UNIVERSITEIT GENT

FACULTY OF PHARMACEUTICAL SCIENCES

CHEMICAL RISKS RELATED TO FOOD AND FEED CONTAINING MASKED *Fusarium* MYCOTOXINS

Apr. Marthe De Boevre

2013

Promotor

Prof. dr. Sarah De Saeger

Co-promotor

Emeritus Prof. dr. Carlos Van Peteghem

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Pharmaceutical Sciences

Proefschrift voorgelegd tot het bekomen van de graad van Doctor in de Farmaceutische Wetenschappen

Promotor and co-promotor

Prof. dr. Sarah De Saeger

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Emeritus Prof. dr. Carlos Van Peteghem

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Members of the examination committee

Dr. ir. Kris Audenaert

Faculty of Applied Bioscience Engineering, University College Ghent, Ghent, Belgium

Dr. ir. Alfons Callebaut

Veterinary and Agrochemical Research Centre (CODA-CERVA), Tervuren, Belgium

Prof. dr. Siska Croubels

Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

Dr. Chem. Chiara Dall'Asta

Department of Food Science, University of Parma, Parma, Italy

Prof. dr. Dieter Deforce (chairman)

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Prof. dr. Mia Eeckhout

Faculty of Applied Bioscience Engineering, University College Ghent, Ghent, Belgium

Prof. dr. Geert Haesaert

Faculty of Applied Bioscience Engineering, University College Ghent, Ghent, Belgium

Dr. Christof Van Poucke

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Dean

Prof. dr. Stefaan De Smedt

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Rector

Prof. dr. Paul Van Cauwenberge

Ghent University

The author and promoters give the permission to make this thesis available for consultation and allow the copying of any part of the manuscript for personal use. Any other use is subjected to the restrictions of copyright laws, in particular in relation to the obligation of explicit mention of the source when any results are taken from the thesis.

Ghent, 2013

Author, Promoters,

Ph. Marthe De Boevre Prof. dr. Sarah De Saeger

Emeritus Prof. dr. Carlos Van Peteghem

Please refer to this work as follows:

M. De Boevre (2013). Chemical risks related to food and feed containing masked *Fusarium* mycotoxins. Dissertation, Ghent University.

ISBN:

The research was funded by the Special Research Fund (BOF) of Ghent University (BOF 05V00709).

This research is linked to the association research group ‘mycotoxins and toxigenic moulds, MYTOX’. More information can be found on www.mytox.be.

There is one thing even more vital to science than intelligent methods; and that is, the sincere desire to find out the truth, whatever it may be.

- Charles Pierce -

TABLE OF CONTENTS

TABLE OF CONTENTS III
INTRODUCTION & OBJECTIVES IX

CHAPTER 1

GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

1.1 MYCOTOXINS: A HISTORICAL PREVIEW 3
1.2 MYCOTOXINS OF INTEREST: FUSARIOTOXINS 4
1.2.1 *Fusarium* mycotoxins: trichothecenes 4
1.2.1.1 Chemistry 4
1.2.1.2 Toxicity 5
1.2.2 *Fusarium* mycotoxins: myco-estrogens 7
1.2.2.1 Chemistry 7
1.2.2.2 Toxicity 8
1.2.3 Fungal diseases related to *Fusarium* 9
1.2.3.1 *Fusarium* head blight 9
1.2.3.2 *Fusarium* ear rot 10
1.2.3.3 *Fusarium* crown rot 11
1.2.4 Role of mycotoxins in the infection process 12
1.3 MASKED MYCOTOXINS 13
1.3.1 Introduction 13
1.3.2 Formation of masked mycotoxins 15
1.3.2.1 Introduction to the conjugation of xenobiotics 15
1.3.2.2 Conjugation of mycotoxins 17
1.3.2.3 Detoxification process of mycotoxins *in planta* 20
1.3.3 Occurrence of masked mycotoxins in food and feed 23
1.3.3.1 Masked trichothecenes 23
1.3.3.2 Masked myco-estrogens 26
1.3.3.3 Other masked mycotoxins 26
1.3.3.4 The fate of processing on mycotoxin conjugates 28
1.3.4 Analytical tools for the detection of masked mycotoxins 30
1.3.4.1 Sampling 31
1.3.4.2 Extraction and clean-up procedures 34
1.3.4.3 Chromatographic methods 37
1.3.4.4 MS fragmentation of trichothecenes and myco-estrogens 43
1.3.4.5 Strategies to detect unknown conjugated mycotoxins 45
1.3.4.6 Immunochemical methods 48
1.4 LEGISLATION AND TOXICOLOGICAL CONSIDERATIONS 52
1.5 RISK ANALYSIS 57
1.5.1 Introduction 57
1.5.2 Risk management 58
1.5.2.1 Preliminary risk management activities 58
1.5.2.2 Evaluation of risk management options 58
1.5.2.3 Implementation of the risk management decision 58
1.5.2.4 Monitoring and review 59

