL/kg for maize and malt, respectively) at 4°C to achieve a water activity of 0.99 (Aqualab Series 3 TE, Decagon Devices Inc., Washington, USA). Three different batches of contaminated grains were prepared: one batch of malt grains and one of maize grains were contaminated with *Fusarium graminearum* (DON and ZEN producer) and another batch of maize grains was contaminated with *Fusarium proliferatum* (FBs producer). For this, the humidified grains were aseptically transferred to Petri dishes

and 1 mL of spore suspensions of *F. graminearum* or *F. proliferatum* (10^6 spores/mL) was sprayed on each dish. Petri dishes with malt and maize were then incubated at 25°C for 30 days. Afterwards, the contaminated grains were dried at 40°C, homogenized and DON, ZEN and FB1 levels were determined.

To obtain the desired mycotoxin contamination level of the malt, spiking was performed by adding to ground malt DON, ZEN and FB1 standard solutions at two different levels each, ensuring the best possible homogenisation for the entire batch. Spiking levels for each toxin were chosen at approximately a half of the legally allowed limit (A1) and at the maximum allowed limit (A2) (Table 1) (European Commission, 2006). For the inoculated malt and maize, an amount of the above *Fusarium* infected grains with the identified concentration of DON, ZEN and FB1 was added to uncontaminated malt. Worth noting is that in both fungal contamination scenarios, malt was the mashed substrate. In the cases when maize was added, its role was only to increase the amount of fermentable sugars present in wort after the mashing stage. Considering the heterogeneity of mycotoxin accumulation in cereals, various batches were prepared (Table 1). All the samples were prepared in triplicate for each concentration level. In order to ensure that the desired concentration was achieved, the mixes were prepared individually for all replica (250 g of malt mashed each time). Samples coding was performed considering the source of contamination, namely the "B" was attributed when the contamination was coming from inoculated malt, the "M" when it was coming from inoculated maize (the mashed malt was free of mycotoxins), the "A" for the spiked samples and "Blank" stands for mashing the uncontaminated malt.

7.3.3 Wort production and sampling

A coarse grinding of malt was performed and, in the case where contaminated maize was added, maize kernels were finely ground and mixed with mycotoxin free malt. For each designed setup, 250 g of contaminated malt, or malt and maize adjunct, were mashed with 1300 mL of water (50% distilled water, 50% deionized water in order to avoid an increase of the pH of the sweet wort). Once mixed, the mashing process was started by holding the temperature of the mix at 45°C for 15 min (M15min), then at 65°C for 1h (M75min) and

finally at 75°C for another 15 min (M90min), using an induction plate (PI 4750, Murcia, Spain) and ensuring a frequent periodical homogenization of the mashed volume. Samples of sweet wort and spent grains were taken at each temperature change. In order to make sure that all the starch was transformed into fermentable sugars, the iodine test was performed, consisting of adding a few drops of 0.1M KI solution in a tube containing 5 mL of wort and observing the change in color (the color changes to blue if starch is present). Afterwards, the wort was decanted, and the density was adjusted to 1005-1010 kg/m^3 with distilled water. Hops were added (10 g of hop pellet/L of wort) and the wort was intensively boiled for 1.5 h, samples being collected every 30 min (B30min, B60min and B90min). All the samples were stored at -18°C until their analysis. The process was repeated per triplicate for each malt sample prepared (Table 21).

7.3.4 Mycotoxin extraction, detection and quantification

Considering the complexity of the two obtained matrices (spent grains and wort), two extraction procedures were used, according to previously validated methods.

7.3.4.1 Spent grains

All solid samples were dried at 40°C for 24h before analysis. Mycotoxin extraction of the spent grains was performed according to Juan et al. (2017), with slight changes. Briefly, 2 g of dried and ground sample was weighted in 50 mL polypropylene tubes, mixed with 10 mL of extraction solvent (acetonitrile:water, 84:16, v/v) and shaken at 200 rpm for 60 min (Infors AG GH-4103, Bottmingen, Switzerland). The mix was then centrifuged at 2336 g for 10 min (Hettich Universal 320R, Tuttlingen, Germany). Five millilitres of supernatant were evaporated under a gentle nitrogen stream (40°C). The dry extract was resuspended in 1 mL of methanol:water (70:30, v/v) and filtered (PTFE syringe filter, 0.22 µm) before the UHPLC-MS/MS analysis (see 7.3.4.3).

Table 21: Initial mycotoxins concentrations obtained in malts for wort production

Contamination scenario	Malt samples ID	Mean concentration, µg/kg± SD							
		DON	***ZEN***	***FB1***	***FB2***	***DON-3-Glc***	***3AcDON***	***15AcDON***	***β-ZEL***
Mycotoxins coming from *F. graminearum* contaminated malt	B1	78±38	4217±1821	ND	ND	<LOQ	2.8±2	<LOQ	<LOQ
	B2	103±17	4969±1189	ND	ND	<LOQ	4.4±0.4	<LOQ	<LOQ
	B3	107±19	5201±827	ND	ND	<LOQ	5±0.8	<LOQ	<LOQ
	B4	209±5	6542±741	ND	ND	<LOQ	9.2±0.8	<LOQ	<LOQ
	B5	1271±1	989	ND	ND	1032±0.4	<LOQ	<LOQ	<LOQ
Mycotoxins coming from the added *F. proliferatum* contaminated maize	M1	12±4	1482±341	43±2	<LOQ	<LOQ	2.02±0.6	<LOQ	6±9.5
	M2	28±6	2605±373	84±28	64±16	<LOQ	3.5±1	4±6	<LOQ
	M3	74±2	5264±772	230±52	200±92	<LOQ	7.8±0.5	14.2±3.5	22±6
	M4	136±35	7053±496	351±103	328±68	<LOQ	15±3.3	13.4±2.1	38.8±7.8
	M5	ND	ND	189±6	84±12	<LOQ	<LOQ	<LOQ	<LOQ
	M6	ND	ND	310±99	200±84	<LOQ	<LOQ	<LOQ	<LOQ
	M7	ND	ND	758±8	332±16	<LOQ	<LOQ	<LOQ	<LOQ
Artificial contamination with mycotoxins standard solutions	A1	50±3	<LOQ	118±7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	A2	750±1	75	400±0.5	<LOQ	120±0.4	<LOQ	<LOQ	<LOQ
Blank		<LOQ	<LOQ	<LOQ	<LOQ	124±0.2	<LOQ	<LOQ	<LOQ

*LOQ=limit of quantification; ND= not defined, DON=deoxynivalenol; ZEN=zearalenone; FB1=fumonisin B_1; DON-3-Glc= deoxynivalenol-3-glucoside; 3-AcDON = 3-acetyl-deoxynivalenol; 15-AcDON = 15-acetyl-deoxynivalenol; β-ZEL= β-zearalenol. The LOQ was: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN, 0.5 µg/kg for 3-AcDON, 15-AcDON and β-ZEL, 45 µg/kg for FB1 and FB2.

7.3.4.2 Wort

For the liquid samples, the QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged and Safe) method developed by Rodríguez-Carrasco et al. (2015), with several modifications, was applied. Ten milliliters of sweet wort were mixed with 10 mL of acetonitrile in a 50 mL polypropylene tube. After mixing it well for 30 seconds, QuEChERS (Phenomenex, California, USA) dehydrating salts were added (4 g of magnesium sulphate, 1 g of sodium chloride, 1 g of sodium citrate tribasic dihydrate, 0.5 g sodium citrate dibasic sesquihydrate) and shaken intensively for 1 min. Afterwards, the tubes were centrifuged at 1413 g for 3 min and 7 mL of supernatant were taken for a pre-mixed clean-up by dispersive solid phase extraction (Phenomenex, California, USA) in a 15 mL tube containing 1.2 g of magnesium sulphate, 0.4 g of PSA (primary secondary amine), 0.4 g of C18 and 0.4 g of activated carbon. It was vortexed for 1 min and centrifuged for 1 min at 1413 g. Finally, 3 mL of supernatant were evaporated under a gentle nitrogen flow (40 °C). The dry extract was also resuspended in 1 mL of methanol:water (70:30, v/v) and filtered (PTFE syringe filter, 0.22 µm) before the UHPLC-MS/MS analysis (see 7.3.4.3).

7.3.4.3 LC-MS/MS analysis

An Agilent Series 1290 RRLC system (Santa Clara, California, USA) equipped with a binary pump (G4220 A) and a thermostatic autosampler (G1330 B), coupled to a triple quadruple mass spectrometer Agilent 6460 A was used for sample analysis. Data acquisition and processing was performed using MassHunter® software (Agilent, Santa Clara, California, USA). Separation was achieved on Agilent Zorbax Plus C18 100x2.1 mm, 1.8 µm column (California, USA).

The mobile phase consisted of methanol (A) and an aqueous solution of ammonium formate at 5mM (B), which was supplied in gradient at a flow rate of 0.2 mL/min. The initial gradient was set at 25% A, increased to 100% by the minute 3.75 and maintained during the following 2.25 min. Within the following 0.5 min it was dropped again to 25% A and maintained at this proportion until the next injection. The injection volume was 5 µL and column temperature was maintained at a constant 25ºC. Total run time was 7.5 min.

Operating ESI (electrospray ionisation) conditions were set up as follows: capillary voltage

3500 V, cone voltage 500 V, nebulizer pressure 45 psi, source temperature 325°C, cone gas flow 5 L/min, drying gas (nitrogen) temperature 400°C, drying gas flow 11 L/min. The mass spectrometer was operated in both positive and negative electrospray ionization modes (ESI) in the multiple reaction monitoring (MRM) mode. The most abundant characteristic product ion was used for quantification and the rest of the product ions served for qualitative confirmation of the analyte (European Commission, 2002). Linearity was checked using both external calibration and matrix-matched calibration plots. The limit of detection (LOD) was considered as three times the signal of blank noise and the limit of quantification (LOQ) as ten times the signal to noise ratio. Table 22 regroups the data concerning the selected ion transition, the settings of the mass detector and the limits of detection and quantification in the injected solutions. The concentration factors during extraction were 1 and 3 for solid and liquid samples, respectively.

7.3.5 Statistical analysis

All weight and volume changes during wort obtention were recorded in order to present the results in µg of toxin and to allow comparison among the transfer rates of *Fusarium* mycotoxins throughout the entire mashing and boiling processes. A post hoc multiple comparison of means, Tukey HSD test, was performed to identify significant changes in mycotoxin content between different stages of production, using JMP Pro 13 Software (SAS Institute, New York, USA). A value of 1/2 LOQ was attributed to the non-quantitated samples. For greater precision, the results from malt mashing were analyzed separately from the results obtained after wort boiling.

Table 22: LC-MS/MS acquisition parameters (MRM mode) used for the analysis of targeted mycotoxins

Mycotoxin	Retention time, min	ESI[1] mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Fragmentor voltage (V)	LOD[2] (µg/L)	LOQ[3] (µg/L)
DON	2.8	ESI+	297.2	**231.1**[4] 203.1	15 15	65	0.9	3.0
DON-3-Glc	2.7	ESI-	476.2	**297.1** 248.9	5 25	85	1.8	6.0
3-Ac-DON	3.7	ESI+	339.2	**231.1** 203.0	15 25	105	0.15	0.5
15-AcDON	3.7	ESI+	339.2	**321.1** 261.1	5 5	125	0.15	0.5
ZEN	5.0	ESI-	317.2	**131.0** 273.1	25 25	185	0.3	1.0
α-ZEL	5.0	ESI-	319.2	**129.9** 174.0	40 40	125	0.15	0.5
β-ZEL	4.8	ESI-	319.2	**174.0** 160.0	40 40	125	0.15	0.5
FB1	4.2	ESI+	722.4	**352.1** 334.1	30 40	175	13.6	45
FB2	4.7	ESI+	706.5	**336.4** 318.4	35 45	125	13.6	45

[1]ESI=electrospray ionisation, [2] LOD=limit of detection; [3] LOQ=limit of quantification; The given LOD and LOQ are corresponding to the injected solutions. [4] Product ions in bold were used for quantification, others for qualitative analysis; DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside;; 3-Ac-DON=3-acetyl-deoxynivalenol; 15-AcDON= 15-acetyl-deoxynivalenol; ZEN= zearalenone; α-ZEL= α-zearalenol; *β*-ZEL= *β*-zearalenol; FB1= fumonisin B1; FB2=fumonisin B2.

7.4 Results and discussion

7.4.1 Blank and spiked malt

In order to obtain complete information regarding the process, the malt used in the present study was checked for all toxins of interest before mashing. It was found to contain only DON-3-Glc, other toxins being below the LOQ (Table 21, Figure 9). Nonetheless, by the end of the mashing process, the levels of DON and DON-3-Glc were significantly increasing in the wort with a simultaneous decrease of DON-3-Glc in the spent grains. This suggested their release from the matrix (particularly obvious for DON, as it was not found in the malt before mashing), due to the increasing temperature and the relative length of the process (90 min of mashing). DON-3-Glc behavior was very similar in the blank and A2 samples. It was not present in A1 sample initially, nor did it appear during the mashing process, either in spent grains or in the wort. A possible explanation of this is heterogeneity in the initial DON-3-Glc contamination of the barley before malting which may be lower compared to the A2 and blank samples, which together with a lower conversion rate of DON to DON-3-Glc in barley (Freire & Sant'Ana, 2018) did not lead to the formation of the masked form DON-3-Glc (Kostelanska et al., 2009; Medina et al., 2006). A significant increase of DON-3-Glc content in the wort was identified after the last stage of mashing (15 min at 75°C). Considering that no decrease of DON was observed after the same production step, no conversion occurred (Lancova et al., 2008). From the above, it can be concluded that the enzymatic activity (macromolecules' hydrolysis) and the high temperature might be the most important catalysts of DON-3-Glc transfer from malt to wort. Boiling led to a 26% reduction in DON level compared to its initial concentration before mashing. DON-3-Glc quantities decreased, with respect to the level after mashing, down to its initial concentration.

In the lower spiking concentration (A1 sample), ZEN was found only in the wort during mashing, with no significant changes between stages, and <LOQ after the first 30 min of boiling (B30min). On the contrary, each step of mashing led to a significant increase in ZEN compared to its initial quantity with 700% in the A2 sample.

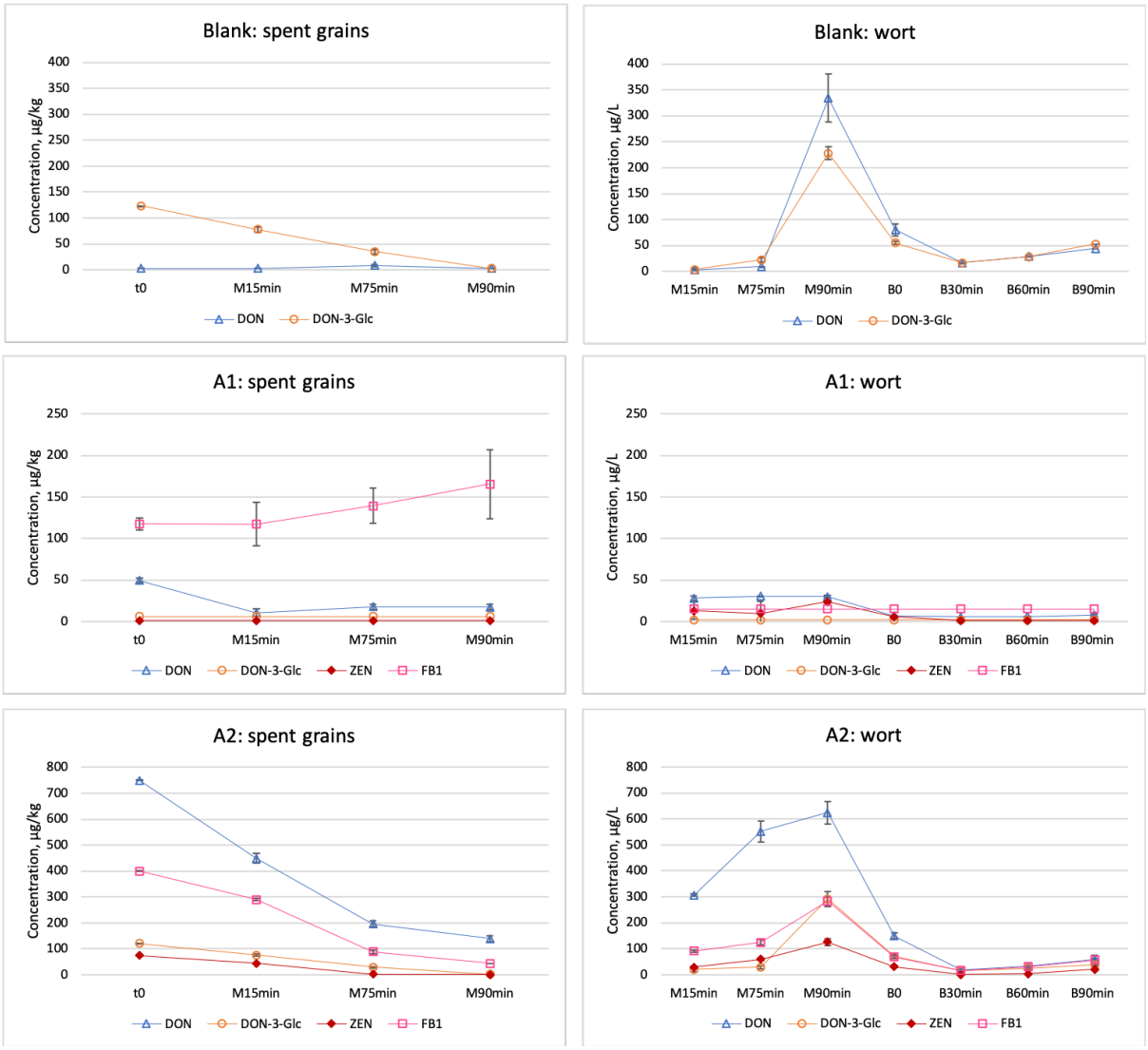

Figure 9: Evolution of mycotoxins concentration originated from malt spiked with standard solutions of DON, ZEN and FB1 throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN and 45 µg/kg for FB1. Wort: 1 µg/L for DON, 2 µg/L for DON-3-Glc, 0.3 µg/L for ZEN and 15 µg/L for FB1.

until it reached its initial level in malt grist. This result confirms those obtained by Inoue et al. (2013), where an almost 20% decrease of ZEN was observed in the spent grains.

FB1 did not transfer to wort in the A1 sample, and was, as a result, eliminated with the spent grains. Worth noting is that its level in the A2 sample almost tripled in the wort after 15 min at 75°C (M90min) compared to the initial quantity present in malt, a similar tendency being observed later in the inoculated samples (M5-M7). Boiling showed a significant reduction of almost 50% of FB1 with respect to the initial contamination level, after the first 30 min of the process.

Inoue et al., (2013) performed a study spiking the malt with 14 different mycotoxins and investigating their fate during brewing. Only the results we obtained in the A2 sample correlate with the ones they observed in their study. The different behavior could be due to the lower spiking concentration that was chosen in the present study for the A1 sample. In Table 1 (Appendix), the data on mycotoxin content is presented in µg of toxin, which makes possible the comparison between the two physical states implied in the process. Altogether, from the obtained results, spiking with mycotoxin standard solution does not take into account the complexity of mycotoxigenic *fungi* propagation into the grain (in the case of fungal contamination) which mainly defines the transfer from malt to wort in *Fusarium* inoculated samples (Freire & Sant'Ana, 2018; Kostelanska et al., 2011).

7.4.2 Transfer of DON and its metabolites from *Fusarium* contaminated malt and maize

Two possible sources of contamination were considered: mycotoxin contaminated malt (Figure 10) and maize (Figure 11). A study of the evolution of DON and its metabolites during mashing and boiling was performed, while also defining initial levels of contamination (Table 21).

As expected, DON passed from malt grist to the wort during mashing process (Lancova et al., 2008). Interestingly, a significant reduction of DON in the spent grains during the first stage of mashing (15 min at 45°C) was observed (93% in B1, 86% in B2, 94% in B3, 86% in B4 and 38% in B5), while the other stages did not significantly influence the level of

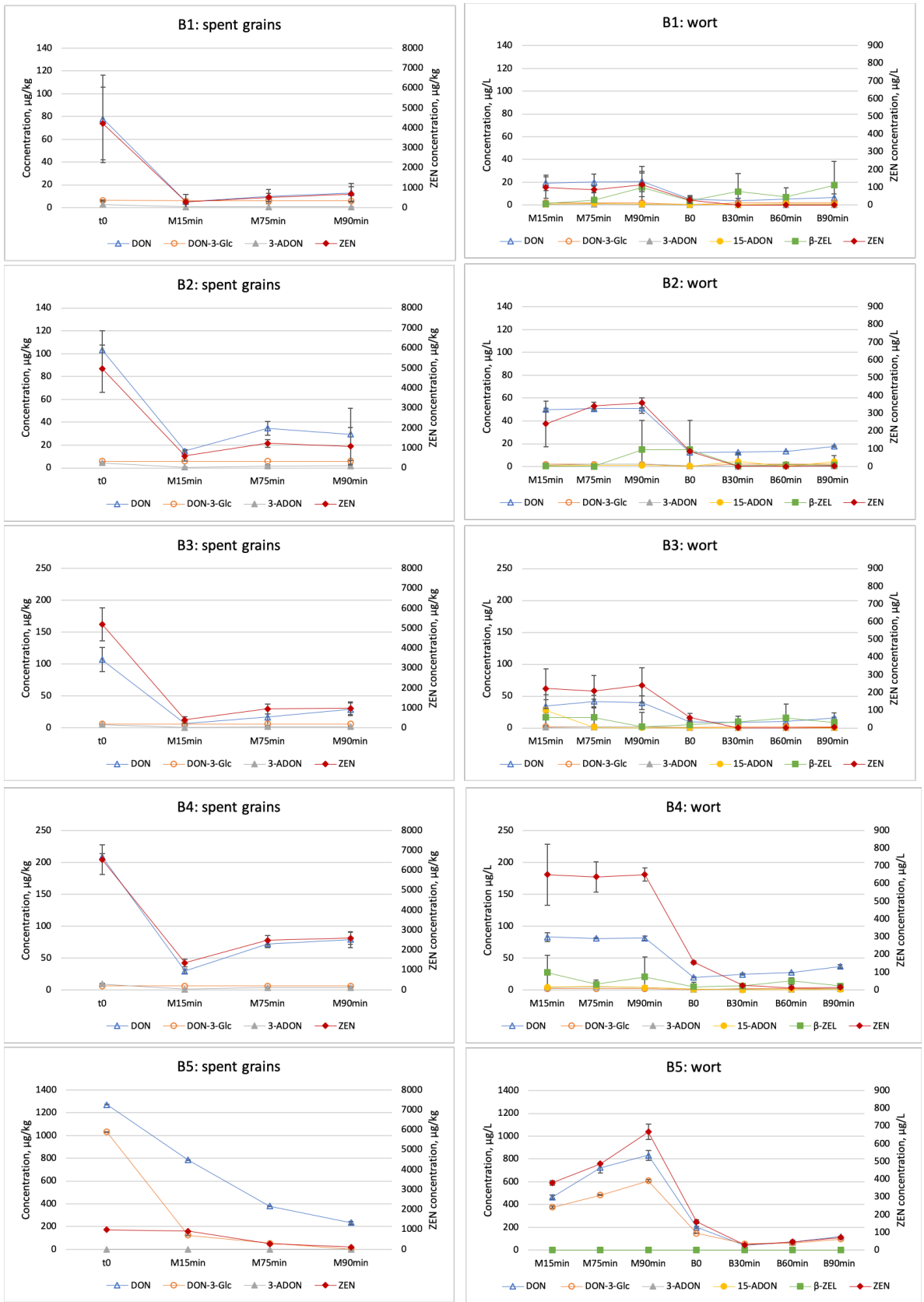

Figure 10: Evolution of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-Glc), 3 and 15-acethyl-deoxynivalenol (3-ADON and 15DON, respectively), β-zearalenol (β-ZEL) and zearalenone (ZEN) concentrations originated from the *F. graminearum* contaminated malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L).

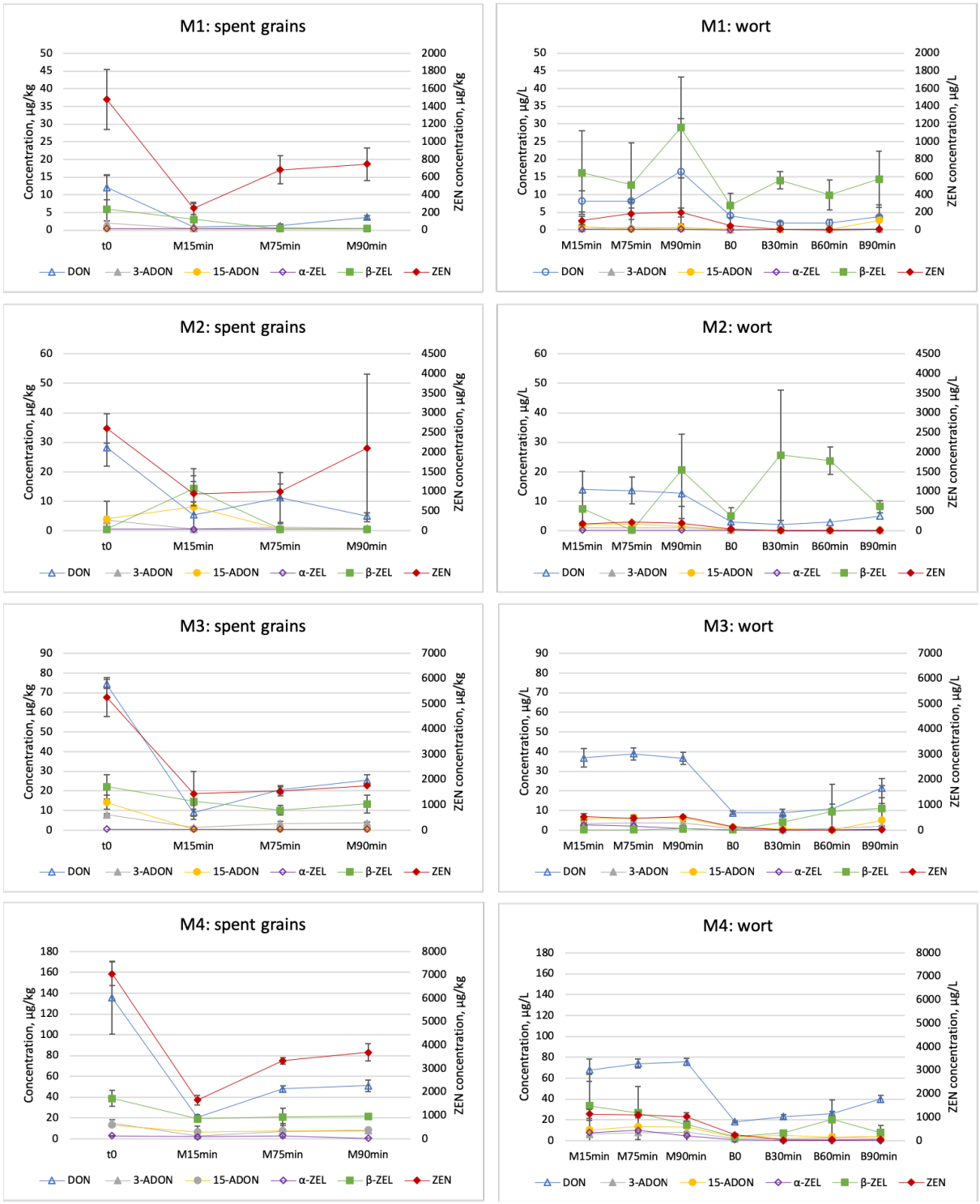

Figure 11: Evolution of deoxynivalenol (DON), 3 and 15-acethyl-deoxynivalenol (3-ADON and 15-ADON, respectively), α- and β-zearalenol (α-ZEL and β-ZEL, respectively) and zearalenone (ZEN) concentrations originated from the *F. graminearum* contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN, 0.5 µg/kg for 3-AcDON, 15-AcDON, α- and β-ZEL, 45 µg/kg for FB1. Wort: 1 µg/L for DON, 2 µg/L for DON-3-Glc, 0.3 µg/L for ZEN, 0.17 µg/kg for 3-Ac-DON, 15-Ac-DON, α- and β-ZEL, 15 µg/L for FB1.

DON contained. The same, but increasing, tendency was observed in the sweet wort in all samples, however in the B5 sample an even greater increase was observed after 60 min at 65°C (35%). In all the samples, except for B1 sample (Appendix, Table 2), the amount of DON found in the wort was twice the initial amount contained in the malt grist and in the case of the samples in which the contamination came from maize (Appendix, Table 3) this increase was even higher (2.5 folds the initial content). The increased amount of DON released into the wort is probably due to the fact that the contact with water and the enzymatic activity during mashing causes DON to be unbound from malt's matrix macromolecules (Kostelanska et al., 2011). The different extracted amounts in B and M samples could be explained by the fact that the maize was ground into a smaller particle size than the malt, which increased the contact surface and, as a result, also aided DON transfer into the water. Boiling led to a reduction in DON levels compared to the last stage of mashing (15 min at 75°C), the most significant change occurring after 30 min of boiling, except for the samples B2, B4 and M4 where an even more significant reduction took place after 60 min of boiling. In none of the studied scenarios was DON entirely eliminated during boiling, however a level significantly lower than the initial was achieved (up to 60% decrease of DON compared to its level before mashing) in malt contaminated samples (Appendix, Tables 2 and 3). This reduction might be related either to its sedimentation with proteins and impossibility of extraction by the method used in this study (Schwarz, 2017) or its chemical modification under temperature action (Rychlik et al., 2014), however supplementary research has to be done to confirm this statement.

DON-3-Glc production is linked either to plant metabolism (Lemmens et al., 2005) or to enzymatic activity during food processing (Vidal et al., 2014). DON distribution in the grain is associated with proteins and β-glucans (Nishio, Takata, Ito, & Tanio, 2010), whose hydrolyzation during mashing would lead to its release into the wort (Kostelanska et al., 2011). DON-3-Glc was found in the malt grist in only two samples of the present study (B5 and M2). At this stage, its origin in the samples certainly derives from the malted barley, probably infested in the field (Medina et al., 2006). Similar to DON, DON-3-Glc in the B5 sample gradually augmented in wort during mashing, a three-fold increase of DON-3-Glc registered in the wort after the last treatment (15 min at 75°C), accompanying a significant

decrease of the toxin in the spent grains during mashing (almost 100% decrease compared to the level before mashing). In the M2 sample no transfer was observed possibly because of the low amount of DON-3-Glc found. Converting the data from concentration to µg of toxin (Appendix, Tables 2 and 3) demonstrates the transfer from the malt matrix to the sweet wort.

3- and 15-Ac-DON production is a function of the fungal strain responsible for contamination (Gauthier et al., 2010), which explains the presence of these metabolites in almost all the samples before mashing, although in relatively low levels (Figures 10 and 11). Both 3-Ac-DON and 15-Ac-DON were transferred to the wort during mashing (Appendix, Tables 2 and 3). For the contamination derived from malt (B1-B5), the decrease of 3-Ac-DON was only significant after 15 min at 45°C, followed by an increase in the sweet wort and maintaining almost same level until the end of the mashing process, with a subsequent, statistically relevant, reduction during the boiling process in all the samples. 15-Ac-DON was found only in the sweet wort at each mashing stage with no significant change up to the end of the boiling process, except for the B3 sample where it was found <LOQ in the steps following the 60 min at 65°C of the mashing stage. Regarding the evolution of 3- and 15-Ac-DON in the samples with contaminated maize adjunct (M1-M4), the two toxins showed a more similar behavior between them, registering an almost 100% decrease in the spent grains. In the samples where the amount of 3-Ac-DON was higher (M3 and M4) the quantity found in the wort after 15 min at 45°C was almost double the initial level in the malt grist (Appendix, Table 3). 15-Ac-DON showed a similarly significant increase in the wort during mashing, however, contrary to 3-Ac-DON, the boiling process did not lead to a reduction in its level, except for the M2 sample, the only one in which both toxins were <LOQ at the end of the process. All the similarities in the changes in the acetylated forms of DON with their parental form were probably due to their sharing of physical and chemical properties (Nagl & Schatzmayr, 2015).

In summary, both DON and its metabolite levels increased in wort, accompanied by a significant reduction in the spent grains. Interestingly, in almost all samples, this change occurred during the first mashing stage (15 min at 45°C), all the following mashing stages not having a significant impact on mycotoxins level, except for a few samples (B2, B5 and

M1). Studies on DON distribution in the kernels showed a positive correlation with ash and protein content, which is higher in the brans due to pericarp and aleurone tissues (Trigo-Stockli, Deyoe, Satumbaga, & Pedersen, 1996). This fact makes the extraction of DON from a contaminated kernel an important issue for analysis (Zheng, Richard, & Binder, 2006). Nevertheless, the aim of enzymatic activity at 45°C during the mashing process is the hydrolysis of β-glucans and proteins; this could lead to a weakening of the bounds between the toxins and the malt matrix and, together with its water solubility, result in an increase in DON and its metabolite content in the wort. From the data presented in supplementary material section (Appendix), it can be seen that 29 to 59.6% of DON remained in the wort after boiling in the case when contamination was coming from the infected malt (B1-B5 samples), 59 to 106.7% of DON were found in the wort of the samples when contaminated maize grits were added (M1-M4) and 26.6 and 58.3% of DON in the case when malt was enriched with DON standard solution. Regarding DON modified forms, besides their low incidence in the analyzed samples, a high variability in the results was observed with 31 and 92% maximum remained in the final wort for DON-3-Glc and 3-Ac-DON, respectively. 15-Ac-DON increased from <LOQ to a maximum of 4,8 µg/L in all the samples coming from malt and maize grits contaminated with *F. graminearum*. Previously published studies have found a correlation between DON and DON-3-Glc accumulation, presumably due to the enzymatic activity (Lancova et al., 2008), however no such correlation was proven in the present research. Also, previous research identified *S. cerevisiae* spp. potential to adsorb mycotoxins (up to 20% of DON can be removed with yeast residue) (Pascari et al., 2018).

7.4.3 Transfer of ZEN and its metabolites in *Fusarium* contaminated malt and maize

In the samples where the contamination came from malt (Figure 9), the amount of ZEN before mashing was almost always very high (except for the B5 sample), nonetheless no such quantity of ZEN was found in the following stages of mashing and boiling.

In most of the samples, the amount of ZEN in the wort was double that in the spent grains at the three stages of mashing, however no significant increase was registered during the

process (Appendix, Table 2). Although ZEN proved to have a low water solubility (Bennett, Klich, & Mycotoxins, 2003), the higher amount in the wort could be explained by the high temperatures and prolonged contact with water during the mashing process (Mastanjević et al., 2018). The level of ZEN in the wort reached after 15 min at 45°C did not change significantly by the end of the mashing process. The boiling process led to a significant reduction of ZEN in the wort compared to its initial quantity, especially in the higher contamination levels (up to 99% reduction compared to the amount of ZEN before boiling). It is worth noting that in the sample with the lower level of ZEN (B5), the reduction during boiling reached 89% compared to the ZEN level before the beginning of the process.

In the case of ZEN derived from maize adjuncts (Figure 11), the amount of the toxin found in the malt grist before mashing was always related to the amount quantified in the spent grains and the sweet wort. A significant reduction in the spent grains was registered after 15 min at 45°C in all the samples, though considering the high variability within ZEN contamination, it is difficult to draw any conclusions from this. Moreover, considering that ZEN distribution in the kernel is similar to DON (Trigo-Stockli et al., 1996), the mashing process might have a partially similar impact beyond the physical parameters of mashing which stimulates the elution of matrix components into the wort (Lancova et al., 2008). There is no proven knowledge on the mechanism of ZEN reduction during boiling, especially considering its thermal stability, and deeper chemistry studies are necessary to complete these findings.

α- ZEL was found only in two of the contamination scenarios (M3 and M4) at low levels, only in the wort with no significant changes during malting, but <LOQ after 30 min of boiling up to the end of the process. β-ZEL, on the contrary, was present in almost all samples (except M2) containing contaminated maize before mashing started. The production of these two metabolites might been attributed to their synthesis by the fungal isolate responsible for the contamination (Bottalico et al., 1985), on one hand, and to ZEN metabolization by *Saccharomyces cerevisiae* cells during fermentation, on the other (Sørensen & Sondergaard, 2014). β-ZEL was predominantly found in the wort of the samples where the contamination came from malt and no stage of the process led to a significant change in its quantity. In the two samples that contained α- ZEL, the levels

were low and remained nearly constant, while boiling led to a decrease down to <LOQ. β-ZEL in the samples with contaminated maize adjunct showed an increasing trend during mashing followed by a decrease during boiling, but never more than 28% compared to the amount before boiling and not statistically significant. No correlation was found between the initial content of β-ZEL and the reduction rate. There are no studies available on the stability of ZEN metabolites during food processing; however, considering that the main metabolite of yeast activity of ZEN transformation is β-ZEL (lower toxicity compared to ZEN (De Saeger & van Egmond, 2012)), together with the results of this study (no α- ZEL present in the wort after boiling), mashing and boiling processes have been shown to lead to a detoxification of the product from ZEN. Finally, in almost all samples, ZEN reduction reached approximately 99 % in the wort at the end of the boiling process.

7.4.4 Transfer of fumonisins B1 and B2 in *Fusarium proliferatum* contaminated maize

The most probable source of fumonisins in beer are maize adjuncts (Marin et al., 2013). Figure 12 represents the data of changes to the FB1 and FB2 during mashing and boiling. There is a slightly different trend between the samples M1 to M4 and M5 to M7, which may be explained by their co-occurrence with other mycotoxins, however there is no evidence to confirm the statement. In the samples from M1 to M4, both FB1 and FB2 were transferred to the spent grains during mashing (no change in concentration in the spent grains and <LOQ in the wort), which suggests their elimination before boiling and decontamination of the product. Nonetheless, the samples M5 to M7 confirm its water soluble properties (Pietri et al., 2010) through the significant increase of FB1 and FB2 in the wort throughout mashing (each of the three stages was described by a significant reduction of FB1 and FB2 in the spent grains with a subsequent significant increase in the wort) (Appendix, Table 4). After the first 30 min of boiling, there was a 90 to 100% reduction in both toxins, which correlates with Pietri et al. (2010) results.

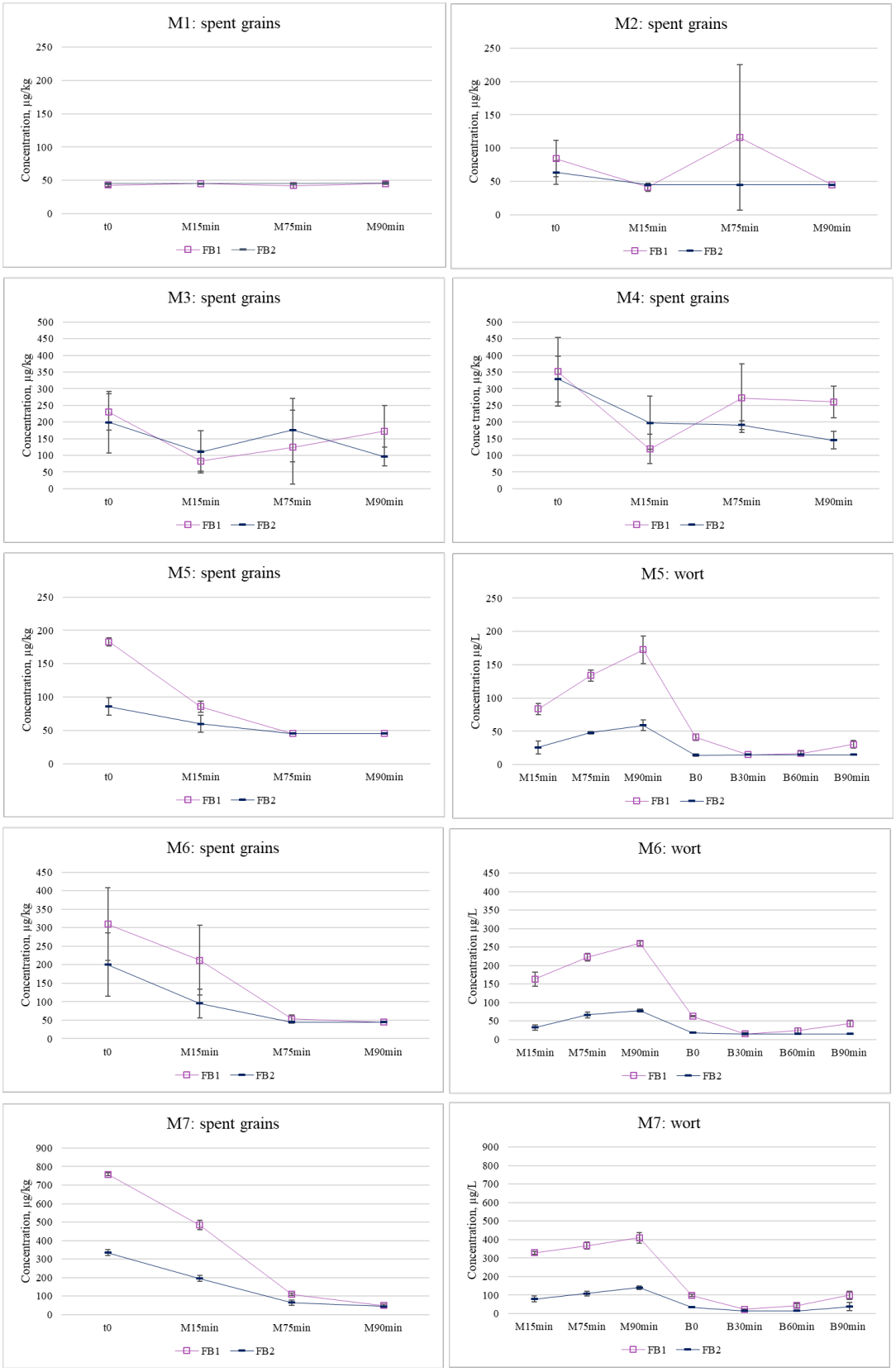

Figure 12: Evolution of fumonisins B_1 (FB1) and fumonisins B_2 (FB2) concentration originated from *F. proliferatum* contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 45 µg/kg for FB1 and FB2. Wort: 15 µg/L for FB1 and FB2.

There is no evidence on the impact of the enzymatic activity on fumonisins content, nonetheless its increase during this process cannot be solely explained by its solubility in warm to hot water (45 to 75°C), suggesting the enzymes may have the indirect effect of facilitating hydrolyzation from macromolecules. At the end of boiling process, a 90% FB1 reduction was observed in the wort containing it before the process started. Also, the different fate of FB1 and FB2 during wort production might be influenced by their co-occurrence with DON and ZEN in the M1-M4 samples, which is not the case for M5-M7 samples, where only FBs are present. Nevertheless, the obtained data are not sufficient to support either of the two abovementioned assumptions and further studies need to be performed to investigate these results.

7.5 Conclusion

From the three contamination scenarios prepared in the present study, some interactions between the process and the level of mycotoxins were identified. Samples spiked with standard mycotoxin solutions (DON, ZEN and FB1) showed changes in contamination similar to the samples in which the contamination was coming from infected malt. DON and its metabolites had a similar behavior, showing a significant transfer from malt to wort during mashing. Moreover, an increase of the extracted amount of toxins was observed through the process, the most significant being observed after 15 min at 45°C. Nonetheless, however great the rise, boiling always led to a reduction down to the initial level (in samples with the addition of the contaminated maize) or even lower (samples containing contaminated malt). Despite the abovementioned decrease, DON is still of a special concern for brewing because of its 30 to 60% remaining in the wort after boiling, which could withstand the following production steps.

Almost 100% reduction in ZEN levels was observed in all the samples (just 30 min of boiling have a significant impact). Very low incidence of α- ZEL was registered along with its complete elimination by the end of the process. β-ZEL was a little more abundant compared to its stereoisomer and showed a low reduction rate at the end of the process.

Both FB1 and FB2 showed transfer into the wort during mashing, however on low levels of contamination it was almost completely removed with the spent grains. Nevertheless,

there is a need for more in-depth studies in order to prove this change in FBs levels.

In summary, the crucial stages that induced significant change in mycotoxins levels were the first 15 min at 45ºC (increase of transfer of mycotoxins into the wort) and the first 30 min of boiling (decrease of mycotoxin level in the wort). Considering the aforementioned, boiling is a crucial step in mycotoxins mitigation but, taking into account that the majority of the mycotoxins are not completely reduced even after 90 min of boiling, more research should be carried out in order to study the changes to mycotoxins and the levels thereof through the next stages of brewing (fermentation, fining and maturation) and identify if there is a reduction in these toxins as expected by European legislation (different maximum allowed limits in raw materials and final products).

7.6 Acknowledgements

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2014-55379-P and AGL2016-77610-R) for funding this work. Y. Rodriguez-Carrasco thanks the University of Valencia for his postdoctoral grant "Atracció de Talent". The authors are grateful to Arthur Kendall for revising English proficiency.

7.7 References

Andrews, S., Pardoel, D., Harun, A., & Treloar, T. (1997). Chlorine inactivation of fungal spores on cereal grains. *International Journal of Food Microbiology*, *35*, 153 162.

Baenziger, P. S., Fredy, J., Nopsa, H., & Bockus, W. W. (2015). Management of Fusarium head blight of wheat and barley. *Crop Protection*, *73*, 100 107.

Beccari, G., Senatore, M. T., Tini, F., Sulyok, M., & Covarelli, L. (2018). Fungal community, Fusarium head blight complex and secondary metabolites associated with malting barley grains harvested in Umbria, central Italy. *International Journal of Food Microbiology*, *273*, 33 42.

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

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. De, Haesaert, G., Karlovsky, P., …

Stroka, J. (2013). Masked mycotoxins: A review. *Molecular Nutrition and Food Research*, *57*, 165–186.

Bolechova, M., Benesova, K., Belakova, S., Caslavský, J., Pospíchalova, M., & Mikulíkova, R. (2015). Determination of seventeen mycotoxins in barley and malt in the Czech Republic. *Food Control*, *47*, 108–113.

Bottalico, A., Visconti, A., Logrieco, A., Solfrizzo, M., Mirocha, C. J., Vegetale, P., & Tossine, I. (1985). Occurrence of Zearalenols (Diastereomeric Mixture) in Corn Stalk Rot and Their Production by Associated *Fusarium* Species. *Applied and Environmental Microbiology*, *49*, 547–551.

De Saeger, S., & van Egmond, H. (2012). Special issue: Masked mycotoxins. *World Mycotoxin Journal*, *5*, 203–206.

European Commission. (2002). COMMISSION DECISION 2002/657 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communitites*, 29.

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

European Food Safety Authority. (2014). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal*, *12*, 107.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189-205.

Gauthier, C. von der O. V., Tamburic-Ilincic, L., Fernando, A. B.-B. W. G. D., Clear, R., Ward, T. J., & Miedaner, T. (2010). A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* isolates with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat. *European Journal of Plant Pathology*, *127*, 407–417.

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. (2013). Fate of Mycotoxins during Beer Brewing and Fermentation. *Bioscience, Biotechnology, and Biochemistry*, *77*, 1410 1415.

JEFCA/FAO. (2011). *Safety evaluation on certain contaminants in food* . Rome: FAO/WHO

Juan, C., Berrada, H., Manes, J., Oueslaty, S. (2017). Multi-mycotoxin determination in barley and derived products from Tunisia and estimation of their dietary intake. *Food and Chemical Toxicology*, *103*, 148 156.

Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., … Edler, L. (2017). Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. *EFSA Journal*, *15*, 347.

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, J., … Krska, R. (2009). Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3- glucoside, in beer and some brewing intermediates. *Journal of Agricultural and Food Chemistry*, *57*, 3187 3194.

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.

Kunze, W. (2006). *Tecnologia para cerveceros y malteros* . Berlin: Westkreuz-Duckerei Ahrens KG.

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 & Contaminants: Part A*, *25*, 732 744.

Lemmens, M., Scholz, U., Berthiller, F., Dall 'asta, C., Koutnik, A., Schuhmacher, 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.

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.

Manova, R., & Mladenova, R. (2009). Incidence of zearalenone and fumonisins in Bulgarian cereal production. *Food Control*, *20*, 362–365.

Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, *60*, 218–237.

Mastanjević, K., Šarkanj, B., Krska, R., Sulyok, M., Warth, B., Mastanjević, K., … Krstanović, V. (2018). From malt to wheat beer: A comprehensive multi-toxin screening, transfer assessment and its influence on basic fermentation parameters. *Food Chemistry*, *254*, 115–121.

Medina, Á., Valle-Algarra, F. M., Mateo, R., Gimeno-Adelantado, J. V., Mateo, F., & Jiménez, M. (2006). Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria*, *Aspergillus* and *Fusarium*. *International Journal of Food Microbiology*, *108*, 196–203.

Morales-Valle, H. (2011). Mohos productores de micotoxinas. In A. J. Ramos (Ed.), *Micotoxinas y micotoxicosis* (pp. 19-44). Madrid: A. Madrid Vicente Ediciones

Nagl, V., & Schatzmayr, G. (2015). Deoxynivalenol and its masked forms in food and feed. *Current Opinion in Food Science*, *5*, 43–49.

Nielsen, L. K., Cook, D. J., Edwards, S. G., & Ray, R. V. (2014). The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK. *International Journal of Food Microbiology*, *179*, 38–49.

Nishio, Z., Takata, K., Ito, M., & Tanio, M. (2010). Deoxynivalenol distribution in flour and bran of spring wheat lines with different levels of Fusarium head blight resistance. *Plant Disease*, *94*, 335–338.

Pascari, X., Ramos, A. J., Marín, S., & Sanchís, V. (2018). Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. *Food Research International*, *103*, 121–129.

Piacentini, K. C., Savi, G. D., Pereira, M. E. V, & Scussel, V. M. (2015). Fungi and the

natural occurrence of deoxynivalenol and fumonisins in malting barley (*Hordeum vulgare L.*). *Food Chemistry*, *187*, 204–209.

Pietri, A., Bertuzzi, T., Agosti, B., & Donadini, G. (2010). Transfer of aflatoxin B1 and fumonisin B1 from naturally contaminated raw materials to beer during an industrial brewing process. *Food Additives & Contaminants. Part A*, *27*, 1431–1439.

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., & Mañes, J. (2015). Occurrence of Fusarium mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry*, *178*, 149–155.

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

Schwarz, P. B. (2017). Fusarium head blight and deoxynivalenol in malting and brewing: successes and future challenges. *Tropical Plant Pathology*, *42*, 153–164.

Sørensen, J. L., & Sondergaard, T. E. (2014). The effects of different yeast extracts on secondary metabolite production in *Fusarium*. *International Journal of Food Microbiology*, *170*, 55–60.

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

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. (2014). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, *40*, 234–242.

Wolf, C. E., & Bullerman, L. B. (1998). Heat and pH alter the concentration of deoxynivalenol in an aqueous environment. *Journal of Food Protection*, *61*, 365–367.

Zachariasova, M., Hajslova, J., Kostelanska, M., Poustka, J., Krplova, A., Cuhra, P., &

Hochel, I. (2008). Deoxynivalenol and its conjugates in beer: A critical assessment of data obtained by enzyme-linked immunosorbent assay and liquid chromatography coupled to tandem mass spectrometry. *Analytica Chimica Acta*, *625*, 77–86.

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.

Zheng, M. Z., Richard, J. L., & Binder, J. (2006). A review of rapid methods for the analysis of mycotoxins. *Mycopathologia*, *161*, 261–273.

Chapter 8. The fate of *Fusarium* mycotoxins (deoxynivalenol and zearalenone) through wort fermenting by Saccharomyces yeast (*S.cerevisiae* and *S. pastorianus*)

Wall-Martinez, H.A., Pascari, X., Bigordà, A., Ramos, A.J., Marin, S., Sanchis, V.

Food Research International 126 (2019), 108587

8.1 Abstract

The aim of this study was to evaluate the effect of 15 commercial yeast in the mitigation of the *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) during the brewing process. *Saccharomyces* strains (10 strains of *S. cerevisiae* and 5 of *S. pastorianus*) were used to ferment DON and ZEN contaminated wort. Wort samples were taken every 24h during fermentation, while mycotoxin analysis in yeast was performed at the end of fermentation (96h); additionally, pH and ethanol content were measured daily. For mycotoxin analysis, after immunoaffinity purification of the sample extracts, analysis was performed using an ultra-high pressure liquid chromatograph coupled with a diode array and fluorescence detectors (UHPLC-DAD/FLD). Mycotoxin presence had no significant effect on ethanol production during brewing. At the end of fermentation, 10-17% of DON and 30-70% of ZEN had been removed, 6% of the total concentration of DON and 31% of ZEN being adsorbed by the yeast. Beermakers must pay careful attention to the raw material since a high percentage of DON could be present at the end of the beer fermentation process. Future studies should focus on the quantification of "masked" mycotoxins that are relevant to food safety.

Keywords:

Beer fermentation, *Fusarium* mycotoxins, *Saccharomyces* yeast, cell wall, mycotoxin adsorption.

8.2 Introduction

Beer is one of the most popular alcoholic beverages in the world (FAO, 2009). By 2017, global beer production had increased to about 1.95 billion hectoliters up from 1.3 billion hectoliters in 1998 (Statista, 2019). It is obtained by fermenting boiled wort (water extract of malt cereal) using yeast (Walther, Ravasio, Qin, Wendland, & Meier, 2015). Cereals used in beer production can be contaminated by different mycotoxins: barley and wheat by ochratoxin A (OTA), trichothecenes, (such as deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins), and zearalenone (ZEN). Corn used as an adjunct during mashing is proven to be mainly contaminated by fumonisins (FBs) and aflatoxins (AFs), but also by trichothecenes (Sanchis, Marín, & Ramos, 2013). All these mycotoxins have been associated with human and animal diseases, some affect the immune system (trichothecenes), while others are hepatotoxic (FBs), nephrotoxic (OTA), estrogenic (ZEN), teratogenic, mutagenic and carcinogenic (AFs). (IARC, 1993; Leblanc, Tard, Volatier, & Verger, 2005; Panel & Chain, 2014; Vila-Donat, Marín, Sanchis, & Ramos, 2018).

Various studies have detected high frequencies and levels of mycotoxins in barley crops (Kroes et al., 2002; Piacentini, Savi, Olivo, & Scussel, 2015; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). Their transfer from barley to malt and to beer subsequently would be expected due to their known resistance to the physical treatments applied during the brewing process (Hazel & Patel, 2004; Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Wolf-Hall, 2007).

However, recent surveys of mycotoxins in beer produced in different geographical regions have mainly detected low levels of DON and its masked forms (DON-3-Glc, 3-Ac-DON), ZEN, OTA and FBs (in order of μg/L) (Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2011; Pascari, Ortiz-Solá, Marín, Ramos, & Sanchis, 2018; Peters et al., 2017; Wall-Martínez, Pascari, Ramos, Marín, & Sanchis, 2019). These small concentrations, together with the available studies, suggest that the various stages of the brewing process (malting, mashing, wort boiling and fermentation) could exert a mitigating effect on mycotoxin contamination or lead to a degradation or transformation to masked forms (Inoue et al., 2013; Kroes et al., 2002; Lancova et al., 2008).

Masked forms of mycotoxins are produced by fungi as well as be formed as part of the defense mechanism of the plant infected with the toxigenic fungi but also are formed during the processing of foods from contaminated raw materials (Freire & Sant'Ana, 2018). Even though toxicological data are scarce, the possibility of modified mycotoxin conversion to its free form may result in a potential risk to human and animal health. The concern is supported as the conversion of modified to free form may lead to increased bioavailability of mycotoxin (Warth et al., 2015).

A study on the transfer of *Fusarium* mycotoxins previously performed by our research group proved that malting could lead to a decrease in the level of DON (from 46.6% up to 78.8%) and an increase in the level of deoxynivalenol-3-glucoside (DON-3-Glc), while ZEN remained almost unchanged (Pascari, Gil-Samarra, Marín, Ramos, & Sanchis, 2019). Also, DON and its metabolites showed a high transfer from malt to the wort during mashing (30 60% of the initial level of DON were identified). During mashing and boiling FBs were reduced by 50 to 100%, and ZEN reduction was higher than 89% (Pascari, Rodriguez-Carrasco, et al., 2019). Considering that the investigated mycotoxins were not completely eliminated after mashing and boiling, there is a need to continue studying mycotoxin levels throughout the fermentation process.

Many species of acid lactic bacteria, yeast and fungi have been shown to enzymatically bind, remove or biodegrade mycotoxins (Faucet-Marquis, Joannis-Cassan, Hadjeba-Medjdoub, Ballet, & Pfohl-Leszkowicz, 2014; Fuchs et al., 2008; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001). It is known that *Gluconobacter oxydans* is able to degrade more than 90 % of patulin (PAT) 80% of OTA and 27% of aflatoxin B_1 (AFB_1) (Markov et al., 2019). Yeast biomass have demonstrated the ability to adsorb mycotoxins due to the presence of mannoproteins and β-glucans in their cell wall, based on physical adsorption, ion exchange and complexation, (Faucet-Marquis et al., 2014; Huwig, Freimund, Käppeli, & Dutler, 2001; Shetty & Jespersen, 2006). Along these lines, Campagnollo et al. (2015) utilized *in vitro* test to determine the capacity of one strain of *S. cerevisiae* to bind mycotoxins: ZEN (77%), OTA (13%), aflatoxin B1 (AFB_1) (38%) and DON (17%) at pH=6, and ZEN (75%), OTA (59%), AFB_1 (48%) and DON (11%) at pH=3.

Few studies investigating the fate of mycotoxins during beer fermentation are available. Garda et al. (2005) identified a 53% reduction of DON and T-2 toxin at the end of the fermentation process with a strain of *Saccharomyces cerevisiae*. Nathanail et al. (2016), using a strain of *Saccharomyces pastorianus* observed a mycotoxin reduction of up to 15 % for DON, 17% for DON-3-Glc, 34% for HT-2, and 31% for T-2 toxins after a 96h period of beer fermentation. From the abovementioned studies, it can be seen that different *Saccharomyces* strains have different abilities in binding mycotoxins. Also, considering the physical nature of the adsorption, this result could be modulated by different physical parameters, such as pH and temperature, that are applied during the process. (Avantaggaito, Solfrizzo, & Visconti, 2005; Huwig et al., 2001). Therefore, the present study focuses on evaluating under single conditions the effect of different starter cultures (10 *S. cerevisiae* and 5 *S. pastorianus* strains) in the mitigation of the *Fusarium* mycotoxins (DON and ZEN) contamination during the beer fermentation process.

8.3 Materials and methods

8.3.1 Chemicals and reagents

Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol and acetonitrile (HPLC grade) were purchased from Scharlab (Sentmenat, Spain). Mycotoxin standards of DON and ZEN were purchased from Romer Labs (Tulln, Austria).

Immunoaffinity columns (IAC) for DON (DONPREP®) and ZEN (EASY-EXTRACT® ZEARALENONE) clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). 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 (Basic 20, CRISON) with hydrochloric acid 1 M. Bacteriological peptone was purchased from Biokar Diagnostics (Allonne, France), magnesium sulfate heptahydrate was acquired from Probus SA

(Badalona, Spain) and agar powder from VWR Prolabo (Leicestershire, UK). Yeast extract peptone dextrose medium (YEPD) was prepared with 10 g/L of yeast extract powder (Bacto, Madrid, Spain), 20 g/L of peptone (Biokar Diagnostics, Allone, France), 20 g/L of glucose (Fisher chemical, UK) and 20 g/L of agar (Prolabo, Leuven Belgium).

8.3.2 Preparation of mycotoxin solutions

DON concentration in the stock solution was checked by UV spectroscopy according to AOAC Official Methods of Analysis, Chapter 49 (AOAC, 2005), obtaining a concentration of the stock solution of 758 and 844 µg/mL, respectively. Standard solutions of DON and ZEN were prepared in methanol at a concentration of 10.0 mg/mL and stored at 4°C. Calibration curves were prepared by appropriate dilution of known volumes of the stock solution with the mobile phase.