1.5.3 Risk Assessment **60**
1.5.3.1 Hazard identification 60
1.5.3.2 Hazard characterization 61
1.5.3.3 Exposure assessment 62
1.5.3.4 Risk characterization 63
1.5.4 Risk communication **64**
1.5.5 Risk management and risk assessment of mycotoxin contamination **66**
1.5.5.1 Planting 66
1.5.5.2 Pre-harvest measures 67
1.5.5.3 (Post)-harvest measures 67
1.5.5.4 Storage 67
1.5.5.5 Control and elimination strategies for mycotoxins 67
1.5.5.6 Application of an HACCP system in mycotoxin control 69

CHAPTER 2
IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

SUMMARY **73**
2.1 INTRODUCTION **74**
2.2 MATERIALS AND METHODS **75**
2.2.1 Reagents and chemicals 75
2.2.2 LC-MS/MS conditions 76
2.2.3 Sample preparation 77
2.2.4 Method validation 79
2.3 RESULTS AND DISCUSSION **81**
2.3.1 Synthesis of masked forms of zearalenone 81
2.3.2 Optimization of the HPLC method 81
2.3.2.1 Optimization of the MS conditions 81
2.3.2.2 Optimization of the LC conditions 82
2.3.2.3 Optimization of the sample preparation and clean-up 87
2.3.2.4 Method validation 88
2.4 CONCLUSION **93**

CHAPTER 3
NATURAL OCCURRENCE OF MYCOTOXINS AND THEIR MASKED FORMS IN FOOD, FEED AND MAIZE: THE ELABORATION OF 3 SURVEYS

SUMMARY **97**
3.1 INTRODUCTION **98**
3.2 MATERIALS AND METHODS **99**
3.2.1 Reagents and chemicals **99**
3.2.2 Collection of samples for (masked) mycotoxin analysis in food and feed **99**
3.2.3 Collection of samples for (masked) mycotoxin analysis in maize: experimental field trial **99**
3.2.4 Determination of (masked) mycotoxin concentrations in food, feed and maize **100**

3.2.5 Statistical analysis **100**
3.3 RESULTS AND DISCUSSION **101**
3.3.1 Part 1: occurrence data for (masked) mycotoxins in foods from the Belgian market **101**
3.3.1.1 Fibre-enriched bread 101
3.3.1.2 Bran-enriched bread 102
3.3.1.3 Breakfast cereals 106
3.3.1.4 Popcorn 107
3.3.1.5 Oatmeal 108
3.3.1.6 Comparison of mycotoxin occurrence in the different food products 109
3.3.1.7 The fate of processing: an additional experiment on baking bread and DON-3G 114
3.3.2 Part 2: occurrence data for (masked) mycotoxins in feed from the Belgian market **116**
3.3.2.1 Deoxynivalenol and derivatives 116
3.3.2.2 T-2 and HT-2 toxin 117
3.3.2.3 Zearalenone and derivatives 120
3.3.3 Part 3: occurrence data for (masked) mycotoxins in maize under natural infection conditions **122**
3.3.3.1 Incidence of (masked) mycotoxins in the maize varieties 122
3.3.3.2 Influence of the maize variety on mycotoxin accumulation 125
3.4 CONCLUSION **130**