8.3.3 DON and ZEN contamination of the malted barley

Twenty kg of malted barley (*Hordeum vulgare*) was supplied by a malting plant (Malteria la Moravia S.L., Bell-lloc d'Urgell, Spain) in March 2018, DON and ZEN were not detected in this malted barley by UHPLC-DAD/FLD analysis. A control batch of malted barley was used to prepare control wort (absent of DON and ZEN), the rest of the malt was contaminated using a toxigenic strain of *Fusarium graminearum* (F.46) (producer of DON and ZEN) obtained from the collection of strains of the Food Technology Department of the University of Lleida, Spain. The grains were disinfected according to Pascari, Rodriguez-Carrasco, et al. (2019). Briefly, 500 g of grains was submerged into 0.4 g/100 mL sodium hypochlorite solution for 2 min and then abundantly rinsed twice with sterile distilled water. Then the grains were placed in hermetically closed sterile ISO bottles and left overnight at 4 °C with a small amount of water to allow the water activity to reach a value close to 0.99 (Aqualab Series 3 TE, Decagon Devices Inc., Washington, USA). Malted barley was aseptically transferred to Petri dishes and 1 mL of a spore suspension of *F. graminearum* (10^6 spores/mL) was transferred to each dish. Petri dishes were incubated at 25°C for 30 days, then contaminated grains were dried at 40°C, homogenized, and DON and ZEN content was determined. The final concentrations in the contaminated

malt were 4600 µg/kg and 2800 µg/kg for DON and ZEN, respectively.

8.3.4 Yeast

Fifteen freeze-dried yeast strains of various commercial brands, 10 *S. cerevisiae* (ale – high fermentation) and 5 *S. pastorianus* (lager – low fermentation) strains were used. All the yeasts used in this study were purchased from a website specializing in the sale of beer preparation supplies (www.cervezasdelmundo.com).

8.3.5 Wort production

Mycotoxin-contaminated wort was prepared by adding appropriate proportions of the contaminated grains previously produced to uncontaminated malted grits. Maceration was carried out by mixing 2.5 L of deionized water with 500 g of malted barley (previously ground) and then maintaining the mix for 15 min at 45°C, followed by 1h at 65°C, ending with 72°C for another 15 min. The wort was decanted, and the density was adjusted to 1005-1010 kg/m^3 with deionized water. The wort was boiled for approximately 2h (up to a density of 1050 kg/m^3) and stored in sterile bottles until fermentation.

A control wort batch was prepared using the malted barley in which the absence of DON and ZEN was determined.

8.3.6 Fermentation

Fermentation assays were performed at 250 mL scale with triplicate samples. The process lasted 96 h at 20 °C. The concentration of yeast used to inoculate the wort was selected following the brand's instructions. Yeast concentration was adjusted at 10^6 CFU/mL for all the wort samples. An initial count was made using a Thoma cell counting chamber. Moreover, a confirmatory initial yeast count on YEPD medium was performed for all the samples. Briefly, 1 mL of the samples was diluted from 10^{-1} to 10^{-7} with sterile saline peptone. Then, 100 µL of each dilution was superficially spread on YEPD agar medium dishes and incubated at 25°C for 48h.

Two samples were taken daily at the following time points: 24h, 48h, 72h, and 96h. The first sample (10 mL) was used to determine the pH and alcohol content (with a portable

densimeter DMA 35, Anton Paar). The second sample (30 mL) was centrifuged at 1869 x g for 10 min to eliminate the yeast from the fermented wort and was used to determine mycotoxins content. After four days' fermentation, the yeast was separated from the remaining wort by centrifugation at 1869 x g for 10 min, lyophilized, weighed and all samples (wort and yeast) were stored at -18ºC until HPLC analysis.

8.3.7 Mycotoxin extraction

DON and ZEN were extracted using specific immunoaffinity columns (DONPREP® and EASY EXTRACT® ZEARALENONE, respectively) following the manufacturer's instructions.

8.3.7.1 DON extraction in malt

Five grams of malt previously ground with an IKA® A11 Basic mill (Darmstadt, Germany) was mixed with 1 g of sodium chloride and 40 mL of Mili Q water in a 250 mL Erlenmeyer flask, followed by 30 min stirring. Then, samples were centrifuged for 10 min at 1846 x g. Supernatant was filtered through a glass microfiber paper filter (Whatman nº 1, Maidstone, UK) and 2 mL of the filtrate was passed through the immunoaffinity column. The column was then washed with 10 mL of bi-distilled water and the toxins were eluted with 3 mL of methanol HPLC-grade (the first 1.5 mL performing back-flushing). Samples were evaporated under a low nitrogen stream at 40°C and resuspended in the mobile phase (acetonitrile:methanol:water, 5:5:90, v/v/v). Every resuspended extract was filtered through a nylon filter (0.4 µm) before being injected (50 µL) into the UHPLC-DAD system.

8.3.7.2 ZEN extraction in malt

Five grams of malt previously ground with an IKA® A11 Basic mill (Darmstadt, Germany) was mixed with 25 mL of acetonitrile:water (75:25, v/v) extraction solvent and stirred for 30 min. Samples were centrifuged for 10 min at 1846 x g and 10 mL of the supernatant was mixed with 40 mL of phosphate buffer saline (PBS) at pH 7.4. The 50 mL obtained from this process was passed through the immunoaffinity column which was afterwards washed with 20 mL of PBS. ZEN was eluted with 3 mL of acetonitrile (the first 1.5 mL performing back-flushing). Samples were evaporated under a low nitrogen stream at 40°C and

resuspended in 1 mL of acetronitrile:Mili Q water (50:50). Every resuspended extract was filtered through a nylon filter (0.4 µm) before being injected (50 µL) into the UHPLC-FLD system.

8.3.8 Mycotoxin analysis in wort

8.3.8.1 DON extraction in the fermented wort

Wort was filtered through glass microfiber filter paper (Whatman no. 1, Whatman, Maidstone, UK) and 1 mL of filtered sample was inserted into an immunoaffinity column at a flow rate of 2 mL per minute. The column was washed by passing 10 mL of Mili Q water at a rate of 5 mL per minute, and finally, air was allowed to pass through the column to remove the residual liquid. DON elution was performed by adding 1.5 mL + 1.5 mL of methanol, performing a back-flushing each time. The samples were evaporated and resuspended in 1 mL of the mobile phase for UHPLC-DAD analysis.

8.3.8.2 ZEN extraction in the fermented wort

Initially, 20 mL of centrifuged wort was mixed with 20 mL of PBS, and pH was adjusted to a pH of 7.4. 20 mL of the mixture was passed through an immunoaffinity column at a flow rate of 2 mL per minute, the column was washed by passing 10 mL of water at a rate of 5 mL per minute and finally, air was allowed to pass through the column to remove the residual liquid. Elution was performed by adding 1.5 mL+1.5 mL of acetonitrile, back-flushing being performed each time. The samples were evaporated and resuspended in 1 mL of acetronitrile:Mili Q water (50:50) for UHPLC-FLD analysis.

8.3.4 DON and ZEN extraction in yeast

DON and ZEN extraction in yeast was performed following the Campagnollo et al. (2015) method. Two hundred milligrams of lyophilized yeast were suspended in 2 mL of 0.1M potassium phosphate buffer pH=6.5, mixed on a rotating shaker (Infors AG-CH-4103 Bottmingen, Switzerland) for 60 min at 25°C and sonicated for 15 min (Brason 2800). The samples were centrifuged (Eppendorf, Hamburg, Germany) at 1846 x g for 10 min at room temperature and 0.8 mL of the supernatant was removed and analyzed by UHPLC. Negative controls (200 mg yeast in buffer solution) were also prepared and analyzed.

8.3.5 UPLC system

The determination of DON was performed using an Agilent Technologies 1260 Infinity UHPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Gemini® C18 column from Phenomenex 150x4.6 mm (California, USA) with a particle size of 5 µm and a pore size of 110 Å was used. Absorption wavelength was setup at 220 nm. The mobile phase was composed of methanol:acetonitrile:water Mili Q (5:5:90, v/v/v) and set at a flow rate of 1 mL/min. For ZEN determination, the same equipment and column were used but the HPLC was coupled to an Agilent 1260 Infinity Fluorescence detector (FLD) (excitation and emission wavelengths were 274 nm and 455 nm, respectively) was used. The mobile phase was acetonitrile:Milli Q water (60:40, v:v) with pH adjusted to 3.2 with acetic acid. Flow rate was set at 0.6 mL/min. The column temperature was 40°C, the injection volume was 50 µL and total run time was 20 min for both mycotoxin analyses.

8.3.6 Validation of the analytical methods

Selectivity was checked by injecting 50 µL of standard solution at least three times (150 µg/L) and comparing retention time and peak resolution between injections. For linearity check, a calibration curve of eight concentration levels for each toxin (20, 30, 50, 100, 250, 500, 1000, 3000 µg/L for DON and 30, 50, 100, 300, 500, 1000, 1500, 3000 µg/L of ZEN solutions) was prepared and injected into the system, generating a linear regression plotting solutions' concentration versus peak area. The limit of detection (LOD) was considered as three times the signal-to-noise ratio. Method performance was assessed according to Commission Regulation (EC) 401/2006 (European Commission, 2006) (Table 23). Finally, precision was evaluated preparing blank wort and yeast samples spiked with DON and ZEN at several concentration levels and recovery percentages were determined: 75 90% (wort), 67 71% (yeast) for DON, and 84 97% (wort), 73 80% (yeast) for ZEN.

The recovery rates of mycotoxins in the yeast were lower than those obtained in the wort, this may be because it was a solid extract and that was extracted without immunoaffinity columns; however, the validation was effective according to Commission Regulation, 2006 for spiked between 100 and 500 µg/kg (60-110%) and higher than 500 µg/kg (70-120% for

Table 23: Validation parameters of the UHPLC method for the mycotoxin analysis in wort and yeast

Mycotoxin	Matrix	Spiking levels (µg/kg)	Replicates	Recovery ± SD (%)	RSD (%)
DON	Wort	50	5	74.58 ± 5.48	7.34
DON	Wort	500	7	82.55 ± 8.17	9.90
DON	Wort	1000	5	89.52 ± 4.12	4.60
DON	Yeast	250	5	67.18 ± 7.15	10.65
DON	Yeast	750	7	70.20 ± 5.55	7.90
DON	Yeast	3000	5	71.27 ± 10.68	14.98
ZEN	Wort	25	5	84.36 ± 6.05	7.17
ZEN	Wort	75	7	86.93 ± 6.99	8.04
ZEN	Wort	100	5	96.52 ± 15.42	15.98
ZEN	Yeast	250	5	72.89 ± 10.52	14.43
ZEN	Yeast	750	7	80.24 ± 8.11	10.11
ZEN	Yeast	3000	5	76.58 ± 8.76	11.44

LOD DON: 20 µg/kg, LOD ZEN: 2.5 µg/kg. SD: Standard deviation. RSD: Relative standard deviation.

Table 24: Alcohol level and pH produced in wort after the fermentation (96 h fermentation)

Yeast strain	pH		Alcohol produced (%)	
	Control	Contaminated	Control	Contaminated
S. cerevisiae 1	3.89 ± 0.05	4.00 ± 0.08	4.07 ± 0.04 [a]	3.85 ± 0.05 [b]
S. cerevisiae 2	3.86 ± 0.05	4.03 ± 0.05	3.89 ± 0.27 [a]	3.88 ± 0.05 [a]
S. cerevisiae 3	3.71 ± 0.16	3.99 ± 0.06	3.55 ± 0.30 [a]	3.83 ± 0.16 [a]
S. cerevisiae 4	3.84 ± 0.03	4.15 ± 0.02	4.11 ± 0.08 [a]	3.98 ± 0.02 [a]
S. cerevisiae 5	3.68 ± 0.11	3.87 ± 0.05	3.23 ± 0.31 [a]	3.96 ± 0.04 [a]
S. cerevisiae 6	3.81 ± 0.05	3.92 ± 0.06	4.11 ± 0.20 [a]	3.81 ± 0.05 [a]
S. cerevisiae 7	3.88 ± 0.02	3.80 ± 0.04	3.81 ± 0.05 [a]	3.88 ± 0.02 [a]
S. cerevisiae 8	4.34 ± 0.02	3.77 ± 0.04	4.22 ± 0.19 [a]	4.29 ± 0.02 [a]
S. cerevisiae 9	3.65 ± 0.06	3.85 ± 0.80	3.95 ± 0.03 [a]	3.96 ± 0.04 [a]
S. cerevisiae 10	3.68 ± 0.03	4.07 ± 0.02	4.33 ± 0.02 [a]	4.18 ± 0.13 [a]
S. pastorianus 1	3.71 ± 0.01	4.07 ± 0.07	3.92 ± 0.06 [a]	3.77 ± 0.07 [a]
S. pastorianus 2	3.71 ± 0.10	3.75 ± 0.08	3.48 ± 0.21 [a]	3.67 ± 0.02 [a]
S. pastorianus 3	3.83 ± 0.08	4.12 ± 0.02	4.08 ± 0.08 [a]	3.77 ± 0.03 [b]
S. pastorianus 4	3.80 ± 0.07	4.03 ± 0.10	4.06 ± 0.14 [a]	3.68 ± 0.10 [b]
S. pastorianus 5	3.85 ± 0.04	3.78 ± 0.02	3.37 ± 0.21 [a]	3.68 ± 0.13 [a]

Values expressed as mean ± standard deviation of samples analyzed in triplicate. [a-b] Levels with different letters within a row are significantly different ($P < 0.05$).

DON and 60-120% for ZEN).

8.3.7 Statistical analysis

All the results are presented as the average of triplicate tests and expressed as mean ± standard deviation. Statistical evaluations were performed by one-way analysis of variance (ANOVA) and Tukey test ($p = 0.05$) using Minitab 18 software.

8.4 Results and discussion

8.4.1 Alcohol production and pH

Control and mycotoxin-contaminated wort had an initial density of 1.043 g/cm^3, whereas initial pH was 4.85 (control) and 5.0 (mycotoxin contaminated). Initial mycotoxin contamination of wort was 1380 µg/kg (DON) and 600 µg/kg (ZEN). Neither DON nor ZEN were detected in the control wort.

Final pH and alcohol produced after 96 h of wort fermentation for the different yeast strains assayed are shown in Table 24.

The pH of the wort at the end of fermentation ranged between 3.80-4.34 (control) and 3.75-4.15 (contaminated). There are no indications of an effect on pH due to the presence of mycotoxins, so the variation in the pH observed in control and contaminated wort could be attributed to the initial difference since pH of the control wort was slightly lower than that of the contaminated wort. Regarding alcohol production, the results agree with those reported by Nathanail et al. (2016) in that the mycotoxins present in the wort had no significant effect on alcohol production during beer fermentation. The progress of alcohol content during the fermentation of the wort using one *S. cerevisiae* and one *S. pastorianus* strain is showed in Figure 13.

Figure 13 shows that in the four sampling points analyzed during the 96 h of beer fermentation no significant differences were found in ethanol content between contaminated and uncontaminated wort.

8.4.2 DON fate

Mycotoxin adsorption on the cell wall structure is an interaction between the toxin and the functional groups of the cell surface. Yeast cell walls contain many different adsorption sites, mainly represented by polysaccharides (glucans and mannans), proteins and lipids (Faucet-Marquis et al., 2014). Considering that mycotoxin adsorption is physical by nature (based on ion exchange and complexation) (Huwig et al., 2001), mycotoxin contamination has been proven to exert little influence on yeast activity (Nathanail et al., 2016).

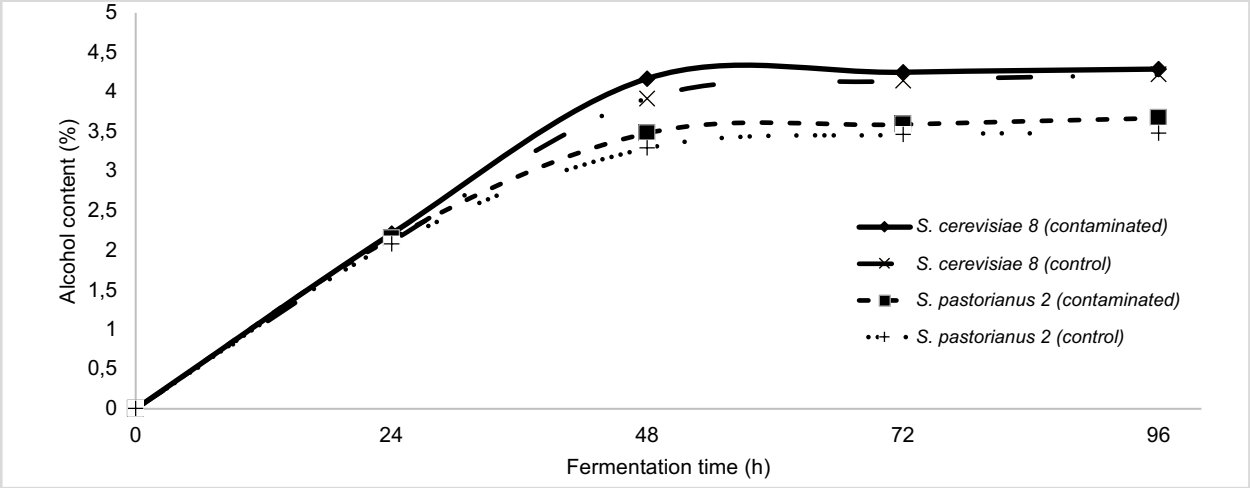

Figure 13: Alcohol production during fermentation of the contaminated and control wort

There are few studies dedicated to investigating the adsorption of DON to yeast cell wall, most of them being performed *in vitro* (Campagnollo et al., 2015; Scott, Kanhere, Daley, & Farber, 1992; Sørensen & Sondergaard, 2014). The present research assesses the adsorption of DON onto brewing yeast cell walls. Table 25 presents DON concentration in yeast and brewing wort throughout the 96 h of fermentation.

The highest effect in DON reduction occurred during the first 24h, resulting in 84% to 95% of the initial content of mycotoxin that remained in the wort. The final concentration of DON in the obtained beers, after 96h of fermentation, ranged from 97.9 82.5% of the initial contamination, with reductions higher than 15% in 9 out of 15 beers. Similar results were obtained by Nathanail et al. (2016) who reported a reduction of 15% in the DON content in a wort using an *S. pastorianus* yeast strain. Garda et al. (2005) observed a reduction of 53.3% in malt spiked with DON and ZEN. Previously, Scott et al. (1992) using

a contaminated wort with DON and ZEN after 7-9 days of fermentation with *S.* cerevisiae reported that DON was stable through the process. The variability in DON reduction in the different studies may be due to various factors including pattern of grain infection, process parameters, interactions with the yeast used or mycotoxin metabolization in a modified form, such as DON-3-Glc, 3-Ac-DON and 15-Ac-DON (Bryła, Ksieniewicz-Woźniak, Waśkiewicz, Szymczyk, & Jędrzejczak, 2018; Kostelanska et al., 2011; Nathanail et al., 2016). Furthermore, it can be seen from Table 25 that the total concentration that was identified in the beer and in the yeast residue suggests that approximately 10% of toxin could be transformed by the yeasts into metabolites, most probably into 3-Ac-DON (Khatibi et al., 2011). This suggests the need for a deeper biological analysis of the yeast strains used in beer fermentation in order to better evaluate the nature of this change.

Regarding DON content in yeast, up to 6.36% of the initial content in the wort was detected. Mycotoxins binding to the yeast is attributed to the formation of both hydrogen bonds and van der Waals interactions between hydroxyl, ketone and lactone groups from β-glucans of the cell wall and the mycotoxin (Faucet-Marquis et al., 2014; Shetty & Jespersen, 2006; Takata et al., 2010; Yiannikouris et al., 2006). In 3 of the 15 samples, the adsorbed mycotoxin was found to be below the detection limit, and the wort fermented by these yeasts presented the lowest decrease in DON after 96 h of fermentation (<11%). In similar works, Campagnollo et al. (2015), performing an *in vitro* test, determined a DON binding in *S. cerevisiae* of 11.6 and 17.6% at pH 3 and 6.5, respectively, while Nathanail et al. (2016) found quantifiable DON values in yeast pellet analyzed after wort fermentation with a high initial DON concentration (10,000 μg/L). The difference in adsorption levels could be explained by strain-dependent cell wall structure, the phase of the cell cycle and growth conditions such as pH, temperature, oxygenation rate, and concentration and nature of carbon source (Jouany, Yiannikouris, & Bertin, 2005).

Table 25:DON concentration in yeast and wort during beer fermentation

	DON in wort (%)												DON in yeast (%)		
Yeast	**24h**			**48 h**			**72 h**			**96 h**			**96 h**		
S. cerevisiae 1	87.27	±	0.36 ^{bc}	87.50	±	0.57 ^{b}	89.82	±	1.45 ^{b}	83.98	±	1.61 ^{c}	6.36	±	1.42
S. cerevisiae 2	87.07	±	2.28 ^{bc}	88.82	±	2.40 ^{b}	84.00	±	2.21 ^{bc}	83.38	±	0.28 ^{c}	4.15	±	1.80
S. cerevisiae 3	86.93	±	3.24 ^{b}	89.06	±	1.08 ^{b}	90.25	±	0.84 ^{b}	88.71	±	2.49 ^{b}	3.37	±	1.25
S. cerevisiae 4	87.44	±	3.57 ^{b}	84.19	±	3.71 ^{b}	83.55	±	2.50 ^{b}	82.51	±	5.03 ^{b}	4.69	±	1.06
S. cerevisiae 5	89.60	±	2.47 ^{b}	87.94	±	1.62 ^{b}	88.09	±	0.98 ^{b}	85.64	±	1.00 ^{b}	4.60	±	0.32
S. cerevisiae 6	85.33	±	0.94 ^{b}	83.63	±	1.08 ^{b}	83.52	±	2.80 ^{b}	83.66	±	1.15 ^{b}	5.32	±	0.86
S. cerevisiae 7	88.06	±	7.99 ^{b}	90.81	±	0.94 ^{ab}	86.70	±	1.82 ^{b}	90.86	±	1.38 ^{ab}		<LOD	
S. cerevisiae 8	84.79	±	5.76 ^{b}	84.39	±	6.84 ^{b}	79.76	±	8.73 ^{b}	83.17	±	2.69 ^{b}	5.92	±	1.95
S. cerevisiae 9	86.75	±	0.61 ^{b}	85.51	±	2.97 ^{b}	84.08	±	2.85 ^{b}	83.86	±	3.36 ^{b}	6.29	±	2.72
S. cerevisiae 10	86.64	±	0.49 ^{b}	86.63	±	1.80 ^{b}	76.91	±	6.68 ^{c}	87.30	±	0.62 ^{b}	3.05	±	0.64
S. pastorianus 1	91.33	±	3.13 ^{a}	92.67	±	3.26 ^{a}	92.86	±	0.78 ^{a}	88.99	±	3.60 ^{a}		<LOD	
S. pastorianus 2	95.10	±	5.05 ^{a}	92.45	±	3.12 ^{ab}	90.25	±	2.69 ^{ab}	83.40	±	5.53 ^{b}	4.68	±	0.57
S. pastorianus 3	89.49	±	3.33 ^{b}	85.89	±	0.60 ^{bc}	84.62	±	1.01 ^{bc}	83.27	±	2.70 ^{c}	2.97	±	1.10
S. pastorianus 4	95.59	±	2.04 ^{a}	98.30	±	2.39 ^{a}	92.09	±	11.16 ^{a}	97.87	±	2.61 ^{a}		<LOD	
S. pastorianus 5	91.51	±	2.41 ^{a}	89.32	±	1.69 ^{ab}	86.49	±	2.34 ^{b}	84.36	±	6.79 ^{b}	3.88	±	0.19

Initial DON contamination in the wort was 1378.89 ± 22.60 μg kg^{-1} (100% ^{a}). Values expressed as mean ± standard deviation of samples analyzed in triplicate. $^{a-b}$ Levels with different letters within a row are significantly different ($P < 0.05$). LOD DON: 20 μg kg^{-1}.

8.4.3 ZEN fate

The ZEN concentration in yeast and brewing wort during 96h of fermentation is presented in Table 26. Unlike DON, whose degradation occurred mainly during the first 24h, the reduction in the ZEN content was continuous throughout the 96h fermentation period. The beer wort showed a total ZEN reduction of between 31 and 72%, and the toxin adsorbed to yeast ranged between 4.5 and 31.3%.

In similar studies, Campagnollo et al. (2015) established the ability of the beer fermentation residue to bind ZEN at acid and neutral pH, with *S. cerevisiae* able to adsorb 75.1% (pH=3) and 77.5% (pH=6.5) of the initial contamination. Previously, Yiannikouris et al. (2004) examined four different compositions of β-(1,3)-D-glucans and β-(1,6)-D-glucan for their ability to adsorb ZEN at pH 3.0, 6.0 and 8.0. At neutral and acid pH, ZEN adsorption ranged from 53.8 to 76.9% (pH=3.0) and from 57.9 to 73.9% (pH=6.0), but at basic pH (8.0) the adsorption ranged from not detected (ND) to 51.8%. In our study, the difference in mycotoxin binding cannot be attributed to the pH differences between the samples, as the pH of the samples ranged only from 3.8 to 4.2.

It is known that the ZEN adsorption capacity of the cell wall of yeast is strongly correlated with the β-D-glucan content of the cell wall; additionally, differences in the binding of mycotoxins with yeast could exist due to charge distribution, the chemical nature of mycotoxin in relation to surface properties, geometry and the available surface of the adsorbent (Bakutis, Baliukonienė, & Algimantas, 2005; Kabak, Dobson, & Var, 2006). In addition to the adsorption to the yeast, the reduction in the ZEN content can be attributed to a possible biodegradation or metabolization of ZEN to other compounds, as β-zearalenol (β-ZEL) and α-zearalenol (α-ZEL) by the brewing strains of *Saccharomyces* during beer production (Keller et al., 2015; Lancova et al., 2008; Mizutani, Nagatomi, & Mochizuki, 2011). This added to the reductions taking place the malting and maceration (higher than 50%) (Pascari, Rodriguez-Carrasco, et al., 2019), could explain the low or no incidence level reported in studies of ZEN in beers (Pascari et al., 2018; Wall-Martínez et al., 2019).

Table 26: ZEN concentration in the wort and yeast during fermentation

	ZEN in wort (%)												ZEN in yeast (%)		
Yeast	**24h**			**48 h**			**72 h**			**96 h**			**96h**		
S. cerevisiae 1	88.72	±	1.06 ^{b}	83.18	±	4.51 ^{c}	75.96	±	2.64 ^{cd}	69.04	±	7.25 ^{d}	14.06	±	4.37
S. cerevisiae 2	86.50	±	2.17 ^{ab}	75.31	±	1.01 ^{bc}	68.13	±	2.52 ^{d}	64.63	±	1.31 ^{d}	6.73	±	5.90
S. cerevisiae 3	89.53	±	4.08 ^{ab}	79.35	±	3.48 ^{bc}	73.66	±	5.15 ^{bc}	64.19	±	13.83 ^{c}	18.43	±	5.88
S. cerevisiae 4	82.28	±	1.21 ^{b}	71.18	±	4.02 ^{c}	65.67	±	1.52 ^{c}	60.40	±	2.07 ^{d}	12.41	±	4.31
S. cerevisiae 5	79.73	±	5.10 ^{b}	77.20	±	4.54 ^{bc}	71.43	±	1.37 ^{bc}	69.18	±	0.44 ^{c}	4.50	±	1.83
S. cerevisiae 6	76.37	±	2.24 ^{b}	64.70	±	4.63 ^{c}	50.75	±	1.40 ^{d}	43.29	±	2.47 ^{e}	31.25	±	11.12
S. cerevisiae 7	61.80	±	1.92 ^{b}	49.40	±	2.98 ^{c}	44.48	±	0.52 ^{cd}	38.36	±	3.88 ^{d}	19.81	±	3.05
S. cerevisiae 8	68.59	±	1.51 ^{b}	50.53	±	4.58 ^{c}	31.53	±	1.63 ^{d}	28.13	±	0.86 ^{d}	28.16	±	8.05
S. cerevisiae 9	83.99	±	2.85 ^{b}	73.94	±	0.59 ^{c}	64.62	±	5.77 ^{d}	62.14	±	4.10 ^{d}	24.49	±	17.10
S. cerevisiae 10	61.45	±	17.61 ^{b}	58.84	±	1.70 ^{bc}	46.75	±	0.96 ^{c}	34.35	±	0.35 ^{c}	28.55	±	7.54
S. pastorianus 1	75.97	±	3.83 ^{b}	88.97	±	3.97 ^{ab}	66.32	±	3.48 ^{c}	63.11	±	10.93 ^{c}	8.19	±	2.68
S. pastorianus 2	83.75	±	3.12 ^{b}	78.12	±	1.02 ^{b}	57.85	±	1.95 ^{c}	53.98	±	1.58 ^{c}	13.79	±	4.51
S. pastorianus 3	66.24	±	4.89 ^{b}	49.74	±	0.96 ^{c}	31.16	±	1.72 ^{d}	29.09	±	2.18 ^{d}	16.75	±	1.67
S. pastorianus 4	81.00	±	1.09 ^{b}	69.10	±	3.40 ^{bc}	67.01	±	2.52 ^{c}	60.40	±	2.07 ^{d}	7.60	±	2.45
S. pastorianus 5	61.39	±	9.75 ^{b}	47.97	±	7.59 ^{bc}	41.10	±	2.31 ^{c}	69.18	±	0.44 ^{c}	15.81	±	2.77

The initial ZEN contamination in the wort was 598.50 ± 4.40 µg/ kg (100% ^{a}). Values expressed as mean ± standard deviation of samples analyzed in triplicate. $^{a-b}$ Levels with different letters in the same row are significantly different ($P < 0.05$).

8.4.4 *S. cerevisiae vs S. pastorianus*

Table 27 shows the yeast performance for the two *Saccharomyces* species in the context of mycotoxin adsorption evaluated in the present study.