CHAPTER 4

DIETARY EXPOSURE TO MYCOTOXINS AND THEIR MASKED FORMS THROUGH CEREAL-BASED FOODS AND FEED IN BELGIUM

SUMMARY **135**
4.1 INTRODUCTION **136**
4.2 MATERIALS AND METHODS **138**
4.2.1 Reagents and chemicals **138**
4.2.2 Collection of samples for (masked) mycotoxin analysis in food and feed **138**
4.2.3 Determination of (masked) mycotoxin concentrations in food and feed **138**
4.2.4 Consumption data **138**
4.2.5 Deterministic exposure assessment **139**
4.2.6 Probabilistic exposure assessment **139**
4.3 RESULTS AND DISCUSSION **141**
4.3.1 Dietary exposure assessment in food **141**
4.3.1.1 Determination of (masked) mycotoxins in foodstuffs 141
4.3.1.2 Consumption data 141
4.3.1.3 Deterministic exposure assessment 141
4.3.1.4 Probabilistic exposure assessment 146
4.3.1.5 Uncertainty related to exposure assessments 153
4.3.2 Dietary exposure assessment in feed **155**
4.3.2.1 Determination of (masked) mycotoxins in feed 155
4.3.2.2 Consumption data 155
4.3.2.3 Deterministic exposure assessment 156
4.4 CONCLUSION **159**

CHAPTER 5

MYCOTOXIN GLUCOSYLATION IN COMMERCIAL WHEAT VARIETIES: IMPACT ON RESISTANCE TO *FUSARIUM GRAMINEARUM* UNDER LABORATORY AND FIELD CONDITIONS

SUMMARY 163
5.1 INTRODUCTION 164
5.2 MATERIALS AND METHODS 166
5.2.1 Plant genotypes, cultivars and fungal isolates 166
5.2.2 *In vivo* ear infection of commercial wheat varieties under laboratory conditions 167
5.2.3 Field experiments to assess FHB resistance and DON-3G presence in commercial wheat varieties 167
5.2.4 Determination of the FHB population composition in the field 168
5.2.5 LC-MS/MS methodology for the quantification of DON, 3-ADON, 15-ADON and DON-3G 168
5.2.6 Statistical analysis 168
5.3 RESULTS 169
5.3.1 Presence and localization of DON and DON-3G after artificial *in vivo* infection of wheat ears 169
5.3.2 Field experiments with commercial wheat varieties in 2010-2011 growing season 172
5.4 DISCUSSION AND CONCLUSION 174

CHAPTER 6

DETACHED LEAF ASSAY FOR MASKED MYCOTOXIN BIOSYNTHESIS AND SUBSEQUENT ANALYSIS OF (UNKNOWN) CONJUGATES BY (UN)TARGETED LC-MS/MS

SUMMARY 181
6.1 INTRODUCTION 182
6.2 MATERIALS AND METHODS 186
6.2.1 Reagents and chemicals 186
6.2.2 Detached leaf assay 186
6.2.3 Sample preparation 188
6.2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions 188
6.2.5 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) conditions 189
6.3 RESULTS AND DISCUSSION 190
6.3.1 Biosynthesis of DON-3G 190
6.3.2 Biosynthesis of masked forms of ZEN 191
6.3.3 Biosynthesis of masked forms of T-2 193
6.4 CONCLUSION 197

CHAPTER 7

GENERAL CONCLUSIONS & FUTURE PERSPECTIVES

GENERAL CONCLUSIONS 201
FUTURE PERSPECTIVES 205

SUMMARY 211
SAMENVATTING 217
BIBLIOGRAPHY 225
LIST OF ABBREVIATIONS 249
LIST OF TABLES 255
LIST OF FIGURES 258
LIST OF EQUATIONS 262
CURRICULUM VITAE 265
ACKNOWLEDGMENTS 273

INTRODUCTION & OBJECTIVES

During the past decades food and feed safety has become an increased concern among consumers and producers resulting from the risen global availability and the intensified public awareness on health and quality. Accessible safe food and feed is a prerequisite for public health and international trade.