In our study fermentation temperature was 20°C, more suitable for *S. cerevisiae*, resulting in a higher quantity of biomass for this yeast after 96h of fermentation and, consequently, higher ethanol production; however, this increase in biomass is hardly significant ($p=0.037$) and did not imply a significant impact on adsorption for DON and ZEN mycotoxins. These factors demonstrate the need to study the process variables (temperature, concentration of yeast) to optimize the removal of mycotoxins during the fermentation process for *S. cerevisiae and S. pastorianus* yeast.

It is recommended that the study be expanded to include the different forms of DON and ZEN, with the aim of ascertaining whether the percentage of mycotoxin that was not detected in wort or yeast could be "masked" and found in sufficient concentration to represent a toxicological risk to the consumer.

8.5 Conclusion

The effect of 15 *Saccharomyces* yeasts in the mitigation of *Fusarium* mycotoxins (DON and ZEN) through brewing was studied, performing a comparative analysis between *S. cerevisiae and S. pastorianus.* ZEN showed higher adsorption (4.50% – 31.25%) than DON (LOD – 6.36%) to *Saccharomyces* yeasts. On the other hand, mycotoxin content in the wort had decreased 11 – 17% for DON and 31 – 72% for ZEN by the end of fermentation. There was no significant difference in the decrease of DON or ZEN when is fermented with *S. cerevisiae* or *S. pastorianus* at 20°C. While mycotoxin presence had no significant effect on ethanol production during the fermentation, brewers must pay special attention to mycotoxin content in raw materials, since a low concentration could be present in the beer or even in the yeast (for unfiltered beers) at the end of brewing process.

Table 27: Properties of mycotoxin contaminated wort fermented with *Saccharomyces*

Yeast	**Fermentation temperature range (°C)**	**Biomass produced (g)**	**Ethanol produced (%)**	**DON adsorbed to cells (%)**	**DON in wort after fermentation (%)**	**ZEN adsorbed to cells (%)**	**ZEN in wort after fermentation (%)**
S. cerevisiae	16-25	1.16 ±0.20[a]	3.99 ± 0.40[a]	3.11 ± 1.18 [a]	85.31 ± 2.77 [a]	18.84 ± 9.34 [a]	47.84 ± 13.74 [a]
S. pastorianus	13-20	0.94 ±0.10[b]	3.71 ± 0.05[b]	4.58 ±1.46 [a]	87.58 ± 6.21 [a]	12.43 ± 4.28[a]	53.37 ± 16.60 [a]
P value	--	0.037	0.017	0.73	0.34	0.17	0.51

Values expressed as mean ± standard deviation of samples analyzed in triplicate. [a-b] Levels with different letters in the same row are significantly different ($P < 0.05$).

Future studies should focus on the analysis of "masked" mycotoxins in wort and yeast, to determine if the reduction in contamination was due to transformation, elimination, or to binding to the yeast, and on the optimization of process conditions, *e.g.* yeast concentration and process temperature, in order to improve the reduction of mycotoxin content during the beer production process.

8.6 Acknowledgement

The authors are grateful to the University of Lleida (JADE Plus 218/2016 grant), to the Spanish Ministry of Economy and Competitiveness (MINECO, AGL2014 55379 P project) for funding this work. Wall-Martínez HA is grateful to the Consejo Nacional de Ciencia y Tecnología (CONACyT) of Mexico for the scholarship.

8.7 References

Avantaggaito, G., Solfrizzo, M., & Visconti, A. (2005). Recent advances on the use of adsorbent materials for detoxification of *Fusarium* mycotoxins. *Food Additives and Contaminants*, *22*, 379 388.

Bakutis, B., Baliukonienė, V., & Algimantas, P. (2005). Use of biological method for detoxification of mycotoxins. *Botanica Lithuanica*, *7*, 123 129.

Bertuzzi, T., Rastelli, S., Mulazzi, A., Donadini, G., & Pietri, A. (2011). Mycotoxin occurrence in beer produced in several European countries. *Food Control*, *22*, 2059 2064.

Bryła, M., Ksieniewicz-Woźniak, E., Waśkiewicz, A., Szymczyk, K., & Jędrzejczak, R. (2018). Co-occurrence of nivalenol, deoxynivalenol and deoxynivalenol-3-glucoside in beer samples. *Food Control*, *92*, 319 324.

Campagnollo, F. B., Franco, L. T., Rottinghaus, G. E., Kobashigawa, E., Ledoux, D. R., Daković, A., & Oliveira, C. A. F. (2015). In vitro evaluation of the ability of beer fermentation residue containing *Saccharomyces cerevisiae* to bind mycotoxins. *Food Research International*, *77*, 643 648.

Commission Regulation. (2006). 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. *Oficial Journal of the European Union*, *2006*(401), 12–34.

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

FAO. (2009). Agribusiness Handbook. *FAO Agribusiness*.

Faucet-Marquis, V., Joannis-Cassan, C., Hadjeba-Medjdoub, K., Ballet, N., & Pfohl-Leszkowicz, A. (2014). Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B1, zearalenone and ochratoxin A. *Applied Microbiology and Biotechnology*, *98*, 7583–7596.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189–205.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., & Knasmüller, S. (2008). Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *Food and Chemical Toxicology*, *46*, 1398–1407.

Garda, J., Martins Macedo, R., Faria, R., Bernd, L., Dors, G. C., & Badiale-Furlong, E. (2005). Alcoholic fermentation effects on malt spiked with trichothecenes. *Food Control*, *16*, 423–428.

Haskard, C. A., El-Nezami, H. S., Kankaanpää, P. E., Salminen, S., & Ahokas, J. T. (2001). Surface Binding of Aflatoxin B_1 by lactic acid bacteria. *Applied and Environmental Microbiology*, *67*, 3086–3091.

Hazel, C. M., & Patel, S. (2004). Influence of processing on trichothecene levels. *Toxicology Letters*, *153*, 51–59.

Huwig, A., Freimund, S., Käppeli, O., & Dutler, H. (2001). Mycotoxin detoxication of animal feed by different adsorbents. *Toxicology Letters*, *122*, 179–188.

IARC - Internationa Agency for research on Cancer. (1993). Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and

mycotoxins. *IARC Monographs on the Evaluation of Carcinogenic Risk to Human*, *56*, 397 444.

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. (2013). Fate of mycotoxins during beer brewing and fermentation. *Bioscience, Biotechnology, and Biochemistry*, *77*(7), 1410 1415.

Jouany, J., Yiannikouris, a, & Bertin, G. (2005). The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified. *Archiva Zootechnica*, (Table 2), 26 50.

Kabak, B., Dobson, A. D. W., & Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. *Critical Reviews in Food Science and Nutrition*, *46*(8), 593 619.

Keller, L., Abrunhosa, L., Keller, K., Rosa, C. A., Cavaglieri, L., & Venâncio, A. (2015). Zearalenone and its derivatives α-zearalenol and β-zearalenol decontamination by *Saccharomyces cerevisiae* strains isolated from bovine forage. *Toxins*, *7*, 3297 3308.

Khatibi, P. A., Montanti, J., Nghiem, N. P., Hicks, K. B., Berger, G., Brooks, W. S., … Iii, D. G. S. (2011). Conversion of deoxynivalenol to 3- acetyl-deoxynivalenol in barley-derived fuel ethanol co-products with yeast expressing trichothecene 3- O - acetyltransferases. *Biotechnology for Biofuels*, *4*, 26.

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 realized by UPLC-TOFMS method. *Food Chemistry*, *126*, 1870 1876.

Kroes, R., Müller, D., Lambe, J., Löwik, M. R. H., Van Klaveren, J., Kleiner, J., … Visconti, A. (2002). Assessment of intake from the diet. *Food and Chemical Toxicology*, *40*, 327 385.

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*, *25*, 732 744.

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 Addit.Contam*, *22*, 652 672.

Markov, K., Frece, J., Pleadin, J., Bevardi, M., Barišić, L., Kljusurić, J. G., … Mrvčić, J. (2019). *Gluconobacter oxydans* potential biological agent for binding or biotransformation of mycotoxins. *World Mycotoxin Journal*, *12*, 1 10.

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

Nathanail, A. V., Gibson, B., Han, L., Peltonen, K., Ollilainen, V., Jestoi, M., & Laitila, A. (2016). The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. *Food Chemistry*, *203*, 448 455.

Panel, E., & Chain, F. (2014). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal*, *12*(8), 3802.

Pascari, X., Gil-Samarra, S., Marín, S., Ramos, A. J., & Sanchis, V. (2019). Fate of zearalenone, deoxynivalenol and deoxynivalenol-3-glucoside during malting process. *LWT – Food Science and Technology*, *99*, 540 546.

Pascari, X., Ortiz-Solá, J., Marín, S., Ramos, A. J., & Sanchis, V. (2018). Survey of mycotoxins in beer and exposure assessment through the consumption of commercially available beer in Lleida, Spain. *LWT Food Science and Technology*, *92*, 87 91.

Pascari, X., Rodriguez-Carrasco, Y., Juan, C., Mañes, J., Marin, S., Ramos, A. J., & Sanchis, V. (2019). Transfer of *Fusarium* mycotoxins from malt to boiled wort. *Food Chemistry*, *278*, 700 710.

Peters, J., Van Dam, R., Van Doorn, R., Katerere, D., Berthiller, F., Haasnoot, W., & Nielen, M. W. F. (2017). Mycotoxin profiling of 1000 beer samples with a special focus on craft beer. *PLoS ONE*, *12*, 1 27.

Piacentini, K. C., Savi, G. D., Olivo, G., & Scussel, V. M. (2015). Quality and occurrence of deoxynivalenol and fumonisins in craft beer. *Food Control*, *50*, 925–929.

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., & Mañes, J. (2015). Occurrence of Fusarium mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry*, *178*, 149–155.

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

Scott, P. M., Kanhere, S. R., Daley, E. F., & Farber, J. M. (1992). Fermentation of wort containing deoxynivalenol and zearalenone. *Mycotoxin Research*, *8*, 58–66.

Shetty, P. H., & Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science and Technology*, *17*, 48–55.

Sørensen, J. L., & Sondergaard, T. E. (2014). The effects of different yeast extracts on secondary metabolite production in *Fusarium*. *International Journal of Food Microbiology*, *170*, 55–60.

Takata, K., Ban, T., Ito, M., Tabiki, T., Yamauchi, H., Tanio, M., & Nishio, Z. (2010). Deoxynivalenol Distribution in flour and bran of spring wheat lines with different levels of *Fusarium* head blight resistance. *Plant Disease*, *94*, 335–338.

Vila-Donat, P., Marín, S., Sanchis, V., & Ramos, A. J. (2018). A review of the mycotoxin adsorbing agents, with an emphasis on their multi-binding capacity, for animal feed decontamination. *Food and Chemical Toxicology*, *114*, 246–259.

Wall-Martínez, H. A., Pascari, X., Ramos, A. J., Marín, S., & Sanchis, V. (2019). Frequency and levels of mycotoxins in beer from the Mexican market and exposure estimate for deoxynivalenol mycotoxins. *Mycotoxin Research*, *35*, 207–216.

Walther, A., Ravasio, D., Qin, F., Wendland, J., & Meier, S. (2015). Development of brewing science in (and since) the late 19th century: Molecular profiles of 110-130 year old beers. *Food Chemistry*, *183*, 227–234.

Warth, B., Fruhmann, P., Wiesenberger, G., Kluger, B., Sarkanj, B., Lemmens, M., …

Schuhmacher, R. (2015). Deoxynivalenol-sulfates: identification and quantification of novel conjugated (masked) mycotoxins in wheat. *Analytical and Bioanalytical Chemistry, 407,* 1033 1039.

Wolf-Hall, C. E. (2007). Mold and mycotoxin problems encountered during malting and brewing. *International Journal of Food Microbiology*, *119*, 89 94.

Yiannikouris, A., François, J., Poughon, L., Dussap, C. G., Jeminet, G., Bertin, G., & Jouany, J. P. (2004). Influence of pH on Complexing of Model β-D-Glucans with Zearalenone. *Journal of Food Protection®*, *67*, 6.

Yiannikouris, Alexandros, André, G., Poughon, L., François, J., Dussap, C. G., Jeminet, G., … Jouany, J. P. (2006). Chemical and conformational study of the interactions involved in mycotoxin complexation with β-D-glucans. *Biomacromolecules*, *7*, 1147 1155.

Chapter 9. The fate of several trichothecenes and zearalenone during roasting and enzymatic treatment of cereal flour applied in cereal-based infant food production

Pascari X., Maul, R., Kemmlein, S., Marin, S., Sanchis, V.

Submitted to Food Control

9.1 Abstract

Cereal-based baby food production process is expected to have an impact on the initial level of *Fusarium* mycotoxins that can contaminate the raw materials. The aim of the present study was to investigate the changes of some of these toxins during roasting and the treatment with amylolytic enzymes, usually applied during the production process. Three different cereal flours contaminated with *Fusarium graminearum* were considered (barley, wheat and oat). The results did not show significant changes in the concentration of any of the studied mycotoxins (up to 5% change in deoxynivalenol concentration after the enzymes were added). The acetyl-deoxynivalenol also showed slight modifications as a result of the applied processes, however their statistical significance was not proved. Zearalenone and T-2 and HT-2 toxins remained almost unaltered throughout the study.

Keywords:

Cereal-based baby food, HPLC-MS/MS, modified mycotoxins, glucoamylase, α-amylase.

9.2 Introduction

Cereal-based products are one of the most important contributors to human diet. Besides their energetic value they are also rich in health promoting compounds such as dietary fiber, antioxidants, B group vitamins and minerals (Gani, SM, FA, & Hameed, 2012). However, also various residues and contaminants may be contained in cereal products. One of the predominant risks associated with their consumption is the exposure to mycotoxins. The major mycotoxins occurring in cereals are *Fusarium* toxins. They are responsible for the contamination with deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins, zearalenone (ZEN), fumonisins (FBs) in maize and small cereals, which besides serious consequences on human and animal health, also cause a huge economic impact for the agriculture sector of many countries (Lee & Ryu, 2017; Marroquín-Cardona, Johnson, Phillips, & Hayes, 2014).

One of the "emerging" food safety issues related to mycotoxins in cereals are the modified forms. They can be produced by the *fungi* or be a part of the plant defense mechanism, which is represented by the activity of plant detoxification enzymes (Berthiller et al., 2013). Considering their proven bioavailability and toxicity in humans and animals, their co-occurrence with parental forms can increase the risk associated to each mycotoxin (De Saeger & van Egmond, 2012). Also, there are several studies investigating their origin of formation from the perspective of an industrial food production process (Freire & Sant'Ana, 2018; Schwarz, 2017; Vidal, Morales, Sanchis, Ramos, & Marín, 2014). As opposite to the parental forms, the modified mycotoxins are not regulated by the European Union legislation. Considering the occurrence and toxicity data, FAO/WHO Joint Expert Committee on Food Additives (JECFA) extended the provisional maximum tolerable daily intake (PMTDI) from DON to a group of DON, 3-acetyl-deoxynivalenol (3-Ac-DON) and 15-acetyl-deoxynivalenol (15-Ac-DON), the information regarding deoxynivalenol-3-glucoside (DON-3-Glc) being insufficient to consider its inclusion in the group (JECFA/FAO, 2011). European Food Safety Authority (EFSA) recently assessed the risk to human and animal health related to the presence of DON, 3-Ac-DON, 15-Ac-DON and DON-3-Glc, estimating the occurring relative concentrations of the modified forms to the

parental form as 10, 15 and 20%, respectively (Knutsen et al., 2017). Also, the report concludes that although the European population exposure to DON and its modified forms is below the acute reference dose (ARfD) of 8µg/kg bw a regular exceedance of the TDI (1µg/kg bw) can present a potential health concern.

Infants are the population group which is the most sensitive to mycotoxin exposure. Moreover, according to EFSA scientific opinion related to the human and animal health risk associated to the presence of mycotoxins, in the case of ZEN, DON and its modified forms, 3-Ac-DON, 15-Ac-DON and DON-3-Glc, infants showed the highest acute and chronic dietary exposure compared to other groups of population (EFSA, 2014; Knutsen et al., 2017). The most frequently used cereals for baby food production are oats, wheat, barley, maize, rye, triticale, sorghum, millet and pseudo-cereals, such as quinoa, buckwheat and amaranth (Klerks et al., 2019). Besides the already mentioned *Fusarium* mycotoxins, cereals were found to contain their modified forms (Bryła et al., 2016; Gottschalk, Barthel, Engelhardt, Bauer, & Meyer, 2007; Lee & Ryu, 2017; Pleadin et al., 2013). The maximum allowed limits for *Fusarium* mycotoxins in cereals and cereal-based baby food are stipulated by the Commission Regulation No 1881/2006 and its following amendments: 750 and 200 µg/kg for DON in flour and cereal based baby food, respectively; 200 and 20 µg/kg for ZEN in cereal flour and cereal-based infant food, respectively (European Commission, 2006). Indicative maximum levels are stipulated for T-2 and HT-2 toxins in cereals and cereal products by the Commission Recommendation 165/2013/EU at 200, 100 and 15 µg/kg for oats, other cereals and cereal-based foods for infants and young children, respectively (European Commission, 2013).

From the technological perspective, cereal-based baby food production process is relatively simple, however the safety requirements are more demanding compared to other production processes in the food industry (European Commission, 2006a). It is characterized by four main steps: flour roasting, pre-gelatinization and enzymatic hydrolysis, enzymes inactivation and drying. Considering the raised questions related to the safety of the enzymes in food products (*e.g.* allergies), the producers of cereal-based baby foods are intending to eliminate the amylolytic treatment from the production

process. Nonetheless, no information is available regarding the nutritional characteristics of a product obtained this way. Thus, the present study will focus on two production stages: (1) flour roasting and (2) pre-gelatinization and enzymatic hydrolysis steps. Flour roasting usually takes place at a temperature range between 105°C and 120ºC, with a duration ranging from 20 min to 40 min. This step is important for the modulation of the organoleptic characteristics of the cereal flour and also improves its dispersibility in water during the next production step (Fernández-Artigas, Guerra-Hernández, & García-Villanova, 1999). Pre-gelatinization represents the loss of crystalline structure of the starch and its swallowing, process that makes it available to the amylolytic enzymes. From the perspective of the possible mitigation effect on mycotoxin contamination of the abovementioned production steps, there are no studies specifically dedicated to cereal-based baby food products, however some available publications deal with the fate of mycotoxins during roasting and enzymatic production steps in other foodstuffs (*e.g.* malting, brewing, breadmaking) (Hazel & Patel, 2004; Lancova et al., 2008; Pietri, Bertuzzi, Agosti, & Donadini, 2010; Vidal et al., 2014). Yumbe-Guevara, Imoto, & Yoshizawa (2003) studied the thermal degradation of DON, ZEN and NIV during the roasting of barley kernels and flour. They observed the first significant effect on the reduction of mycotoxins at 180 °C treatment, achieving up to 90 and 80% reduction in DON (11 min treatment) and NIV (60 min treatment), respectively. For ZEN, no significant effect on toxin concentration was observed at 140 and 160ºC, however 60 min at 220ºC were needed to achieve an 85% reduction in barley flour. With regard to the effect of the enzymatic reactions applied in food industry, α-amylase was proven to lead to up to 10% increase in DON (Vidal, Ambrosio, Sanchis, Ramos, & Marín, 2016), result also found by Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova (2012) during brewing an breadmaking. This increase was partially explained by the cleavage of DON-3-Glc under the enzymatic activity in both studies mentioned above, factor to be possibly considered for the initial contamination of the selected batches of raw materials. For the loss of T2-toxin parallel to increased values for HT2-toxin in grain analysis it is assumed that the conversion is an enzyme mediated process due to deacetylation of T2-toxin (Maul, Pielhau, & Koch, 2014). Also, for mycotoxin detoxification enzymes may be utilized (Karlovsky et al., 2016).

Consequently, various thermal or enzymatic processed may interfere in different ways with the fusarium mycotoxin content of a processed grain sample.

The present study aims to identify the impact of roasting and enzymatic hydrolysis processes on DON, acetylated DON (3- and 15-Ac-DON), DON-3-Glc, ZEN and T-2 and HT-2 toxins in *Fusarium* infected cereal flour under typical conditions used in infant food production. The three most frequently used cereal flours will be used for the experiment: oats, wheat and barley. The treatment parameters will be set as close as possible to the ones used during the cereal-based baby food production process. Results will help understand the possible changes in mycotoxin concentration during these production steps considering the compositional differences in the used cereal flours.

9.3 Materials and methods

9.3.1 Chemicals and reagents

All mycotoxin standards (DON, 3-Ac-DON, 15-Ac-DON, ZEN, T-2 and HT-2 toxins) including the unlabeled and the U-[^{13}C]-labelled were purchased from Romer Lab Diagnostic (Tulln, Austria). Methanol and acetonitrile as well as magnesium sulfate, formic acid and ammonium formate were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The amylolytic enzymes (α-amylase and amyloglucosidase) were kindly provided by Hero Spain (Alcantarilla, Spain). Malic acid and sodium hydroxide were purchased from VWR Chemicals (Radnor, Pennsilvania, USA); sodium chloride, glucose and sodium potassium tartrate were bought from Fisher Scientific (Hampton, New Hampshire, USA); sodium azide and sodium citrate dihydrate were acquired from Scharlab (Sentmenat, Spain). The 3,5-dinitrosalicylic acid (DNS) was bought from Acros Organics (New Jersey, USA), the citric acid was acquired from PRS Panreac (Barcelona, Spain) and the starch was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

9.3.2 Preparation of mycotoxin contaminated flour

Three different cereal flours were purchased for the experiment from a local supermarket in Lleida (Spain), namely barley, oat and wheat flour. A part of each was inoculated with *Fusarium graminearum* strain (F.46) obtained from the strains collection of the Food

Technology Department of the University of Lleida.

Before inoculation the flours (500 g portions) were put in ISO bottles and sterilized in an autoclave. Afterwards, the flours were aseptically transferred to Petri dishes and 2 mL of sterile MilliQ water was added to each Petri dish, which were later stored overnight at 4ºC to achieve a water activity of 0.99 (Aqualab, Serie 3 TE, Decagon Devices, Washington, USA). Then, 1 mL of spore suspensions of *F. graminearum* (10^6 spores/mL) was sprayed on each dish. Petri dishes with barley, wheat and oat flour were then incubated at 25 °C for 21 days. Afterwards, the contaminated flours were dried at 40 °C, homogenized and DON and ZEN levels were determined.

To obtain the required mycotoxin concentration, the contaminated flour was mixed with uncontaminated one in certain proportions. For each of the treatments a separate flour mix was prepared. Table 28 regroups the initial concentrations of the analyzed mycotoxins in the three matrices.

Table 28: Initial mycotoxin concentrations obtained in barley, wheat and oat flours

Matrix	Mean concentration, µg/kg±SD						
	DON	DON-3-Glc	3-Ac-DON	15-Ac-DON	ZEN	T-2	HT-2
Barley	4610±180	<LOD	83.5±6.2	201±14.7	2991±593	<LOD	<LOD
Wheat	1254±100	<LOD	<LOD	150±12	1209±152	<LOD	<LOD
Oat	1390±72	<LOD	<LOD	482±13.3	5027±219	1.4±0.07	3.85±0.29

LOD=limit of detection; DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside; 3-Ac-DON=3-acetyl-deoxynivalenol; 15-Ac-DON=15-acetyl-deoxynivalenol; ZEN=zearalenone.

9.3.3 Flour roasting

The roasting of the contaminated flour was performed using a convection oven (Eurofred, Barcelona, Spain). To confirm that the right temperature was applied throughout the entire treatment time, a temperature logger (Plug&Track, Progres Plus, Willems, France) was used inside the oven. For each designed setup (temperature *vs* time), 50g of contaminated sample were weighted and placed in aluminum trays (32x21x5cm) in order to form a very thin layer (approximately 1 cm) to ensure that the oven temperature is easily reached inside the sample. Two temperature levels were chosen, namely 105 and 120ºC with a treatment length of 30 and 40 min each. Every sample was processed in triplicate

and collected at the end of the programmed time. A total of 12 samples of each flour type (barley, wheat and oat) were obtained. The treated samples were cooled down and stored in a dry place at room temperature until their analysis.

9.3.4 Enzymatic treatment

Before proceeding to the enzymatic treatment of the cereal flour, it was roasted at 105ºC for 30 min. Then, the obtained roasted flour was cooled and 30 g samples were prepared by mixing a respective amount of the contaminated flour with the roasted blank flour. The mix was placed in a 100 mL beaker and the enzymes (a-amylase and glucoamylase) were added. The available information of the used doses in cereal-based baby food production process is scarce and non-specific, thus it was decided to add the chosen enzymes simultaneously at the same dose in each sample: each of the enzymes was added in two dosages to the samples: 2.1 and 4 g of enzyme/kg of cereal flour. The 53ºC temperature was chosen for the present study as it corresponds to the temperature allowing the activity of both enzymes. Once the flour and enzyme mix was ready, 70 mL of distilled water at 53ºC was added and the slur was put into a water bath for 10, 50 and 90 min. Samples for each treatment were prepared in parallel and per triplicate for every designed setup. When the time expired, the samples were rapidly cooled in an ice bath, frozen and lyophilized (Telstar LyoBeta 15, Terrassa, Spain). The obtained lyophilized samples were then packed individually and stored in a dry place at room temperature until analysis.

9.3.5 Assessment of the enzymatic activity

The activity of the added α-amylase and glucoamylase was quantified throughout the experiment to evaluate if there were differences in starch hydrolysis related to each studied matrix.

9.3.5.1 α-amylase activity

The activity of the α-amylase used during the experiment was determined according to Vidal et al. (2016). Briefly, 1.5 g of treated flour was weighted in 12 mL polypropylene tubes and 10 mL of extraction solvent (0.2M malic acid:0.35M sodium hydroxide:0.2M sodium chloride:0.003M sodium azide, 1:1:1:1, *v/v/v/v*) was added. The obtained mix was incubated

in a water bath for 20 min at 40°C. Then, 0.5 mL of the supernatant was mixed with 1.5 mL substrate (1g of starch in 20 mL of 1M sodium hydroxide, 10 mL of 1.06M sodium and potassium tartrate and 70 mL water). It was left to interact for 3 min at 25°C, followed by the addition of 1 mL of DNS reagent (1g of DNS, 20 mL of 2M sodium hydroxide, 10 mL of 1.06M sodium and potassium tartrate and 70 mL water) and boiled in a water bath for 5 min. The whole mix was cooled down in a cold-water bath and 10 mL of distilled water were added. The absorbance of the obtained solution was measured in an UV-visible spectrophotometer (Helios γ, Thermo Electron Corporation, Waltham, Massachusetts, USA) at a wavelength of 540 nm. The results were compared to a glucose standard curve with concentrations ranging from 1 to 10 mg of glucose/mL (r^2=0.99). The corresponding dilution factor was applied to the analyzed samples.

9.3.5.2 Glucoamylase activity

Glucoamylase activity in all the samples was evaluated according to the method described by Puri et al. (2013). The enzymatic activity was expressed in international units (IU), one IU being defined as μmol of glucose per min and milliliter under the standard assay conditions using a glucose standard curve. Briefly, 1g of treated flour was added to 10 mL of 0.1M citrate buffer (pH=5) and stirred at 250 rpm for 30 min. Then, the mix was centrifuged at 5000 rpm for 20 min (Hettlich Universal 320R, Tuttlingen, Germany) and 1 mL of supernatant was mixed with 1mL of 1% maltose in 0.1M citrate buffer (pH=5). The obtained extract was then incubated in a water bath for 30 min at 45°C. The reaction was stopped by adding 3 mL of DNS reagent (composition described above) and submitted to a boiling water bath for 15 min. One mL of 40% solution of sodium potassium tartrate and 1 mL of distilled water were added prior to cooling the extract to room temperature. Afterwards, absorbance was measured at 575 nm using an UV-visible spectrophotometer and the results were compared to a standard curve using glucose at concentrations between 1 to 10 mg of glucose/mL (r^2=0.99), applying the appropriate dilution factor.

9.3.6 Mycotoxin extraction, detection and quantification

9.3.6.1 Sample preparation

The sample preparation method is based on the draft WI 00275287 of the pre-norm FprN 17279 (DIN, 2018), describing a LC-MS/MS screening method developed by CEN/TC 275/WG5. Briefly, 5g of sample were weighted in a 50 mL polypropylene tube, mixed with 20 mL acetonitrile/water (50:50, *v/v*), shaken for 30 min (Multi Reax, Heidolph Instruments GmbH & Co.KG, Schwabach) and centrifuged for 30 min at 4500 rpm (Microfuge R, Beckman Coulter, Brea, California, USA). Then, 1 mL of the supernatant was taken and mixed with 100 µL of the internal standard solution and 100µL of MiliQ water. Afterwards, 250 mg of anhydrous magnesium sulfate was added, vortexed for another 30 seconds, centrifuged for 5 min at 14000 rpm and 200 µL of supernatant were diluted with 400 µL water and submitted to HPLC MS/MS analysis.

In order to minimize the losses of the more hydrosoluble analytes during phase separation step, the sample preparation before injection for the initial screening of the mycotoxins followed only the first four steps of the protocol described above, namely mixing the 5g of sample with 10 mL water and 10 mL acetonitrile, followed by shaking, centrifuging and taking 1 mL of the supernatant for injection into the LC MS/MS.

9.3.6.2 LC MS/MS analysis

A Shimadzu Nexera X2 HPLC system (Kyoto, Japan) equipped with a binary pump and a thermostatic autosampler, coupled with a triple quadrupole mass spectrometer QTRAP 6500+ (SCIEX, Framingham, Massachusetts, USA). Data acquisition and processing was achieved using Analyst® and MultiQuant® software (Sciex, Framingham, Massachusetts, USA). Separation was achieved on Restek Raptor Fluoro phenly100x2.1 mm, 5µm column (Bellefonte, Pennsylvania, USA).

The mobile phase consisted of water (A) and methanol (B), both containing 0,1% formic acid and 300 mg/L ammonium formate, which was supplied at a gradient with a flow rate of 0.5 mL/min. The initial gradient was kept at 2% B for 0.8 min, after 4 min B was increased to 53%, after 6 min B was set at 60%, after 11 min B was 95%, followed by 1.5

min washout at 95% B and a 5 min equilibration period at the initial conditions. Total run time was 17.5 min.

The detector was operated in both positive and negative electrospray ionization (ESI) modes under multiple reaction monitoring (MRM). Operating ESI conditions were setup as follows: curtain gas: 40 psi, gas 1: 60 psi, gas 2: 35 psi, collision gas flow: medium and source temperature: 300°C. Ion spray voltage (IS) was set at 4500 V and -4000 V in positive and negative ionization mode respectively. Two characteristic ions were chosen for the assessment of the mycotoxins in the samples for quantification and for qualitative confirmation of the analytes. To compensate for matrix effect, stable isotope dilution assay was performed using U-[^{13}C]-labelled mycotoxins. The recovery rate for DON, ZEN, AcDON, T2 and HT2 toxin was determined by tenfold spiking and analysis of blank oat samples. Table 29 regroups the data concerning the selected ion transitions, the parameters of the mass detector specific for each fragment ion as well as the individual recoveries.

9.3.7 Data analysis

MultiQuant™ software (SCIEX, Framingham, Massachusetts, USA) was used for the LC-MS/MS data handling and evaluation of the analyzed samples. ANOVA analysis was applied to the obtained results to determine the significance ($p=0.05$) of the observed changes.

9.4 Results and discussion

9.4.1 Initial screening of mycotoxins

Barley, wheat and oat flours used in the present study were intentionally contaminated with a *Fusarium graminearum* strain, mainly producing DON and ZEN. Reports also suggested the ability of *F. graminearum* strains of producing some of their modified forms, such as 3-Ac-DON, 15-Ac-DON, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and others (Hagler, Mirocha, Pathre, & Behrens, 1979; Mirocha, Abbas, Windels, & Xie, 1989). Besides the abovementioned "expected" mycotoxins, the study seeks to evaluate the possible presence of the "emerging" mycotoxins, such as enniatins, beauvericin,

sterigmatocystin, which might occur in high frequency or high concentration in cereals and cereal products originating from a natural field contamination (Gruber-Dorninger, Novak, Nagl, & Berthiller, 2017).

To achieve this aim and decide on the mycotoxins that, additional to DON and ZEN, should be focused on in this study, an initial screening of mycotoxins was performed on two groups of samples for each matrix (barley, wheat and oat): (i) samples before treatment and (ii) samples treated with a dosage of 4.0 g of enzyme/kg of flour, during 90 min. This screening was also aiming to search for the presence of DON-3-Glc, previously reported as being formed as a result of plant enzymatic activity (Ronald Maul et al., 2012; Vidal et al., 2016).

The initial screening omitting the phase separation step that would discriminate the most polar compounds led to the identification of the presence of several emerging mycotoxins, mainly produced by *Fusarium* species. The mycotoxins found in all the samples were DON, 3-Ac-DON, 15-Ac-DON, and ZEN. T-2 and HT-2 toxins were only found in oat flour, both before and after the enzymatic treatment. In the case of the "emerging" mycotoxins, enniatin B (ENN B) was present in all the samples, however it showed a relatively low signal intensity. Beauvericin (BEA) was found in barley and oat samples but not in wheat. Sterigmatocystin and enniatin B1 (ENN B1) were only present in oat samples, however also in very low amounts. The findings of the initial screening of mycotoxins were in accordance to the occurrence studies available (Bryła et al., 2016; Lindblad et al., 2013; Santini, Meca, Uhlig, & Ritieni, 2012).

At the end of the present screening, it could be seen that the analyzed samples presented a certain heterogeneity, especially in contamination with "emerging" mycotoxins, which brought the work to the need of focusing the study to the quantification of the most abundant following analytes: DON, 3-and 15-Ac-DON, ZEN and, in the case of oat flour, T-2 and HT-2 toxins were included, but the data need to be considered as only semi-quantitatively due to their low content levels.

Table 29: Acquisition parameters used for the targeted analytes

Mycotoxin	Retention time, min	ESI[1] mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Declustering potential (V)	LOD[2] (µg/kg)	LOQ[3] (µg/kg)
DON	1.4	ESI+	297.1	249.0[4]	17	66	60.4	199.5
				203.0	21	66		
3-Ac-DON	3.4	ESI+	339.1	231.0	15	31	17.3	57.1
				203.1	19	31		
15-Ac-DON	3.3	ESI+	356.1	137.0	21	31	9.51	31.4
				321.0	17	31		
ZEN	5.9	ESI-	317.1	131.0	-40	-75	17.9	59.1
				175.0	-34	-75		

DON=deoxynivalenol; 3-Ac-DON=3-acetyl-deoxynivalenol; 15-Ac-DON=15-acetyl-deoxynivalenol; ZEN=zearalenone; [1]Electrospray ionization; [2] Limit of detection; [3]Limit of quantification.

9.4.2 Roasting

In food processing, roasting is an important step in flavor modulation and enhancement through caramelization and Maillard reactions taking place on the surface of the product. Besides, cereal roasting is one of the processes thought as potentially being able to reduce the initial mycotoxin charge. Table 28 regroups the initial concentration of mycotoxins in the prepared contaminated flours which were submitted to roasting. Figure 14 shows the changes in mycotoxin concentration in the three flours after applying each combination of time and temperature.

For DON, no significant change in concentration was observed in none of the three matrices studied. Although in barley the effect of the treatment was statistically significant at 105°C after 30 and 40 min roasting ($p=0.05$), the observed trend is not aligned with the one in wheat and oat flours (no significant change in the level of mycotoxin), which suggests a possibility of sampling a fraction which had a higher level of mycotoxins accumulated that could have caused the observed apparent change. Similar results (no change of DON during roasting) were reported several times (Milani & Maleki, 2014; Yumbe-Guevara et al., 2003), showing that at least a heat treatment of 160°C for 15 min is needed in order to achieve a reduction of 10% in bread and biscuits baking. However, Kaushik (2015) in its review reported studies showing no effect of baking in breadmaking process on DON concentration nor after 30 min at 170°C, nor after 30 min at 205°C.

There are many recent studies investigating the fate of DON during food processing (Malachova et al., 2010; Pronyk, Cenkowski, & Abramson, 2006; Vidal et al., 2014), however regardless its frequent co-occurrence with 3-AcDON and 15-AcDON, the studies on the evolution of their concentration are scarce (Wu & Wang, 2016). In the present work, 15-AcDON was present in the three matrices, however 3-AcDON (Fig. 15) was present only in barley, probably due to small differences in toxin production of the fungus used for flour treatment related to individual matrix composition of barley, wheat and oat (Mirocha et al., 1989). 3-AcDON showed a very similar trend of a slight toxin level decrease like DON in barley, which can be explained by its conversion into the parent compound, nonetheless its relatively low amounts compared to DON levels present and presence

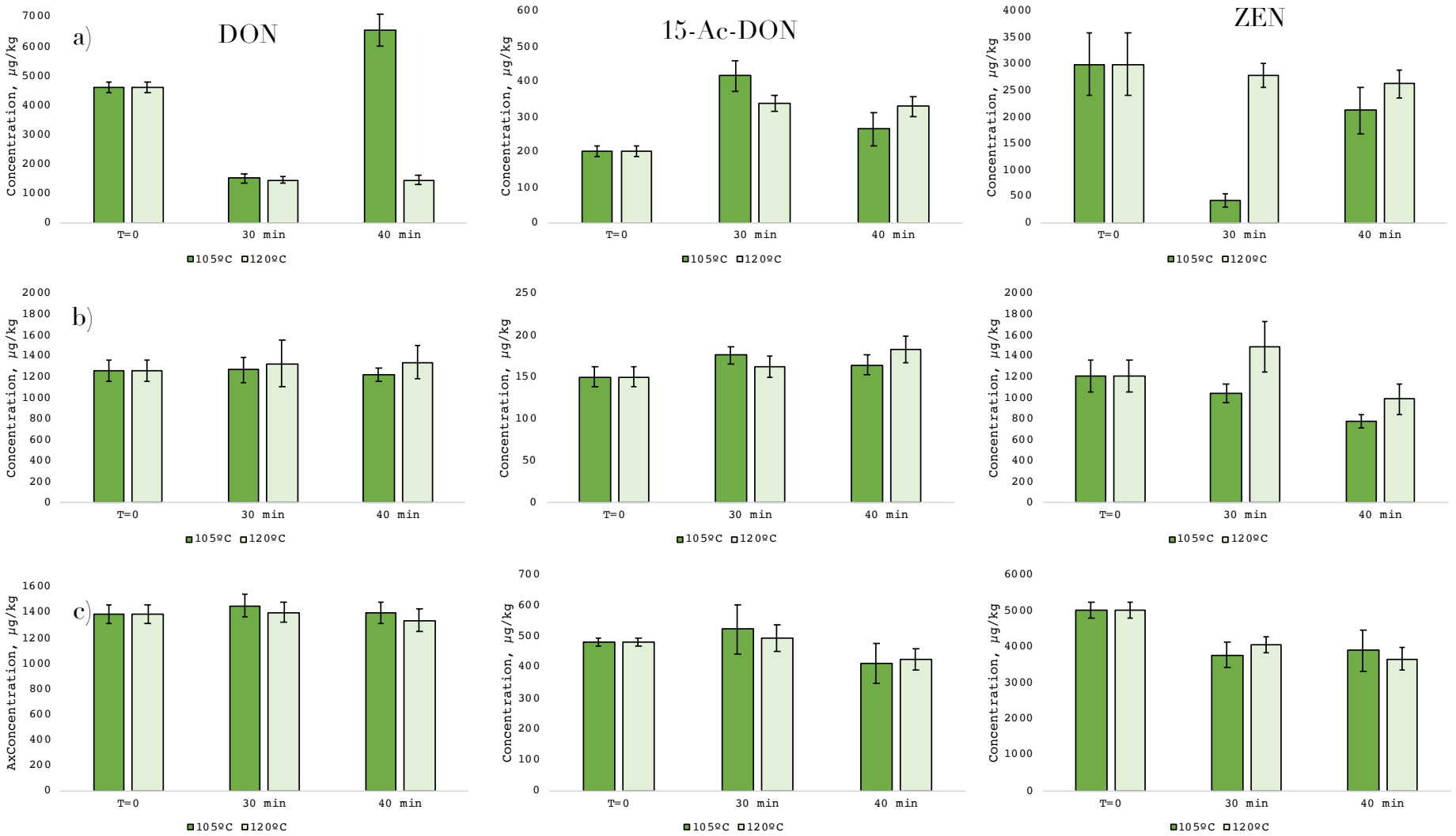

Figure 14: Concentrations of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) (±SD) in barley (a), wheat (b) and oat (c) during roasting at 105 and 120°C for 30 and 40 min.

only in treated barley flour cannot definitively confirm this statement. No significant differences related to the applied treatment are observed for 15-Ac-DON in none of the flours. Likewise for DON, the graphs show a change in the levels of both acetylated DON in barley flour during roasting, which can be seen in the reduction of the level of toxin after 30 min at 105°C and its increase after 40 min at the same temperature, which confirms the possibility of a different starting level of toxins present in the flour before roasting.

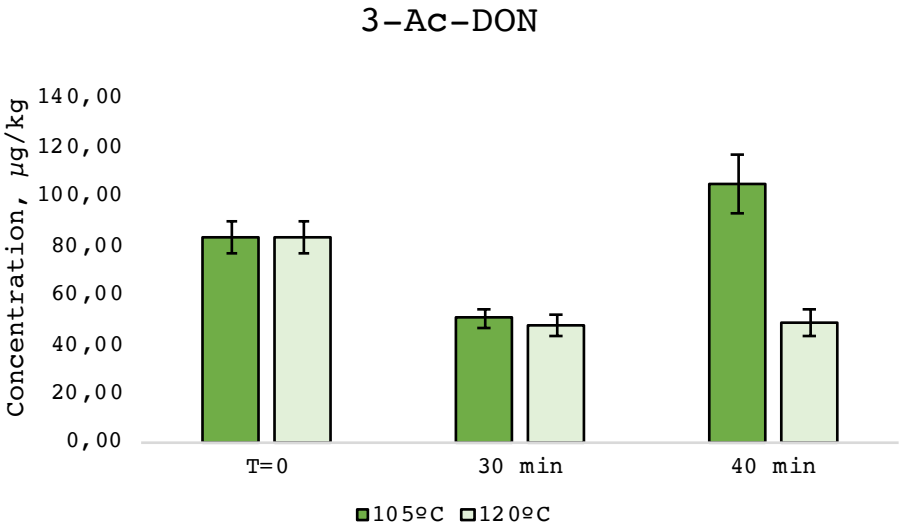

Figure 15: Changes in 3-acetyl-deoxynivalenol (3-Ac-DON) concentration (±SD) during barley roasting at 105 and 120°C for 30 and 40 min.

ZEN shows a decreasing tendency during the roasting process at both temperatures, however none of the changes in concentration were significant. This matches the previous studies investigating ZEN thermal stability during roasting, which had proven its high resistance to temperature treatment (Kabak, 2009; Yumbe-Guevara et al., 2003).

Besides the *F. graminearum* toxins coming from the intended contamination in the laboratory, the analysis of the oat flour identified the presence of T-2 and HT-2 toxins. Due to the low levels identified, the derived content data have to be considered as a semi-quantitative assessment. The toxins were proven as relatively stable with only slight changes compared to the initial level found before the treatment was applied. The result shows more stability compared to the study performed by Kuchenbuch, Becker, Schulz, Cramer, & Humpf (2018), where the decrease of the toxins during roasting represented approximately 5%, however their experiment included two previous operations which led to the main decrease in both toxins. Perhaps, an intended contamination with these toxins

could help better explain their fate during roasting.

Despite the expected effect of the relatively long treatment time compensating for the low roasting temperature from the perspective of mycotoxins mitigation, no significant impact of the roasting process on mycotoxin levels was observed.

Additionally, no significant correlation between different mycotoxins was recorded.

9.4.3 Enzymatic treatment

There are no studies available on the impact of enzymatic processes on *Fusarium* mycotoxin levels during baby food production process, however enzymes are also commonly used in food production processes either with the aim of achieving the specific characteristics of the final product (*e.g.* brewing) or for the improvement of the technological quality and shelf life (*e.g.* breadmaking, production of the fruit juice) (Juodeikiene, Basinskiene, Vidmantiene, & Bartkiene, 2009; Kostelanska et al., 2011; Malachova et al., 2011; Vidal et al., 2016).

Two enzymes were used in the present study (α-amylase and glucoamylase) at two doses (2.1 and 4.0 g of enzyme/kg of flour). For practical reasons related to the homogeneity of the contamination, the reference value of the initial mycotoxin concentration was chosen at 10 min of incubation with the enzymes. No strong impact on the mycotoxin levels was expected after such a short time. Table 30 regroups the information on the units of glucose per minute transformed as a result of the presence of the amylolytic enzymes in 1 g of flour. It can be seen that the activity changes depending on the matrix used, which is probably related to the differences in the structure of the starch in the different cereals which has implications on the efficacy of the reaction (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003).

Figure 16 illustrates the changes in concentration of DON, 15-Ac-DON and ZEN during enzymatic hydrolysis caused by two amylolytic enzymes in barley, wheat and oat flours. The obtained results indicate a relatively unchangeable levels of DON in the three matrices (barley, wheat and oat) and even a slight decrease when 2.1 g of enzyme/kg flour was added (between 1.5 and 4%), however it is not proved statistically significant.

One similar study of Vidal et al. (2016) reports on the increase in DON (up to 10%) due to α-amylase activity, mainly as a result of their release from the cereal matrix, however the temperature used for bread fermentation was 30 and 45°C, which could have been the reason of the different result obtained.

In the case of the 15-Ac-DON, which was found in all of the three studied flours, a deviating behavior during the process was observed, manifesting a slightly increasing trend in barley flour and decreasing in wheat and oat flour. For barley flour, the tendency was better shaped at a higher enzymatic concentration. Wheat flour was characterized by a higher and significant decrease of 15-Ac-DON at 2.1 g of enzyme/kg flour dose compared to the 4.0 g/kg after 90 min treatment.

Table 30: Activity of α-amylase and glucoamylase in barley, wheat and oat flours after each sampling step during enzymatic treatment

Sample treatment		**Enzymatic activity, mg glucose/g sample*min**			
		α-amylase		**Glucoamylase**	
		2.1 g/kg[a]	***4.0 g/kg***	***2.1 g/kg***	***4.0 g/kg***
Barley					
	10 min	ND[b]	ND	0.26±0.01	0.29±0.01
	50 min	ND	0.05±0.007	0.21±0.02	0.18±0.03
	90 min	0.03±0.003	0.14	0.19±0.01	0.19±0.01
Wheat					
	10 min	0.79±0.06	1.11±0.05	0.2±0.01	0.22±0.01
	50 min	1.07±0.04	1.22±0.07	0.23±0.01	0.24±0.01
	90 min	1.28±0.09	1.42±0.1	0.23±0.01	0.21±0.02
Oat					
	10 min	3.55±0.38	1.16	0.1±0.007	0.09
	50 min	4.54±0.25	0.88±0.1	0.11±0.01	0.10±0.01
	90 min	5.06±0.04	0.76±0.07	0.12±0.01	0.1±0.01

[a] dose of enzyme added to the flour for the treatment 2.1 and 4.0 g of enzyme/kg of flour. [b] ND=not detected

In the oat flour, a trend similar to wheat flour was registered, however at a much smaller extent. 3-Ac-DON which was found only in barley flour, was following a very similar trend to its isomer 15-Ac-DON during the enzymatic treatment (data not shown). There are no available studies focusing on these modified forms of DON which the present work could relate to in order to explain the observed, although small, changes in concentration during the enzymatic process applied. However, the natural presence of other individual enzymes

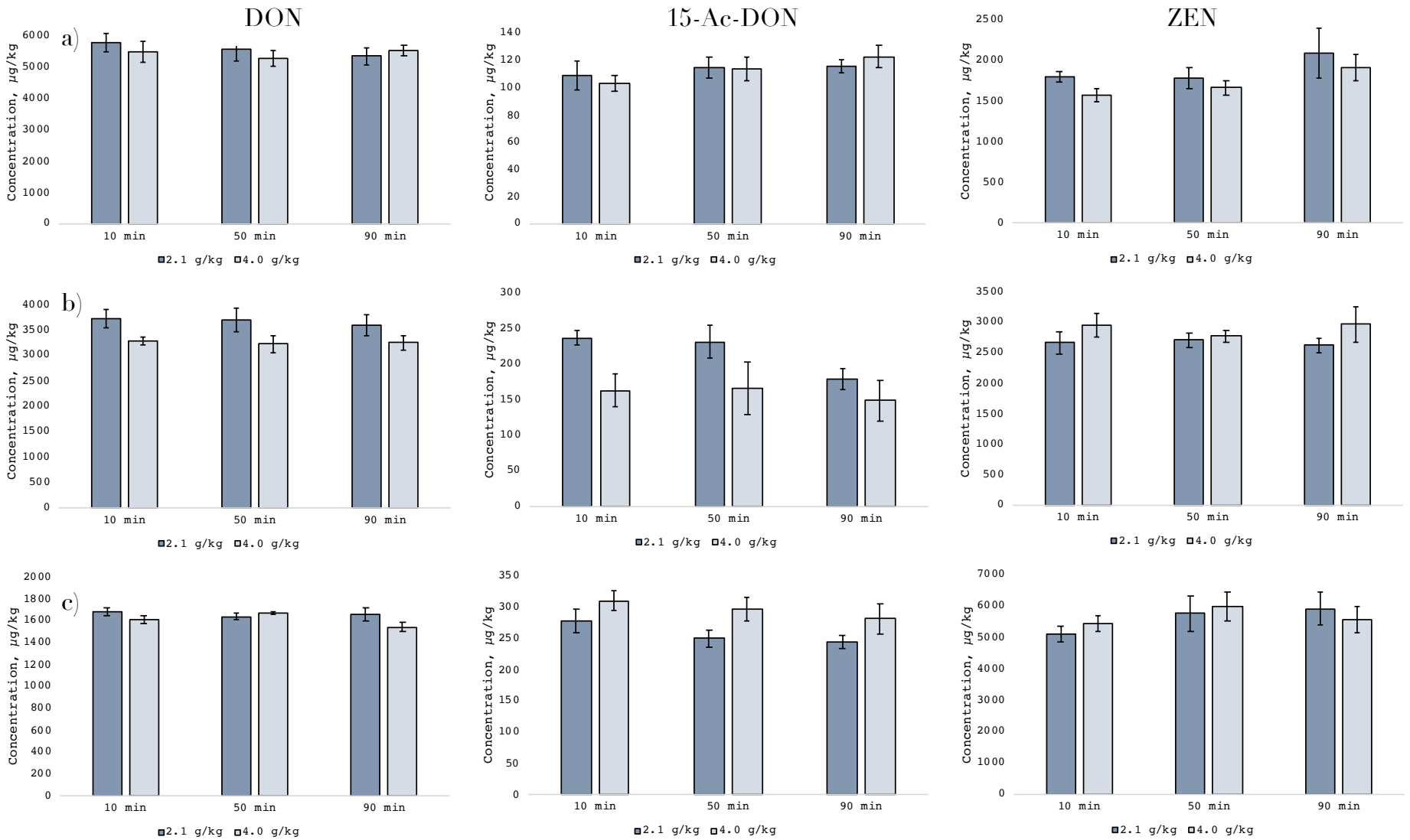

Figure 16: Concentrations (±SD) of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) in barley (a), wheat (b) and oat (c) during enzymatic treatment (α-amylase and glucoamylase) at a dose of 2.1 and 4.0 g/kg during 10, 50 and 90 min.

together with a different enzymatic activity observed in the three flours could explain the observed changes in mycotoxin levels during the process.

ZEN content remained almost unchanged throughout the whole process at both doses of the enzymes applied in barley, wheat and oat flours. Considering the high concentration of ZEN accumulated in the samples, more studies are needed in order to identify more certainly the behavior of the toxin during enzymatic processes in food industry.

T-2 and HT-2 toxin contents in oat did not undergo any significant change at none of the two applied doses of enzymes. Also, their identified low levels were of no concern from the safety perspective investigated by the present study. There are no available studies showing a possible effect of a treatment with amylolytic enzymes on the levels of these toxins and no release from the matrix could be observed in the present study. Other enzymes (such as esterase) are however proven to have the ability of converting T-2 toxin into HT-2 toxin (Lattanzio, Visconti, Haidukowski, & Pascale, 2012).

A confirmatory small-scale experiment repeating the enzymatic treatment at 4.0 g enzyme/kg flour and 90 min incubation time was performed in the three studied flours (Table 31). All the results obtained before were confirmed and the same tendencies were shown but for DON, which exhibited an increase of approximately 4.5 and 10% in wheat and barley, respectively, nonetheless its concentration in oat flour remained stable after the applied treatment. As the obtained data goes in line with an already published report (Simsek, Burgess, Whitney, Gu, & Qian, 2012), more detailed studies of DON under these conditions might be considered.

Table 31: Mycotoxin concentration after repeating the enzymatic treatment (4.0 g enzyme/kg flour, 90 min)

Analyte	Process step	Concentration, µg/kg±SD		
		Barley	Wheat	Oat
Deoxynivalenol	B.T.[a]	4728	1225	911
	E.T.[b]	5217±101	1280±26.5	920±68
3-Acetyl-deoxynivalenol	B.T	84.7	36.5	79.2
	E.T.	98.8±2,4	18.3±2.5	76.6±5.4
15-Acetyl-deoxynivalenol	B.T.	179	178	237
	E.T.	209±34	77±16.1	233±33
Zearalenone	B.T.	1606	1380	1931
	E.T.	2151±141	1329±79	2087±271
HT-2 toxin	B.T.	<LOD	<LOD	6.2
	E.T.	<LOD	<LOD	5.1±0.9
T-2 toxin	B.T.	<LOD	<LOD	2.03
	E.T.	<LOD	<LOD	1.8±0.2

[a] B.T. = before treatment; [b] E.T. = enzymatic treatment applied.

9.5 Conclusion

Cereal based baby food products are an important part of the diet for a very sensitive group of population. The safety of these products is a priority for the food industry, especially regarding their contamination with mycotoxins. The present study investigated the change of concentration of *Fusarium* mycotoxins during two steps of the production process: flour roasting and treatment with amylolytic enzymes (glucoamylase and α-amylase).

Roasting did not have any significant impact on none of the analyzed mycotoxins, most probably due to the relatively low temperatures applied during the process. It is important to mention also the stability of the acetylated forms of DON, which were still present in the flours after the treatment was applied. Thus, also in treated infant food samples conventional analytical methods do not cover this additional source of toxic potential as acetylated DON remains modified and does not add to DON following deacetylation. Enzymatic treatment did not show a significant effect on DON level in none of the cereal flours studied. The 15-Ac-DON showed a slight increase in barley flour and a relatively small decrease in oat and wheat flours, however these changes could not be confirmed after the statistical evaluation of the data. ZEN remained stable throughout both treatments applied. The repeated small-scale enzymatic treatment (using the highest

enzyme amount and the longest treatment time) only confirmed and strengthened the obtained results.

In summary, it can be concluded that the cereal based baby food production process does not lead to a significant decrease of *Fusarium* mycotoxins during the studied steps. Also, no conversion of the acetylated DON into DON was observed. More extreme physical parameters could enhance the impact on the studied mycotoxins, however the applicability of the new conditions from the technological (i.e. cost, yield, organoleptic properties) and safety perspectives (i.e. acrylamide formation) should be considered first. However, also no additional toxin amounts were deliberated from naturally contaminated matrix due to the applied techniques. This results in the need of a particularly careful evaluation of the quality of the raw materials with regards to mycotoxin contamination, especially considering that there is a gap in the legislation (EC 1881/2006) between the maximum allowed limits of mycotoxins in cereals intended for human consumption and cereal-based infant food. Also, the evaluation of the impacts of the following drying step has to be assessed, yet that there are published studies suggesting the promising potential of this step in reducing the mycotoxin contamination due to the high efficiency of the temperature delivery throughout the food materials

9.6 Acknowledgement

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2017-87755-R) for funding this work. The authors are also grateful to Montserrat Prim for technical support in the assessment of the enzymatic activity.

9.7 References

Berthiller, F., Crews, C., Dall 'asta, C., De Saeger, S., Haesaert, G., Karlovsky, P., … Stroka, J. (2014). Masked mycotoxins: A review. *Zeitschrift Fur Gefassmedizin*, *11*, 6 8.

Bryła, M., Waśkiewicz, A., Podolska, G., Szymczyk, K., Jędrzejczak, R., Damaziak, K., & Sułek, A. (2016). Occurrence of 26 mycotoxins in the grain of cereals cultivated in Poland. *Toxins*, *8*, 20.

De Saeger, S., & van Egmond, H. (2012). Special issue: Masked mycotoxins. *World Mycotoxin Journal*, *5*, 203–206.

EFSA. (2014). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal*, 107.

Erkekoğlu, P., & Baydar, T. (2010). Toxicity of acrylamide and evaluation of its exposure in baby foods. *Nutrition Research Reviews*, *23*, 323–333.

European Commission. (2006a). Commission Directive 2006/125/EC on processed cereal-based foods and baby foods for infants and young children. *Official Journal of the European Union*, 396, 16–35.

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

European Commission. (2013). Commission Recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxins in cereals and cereal products. *Official Journal of the European Union*, *9*, 12–15.

Fernández-Artigas, P., Guerra-Hernández, E., & García-Villanova, B. (1999). Browning indicators in model systems and baby cereals. *Journal of Agricultural and Food Chemistry*, *47*, 2872–2878.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology, 111*, 189–205.

Gani, A., SM, W., FA, M., & Hameed, G. (2012). Whole-Grain Cereal Bioactive Compounds and Their Health Benefits: A Review. *Journal of Food Processing & Technology*, *03*, 10.

Gottschalk, C., Barthel, J., Engelhardt, G., Bauer, J., & Meyer, K. (2007). Occurrence of type a trichothecenes in conventionally and organically produced oats and oat products. *Molecular Nutrition and Food Research*, *51*, 1547–1553.

Gruber-Dorninger, C., Novak, B., Nagl, V., & Berthiller, F. (2017). Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. *Journal of Agricultural and Food Chemistry*, *65*, 7052 7070.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, *38*, 1599 1616.

Hagler, W. M., Mirocha, C. J., Pathre, S. V., & Behrens, J. C. (1979). Identification of the naturally occurring isomer of zearalenol produced by Fusarium roseum "Gibbosum" in rice culture. *Applied and Environmental Microbiology*, *37*, 849 853.

Hazel, C. M., & Patel, S. (2004). Influence of processing on trichothecene levels. *Toxicology Letters*, *153*, 51 59.

JECFA/FAO. (2011). Deoxynivalenol. *Safety evaluation of certain contaminants in food* (Vol. 63, pp. 317 385).

Juodeikiene, G., Basinskiene, L., Vidmantiene, D., & Bartkiene, E. (2009). Reduction of deoxynivalenol (DON) using xylanolytic enzymes during alcoholic fermentation of Fusarium contaminated wheat. *PTEP J. Process Ener. Agric.*, *13*, 107 110.

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

Kaushik, G. (2015). Effect of Processing on Mycotoxin Content in Grains. *Critical Reviews in Food Science and Nutrition*, *55*(12), 1672 1683.

Klerks, M., Bernal, M. J., Roman, S., Bodenstab, S., Gil, A., & Sanchez-Siles, L. M. (2019). Infant cereals: Current status, challenges, and future opportunities for whole grains. *Nutrients*, *11*, 1 25.

Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., … Edler, L. (2017b). Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. *EFSA Journal*, *15*, 347.

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.

Kuchenbuch, H. S., Becker, S., Schulz, M., Cramer, B., & Humpf, H. U. (2018). Thermal stability of T-2 and HT-2 toxins during biscuit-and crunchy muesli-making and roasting. *Food Additives and Contaminants - Part A*, *35*, 2158 2167.

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 & Contaminants: Part A*, *25*, 732 744.

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.