Several highly publicized global incidents relating to chemical hazards in food and feed have attracted much media attention. Trace levels of chemical contaminants can originate from natural sources such as marine biotoxins and mycotoxins. Mycotoxins are secondary metabolites produced by fungi on agricultural commodities in the field, and during storage under a wide range of climatic conditions. The Food and Agricultural Organization (FAO) estimated that 25% of the world's food crops are affected by mycotoxins resulting in an enormous impact on global economy.

More than 400 mycotoxins have been identified with widely different chemical structures, ensuring differing modes of action and affecting variable targets. Potential health risks to animals and humans, resulting from food and feed borne mycotoxin intoxications, have been recognized by national and international institutions such as the European Commission (EC), Food and Drug Administration (FDA), the World Health Organization (WHO) and FAO. These instances have addressed the problem by adopting regulatory limits for major mycotoxin classes as for selected individual mycotoxins.

In the mid 80ies conjugated or masked mycotoxins first came to attention, when animals, fed with seemingly low mycotoxin contaminated feed, showed high severity of mycotoxicosis. The unexpected high toxicity was attributed to the presence of conjugated forms of mycotoxins, possibly generated by the plant's metabolism. These derivatives were referred to as masked mycotoxins as they remained undetected by the, at that time, commonly-used analytical methods, causing a general underestimation of the total mycotoxin load. Masked mycotoxins can be hydrolyzed to their parent toxins in the digestive tract of the animals or can exert toxic effects comparable to those imputable to parent mycotoxins. Since little is known about the occurrence, bioavailability and metabolism of these masked compounds, the execution of a comprehensive study was imperative.

The goal of this PhD-dissertation is to unravel the true problematic nature of masked mycotoxins, and to contribute to the evaluation of the applicability of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high resolution mass spectrometry (HRMS). Several objectives have been set to reach this goal and are detailed here.

Current safety concerns on mycotoxin control demand the availability of a fast, reliable, low-cost control system to measure the content of mycotoxins present in cereal-based foods and feed. The **first objective** was to develop an extraction, purification and quantification method based on LC-MS/MS for the simultaneous determination of parent and masked *Fusarium* mycotoxins in maize, wheat, oats and their assimilated products (*Chapter 2*). The developed analytical methodology has been the foundation for the accomplishment of the second, third and fourth objective.

Literature reviews have clearly shown that mycotoxins are common contaminants of cereals and cereal-based food and feed commodities. The **second objective** of this research was to provide quantitative data on the occurrence of masked *Fusarium* mycotoxins and the circumstances in which they are formed. The main research focus was the glucosylated conjugates of deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin (T-2) in cereals and cereal-based food and feed. Also, the role of the cereal variety and genotype on the occurrence of masked mycotoxins was determined (*Chapter 3*).

The **third objective** of this PhD-dissertation was to gain insight into the risks related to the dietary intake of parent and masked mycotoxins. The extent in which animals and humans are exposed to masked mycotoxins was assessed, and a preliminary risk assessment was carried out (*Chapter 4*).

The **fourth objective** was to unravel the glucosylation capacity among the Belgian commercial wheat cultivars and to determine the importance of DON glucosylation in the complex background of natural field circumstances (*Chapter 5*).

Masked mycotoxins can be identified due to their molecular mass and their collision-induced dissociation (CID) fragmentation behaviour. Therefore, a **fifth objective** was to identify unknown mycotoxin conjugates with HRMS, and to investigate the occurrence of these unknown masked mycotoxins in naturally contaminated cereals (*Chapter 6*).

The complete research was carried out to achieve the objectives and has been presented throughout 6 chapters. Finally, general conclusions and future perspectives regarding masked mycotoxins are presented in *Chapter 7*. A schematic outline of the research carried out in this PhD-dissertation is pointed out in **Figure I**.