Lee, H. J., & Ryu, D. (2017). Worldwide Occurrence of Mycotoxins in Cereals and Cereal-Derived Food Products: Public Health Perspectives of Their Co-occurrence. *Journal of Agricultural and Food Chemistry*, *65*, 7034 7051.

Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., & Fredlund, E. (2013). Deoxynivalenol and other selected fusarium toxins in swedish wheat - occurrence and correlation to specific fusarium species. *International Journal of Food Microbiology*, *167*, 284 291.

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., 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.

Marroquín-Cardona, A. G., Johnson, N. M., Phillips, T. D., & Hayes, A. W. (2014). Mycotoxins in a changing global environment - A review. *Food and Chemical*

Toxicology, *69*, 220–230.

Martinez, M. M., & Gómez, M. (2016). Insight of the α-amylase Family of Enzymes. In *Microbial Enzyme Technology in Food Applications* (pp. 21–45).

Maul, R., Müller, C., Rieß, S., Koch, M., Methner, F. J., & Irene, N. (2012). Germination induces the glucosylation of the Fusarium mycotoxin deoxynivalenol in various grains. *Food Chemistry*, *131*, 274–279.

Milani, J., & Maleki, G. (2014). Effects of processing on mycotoxin stability in cereals. *Journal of the Science of Food and Agriculture*, *94*, 2372–2375.

Mirocha, C. J., Abbas, H. K., Windels, C. E., & Xie, W. (1989). Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by Fusarium graminearum isolates. *Applied and Environmental Microbiology*, *55*, 1315–1316.

Pietri, A., Bertuzzi, T., Agosti, B., & Donadini, G. (2010). Transfer of aflatoxin B1 and fumonisin B1 from naturally contaminated raw materials to beer during an industrial brewing process. *Food Additives & Contaminants. Part A*, *27*, 1431–1439.

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.

Pronyk, C., Cenkowski, S., & Abramson, D. (2006). Superheated steam reduction of deoxynivalenol in naturally contaminated wheat kernels. *Food Control*, *17*, 789–796.

Puri, S., Arora, M., & Sarao, L. (2013). Production and optimization of amylase and glucoamylase using Aspergillus oryzae under solid state fermentation. *International Journal of Research in Pure and Applied Microbiology*, *3*, 83–88.

Santini, A., Meca, G., Uhlig, S., & Ritieni, A. (2012). Fusaproliferin, beauvericin and enniatins: occurrence in food – a review. *World Mycotoxin Journal*, *5*, 71–81.

Schwarz, P. B. (2017). Fusarium head blight and deoxynivalenol in malting and brewing: successes and future challenges. *Tropical Plant Pathology*, *42*, 153–164.

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.

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.

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.

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.

Yumbe-Guevara, B. E., Imoto, T., & Yoshizawa, T. (2003). Effects of heating procedures on deoxynivalenol, nivalenol and zearalenone levels in naturally contaminated barley and wheat. *Food Additives and Contaminants*, *20*, 1132 1140.

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.

Chapter 10. Detection and quantification of zearalenone, zearalenone-14-sulfate, α- and β-zearalenol and α- and β-zearalenol-14-sulfate in cereal flour

Pascari X., Maul, R., Kemmlein, S., Marin, S., Sanchis, V.

Preparing for submission

10.1 Abstract

An analytical method for the analysis of ZEN and its modified forms was developed. Sample preparation was performed based on a modified QuEChERS method combined with liquid chromatography coupled to a triple quadrupole MS/MS detector. The method was tested for linearity, precision, limits of detection and quantification and recoveries. The evaluation of the abovementioned parameters was performed on oat flour. The applicability of the method was tested by analyzing oat and wheat flours coming from an experiment of the effects of an amylolytic treatment (α-amylase and glucoamylase) similar to the one used in cereal-based baby food production process on mycotoxins. It showed a decrease in β-zearalenol and β-zearalenol-14-sulfate of approximately 40% after 90 min incubation, the other analytes did not show any significant changes. To our knowledge, this is the first study that approaches the identification and assessment of ZEN sulfate-derivates in cereal matrix.

Keywords:

Zearalenone, MS/MS, QuEChERS, zearalenone-sulfate, amylolytic enzymes.

10.2 Introduction

Cereals are an important source of energy, minerals and vitamins for many people around the world. Their contribution to human diet is enormous as is the variety of food products that are based on cereals. One of the main safety issues related to cereals and cereal-based products is fungal infestation and contamination with mycotoxins (Cheli et al., 2014). According to the survey performed by Mousavi Khaneghah et al. (2019), during the last 24 years of investigation on mycotoxin occurrence, *Fusarium* mycotoxins are the most frequently encountered, deoxynivalenol (DON) and zearalenone (ZEN) being the predominant mycotoxins in cereals and cereal-based products.

ZEN is a non-steroidal oestrogenic mycotoxin, produced by several *Fusarium* species, mainly *F. graminearum*, *F. culmorum*, *F. crookwellense, F. cerealis, F. semitectum* and *F. equiseti,* which are common contaminants of cereal crops worldwide (Ismaiel and Papenbrock 2015). These *fungi* can contaminate cereals like maize, barley, wheat, oats, sorghum and, if the climate and storage conditions are favorable, they can produce relatively high amounts of ZEN (Marroquín-Cardona et al. 2014; Zinedine et al. 2007). According to the Scientific Opinion published by EFSA (European Food Safety Authority) Panel on Contaminants in the Food Chain (2011), ZEN might represent an important health risk due to its estrogenic activity. Based on the exposure data, EFSA established a tolerable daily intake (TDI) of ZEN at 0.25 μg/kg body weight (EFSA, 2011). Also, available studies suggested two possible ways of exposure to this toxin that can occur: (i) the consumption of contaminated cereals and cereal-based products or (ii) the consumption of animal-derived products like meat or milk (Rogowska et al. 2019). According to EFSA, the main products contributing to the exposure to ZEN are breakfast cereals (EFSA, 2011). ZEN was classified as a endocrine disruptor compound due to its molecular similarity with 17β-estradiol (Kowalska, Habrowska-Górczyńska, and Piastowska-Ciesielska, 2016) and was proven as being able to induce various hormonal deregulations in humans and animals of both sexes, such as disruption of hormone management, strong embryonic toxicity, apoptosis and oxidative stress in human embryonic stem cells etc. (Rogowska et al., 2019).

In addition to the already known and thoroughly described fungal mycotoxins, ZEN modified mycotoxins have been detected in various cereals and cereal-based products (Bryła et al., 2018). The most commonly detected ZEN modified forms are α- and β-zearalenol (α-ZEL and β-ZEL) and zearalenone-14-sulfate (ZEN-14-Sulf), all found to be also natural fungal metabolites (Marthe De Boevre et al., 2013; Kovalsky et al., 2016). Also new modified forms of ZEN were recently identified by Brodehl et al. (2014), while studying the biotransformation of ZEN by the *fungi* of genera *Rhizopus* and *Aspergillus*, namely α-zearalenol-14-sulfate (α-ZEL-14-Sulf) (Figure 17).

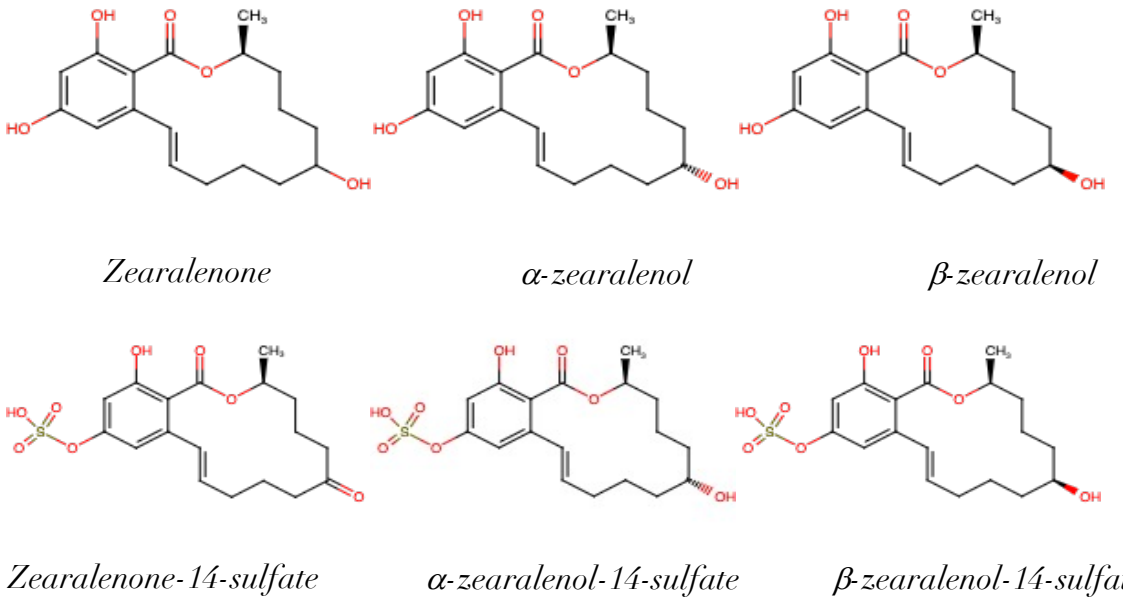

Figure 17: Chemical structure of zearalenone and its modified forms

Due to the fact that the modified forms are easily transformed into their parental form during mammalian digestion, the exposure to ZEN without studying these toxins can be underestimated (De Boevre et al., 2012). Nonetheless, until recently there were no available standards of ZEN sulfate-conjugates of sufficient purity, which made their quantification a challenge for the researchers. The available literature reports several high performance liquid chromatography (HPLC) methods tandem with mass spectrometric (MS) detection for the analysis of ZEN and its major modified forms, sample preparation generally consisting of a hydrolysis step which involves a sulfatase (Baere et al., 2012; Brodehl et al., 2014). Catteuw et al. (2019) used enzymatic synthesis to obtain ZEN-14-Sulf for their study,

which was purified, and its identity was then verified by NMR (Nuclear Magnetic Resonance) techniques. Considering the abovementioned implication of the enzymes in the formation of ZEN- and ZEL-sulfates together with the EFSA opinion on ZEN toxicity (EFSA, 2011) that highlights the lack of sufficient information related to the modified forms and their toxic effects, the need in investigating the role of food processes involving naturally occurring and added enzymes becomes more evident.

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) extraction is frequently employed for the samples preparation in multi-mycotoxin analysis (Lu et al., 2013; Pereira, Fernandes, and Cunha, 2015). The main advantages of the technique are the fastness and good recoveries obtained for the majority of the mycotoxins. The present study proposes a modified QuEChERS based method for sample preparation, followed by the simultaneous analysis of ZEN, α-ZEL, β-ZEL, ZEN-14-Sulf, α-ZEL-14-Sulf and β-zearalenol-14-sulfate (β-ZEL-14-Sulf) in a HPLC system tandem mass spectrometry detector. The interest of the proposed method is valuable also due to its good performance in the case of ZEN- and ZEL-sulfates for which internal standards (IS) are not yet available. To our knowledge, this is the first study integrating the simultaneous identification and the quantification of three sulfate-conjugates of ZEN. The analytical method was validated in oat flour matrix. To confirm the applicability of the analytical method, the effect of the enzymatic (amylolytic) treatment of the oat and wheat flour during cereal-based baby food production process on the concentration of ZEN and its modified forms were analyzed. Considering the natural occurrence of sulfate-conjugates of ZEN in infected wheat products and that infants represent the population group which is the most exposed to ZEN, the development of a simple and accurate analytical method which allows the quantification of these compounds, is of a particular importance.

10.3 Materials and methods

10.3.1 Chemicals and reagents

ZEN, α-ZEL, β-ZEL and their U-[^{13}C]-labelled standards were purchased from Romer Lab Diagnostics (Tulln, Austria). ZEN-14-Sulf ammonium salt, α-ZEL-14-Sulf sodium salt and β-ZEL-14-Sulf sodium salt were acquired from ASCA GmbH (Berlin-Adlershof, Germany). Methanol and acetonitrile as well as magnesium sulfate, formic acid and ammonium formate were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The amylolytic enzymes (α-amylase and amyloglucosidase) were kindly provided by Hero Spain (Alcantarilla, Spain). Malic acid and sodium hydroxide were purchased from VWR Chemicals (Radnor, Pennsilvania, USA); sodium chloride, glucose and sodium potassium tartrate were bought from Fisher Scientific (Hampton, New Hampshire, USA); sodium azide and sodium citrate dihydrate were acquired from Scharlab (Sentmenat, Spain). The 3,5-dinitrosalicylic acid (DNS) was bought from Acros Organics (New Jersey, USA), the citric acid was acquired from PRS Panreac (Barcelona, Spain) and the starch was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

10.3.2 Samples

Oat and wheat flours were purchased in a local supermarket in Lleida (Spain). The *Fusarium graminearum* strain (F.46) used for the present study was obtained from the strains collection of the Food Technology Department of the University of Lleida (Spain).

Before inoculation, the flours were placed into ISO bottles and autoclaved. Then, they were aseptically transferred into Petri dishes (20 g of flour each) and 2 mL of sterile Mili Q water was added to each of them to reach a water activity suitable for the fungal growth (approximately 0.98-0.99). The prepared Petri dishes were stored at 4ºC overnight, water activity being measured with Aqualab Serie 3 TE (Decagon Devices, Washington, USA). One milliliter of *F. graminearum* spore suspension (10^6 spores/mL) was added to the flour and slightly mixed for a better spreading of the contamination within the Petri dish. The plates were stored at 25ºC for 21 days. The obtained contaminated flour was dried at 40ºC, homogenized and the resulting mycotoxin concentration was determined.

The developed chromatographic method was applied to analyzing the mycotoxins in both oat and wheat flour, submitted to a treatment with two amylolytic enzymes (α-amylase and glucoamylase), under the conditions applied during cereal-based baby food production process. Briefly, 30g samples were prepared by mixing a respective amount of the contaminated flour with the roasted blank flour. The mix was placed in a 100 mL beaker and the enzymes were added. Each of the enzymes was added in two dosages to the samples: 2.1 and 4 g of enzyme/kg of cereal flour. The 53°C temperature was chosen for the present study as it corresponds to the temperature allowing the activity of both enzymes. After the flour and enzyme mix was ready, 70 mL of distilled water at 53°C was added and the slur was put into a water bath for 10, 50 and 90 min. Samples for each treatment were prepared in parallel and per triplicate for every designed setup. When the time expired, the samples were rapidly cooled in an ice bath, frozen and lyophilized (Telstar LyoBeta 15, Terrassa, Spain). The obtained lyophilized samples were then packed individually and stored in a dry place at room temperature until analysis.

10.3.3 Instrumental conditions

Detection and quantification of the analytes was achieved using a Shimadzu Nexera X2 HPLC system (Kyoto, Japan) equipped with a binary pump and a thermostatic autosampler, coupled with a triple quadrupole mass spectrometer Sciex QTRAP 6500+ (Framingham, Massachusetts, USA). Data acquisition and processing was performed using Analyst® software (Sciex, Framingham, Massachusetts, USA). Separation was achieved at 40°C on Restek Raptor Fluoro phenyl 100x2.1 mm, 5µm column (Bellefonte, Pennsylvania, USA).

The mobile phase consisted of water (A) and methanol (B), both containing 0,1% formic acid and 300 mg/L ammonium formate, which was supplied at a gradient with a flow rate of 0.5 mL/min. The initial gradient was kept at 2% B for 0.8 min, after 4 min B was increased to 53%, after 6 min B was set at 60%, after 11 min B was 95%, followed by 1.5 min washout at 95% B and a 5 min equilibration period at the initial conditions. Total run time was 17.5 min.

The detector was operated in both positive and negative electrospray ionization (ESI)

modes under multiple reaction monitoring (MRM). Operating ESI conditions were setup as follows: curtain gas: 40 psi, gas 1: 60 psi, gas 2: 35 psi, collision gas flow: medium and source temperature: 300 °C. Ion spray voltage (IS) was set at 4500 V and -4000 V in positive and negative ionization mode respectively. Two characteristic ions were chosen for the assessment of the mycotoxins in the samples for quantification and for qualitative confirmation of the analytes. To compensate for matrix effect, stable isotope dilution assay was performed using U-[^{13}C]-labelled mycotoxins. The recovery rate for DON, ZEN, AcDON, T2 and HT2 toxin was determined by tenfold spiking and analysis of blank oat samples. Table 32 regroups the MS/MS parameters applied.

The optimization of the compound related signal was performed by a direct infusion of the standards of ZEN-14-Sulf and α-ZEL-14-Sulf at a concentration of 10 µg/L (dissolved in methanol:water, 1:3, *v/v*) into the MS/MS detector at a flow rate of 10 µL/min via the syringe pump integrated into it.

Table 32: MS/MS parameters for zearalenone (ZEN), α-zearalenone (α-ZEL), β-zearalenone (β-ZEL), α-zearalenone-14-sulfate (α-ZEL-14-Sulf) and β-zearalenone-14-sulfate (β-ZEL-14-Sulf).

Mycotoxin	Retention time, min	ESI[1] mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
ZEN	5.9	ESI-	317.1	131.0	-40
				175.0	-34
α-ZEL	6.0	ESI-	319.1	174.0	-37
				160.0	-44
β-ZEL	5.3	ESI-	319.2	174.0	-37
				160.0	-44
ZEN-14-Sulf	4.8	ESI-	397.1	317.1	-25
				175.0	-46
α-ZEL-14-Sulf	4.8	ESI-	399.1	319.1	-25
				275.0	-42
β-ZEL-14-Sulf	4.3	ESI-	399.2	319.1	-30
				275.1	-44

[1]Electrospary ionization.

10.3.4 Procedure

10.3.4.1 Calibration solutions

To improve the accuracy of the measurements, matrix-matched calibration and standard isotope dilution assay (SIDA) were performed. For that, several blank samples were prepared by weighting 5 g of oat flour and mixing it with 10 mL acetonitrile and 10 mL water, shaking for 30 min, centrifuging at 4500 rpm for 30 min, collecting 1 mL of the supernatant and mixing it with 250 mg of anhydrous magnesium sulfate. Then, the samples were centrifuged at 14000 rpm for 5 min and all the resulting extract was collected in a vial for further use. Finally, three series of calibration solutions were prepared. One was containing ZEN, α-ZEL, β-ZEL and their respective U-[^{13}C]-labelled homologues, dissolved in acetonitrile:water (25:75, *v/v*); one was a series of calibration solutions of ZEN-14-Sulf and another one was containing α-ZEL-14-Sulf and β-ZEL-14-Sulf, both dissolved in blank matrix extract (25%).

10.3.4.2 Sample preparation

The sample preparation method is based on the draft WI 00275287 of the pre-norm FprN 17279 (DIN, 2018), describing a LC-MS/MS screening method developed by CEN/TC 275/WG5. Briefly, 5g of sample were weighted in a 50 mL polypropylene tube, mixed with 20 mL acetonitrile/water (50:50, *v/v*), shaken for 30 min (Multi Reax, Heidolph Instruments GmbH & Co.KG, Schwabach) and centrifuged for 30 min at 4500 rpm (Microfuge R, Beckman Coulter, Brea, California, USA). Then, 1 mL of the supernatant was taken and mixed with 100 µL of the internal standard solution and 100µL of MiliQ water. Afterwards, 250 mg of anhydrous magnesium sulfate was added, vortexed for another 30 seconds, centrifuged for 5 min at 14000 rpm and 200 µL of supernatant were diluted with 400 µL water and submitted to HPLC-MS/MS analysis.

10.3.4.3 Validation

For the validation of the method, blank oat flour samples (2.5 g) were spiked with ZEN and its modified forms and left overnight at ambient temperature to allow the evaporation of the solvent and the establishment of an equilibrium between the analytes and the

matrix. The sample preparation procedure (section 10.3.4.2) was carried out on one concentration level and repeated three consecutive days with 3, 3 and 10 replicates on each day, respectively, according to Wenzl et al. (2016).

10.3.4.4 Data evaluation

MultiQuant™ software version 3.1.3 (Sciex, Framingham, Massachusetts, USA) was used for the regression analysis and the assessment of method performance. Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on signal to noise ratio (S/N) of 3 and 10, respectively. Also, intra- and inter- day precision were evaluated.

10.4 Results and discussion

10.4.1 MS/MS detection

MS/MS parameters (selection of the most abundant MRM transition, declustering potentials, collision energies) for ZEN-14-Sulf, α- and β-ZEL-14-Sulf were optimized using the automatic routine provided by Analyst in the negative ESI mode. The parameters for the other analytes have been optimized previously. Table 32 summarizes the set conditions of the optimized MRM transitions. ZEN-14-Sulf and α-ZEL-14-Sulf showed a relatively poor chromatographic separation on a regular column, which together with the similarity of the ionization and the insufficient specificity of the most abundant product ions between ZEN-14-Sulf and α-ZEL-14-Sulf, required two different spiked series of oat flour to be prepared: one containing ZEN-14-Sulf and another one containing ZEN, α-ZEL, β-ZEL, α-ZEL-14-Sulf and β-ZEL-14-Sulf. The product ions selected for the identification and quantification of α- and β-ZEL-14-Sulf are similar to the ones described by Brodehl et al. (2014).

Thus, the quantification of ZEN-14-Sulf and α-ZEL-14-Sulf required a different approach. As a result of the optimization procedure, it was concluded that the ionization of the ZEN-14-Sulf leads to the formation of the product ions specific for α-ZEL-14-Sulf. Thus, due to the fact that the two were present in the samples that were to be analyzed, a mathematical subtraction of the part of the area of the signal produced by ZEN-14-Sulf from the area of the α-ZEL-14-Sulf was required. Thus, individual solutions of the two

sulfate-derivates of ZEN, at three different concentration levels (2, 5 and 10 µg/L in spiked blank sample extract and liquid standard solution with 25% acetonitrile) were injected into the system. The four product ions were monitored on the ZEN-14-Sulf chromatograms and the ratio of the areas of the quantifier ion for α-ZEL-14-Sulf/area of the quantifier for ZEN-14-Sulf was calculated (2.9% in all concentrations). Considering that the two analytes were expected to be contained simultaneously in the samples, the following formula was used to determine the final area of the α-ZEL-14-S quantifier ion:

$$Final\ A_{\alpha-\mathrm{ZEL}-14-\mathrm{Sulf}} = A_{\alpha-\mathrm{ZEL}-14-\mathrm{Sulf}} - (2.9\% * A_{ZEN-14-Sulf}),$$

where $Final\ A_{\alpha\text{-}ZEL\text{-}14\text{-}Sulf}$ the resulting corrected area of α-ZEL-14-Sulf peak; $A_{\alpha\text{-}ZEL\text{-}14\text{-}Sulf}$ the area of α-ZEL-14-Sulf peak that was recorded by the MS/MS detector and $A_{ZEN\text{-}14\text{-}Sulf}$ peak area of ZEN-14-Sulf. The concentration of α-ZEL-14-Sulf was determined by substituting the obtained final area in the linear regression equation.

10.4.2 Method performance

The QuEChERS based method used for the present study showed relatively good results. To account for possible interferences from the oat flour components, stable isotope dilution assay was used for the assessment of the concentration of ZEN, α- and β-ZEL. For the determination of ZEN sulfate-derivates (ZEN-14-Sulf, α- and β-ZEL-14-Sulf), for which the use of internal standard was impossible, matrix-matched calibration plots were constructed. Table 33 summarizes the obtained results. The linearity was interpreted graphically using a scatter plot and confirmed through Mandel test for all analytes. The precision was evaluated in terms of relative standard deviation (RSD). It ranged between 2.5 and 8.3% for intra-day precision and from 0.1 to 9.6% between the three days. Method recoveries were in the range between 72.4 and 85.7 % for ZEN sulfato-derivates and from 90.7 to 95.6 % for other analytes studied.

Table 33: Overview of the limits of detection (LOD), limits of quantification (LOQ), recovery (%), intra- and inter-day precision (RSD_r and RSD_R, respectively) of the analytical method validated in oat flour (n=16)

Mycotoxin	LOD[2] (µg/kg)	LOQ[3] (µg/kg)	Recovery, %	RSD_r, %	RSD_R, %
ZEN	17.9	59.1	95.6	2.5	0.6
α-ZEL	5.6	18.46	92.6	3.8	2.4
β-ZEL	12.16	40.14	90.7	2.5	0.01
ZEN-14-S	0.72	2.39	72.6	7.3	9.6
α-ZEL-14-S	0.31	1.03	72.4	7.6	2.3
β-ZEL-14-S	0.3	1.0	85.7	8.3	7.1

10.4.3 Analysis of the treated flour samples

Cereal-based baby food is a highly important part of human diet during the first years of life (European Commission 2006). Its production process aims to partially breakdown the starch contained in the cereals and to facilitate the assimilation of the nutrients by the digestive system of a child. Amylolytic enzymes are the ones usually used to perform this task, mainly due to the high efficiency of the reaction (Martinez and Gómez, 2016).

Previous studies have reported the presence of mycotoxins in cereal-based baby food (Cano-Sancho et al., 2011; Dombrink-Kurtzman, Poling, and Kendra, 2010; Juan et al., 2014). The developed method was applied to *Fusarium* contaminated wheat and oat flours, which were submitted to an enzymatic treatment with α-amylase and glucoamylase (enzymes characteristic for cereal-based infant food production process). The aim was to investigate the effect of different incubation times on ZEN and its modified forms. Due to the fact that infants are particularly sensible to the effect of cereal contaminants and particularly the ones targeting their in-development hormonal system (as is the case of ZEN and its modified forms), a special attention was given to following the changes in the levels of α-ZEL and α-ZEL -14-Sulf. Figure 18 is a graphical representation of the obtained results. It can be seen that ZEN and α-ZEL are not showing any significant change during the applied process in none of the two matrices, although a slightly increasing trend can be observed. For β-ZEL, a significantly decreasing trend can be

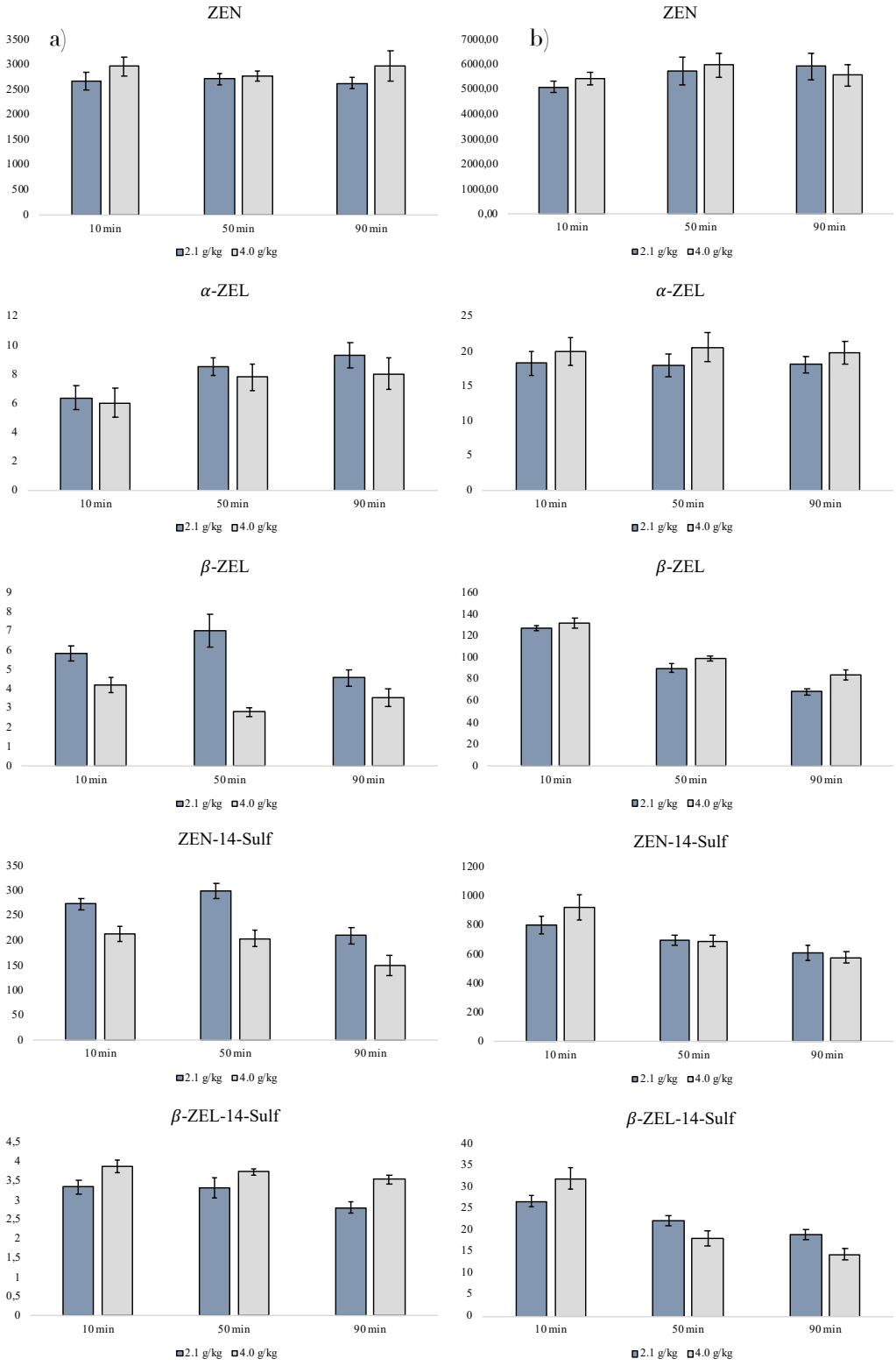

Figure 18: Concentration of zearalenone (ZEN) and its modified forms α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-sulfate (ZEN-14-Sulf) and β-zearalenol-14-sulfate (β-ZEL-14-Sulf) in a) wheat and b) oat flour during enzymatic treatment at a dose of 2.1 and 4.0 g of enzyme/kg of flour during 10, 50 and 90 min. The error bars represent the standard deviation of the mean concentration

observed during the treatment at both concentrations of enzymes, 2.1 and 4.0 g enzyme/kg flour in oats with up to 46 and 37% after 90 min of incubation, respectively. The variable changes of β-ZEL level observed during the treatment of wheat flour cannot help the study confirm the results observed in oat and more research would be needed in order to achieve it. Regarding the fate of ZEN sulfate-derivates, ZEN-14-Sulf shows a relatively significant decrease after 90 min at both doses of enzymes (up to 37% in oat and up to 29% in wheat flours). The variable results for ZEN do not allow us to claim a correlation between the opposite behavior of the two toxins. -ZEL-14-Sulf was not identified in any of the analyzed samples. -ZEL-14-Sulf showed a very similar behavior compared to its free form (-ZEL) in both matrices, namely no increase nor reduction compared to the initially identified levels of the toxin.

10.5 Conclusion

The developed LC-MS/MS method for the analysis of ZEN and modified forms demonstrated good performance criteria and applicability. The QuEChERS based method used for sample preparation also proved itself suitable for the quantification of the analytes showing good recoveries. The very similar ionization products of ZEN-14-S and α-ZEL-14-S could require either an indirect calculation of the concentration or finding a stationary phase able to chromatographically separate the two analytes and allow their direct identification. The analysis of wheat and oat flours after the enzymatic treatment did not show any changes for ZEN and α-ZEL levels, however up to 46% decrease of β-ZEL was observed after 90 min of incubation. A quite similar behavior was observed for ZEN-14-S and β-ZEL-14-S, which proves no correlation between the change of the two modified forms under the performed treatment. α-ZEL-14-S was not identified in any of the analyzed samples. Taking into account that, according to EFSA report on the risk to the public health related to the presence of ZEN in food products (2011) infants are a population group which is already overexposed to ZEN, monitoring the modified forms, of which the toxicity and the metabolism in the human body is not fully understood is of a great importance and requires more attention.

10.6 Acknowledgement

The authors are grateful to the University of Lleida (grant JADE Plus 218/2016), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2017-87755-R) for funding this work.

10.7 References

De Baere, S. et al. (2012). Development of a Liquid Chromatography Tandem Mass Spectrometry and Ultra-High-Performance Liquid Chromatography High-Resolution Mass Spectrometry Method for the Quantitative Determination of Zearalenone and Its Major Metabolites in Chicken and Pig Plas. *Analytica Chimica Acta, 756*, 37 48.

Berthiller, F. et al. (2007). Short Review: Metabolism of the *Fusarium* mycotoxins deoxynivalenol and zearalenone in plants. *Mycotoxin Research, 23,* 68 72.

Binder, S. B. et al. 2017. Metabolism of zearalenone and its major modified forms in pigs. *Toxins, 9,* 1 15.

De Boevre, M. et al. (2013). Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218*, 281 92.

Brodehl, A. et al. (2014). Biotransformation of the mycotoxin zearalenone by fungi of the genera *Rhizopus* and *Aspergillus*. *FEMS Microbiology Letters, 359*, 124 30.

Bryła, M. et al. (2018). Modified *Fusarium* mycotoxins in cereals and their products metabolism, occurrence, and toxicity: an updated review. *Molecules, 23*, 1 34.

Cano-Sancho, G. et al. (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.

Catteuw, A. et al. (2019). Insights into in vivo absolute oral bioavailability, biotransformation, and toxicokinetic of zearalenone, α-zearalenol, β-zearalenol, zearalenone-14-glucoside and zearalenone-14-sulfate in pigs. *Journal of Agricultural and Food Chemistry, 67*, 3448 58.

Cheli, F., Battaglia, D., Gallo, R., and Dell'Orto, V. (2014). EU legislation on cereal safety: an update with a focus on mycotoxins. *Food Control, 37*, 315 25.

Dombrink-Kurtzman, M. A., Poling, S.T.M., and Kendra, D.F. (2010). Determination of deoxynivalenol in infant cereal by immunoaffinity column cleanup and High-Pressure Liquid Chromatography UV detection. *Journal of Food Protection, 73*, 1073 76.

EFSA. (2011). Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal, 9*, 2197 2321.

European Commission. (2006). Commission Directive 2006/125/EC on Processed cereal-based foods and baby foods for infants and young children. *Official Journal of the European Union*: 16 35.

Ismaiel, A.A., and Papenbrock, J. (2015). Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture, 5*, 493 537.

Juan, C., Raiola, A., Mañes, J., and Ritieni, A. (2014). Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control, 39*, 227 36.

Kovalsky, P. et al. (2016). Co-occurrence of regulated, masked and emerging mycotoxins and secondary metabolites in finished feed and maize an extensive survey. *Toxins, 8*, 1 29.

Kowalska, K., Habrowska-Górczyńska, D.E., and Piastowska-Ciesielska, A.W. (2016). Zearalenone as an endocrine disruptor in humans. *Environmental Toxicology and Pharmacology, 48*, 141 49.

Lu, H., Leal Ruiz, M.J., Míguez, M.P., and Fernández-Franzón, M. (2013). Detection of seven trichothecene mycotoxins in infant cereal foods by QuEChERS extraction and liquid chromatography coupled to tandem mass spectrometry. *Revista de Toxicologia, 30*, 203 8.

Marroquín-Cardona, A. G., N. M. Johnson, T. D. Phillips, and A. W. Hayes. (2014). Mycotoxins in a changing global environment - A review. *Food and Chemical Toxicology, 69*, 220 30.

Martinez, M. M., and Gómez, M. (2016). Insight of the α-amylase family of enzymes. In *Microbial Enzyme Technology in Food Applications*, 21 45.

Mousavi Khaneghah, A. et al. (2019). Mycotoxins in cereal-based products during 24 years (1983 2017): a global systematic review. *Trends in Food Science and Technology, 91*, 95 105.

Pereira, V. L., Fernandes, J. O. and Cunha, S. C. (2015). Comparative assessment of three cleanup procedures after QuEChERS extraction for determination of trichothecenes (type A and type B) in processed cereal-based baby foods by GC MS. *Food Chemistry, 182*, 143 49.

Rogowska, A., Pomastowski, P., Sagandykova, G., and Buszewski, B. (2019). Zearalenone and Its Metabolites: Effect on Human Health, Metabolism and Neutralisation Methods. *Toxicon, 162*, 46 56.

Wang, N., Wu, W., Pan, J., and Long, M. (2019). Detoxification strategies for zearalenone using microorganisms: A review. *Microorganisms, 7*, article No. 208, pages 14.

Wenzl, T. et al. (2016). European Union Reference Laboratory. Publications Office of the European Union, Luxembourg, ISBN 978-92-79-61768-3; *Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. EUR 28099 EN*.

Zinedine, A., Soriano, J.M., Moltó, J.C., and 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

Chapter 11. General discussion

Mycotoxins are secondary metabolites produced by filamentous *fungi* in plants, which if accumulated are able to cause diseases in humans and animals (Zinedine, Soriano, Moltó, & Mañes, 2007). The main fungal genera involved in mycotoxin production are *Fusarium*, *Aspergillus* and *Penicillium* (Lee & Ryu, 2017). Very few plant diseases have had the impact mycotoxigenic *fungi* have on cereal industry, being mainly related to production losses and mycotoxin management costs (Robens & Cardwell, 2003). *Fusarium* mycotoxins are commonly occurring in different cereals and cereal-based products. The infection with *Fusarium* strains takes place in the field (before harvesting), which lead not only to the contamination with mycotoxins but also to the formation of various modified forms of these as a result of plant or fungal metabolisms (Berthiller et al., 2013). Many of these modified forms are less toxic than their parental compounds, however their toxicity is restored once the added radical is cleaved during animal or human digestion (EFSA, 2011).

Various steps applied during food production are proven as being able to decrease the contamination with mycotoxins, mostly due to the elimination of the most contaminated parts (e.g. sorting and dehulling) (Ryu et al., 2008). Nonetheless, the severity of the fungal infection is directly correlated with the degree of propagation of the toxin inside the cereal kernels (Habschied et al., 2011), which, considering their high thermal and chemical stability during processing (Zinedine et al., 2007), still puts the consumer at a risk of exposure to these contaminants.

Considering all of the abovementioned, the present work aims to investigate the effect of processing steps applied in food industry on the initial level of mycotoxins. Two food products were chosen: (i) beer, which is the most consumed alcoholic beverage worldwide and for which maximum limits are set only for FBs and only in the case of using maize adjuncts; (ii) cereal-based baby food, product which represents the main element of infants diet, infants being considered by the EFSA as the most exposed and at the same time the group of population most sensitive to the exposure to mycotoxins. The two production processes are characterized by stages where physical (e.g. kilning, roasting) and biochemical (e.g. enzymatic hydrolyzation, alcoholic fermentation) treatments are applied. The final results of the proposed study will be discussed in the following section of the present chapter.

11.1 Beer

Barley is the main commodity used in beer production together with water, yeast and hops. The greatest malting barley producer is Europe, accounting for more than 60% of the world malt trade (Euromalt, 2019). Fusarium Head Blight disease (FHB) is highly occurring on barley crops and represent an important issue for farmers and brewers, especially because the disease affects grain quality and, as a result, its suitability for brewing (Lancova et al., 2008). On the regulatory side, EU establishes specific maximum allowed limits for mycotoxins throughout the Regulation EC 1881/2006, taking into account criteria such as mycotoxin toxicity, occurrence in food products and the intake of those foods by the population (European Commission, 2006).

11.1.1 Occurrence of mycotoxins in barley and beer

The most frequently encountered mycotoxin in malting barley and beer are DON, followed by ZEN, NIV, T-2 and HT-2 toxins, FBs, AFs, OTA (Nielsen, Cook, Edwards, & Ray, 2014). The maximum allowed limits of mycotoxins in barley are stipulated by the Regulation EC 1881/2006 and set as follows: 1250 µg/kg for DON, 100 µg/kg for ZEN, 5 µg/kg for OTA, 2 µg/kg for aflatoxin B_1 (AFB1) and 4 µg/kg for total AFs. Beer is only subjected to legal limits in FBs content (400 µg/L for the sum of fumonisin B_1 and B_2) and only in the case in which maize is used as adjunct, other mycotoxins not being included. Hence, the legislation expects a nearly complete elimination and/or reduction of mycotoxins present in raw materials during malting and brewing, which in the context of a moderate beer consumption would not represent any risk even if variable reduction factors are experienced. Nonetheless, the available surveys on the occurrence of mycotoxins in barley and beer are showing relatively worrying results with high frequencies, although not too high concentration levels (Běláková, Benešová, Čáslavský, Svoboda, & Mikulíková, 2014; Malachova, Varga, Schwartz, Krska, & Berthiller, 2012; Malachova et al., 2010; Piacentini, Savi, Pereira, & Scussel, 2015; Zachariasova et al., 2008). The reported DON frequency is slightly lower in beer compared to barley (30-100 % *vs* 50-100%, respectively) and so are the encountered levels (3-570 µg/L in beer and 40-50000 µg/kg in malting barley) (Malachova et al., 2010; Niessen, Böhm-Schrami, Vogel, &

Donhauser, 1993; Ruan, Li, Lin, & Chen, 2002; Tabuc, Marin, Guerre, Sesan, & Bailly, 2009; Varga, Malachova, Schwartz, Krska, & Berthiller, 2013). Similar is the case of OTA (frequency decreased from 70 to a maximum of 20% and the found levels from 50 to 7 µg/L) (Medina et al., 2006; Rubert, Soler, Marín, James, & Mañes, 2013) and T-2 and HT-2 toxins with almost 50% decrease of the frequency and almost 20% decrease of the reported levels. AFs in barley were always below the maximum allowed limits and with more than 40% occurrence between the analyzed samples, however in beer they were almost always reported <LOD (limit of detection). Higher levels of ZEN and NIV were observed in beer compared to barley but only three surveys are available, which makes it difficult to draw a conclusion on the relationship between the results in the raw material and in the final product (Gil-Serna et al., 2013; Malachova et al., 2010; Shim, Kim, Seo, & Lee, 1997).

In order to evaluate the risk for the consumer, many surveys also included assessments of the exposure to mycotoxins through beer consumption (Cano-Sancho, Gauchi, Sanchis, Marín, & Ramos, 2011; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015; Varga et al., 2013). Interestingly, the identified contribution of beer consumption to the exposure to mycotoxins was found relatively low in both analyzed scenarios (lower and upper bound) and is not a matter of concern for an average beer consumption, however for the heavy consumers the intake of mycotoxins only from drinking beer might exceed the TDI by up to 5-folds. Since the modified forms of mycotoxins became an emerging issue, they were more and more included in the surveys and exposure assessments (Habler & Rychlik, 2016; Kostelanska et al., 2011). In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) extended the group of DON to its modified forms, 3- and 15-Ac-DON, establishing a TDI equivalent to 1µg/kg body weight for the sum of three toxins (JECFA/FAO, 2011).

In the present thesis, two studies were performed analysing beer samples available in supermarkets from Lleida (Spain) and from Veracruz (Mexico). In order of their prevalence, the identified mycotoxins were DON-3-Glc, DON, FB1 and 3-Ac-DON in the samples from Veracruz (26.2% of contamination), and ZEN, DON, DON-3-Glc, HT-2 toxin and FB1 in the samples from Lleida (20.3% of contamination). Similar to previous studies, the beers with higher alcohol content and the ones from craft breweries that were

analyzed, showed the highest levels of contamination (Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2018; Varga et al., 2013). As in the study performed by Marin, Ramos, Cano-Sancho, & Sanchis (2013), a correlation between the contamination with FB1 and the presence of maize adjuncts was found in the beers from Mexico, and not in the ones from Lleida (only one sample was contaminated with FB1). Another aspect of mycotoxin contamination and consumer safety that was underlined in these studies was the co-occurrence of mycotoxins: one sample in each study registered a simultaneous occurrence of DON and FB1 in the same bottle. Besides, a co-occurrence of DON and its modified form DON-3-Glc was registered , which could be the result of either its release from the matrix or its formation during mashing or malting (Lancova et al., 2008). A preliminary exposure assessment to DON and its modified forms was performed in both studies. DON intake through beer consumption in Lleida contributed with a maximum of 5.8% of the TDI (upper bound scenario). In the Mexican beers, this contribution represents almost 20%, and rises up to 50% in the case of a high consumption scenario. Considering the obtained results and the available studies (Rodríguez-Carrasco et al., 2015; Varga et al., 2013), Mexican adult population looks to be slightly more exposed to DON through beer consumption compared to the Spanish. Nonetheless, it is difficult to make a fair assessment of the exposure, firstly because the available consumption data is scarce and, besides the unavailability for a larger sampling, it is impossible to have a suitable picture of the consumption by groups of population (different consumption habits in accordance with age, sex and social position). From the performed studies, a transfer of mycotoxins from raw materials to beer is possible and, considering the diversity of consumers and their habits, more research is needed to identify production stages with importance to the mycotoxin levels changing and, as a result, the evaluation of the risk for the population.

11.1.2 Fate of *Fusarium* mycotoxins during beer production

From the technological point of view, beer production is considered one of the more complex and delicate processes in food industry from biochemical and physical perspective. It includes steps such as germination, mashing, boiling, fermentation. As it was described in the present work, it is also subjected to the contamination with various mycotoxins. Thus, this study highlights the most important stages in malting and brewing,

that according to the available literature have or could have an impact on the levels of *Fusarium* mycotoxins in the case of processing a batch of contaminated barley. The designed experiments intended to simulate at best the physical parameters applied during malting and brewing, also using *Fusarium* infested raw material.

11.1.2.1 Malting

Malting is a controlled germination process to produce malt. It consists of three stages (steeping, germination and kilning), which are initiated under specific conditions of humidity and temperature. This is one of the most important production stages for brewing because the quality of the obtained malt will define the quality of the wort and beer, subsequently. Steeping is aiming to create favourable humidity conditions for germination, where the activated enzymes will break starch and proteins. Kilning process will inactivate the enzymes before an excessive hydrolysis may take place. Also, kilning is decisive for flavour and colour formation.

The present work investigated the transfer of DON, DON-3-Glc and ZEN in naturally contaminated and in laboratory infected barley through malting process. Firstly, the results on naturally contaminated barley will be discussed. The effect on the three mycotoxins had a similar tendency and was in accordance with the available studies, proving malting an important production stage with an impact on fungal infection and mycotoxin contamination. DON was washed out during steeping, registering from 75 to 85% of reduction, which was considerably higher compared to the results obtained by Lancova et al. (2008) who identified a 10% decrease of DON after this step. It could be explained by its solubility in water and by the fact that the higher part of the toxin is located on the outer layers of the kernel allowing it to be washed out. Vegi, Schwarz, & Wolf-Hall (2011) identified that water flow could spread the *Fusarium* infection among the grains by 15-90%, however grain storage have a drastic effect on *Fusarium* viability (Beattie, Schwarz, Horsley, Barr, & Casper, Howard, 1998), which decrease the risk related to this step. In the present work, no *Fusarium* colony was observed in the culture study, fact that proves again that it does not survive the low water activity maintained during grains storage.

Germination process led to an increase in DON concentration after 48h of the process up to 30%, followed by a significant decrease by the end of it (the resulted concentration was lower compared to the initial before kilning). An increase in DON-3-Glc levels accompanied this decrease of DON. It could have two origins: first, DON-3-Glc was released from the matrix thanks to the activation of the hydrolytic enzymes during germination, and secondly, it was formed under the activity of glucosyltransferase (Lemmens et al., 2005; Maul et al., 2012).

As it was proven in previous publications (Oliveira, Mauch, Jacob, Waters, & Arendt, 2012; Vegi et al., 2011), kilning did not have any destructive effect on neither DON nor DON-3-Glc, moreover an increase of the concentration of both mycotoxins compared to the last day of germination was registered (21.5 and 4.5% increase in DON in level 1 and 2, respectively; 107.3 and 33.2% increase in DON-3-Glc for contamination levels 1 and 2, respectively). However, roasting at temperatures above 150 °C, if applied, could induce a reduction in DON level (Kostelanska et al., 2011).

There is scarce information concerning the fate of other *Fusarium* toxins during malting, such as ZEN, AFs or NIV, which could be probably due to their lower occurrence in barley (Medina et al., 2006). More than 40% decrease in ZEN was observed after steeping. Germination showed an increase in ZEN levels during the first 48h followed by a significant decrease by the end of the process (the concentration at the end of germination was lower compared to the initial level of ZEN before malting). Kilning process did not lead to a significant change in ZEN concentration. The study on barley mycobiota performed did not find any *Fusarium* on the plated kernels, which would suggest that the possible origins of the abovementioned increases are either their release from the matrix or their formation under the enzymatic activity.

In the case of laboratory infected barley, fungal growth was promoted by the increased water humidity during the process, which led to a greater rise of mycotoxins concentrations during steeping and germination processes (almost 10000% rise of DON concentration, and up to 2959 % rise of DON-3-Glc level). Kilning did not result in any significant change of concentration, except for a 78% decrease of DON-3-Glc in the level 3 of contamination, which could be explained by the high variability in mycotoxin

accumulation between samples. ZEN concentration in laboratory infected barley did not show any significant change at the end of malting process compared to its level before the process.

A definite increase in mycotoxin levels during malting can be concluded, most probably coming from their release from barley matrix due to enzymatic activity. Also, the conversion of DON into DON-3-Glc during germination was observed, which considering the higher solubility in water of the latter will bring to a higher transfer of it into the wort during mashing, thus increasing the exposure rate to DON toxins.

11.1.2.2 Mashing and boiling

Mashing is the mix of coarse ground malt with a high amount of water (17 kg of malt are needed for 1 hL of beer) under specific temperatures to activate all the enzymes present (inactivated during kilning) and to allow the conversion of starches into fermentable sugars, and of the proteins into aminoacids, the aim being to ensure a correct fermentation process and the achievement of the proposed technological quality of the product. Mashing is followed by wort separation and boiling (1.5 to 3 h), accompanied by hops addition. During this step, the following processes take place: enzyme inactivation, protein precipitation, sterilization, isomerisation of hop α-acid, evaporation of water and undesirable volatile compounds (*e.g.* dimethyl sulphides) etc.

In terms of impact on mycotoxin contamination, only mashing was proved as having a certain impact, boiling process not being considered individually in the available literature. From the three contamination scenarios prepared in the present study, some interactions between the process and the level of mycotoxins were identified. Samples spiked with standard mycotoxin solutions (DON, ZEN and FB1) showed changes in contamination similar to the samples in which the contamination was coming from infected malt. DON and its metabolites had a similar behaviour, showing a significant transfer from malt to wort during mashing. Moreover, an increase of the extracted amount of toxins was observed through the process, the most significant being registered after 15 min at 45 °C. Wolf-Hall (2007) reported a possible release of DON from protein complex under proteolysis during mashing, and considering its solubility in water, once released,

it would probably pass into the wort. However, Inoue, Nagatomi, Uyama, & Mochizuki (2013) determined almost 20% of mycotoxins eliminated with the spent grains. Interestingly, however great the rise, boiling always led to a reduction down to the initial level (in samples with the addition of the contaminated maize) or even lower (samples containing contaminated malt). Despite the abovementioned decrease during boiling, DON is still of a special concern for brewing because of its 30 to 60 % remaining in the wort after the process, which could withstand the following production steps.

ZEN was characterized by a relatively high transfer to the wort during mashing, with the changes occurring gradually over the three temperatures steps applied. This effect does not go entirely in line with the findings reported by Inoue, Nagatomi, Uyama, & Mochizuki, (2013), showing more than 60% of the toxin retained in the spent grains, nonetheless this variations of the results can be accounted for the different approaches between the two studies. The following boiling step led to an almost 100% reduction of ZEN level after only 30 min of treatment, the next 60 min not having a significant impact on the remaining amount toxin. Very low incidence of α-ZEL was registered along with its complete elimination by the end of the process. β-ZEL was a little more abundant compared to its stereoisomer and showed a low reduction rate at the end of the process.

Both FB1 and FB2 showed transfer into the wort during mashing, however on low levels of contamination it was almost completely removed with the spent grains. Also, in the case of the FBs, the first 30 min of boiling reduced between 90 to 100% of the toxins that were transferred during mashing. There is a need of in-depth studies in order to identify the exact behavior of the FBs and the factors involved in it.

In summary, the crucial stages that induced significant change in mycotoxins levels were the first 15 min at 45 °C (increase of transfer of mycotoxins into the wort) and the first 30 min of boiling (decrease of mycotoxin level in the wort). Considering the aforementioned, boiling is a crucial step in mycotoxins mitigation but, taking into account that the majority of the mycotoxins are not completely reduced even after 90 min of boiling. The study does not search for other modified forms of the targeted analytes, which could better explain the direction of the changes and their impact on the assessment of the mitigation role of these two brewing stages.

11.2.2.3 Fermentation

Yeast are critical to beer making process and specifically, the fermentation stage. Their activity is not only limited to transforming malt sugars into alcohol, their enzymes are also crucial in shaping beer flavour and aroma by creating volatile compounds such as esters and higher alcohols. Two fermentation styles are known worldwide: ale (top fermentation) and lager (bottom fermentation), performed by two different strains of *Saccharomyces* yeast.

The present work focused on 15 different *Saccharomyces* strains and two *Fusarium* toxins, DON and ZEN. Interestingly, the adsorption dynamics of the two toxins are relatively different, most of the adsorbed DON being retained on the yeast cell wall during the first 24h of fermentation, however ZEN adsorption took place gradually during the 96h of the process. This difference can be due to various factors such as physical and chemical parameters of the fermentation process (temperature, pH, duration etc.), the nature of the contamination (natural or spiked), and due to the different chemical properties of each targeted mycotoxin. The ratios of the observed changes are in line with the available studies (Keller et al., 2015; Scott, Kanhere, Daley, & Farber, 1992; Sørensen & Sondergaard, 2014), namely from 5 to 15% DON and from 31 to 72% ZEN retained on the yeast cell wall. To identify the role of the viability of the yeast cells in the adsorption process, other studies investigated the ability of yeast residue to adsorb mycotoxins reporting the reduction of ZEN (75%), AFB1 (48%) and OTA (59%) due to the β-glucans contained in the cell wall (Campagnollo et al., 2015). Interestingly, DON was not proven to be efficiently adsorbed by the yeast, reaching a maximum of 17%, which considering its high occurrence and transfer rate to the wort, can be a subject of concern (Nathanail et al., 2016). However, the study performed by Garda et al. (2005) shows a 53% reduction in DON levels. Studies are available reporting a partial metabolization of the mycotoxins by the yeast and the formation of α- and β-ZEL from ZEN (Keller et al., 2015), and the formation of acetylated-deoxynivalenol and DON-3-Glc (Khatibi et al., 2011). This can explain the differences in the identified mycotoxin concentration in the intermediary samples, nonetheless the focus of the study would have to be expanded using additional tools in order to prove this hypothesis. Also, the present work identified a slightly significant difference related to mycotoxin adsorption between the two *Saccharomyces* strains, *S.*

cerevisiae showing a slightly higher adsorption rates in both toxins. This effect can be explained by the production of a higher amount of the biomass which increased the active sites for mycotoxin binding. As it was reported previously in the available literature, the contamination of the wort with mycotoxins did not have any effect on the biochemical and technological performance of the yeast.

11.2 Cereal-based baby food

Cereals represent an important part of human diet. One of the main risks associated to cereals are the contaminants, predominantly the mycotoxins. In the case of infants, the most sensitive to contaminants population group, mycotoxins are of a special concern and strict maximum limit are set by the European Commission (EC 1881/2006). The most frequently used cereals for baby food production are oats, wheat, barley, maize, rye, triticale and pseudo-cereals, such as quinoa, buckwheat and amaranth (Klerks et al., 2019). Very few studies are available investigating the occurrence or the problem of the contamination with *Fusarium* mycotoxins in cereal-based infant food, considering the high importance that it has in their diet. Also, it is worth mentioning that there are no works describing the effect of the production process of this foodstuff, especially considering that it includes steps which were proven as having a mitigation impact related to mycotoxins.

For the present study, barley, wheat and oat flours were bought in a local supermarket. Before proceeding with the assessment of the effect of the two processes applied (roasting and enzymatic treatment), a screening of the mycotoxins present in the flours was performed. DON, 3- and 15-Ac-DON and ZEN were identified in all the matrices. Besides, T-2 and HT-2 toxins were contained in oat flour and ENN B and BEA were present in barley and oat flour, however in relatively low concentrations. The findings of the screening procedure are aligned with the results reported by (Bryła et al., 2016; Lindblad et al., 2013). The intensities of the signals obtained during this phase, focused the present study to the analysis of the following analytes: DON, 3- and 15-Ac-DON, ZEN in three matrices and a semiquantitative assessment of T-2 and HT-2 toxins was performed for oat flour.

11.2.1 Fate of *Fusarium* mycotoxins during cereal-based baby food production

11.2.1.1 Roasting

Roasting is an important flavor modulator process extensively used in food industry. Besides, it is important from the safety perspective, yet that many biological contaminants are sensitive to temperature treatments. Mycotoxins are proved as compounds with high thermal stability, only temperature ranging from 160°C and above can lead to a significant impact on their initial concentration (Israel-Roming & Avram, 2010; Yumbe-Guevara, Imoto, & Yoshizawa, 2003). The roasting process of the cereal-based baby food is performed at temperatures between 105 and 120°C, the latter one being an important point against the formation of the acrylamide (toxic carcinogenic product of Maillard reactions), to which infants are particularly sensitive (EFSA, 2015; Erkekoğlu & Baydar, 2010).

Barley flour contamination pattern and impact of the applied processing on it was different compared to wheat and oat flours. DON and its acetylated forms showed significant changes in their concentrations after the roasting process, however not being aligned with other matrices makes these results inconclusive and imply the need in a deeper study of the observed effects. Similarly to the available reports, the present study proved the stability of DON and its modified forms during flour roasting at 105 and 120°C for 30 and 40 min (Kabak, 2009; Yumbe-Guevara et al., 2003; Zinedine et al., 2007). Considering the abovementioned, this study proves also the stability of 3- and 15-Ac-DON, which strengthen the food safety concern related to these analytes.

ZEN showed a slightly decreasing trend after the process; but the decrease was not great enough to be assessed significantly important. The present study also focused on the effects roasting had on T-2 and HT-2 toxins in oat flour. Their stability was higher compared to the one reported by Kuchenbuch, Becker, Schulz, Cramer, & Humpf (2018), where an almost 5% decrease was registered. Nonetheless, the differences in the experimental designs do not lead to a conclusive statement with regard to their stability.

11.2.1.2 Enzymatic treatment

Amylolytic enzymes are widely used in food processing, cereal-based baby foods being one of them. Their role is to ensure a partial digestion of the starch present in the flour and help the infants still underdeveloped digestive tract to assimilate more of the energy provided by the cereals. There are no available studies investigating the fate of the mycotoxins in cereal-based infant food, however the impact of the same enzymes was studied in breadmaking and brewing (Lancova et al., 2008; Vidal, Ambrosio, Sanchis, Ramos, & Marín, 2016; Zachariasova et al., 2008).

The impact of α-amylase and glucoamylase were studied on the level of *Fusarium* mycotoxins in barley, wheat and oat flour. The results show a relatively unchangeable trend of DON concentration in the three matrices. DON-3-Glc was not found in any of the sample, as it would be expected from the previous reports (Vidal et al., 2016; Vidal, Morales, Sanchis, Ramos, & Marín, 2014). The acetylated forms of DON were also investigated, and no significant changes could be identified after applying the treatment, which also show no transformation or release of DON during the process. Nonetheless, slight difference between the three matrices were observed, probably explained by different enzymes naturally present in each of the studied flours which could have changed slightly the trends in mycotoxin levels (DON and 3- and 15-Ac-DON). There are no studies pointing it out, however the results obtained by Vidal et al. (2016) already proved that enzymes can lead to a change in the level of mycotoxins.

The obtained concentrations of ZEN were too high to be able to draw any conclusions regarding its fate; nonetheless, it is shown to be quite stable. The treatment also did not show any significant change in α-ZEL level, however an almost 50% decrease was reported for β-ZEL at both doses of the enzymes (46 and 37% decrease after the treatment at 2.1 and 4.0 g of enzyme/kg of flour, respectively). The EFSA (2011) opinion on the risk related to the presence of ZEN in food states that the lack of sufficient toxicokinetic data in young animals suggests that infants can be more sensitive to the effect of the phenolic oestrogen of the toxin and considering the common presence of α- and β-ZEL in food products, this risk is even higher.