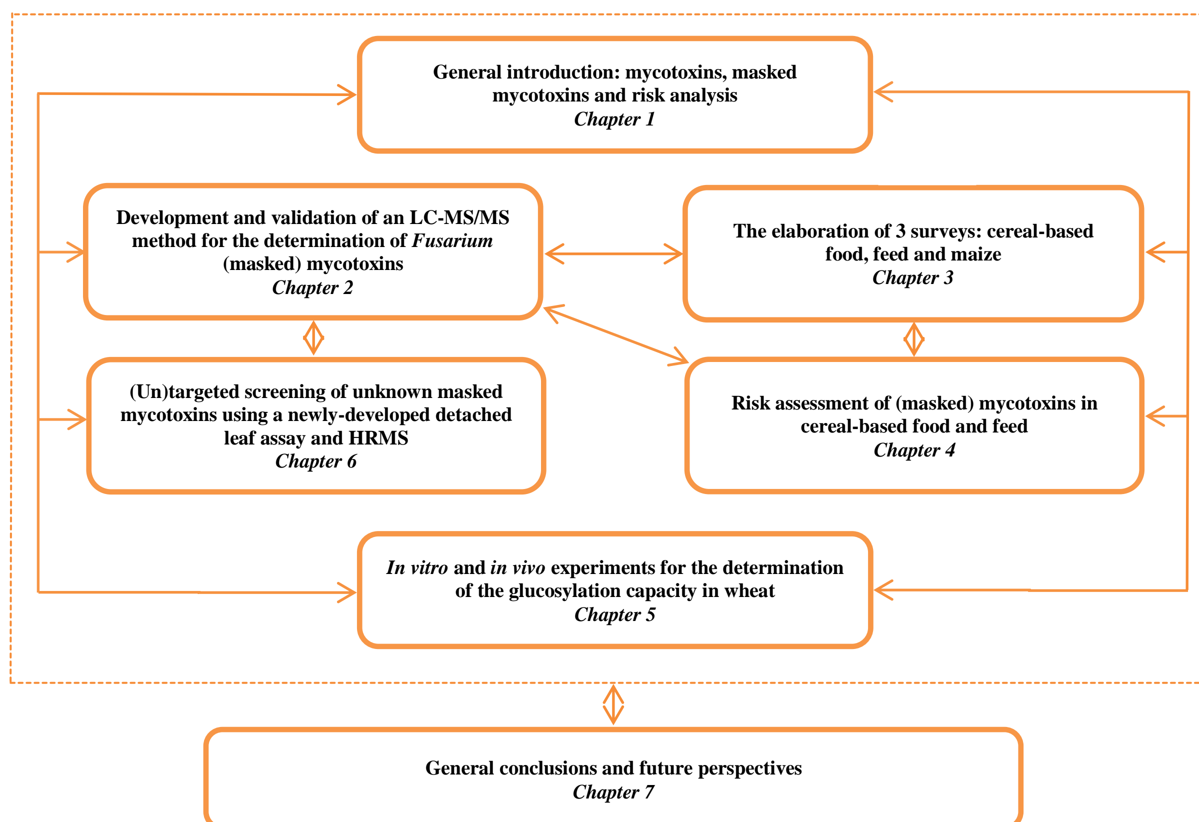


Figure I Schematic outline of the research carried out in this PhD-dissertation

CHAPTER 1

GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

CHAPTER 1 GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

1.1 MYCOTOXINS: A HISTORICAL PREVIEW

Mycotoxins are toxic, low-molecular-weight, secondary metabolites produced by molds. They function as insecticides, play a role in fighting against plant defense to the fungus, and they assist the fungus in some way to compete for their ecological niche in nature (Richard, 2012). Some mycotoxins are harmful to other micro-organisms such as other fungi or even bacteria, like the antibiotic penicillin, produced by *Penicillium chrysogenum* (Fleming, 1929), others are potent carcinogens like aflatoxins. The most important fungal genera producing mycotoxins that are found in food products are *Aspergillus, Fusarium, Alternaria, Claviceps* and *Penicillium*.

The term mycotoxins was adopted in 1962 in the aftermath of an unusual veterinary crisis in the United Kingdom during which approximately 100,000 turkeys died. The mysterious Turkey X-disease was linked to a peanut meal contaminated with secondary metabolites of *Aspergillus* (*A.*) *flavus* (*i.e.* aflatoxins) (Bennett and Klich, 2003). In history the involvement of mycotoxins is conjectural as contamination events were not well documented. However, the major outcomes of these events were caused by the incidence of *mildew*.

In the Middle Ages, the deterioration of rye caused *St. Anthony's Fire* or ergotism (ergot alkaloids), which was frequently correlated with bewitchment, as strange sensations of fire shooting were observed (Richard, 2012).

In the 30ies Henrici stated the following: "It is not clearly understood how the pathogenic fungi injure tissues. Although fibrosis and giant cell reactions about some lesions bear a resemblance to a foreign body reaction, the extensive necrosis and suppuration which occur in the centre of most lesions cannot be readily explained in this way. Moreover, the experimental lesions produced with freshly isolated and highly virulent strains of some species, as *A. fumigatus* and *Candida albicans* are so acute as to suggest that these diseases may be caused by the same mechanisms as those found in bacterial infections" (Henrici, 1930).

Three major episodes of lethal diseases made clear that mycotoxins were harmful both to humans and animals: Alimentary Toxic Aleukia (ATA)-outbreak in Russia caused by *Fusarium (F.) sporotrichioides*, *F. poae* and *Cladosporium epiphyllum*; stachybotryotoxicosis or moldy corn toxicosis in humans and horses by *Stachybotrys*, and facial eczema in sheep by the fungus *Sporidesmium bakeri* (sporidesmin) (Thorton, 1959; Forgacs, 1962).

Mycotoxins are therefore stated to be a major public health concern because they are the most hazardous of all food contaminants in terms of chronic toxicity, and main clinical outcomes include carcinogenicity, genotoxicity, nephrotoxicity, hepatotoxicity, estrogenicity, immunosuppression and dermal infestations (Sulyok *et al.*, 2006).

1.2 MYCOTOXINS OF INTEREST: FUSARIOTOXINS

In the scope of this PhD-dissertation 2 major groups of mycotoxins were investigated in terms of economic impact and scientific interest. The fusariotoxins trichothecenes, fumonisins and myco-estrogens are undoubtedly key substances in the mycotoxin incidence in cereals and cereal-based commodities. Fusariotoxins are secondary metabolites produced by toxigenic micromycetes of the genus *Fusarium* (Conkova *et al.*, 2003). *Fusarium* species might endanger human health through the action of their toxic metabolites, mycotoxins (Bauer *et al.*, 1980). Besides fumonisins, most dominant mycotoxin production includes trichothecenes and myco-estrogens. A general overview concerning chemistry and toxicity of these two research subjects follows below.

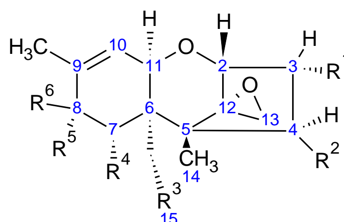
1.2.1 *Fusarium* mycotoxins: trichothecenes

1.2.1.1 Chemistry

To date, more than 400 mycotoxins have been reported (Grovey, 2007). Trichothecenes are a family of naturally occurring tetracyclic sesquiterpenoids and in terms of their functional groups, they are divided into four groups (A, B, C and D). Most compounds contain an epoxide ring and a double bound at C_{12}-C_{13} and C_9-C_{10}, respectively (12,13-epoxytrichothec-9-ene) (Ueno, 1985). It is the epoxide ring, when present, that exerts the toxic activity of the trichothecenes.

Type A trichothecenes, mainly produced by *F. poae, F. langsethiae* and *F. sporotrichioides*, include the highly toxic T-2 toxin (T-2), its deactylated form HT-2 toxin (HT-2), diacetoxyscirpenol (DAS) and neosolaniol (NEO) (Thrane *et al.*, 2004). Type B forms are distinguished from type A by the presence of a keto-group at C_8 and include the important trichothecenes nivalenol (NIV), deoxynivalenol (DON), the acetylated forms 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), and fusarenon-X (FUS-X) (**Figure 1.1**). They are produced by *F. graminearum*, *F. equiseti* and *F. culmorum* (Richard *et al.*, 2007). Type C-trichothecenes, like crotocin, contain an additional C_7-C_8 epoxide, however, they are not produced by *Fusarium* species. Type D are also non-*Fusarium* mycotoxins and contain a macrocyclic ring linking at C_4 and C_{15} with di- or triesters. These

airborne *Stachybotrys* mycotoxins include satratoxins, roridins and verrucarins and are prevalent in indoor environments (Brasel *et al.*, 2005). Type A and B are widely distributed in cereals as natural pollutants, whereas the macrocyclic trichothecenes rarely occur in food and feed.