From the perspective of the abovementioned, ZEN sulfate-derivates were also considered in the present study. A relatively significant decrease of ZEN-14-Sulf concentration was registered after 90 min of incubation with the enzymes in the three flours used; however, the low ratio of ZEN-14-Sulf compared to ZEN present did not prove any relationship between the two forms of the mycotoxin. α-ZEL-14-Sulf was not found in any of the samples and β-ZEL-14-Sulf demonstrated a decreasing trend similar to β-ZEL at both enzyme doses. This suggests that no cleavage of the sulfate takes place during the process. There are no available publications focused on the fate of ZEN and its modified forms under an enzymatic process which could prove the obtained observations. Thus, the assumed high sensibility of this population segment to the estrogenic effects of the toxin justifies the need in more studies on this topic. An important aspect to consider related to the use of milk in the preparation of the cereal-based baby food, is the fact that ZEN is a toxin that is demonstrated to be carried over to the milk in the case of the exposure of the cattle through feed; α- and β-ZEL representing a high share of this transfer (EFSA, 2011). Thus, the future studies, besides investigating the impact of different processes on mycotoxin levels, should take into account consumers behavior after purchasing the foodstuff.

11.3 References

Beattie, S., Schwarz, P. B., Horsley, R., Barr, J., & Casper, Howard, H. (1998). The effect of grain storage conditions on the viability of *Fusarium* and deoxynivalenol production in infested malting barley. *Journal of Food Protection*, *61*, 103–106.

Běláková, S., Benešová, K., Čáslavský, J., Svoboda, Z., & Mikulíková, R. (2014). The occurrence of the selected fusarium mycotoxins in czech malting barley. *Food Control*, *37*, 93–98.

Bertuzzi, T., Rastelli, S., Mulazzi, A., Donadini, G., & Pietri, A. (2018). Known and emerging mycotoxins in small- and large-scale brewed beer. *Beverages*, *4*, 46.

Bryła, M., Waśkiewicz, A., Podolska, G., Szymczyk, K., Jędrzejczak, R., Damaziak, K., & Sułek, A. (2016). Occurrence of 26 mycotoxins in the grain of cereals cultivated in Poland. *Toxins*, *8*, 20.

Campagnollo, F. B., Franco, L. T., Rottinghaus, G. E., Kobashigawa, E., Ledoux, D. R., Daković, A., & Oliveira, C. A. F. (2015). In vitro evaluation of the ability of beer fermentation residue containing *Saccharomyces cerevisiae* to bind mycotoxins. *Food Research International*, *77*, 643–648.

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.

EFSA (2011). Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal*, 1-124.

EFSA. (2015). Scientific Opinion on acrylamide in food. *EFSA Journal*, *13*(6), 4104.

Erkekoğlu, P., & Baydar, T. (2010). Toxicity of acrylamide and evaluation of its exposure in baby foods. *Nutrition Research Reviews*, *23*, 323–333.

Euromalt. (14 de January de 2019). *Euromalt statistics*. Obtenido de Euromalt: http://www.euromalt.be/list_infos/euromalt%20statistics/1011306087/list1353668317.html

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

Garda, J., Martins Macedo, R., Faria, R., Bernd, L., Dors, G. C., & Badiale-Furlong, E. (2005). Alcoholic fermentation effects on malt spiked with trichothecenes. *Food Control*, *16*, 423–428.

Gil-Serna, J., Mateo, E. M., González-Jaén, M. T., Jiménez, M., Vázquez, C., & Patiño, B. (2013). Contamination of barley seeds with *Fusarium* species and their toxins in Spain: an integrated approach. *Food Additives & Contaminants: Part A*, *30*, 372–380.

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

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. (2013). Fate of Mycotoxins during Beer Brewing and Fermentation. *Bioscience, Biotechnology, and Biochemistry*, *77*, 1410 1415.

Israel-Roming, F., & Avram, M. (2010). Deoxynivalenol stability during wheat processing. *Romanian Biotechnological Letters*, *15*, 47 50.

JECFA/FAO. (2011). *Safety evaluation of certain contaminants in food. Prepared by the Sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). FAO JECFA Monographs 8* (Vol. 82).

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

Keller, L., Abrunhosa, L., Keller, K., Rosa, C. A., Cavaglieri, L., & Venâncio, A. (2015). Zearalenone and its derivatives α-zearalenol and β-zearalenol decontamination by *Saccharomyces cerevisiae* strains isolated from bovine forage. *Toxins*, *7*, 3297 3308.

Khatibi, P. A., Montanti, J., Nghiem, N. P., Hicks, K. B., Berger, G., Brooks, W. S., … Schmale, D. G. (2011). Conversion of deoxynivalenol to 3-acetyldeoxynivalenol in barley-derived fuel ethanol co-products with yeast expressing trichothecene 3-O-acetyltransferases. *Biotechnology for Biofuels*, *4*, 1 13.

Klerks, M., Bernal, M. J., Roman, S., Bodenstab, S., Gil, A., & Sanchez-Siles, L. M. (2019). Infant cereals: Current status, challenges, and future opportunities for whole grains. *Nutrients*, *11*, 1 25.

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.

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 & Contaminants: Part A*, *25*, 732 744.

Lee, H. J., & Ryu, D. (2017). Worldwide Occurrence of Mycotoxins in Cereals and Cereal-Derived Food Products: Public Health Perspectives of Their Co-occurrence. *Journal*

of Agricultural and Food Chemistry, *65*, 7034 7051.

Lemmens, M., Scholz, U., Berthiller, F., Dall 'asta, C., Koutnik, A., Schuhmacher, 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.

Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., & Fredlund, E. (2013). Deoxynivalenol and other selected *Fusarium* toxins in Swedish wheat - occurrence and correlation to specific fusarium species. *International Journal of Food Microbiology*, *167*, 284 291.

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.

Malachova, Alexandra, 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.

Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, *60*, 218 237.

Maul, R., Müller, C., Rieß, S., Koch, M., Methner, F. J., & Irene, N. (2012). Germination induces the glucosylation of the *Fusarium* mycotoxin deoxynivalenol in various grains. *Food Chemistry*, *13*, 274 279.

Medina, Á., Valle-Algarra, F. M., Mateo, R., Gimeno-Adelantado, J. V., Mateo, F., & Jiménez, M. (2006). Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria, Aspergillus* and *Fusarium*. *International Journal of Food Microbiology*, *108*, 196 203.

Nathanail, A. V., Gibson, B., Han, L., Peltonen, K., Ollilainen, V., Jestoi, M., & Laitila, A. (2016). The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. *Food Chemistry*, *203*, 448 455.

Nielsen, L. K., Cook, D. J., Edwards, S. G., & Ray, R. V. (2014). The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK. *International Journal of Food Microbiology*, *179*, 38–49.

Niessen, L., Böhm-Schrami, M., Vogel, H., & Donhauser, S. (1993). Deoxynivalenol in commercial beer - screening for the toxin with an indirect competitive ELISA. *Mycotoxin Research*, *9*, 99–109.

Oliveira, P. M., Mauch, A., Jacob, F., Waters, D. M., & Arendt, E. K. (2012). Fundamental study on the influence of *Fusarium* infection on quality and ultrastructure of barley malt. *International Journal of Food Microbiology*, *156*, 32–43.

Piacentini, K. C., Savi, G. D., Pereira, M. E. V, & Scussel, V. M. (2015). Fungi and the natural occurrence of deoxynivalenol and fumonisins in malting barley (*Hordeum vulgare L.*). *Food Chemistry*, *187*, 204–209.

Robens, J., & Cardwell, K. (2003). The costs of mycotoxin management to the USA: Management of aflatoxins in the United States. *Journal of Toxicology - Toxin Reviews*, *22*, 139–152.

Rodríguez-Carrasco, Y., Fattore, M., Albrizio, S., Berrada, H., & Mañes, J. (2015). Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population. *Food Chemistry*, *178*, 149–155.

Ruan, R., Li, Y., Lin, X., & Chen, P. (2002). Non-Destructive determination of deoxynivalenol in barley using near-infrared spectroscopy. *Applied Engineering in Agriculture*, *18*, 549–553.

Rubert, J., Soler, C., Marín, R., James, K. J., & Mañes, J. (2013). Mass spectrometry strategies for mycotoxins analysis in European beers. *Food Control*, *30*, 122–128.

Scott, P. M., Kanhere, S. R., Daley, E. F., & Farber, J. M. (1992). Fermentation of wort containing deoxynivalenol and zearalenone. *Mycotoxin Research*, *8*, 58–66.

Shim, W., Kim, J., Seo, J., & Lee, Y. (1997). Natural occurrence of trichothecenes and zearalenone in Korean and imported beers. *Food Additives and Contaminants*, *14*, 1–5.

Sørensen, J. L., & Sondergaard, T. E. (2014). The effects of different yeast extracts on

secondary metabolite production in *Fusarium*. *International Journal of Food Microbiology*, *170*, 55 60.

Tabuc, C., Marin, D., Guerre, P., Sesan, T., & Bailly, J. D. (2009). Molds and mycotoxin content of cereals in southeastern Romania. *Journal of Food Protection*, *72*, 662 665.

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*, *13*, 750 757.

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.

Vegi, A., Schwarz, P., & Wolf-Hall, C. E. (2011). Quantification of Tri5 gene, expression, and deoxynivalenol production during the malting of barley. *International Journal of Food Microbiology*, *150*, 150 156.

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.

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.

Wolf-Hall, C. E. (2007). Mold and mycotoxin problems encountered during malting and brewing. *International Journal of Food Microbiology*, *119*, 89 94.

Yumbe-Guevara, B. E., Imoto, T., & Yoshizawa, T. (2003). Effects of heating procedures on deoxynivalenol, nivalenol and zearalenone levels in naturally contaminated barley and wheat. *Food Additives and Contaminants*, *20*, 1132 1140.

Zachariasova, M., Hajslova, J., Kostelanska, M., Poustka, J., Krplova, A., Cuhra, P., & Hochel, I. (2008). Deoxynivalenol and its conjugates in beer: A critical assessment of data obtained by enzyme-linked immunosorbent assay and liquid chromatography coupled to tandem mass spectrometry. *Analytica Chimica Acta*, *625*, 77 86.

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.

Chapter 12. Conclusion and future development

12.1 Conclusion

12.1.1 Beer

During this work, several steps of the beer production process were proven to have a mitigation effect on the initial level of mycotoxins. DON and its modified forms are shown to be the most stable, although some of the steps led to their transformation from one form to another. This work proves a total reduction of DON of about 50 to 70%, a little of it being achieved at different production steps. ZEN reduction was achieved during boiling. Their modified forms were proven almost as stable as the parental toxins.

Mycotoxins occurrence in beer. The results showed a relatively low level of mycotoxins in beer, nonetheless the biggest concern in the two performed surveys was the co-occurrence of two and more analytes. In order of their prevalence, the beer samples purchased in Lleida contained ZEN, DON, DON-3-Glc, HT-2 toxin and FB1, whereas the ones from Veracruz were contaminated with DON-3-Glc, DON, FB1 and 3-Ac-DON. The proportion of the mycotoxin contaminated samples was 20.3 and 26.2% of beers from Lleida and Veracruz, respectively. An assessment of the exposure of an average consumer was performed for DON in both groups of samples, which showed a 5.8% contribution to the TDI in the upper bound scenario in Lleida. The data from Veracruz are more alarming showing an almost 20 and 50% contribution to the TDI in the lower and upper bound scenarios, respectively.

Malting. The fate of DON during malting was characterized by several effects. Firstly, its partial washout during the steeping process was proven (approximately 50%), most probably due to its solubility in water. Secondly, its conversion to DON-3-Glc was identified statistically significant in the two tested setups during germination (the sum level of DON and DON-3-Glc in the lower level of contamination was constant until the end of malting). ZEN showed an increasing and then decreasing trend during steeping and germination returning to its initial concentration. Kilning process did not show any impact on none of the tested mycotoxins.

Mashing and boiling. Up to 35% of DON was transferred from malt to wort during the first step of the mashing process (15 min at 45ºC). Also, almost 80% of DON-3-Glc was

transferred to wort during mashing together with FB1 and FB2 in the case of the samples containing maize adjuncts. Despite its lower solubility in water compared to DON and FBs, ZEN and its modified forms were also transferred to wort in high proportion, probably due to the high temperature and the duration of the process.

Boiling did not lead to a complete reduction in any of the toxins, however the most significant impact was achieved after the first 30 min of the process. α-ZEL was the only toxin completely eliminated during this step, but its incidence and levels were also low compared to other contaminants present. From 30 to 60% DON remained in wort after 90 min of boiling but an almost 99% reduction of ZEN could be achieved. β-ZEL was more abundant compared to its stereoisomer and its concentration remained almost unchanged at the end of boiling process.

Fermentation. The fermentation process was proven an important step in mycotoxin mitigation by achieving a significant reduction of 11 to 17% of DON and up to 72% adsorption of ZEN. *S. pastorianus* strains were able to produce a higher amount of the biomass which led to a higher alcohol concentration at the end of the process and a slightly better adsorption ability, however no significant difference with *S. cerevisiae* was reported. The contamination with mycotoxins did not have any impact of yeast alcohol production and fermentation dynamics compared to the uncontaminated control samples.

12.1.2 Cereal-based baby food

The two studied stages of the production process did not prove a significant impact on the levels of *Fusarium* mycotoxins; nonetheless, while studying three cereal matrices, the differences between their compositions and properties were highlighted. There is one more step to be investigated for its possible mitigation effect on the contamination with mycotoxins, which could explain the existing difference between the maximum limits set for mycotoxins in raw materials and final product.

Flour roasting. No significant changes in any of the studied mycotoxins, including T-2 and HT-2 toxins identified only in oat flour, was observed after roasting. 3- and 15-Ac-DON were found to be equally stable as their parental form DON, which increase the concern related to their often co-occurrence in cereal grains.

Enzymatic treatment. The activity of the enzymes was different in the three matrices studied, nonetheless no significant changes in the initial concentration of the mycotoxins contained in barley, wheat or oat were identified. A decreasing trend for ZEN and its sulfate-derivates was observed, but the high proportion between the concentration of the two groups of compounds could not prove these changes statistically significant. Particularly, up to 46% decrease in the level of β-ZEL was observed, β-ZEL-14-S following a very similar trend compared to it. α-ZEL-14-Sulf was not found in any of the samples.

12.2 Future development

- Craft beer industry is characterized by a system which does not always allow an appropriate control of the raw materials used regarding the contamination with mycotoxins. Considering the progressive and fast development of this industry, more research on the applied brewing techniques, especially related to the fermentation process, have to be considered for their potential to mitigate these contaminants.
- Lactic acid bacteria are used in some traditional brewing recipes to improve the quality and the safety of the final product due to their ability to produce antimicrobial substances. They were also proved able to metabolize and modify some of the *Fusarium* mycotoxins, nonetheless little information is available so far regarding their application in brewing with a mycotoxin mitigation aim.
- Cereal-based baby foods are subjected to a strict regulation regarding the maximum limits of the mycotoxins; however, this is not the case for the modified forms. Deeper study of the possibly occurring modified mycotoxins and their fate under several variations of the production process could improve the knowledge and help assess the risk related to mycotoxins for this product.
- Studies investigating not only the occurrence of the mycotoxins in different food products but also the particularity and geographical/cultural differences in their preparation that could have an impact on the risks related to mycotoxin presence in food.

Appendix

Table 1: Evolution of mycotoxins originated from the malt spiked with standard solutions of DON, ZEN and FB1 (mean content, µg ±SD) throughout mashing and wort boiling processes.

Samples	Mycotoxin	Malt before mashing	15 min 45 °C		60 min 65 °C		15 min 75 °C		Boiling			
			Solid	Liquid	Solid	Liquid	Solid	Liquid	T=0	30 min	60 min	90 min
Blank	DON	<LOQ	<LOQ	<LOQ	2.1±0.5c	12±0.6b	<LOQ	435±60a	109±15A	44±3.4B	40B	45B
	DON-3-Glc	31	20±1.3c	5.1±2.7d	8.8±1.2d	29±5b	<LOQ	296±16a	74±4A	44±3B	40B	44B
A1	DON	12±1b	3±1c	36±3a	5±1c	38±1a	4±1c	36±2a	36±2A	14±1B	9±0.3C	7±1C
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	ZEN	<LOQ	<LOQ	16±14a	<LOQ	11±19a	<LOQ	29±0.5a	<LOQ	<LOQ	<LOQ	<LOQ
	FB1	29±2a	29±6a	<LOQ	35±5a	<LOQ	41±10a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
A2	DON	188c	112±5cd	3999±6b	49±3de	690±51a	35±3e	749±52a	749±52A	48±21B	46±19B	50±13B
	DON-3-Glc	30b	19±1b	26±12b	7±0.5b	36±8b	<LOQ	350±36a	350±36A	39±13B	34±1B	32±2B
	ZEN	19d	11±0.5de	37±4c	0.72±0.1de	74±6b	<LOQ	151±15a	151±15A	<LOQ	4±1B	17±2B
	FB1	100c	73±1d	119±6c	22±2e	157±13b	<LOQ	341±19a	341±19A	35±14B	44±8B	49±4B

LOQ=limit of quantification; Connecting letters for Tukey HSD test (levels not connected by the same letter within a row are significantly different); Letters in the same case are analyzed within the same dataset; DON=deoxynivalenol; DON-3-Glc= deoxynivalenol-3-glucoside; ZEN=zearalenone; FB1= fumonisin B_1.

Table 2: Evolution of DON, ZEN and their metabolites originated from the contaminated malt (mean content, µg ±SD) throughout mashing and wort boiling processes.

Samples	Mycotoxin	Malt before mashing	15 min 45 °C		60 min 65 °C		15 min 75 °C		Boiling			
			Solid	Liquid	Solid	Liquid	Solid	Liquid	T=0	30 min	60 min	90 min
B1	DON	19±10ab	0.55±0.3b	25±9a	2.2±1.7b	25±9a	3.2±2.2b	25±11a	25±11A	11±6B	7±3B	5.5±3B
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	0.7±0.5a	<LOQ	0.7±0.5a	<LOQ	0.8±0.5a	<LOQ	0.6±0.8a	0.6±0.8A	<LOQ	<LOQ	0.2±0.1A
	15-AcDON	<LOQ	<LOQ	1±1.3a	<LOQ	2±1.6a	<LOQ	0.8±1.1a	0.8±1.1A	<LOQ	1.5±2.1A	0.7±1A
	ZEN	1053±455a	70±94b	128±78b	128±49b	108±23b	170±92b	137±81b	137±81A	<LOQ	<LOQ	<LOQ
	α -ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β -ZEL	<LOQ	<LOQ	1.5±2.2a	<LOQ	5.4±9a	<LOQ	18±22a	18±22A	31±40A	10±11A	15±18A
B2	DON	26±4b	3.7±0.3c	65±1a	8.7±1.5c	63±3a	7.2±6c	61±5a	61±5A	33±1B	19±0.1C	15±0.6C
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	1.1±0.1b	<LOQ	1.4±1.1ab	0.4±0.2b	2.7±0.5a	0.5±0.3b	2.8±0.1a	2.8±0.1A	<LOQ	0.8±0.05B	0.4±0.4B
	15-AcDON	<LOQ	<LOQ	0.8±1.1a	<LOQ	1.3±1.8a	<LOQ	1.4±2.1a	1.4±2.1A	12±16A	0.7±0.8A	3.7±4.5A
	ZEN	1242±297a	151±63b	313±166b	307±47b	427±23b	271±237b	429±33b	429±33A	<LOQ	<LOQ	3.6±6B
	α-ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β-ZEL	<LOQ	2.1±0.5a	0.7±0.7a	<LOQ	<LOQ	<LOQ	18±30a	18±30A	7.7±12.6A	2.2±3.4A	1.2±1.9A
B3	DON	27±5bc	1.7±1d	44±13ab	4.1±3cd	52±13a	7.2±2.5cd	48±13ab	48±13A	23±7B	14±5B	14±7B
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	1.25±0.2abc	<LOQ	1.5±0.9ab	0.4±0.03bc	2±0.6a	0.4±0.2bc	2.1±0.4a	2.1±0.4A	0.5±0.2b	<LOQ	<LOQ
	15-AcDON	<LOQ	<LOQ	36±31a	<LOQ	1.4±2b	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	ZEN	1300±207a	99±43b	289±144b	238±63b	260±110b	245±80b	289±117b	289±117A	<LOQ	<LOQ	3±5B
	α-ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β -ZEL	<LOQ	<LOQ	21±37a	<LOQ	21±36a	<LOQ	26±27a	26±27A	25±22A	22±30A	8±6A

Appendix

Samples	Mycotoxin	Malt before mashing	15 min 45 ºC		60 min 65 ºC		15 min 75 ºC		Boiling			
			Solid	Liquid	Solid	Liquid	Solid	Liquid	T=0	30 min	60 min	90 min
B4	DON	52±1b	7.3±0.8d	108±9a	18±1cd	101±1a	20±3c	97±4a	97±4A	63±3A	38±1C	31±2C
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	2.3±0.2b	0.2±0.1c	4.8±0.5a	0.9±0.1c	5.3±0.5a	1±0.3c	4.8±0.7a	4.8±0.7A	2.3±0.1B	0.7±0.7C	1.5±0.3BC
	15-AcDON	<LOQ	<LOQ	5.9±2.1a	<LOQ	6.9±1.3a	<LOQ	4.2±3.5a	4.2±3.5A	<LOQ	1±1.4A	0.9±1.3A
	ZEN	1635±185a	338±47c	845±224b	622±58bc	798.3±106.5b	648±77bc	780±45b	780±45A	66±6B	15±13B	12±10B
	α -ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β -ZEL	<LOQ	<LOQ	35±36a	<LOQ	11±8a	<LOQ	24±38a	24±38A	15±7A	19±7A	5±3A
B5	DON	318±0.3d	196±0.4e	604±21c	95±0.4ef	903±54b	58±3f	998±51a	998±51A	114±17B	97±11B	101±3B
	DON-3-Glc	258±0.1a	31±0.5b	488±12c	14±1c	603±7.4b	1±1.2d	729±11a	729±11A	90±11B	88±19B	81±1B
	3-AcDON	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	15-AcDON	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	ZEN	247d	227±5d	493±15c	71±2e	608±6b	29±1e	801±51a	801±51A	77±15B	65±13B	61±1B
	α -ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β -ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

LOQ=limit of quantification; Connecting letters for Tukey HSD test (levels not connected by the same letter within a row are significantly different); Letters appearing as the same case are analyzed within the same dataset; DON=deoxynivalenol; DON-3-G= deoxynivalenol-3-glucoside; 3-AcDON = 3-acetyl-deoxynivalenol; 15-AcDON = 15-acetyl-deoxynivalenol; ZEN=zearalenone; α- ZEL= α-zearalenol; β-ZEL= β-zearalenol.

Appendix

Table 3: Evolution of DON, ZEN and their metabolites originated from the contaminated maize (mean content, µg ±SD) throughout mashing and wort boiling processes.

Samples	Mycotoxin	Malt before mashing	15 min 45 °C		60 min 65 °C		15 min 75 °C		Boiling			
			Solid	Liquid	Solid	Liquid	Solid	Liquid	T=0	30 min	60 min	90 min
M1	DON	3±1a	<LOQ	11±4a	<LOQ	10±0.5a	0.9±0.1a	20±18a	20±18A	5±1.4A	2.8±1.3A	3.2±0.2A
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	0.5±0.2ab	<LOQ	0.5±0.4ab	0.2±0.1b	0.9±0.1a	<LOQ	0.6±0.4ab	0.6±0.4A	<LOQ	<LOQ	0.2±0.1A
	15-AcDON	<LOQ	<LOQ	1.4±1.6a	<LOQ	<LOQ	<LOQ	0.9±1.2a	0.9±1.2A	<LOQ	<LOQ	2.3±3.8A
	ZEN	370±85a	62±17c	137±60bc	171±40bc	230±848abc	187±45bc	237±63ab	237±63A	16.5±28B	<LOQ	4.7±8B
	α-ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β-ZEL	1.5±2.4b	0.8±1.1b	21±15ab	<LOQ	16±15ab	<LOQ	35±17a	35±17A	36±6A	14±6A	12±7A
M2	DON	7.1±2b	1.1±1.3c	18±1a	2.7±2.3c	17±1a	1.2±0.3c	15±0.2a	15±0.2A	5.6±1.5B	4±0.3B	4.2±0.8B
	DON-3-Glc	2.3±1a	1±0.5ab	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	0.9±0.2a	<LOQ	1.3±0.1a	0.3±0.3b	1.3±0.2a	0.2±0.1b	1.3±0.1a	1.3±0.1A	<LOQ	<LOQ	<LOQ
	15-AcDON	1±1.5a	2±3.3a	2.6±0.04a	<LOQ	2.7±0.5a	<LOQ	1.7±1.3a	1.7±1.3A	<LOQ	<LOQ	<LOQ
	ZEN	651±93a	235±78a	226±46a	249±46a	266±28a	527±468a	227±37a	227±37A	<LOQ	<LOQ	<LOQ
	α -ZEL	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	β-ZEL	<LOQ	3.6±1.1b	9.7±7ab	<LOQ	<LOQ	<LOQ	25±15a	25±15A	67±57A	33±6A	7±1.7A
M3	DON	19±0.6b	2.3±0.9c	48±6a	5.2±0.5c	46±4a	6.4±0.7c	44±4a	44±4A	23±5B	15±4B	18±4B
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.5±1.2a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	2±0.1b	0.3±0.2c	4.3±0.5a	0.9±0.2b	4.5±0.6a	0.9±0.2b	4.3±1a	4.3±1A	0.9±0.9B	1±0.7B	1.8±0.1B
	15-AcDON	3.6±0.9b	<LOQ	7.1±1.4a	<LOQ	8±2a	<LOQ	7.4±0.8a	7.4±0.8A	2.4±1.8A	<LOQ	4.3±7.3A
	ZEN	1316±193a	361±222b	687±157b	387±45b	590±120b	439±13b	644±35b	644±35A	<LOQ	10±8B	21±4B
	α -ZEL	<LOQ	<LOQ	3.5±4.3a	<LOQ	2.7±3.3a	<LOQ	1.1±1.5a	1.1±1.5A	<LOQ	<LOQ	<LOQ
	β-ZEL	5.5±1.6a	3.7±1ab	<LOQ	2.5±0.6bcd	<LOQ	3.3±1.1abc	0.9±1.1cd	0.9±1.1A	11±10A	13±19A	9.5±9.8A
M4	DON	34±9b	5±0.7c	88±14a	12±1c	92±5a	13±1c	90.4±4.1a	90.4±4.1A	60±5B	37±2C	34±3C
	DON-3-Glc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3-AcDON	3.8±0.8b	0.7±0.02c	8±1.6a	1.8±0.1bc	10±1a	1.9±0.2bc	9.8±0.4a	9.8±0.4A	5.4±0.9B	4.1±0.6C	3.5±0.4C
	15-AcDON	3.3±0.5a	1.7±1.3a	13±2a	1.9±1.8a	17±2a	2.1±0.3a	16±0.9a	16±0.9A	14±9A	4.9±4A	3.3±2.8A
	ZEN	1763±124a	412±53d	1474±246ab	834±34c	1371±143ab	923±90b	1225±207bc	1225±207A	71±21B	58±14B	50±9B
	α -ZEL	0.7±0.1a	0.5±0.6a	10±15a	0.7±1a	12±21a	<LOQ	5.8±9.7a	5.8±9.7A	<LOQ	<LOQ	<LOQ
	β-ZEL	10±2a	5±0.6a	44±39a	5.3±2.1a	33±32a	5.4±0.5a	18.3±7.9a	18.3±7.9A	19±3A	29±26A	6.5±6A

LOQ=limit of quantification; Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different); Letters appearing as the same case are analyzed within the same dataset; DON=deoxynivalenol; DON-3-Glc= deoxynivalenol-3-glucoside; 3-AcDON = 3-acetyl-deoxynivalenol; 15-AcDON = 15-acetyl-deoxynivalenol; ZEN=zearalenone; α- ZEL= α-zearalenol; β-ZEL= β-zearalenol.

Table 4: Evolution of fumonisins $B_1$and B_2 (mean content, µg ±SD) throughout mashing and wort boiling processes

Samples	Mycotoxin	Malt before mashing	15 min 45 ºC		60 min 65 ºC		15 min 75 ºC		Boiling			
			Solid	Liquid	Solid	Liquid	Solid	Liquid	T=0	30 min	60 min	90 min
M1	FB1	11±0.5a	<LOQ	<LOQ	10±1a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	FB2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	12±0.5a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
M2	FB1	21±7a	10±2a	<LOQ	29±27a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	FB2	16±4a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
M3	FB1	57±14a	21±8a	<LOQ	31±2a	<LOQ	43±19a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	FB2	50±23a	27±16a	<LOQ	44±24a	<LOQ	24±7a	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
M4	FB1	88±26a	30±11b	<LOQ	68±26ab	<LOQ	65±12ab	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	FB2	82±17a	49±20b	24.±8bc	48±3bc	<LOQ	36±7bc	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
M5	FB1	46±2d	21±2d	109±11c	<LOQ	167±10b	<LOQ	207±25a	207±25A	<LOQ	17±11B	26±5B
	FB2	21±3bc	15±3c	34±13b	<LOQ	60±2a	<LOQ	71±9a	701±9A	<LOQ	<LOQ	<LOQ
M6	FB1	77±25c	53±23cd	212±25b	11±5d	280±13a	<LOQ	312±8a	312±8A	27±13B	34±10B	37±7B
	FB2	50±21b	24±10bc	42±10b	<LOQ	83±10a	<LOQ	93±4a	<LOQ	<LOQ	<LOQ	<LOQ
M7	FB1	190±2c	121±7d	428±10b	27±2e	459±24ab	8.5±5e	491±35a	491±35A	58±6B	60±14B	84±19B
	FB2	83±4c	49±4d	103±21c	16±4e	136±17b	<LOQ	168±11a	168±11A	<LOQ	<LOQ	32±19B

LOQ=limit of quantification; Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different); Letters appearing as the same case are analyzed within the same dataset; FB1= fumonisin B_1; FB2= fumonisin B_2.