Type A trichothecenes						
	R^1	**R^2**	**R^3**	**R^4**	**R^5**	**R^6**
T-2 toxin (T-2)	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	H
HT-2 toxin (HT-2)	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	H
Diacetoxyscirpenol (DAS)	OH	$OCOCH_3$	$OCOCH_3$	H	H	H
Neosolaniol (NEO)	OH	$OCOCH_3$	$OCOCH_3$	H	OH	H
Type B trichothecenes						
	R^1	**R^2**	**R^3**	**R^4**	**R^5 and R^6**	
Nivalenol (NIV)	OH	OH	OH	OH	O	
Deoxynivalenol (DON)	OH	H	OH	OH	O	
3-acetyldeoxynivalenol (3-ADON)	$OCOCH_3$	H	OH	OH	O	
15-acetyldeoxynivalenol (15-ADON)	OH	H	$OCOCH_3$	OH	O	
Fusarenon-X (FUS-X)	OH	$OCOCH_3$	OH	OH	O	

Figure 1.1 Basic structure of trichothecenes and their respective structures

1.2.1.2 Toxicity

The clinical outcome of trichothecenes is known as mycotoxicosis, which shows a variety of clinical symptoms where synergistic or additive effects between several mycotoxins can enhance the adverse health effects for the exposed organism (Grenier and Oswald, 2011). Acute exposure induces radiomimetic effects and gastro-intestinal manifestations such as diarrhea, leucocytosis, vomiting and melena with extremely high doses causing a shock-like syndrome ultimately resulting in death (Pestka *et al.*, 2005). Chronic exposure reported effects such as anorexia, reduced weight gain, retarded growth, nausea and degeneration of the immune, neural and reproductive systems. Emesis, reduced weight gain and other gastro-intestinal disorders are the most sensitive functional manifestations of the type B trichothecenes, while immunotoxicity, cytotoxicity and neurotoxicity are caused by the type A trichothecenes (van der Fels-Klerx and Stratakou, 2010).

According to the International Agency for Research on Cancer (IARC) there is an inadequate evidence in humans for the carcinogenicity of toxins derived from *F. graminearum*. Therefore, mycotoxins (T-2, DON, FUS-X and NIV) derived from *F. graminearum*, *F. culmorum* and *F.*

crookwellense are not classifiable as to their carcinogenicity to humans (*Group 3*) (IARC, 2002).

Trichothecenes bind readily to the 60S-ribosomal peptidyltransferase site of eukaryotic ribosomes and are potent inhibitors of the translation process, inducers of apoptosis in lymphatic and hematopoietic tissues, and cause damage to cellular membranes (Ostry, 1998; Pestka & Smolinski, 2005). Macrophages, T-cells, and B-cells of the immune system are central targets of the trichothecenes. The toxic effects are known to involve ribotoxic stress responses that activate protein kinases/cJun N-terminal kinases (SAPK/JNK) (*i.e.* mitogen-activated protein kinases (MAPKs)) of a signaling cascade that regulates cell survival in response to stress and induce rapid apoptosis, rather than a simple translational arrest (Jimenez and Vazquez, 1975; Shifrin and Anderson, 1999; Moon and Pestka, 2003; Pestka, 2004).

Immunostimulatory or immunosuppressive effects depend on dose, exposure frequency and timing of functional immune assay. Whereas high concentrations of trichothecenes completely shut down translation and induce apoptosis by the activation of caspases (cysteine-aspartylprotease) with concomitant immune suppression; subinhibitory concentrations of these mycotoxins up-regulate cytokines, chemokines and inflammatory genes production. This is accomplished by elevating the transcription factor activity and increasing mRNA stability with concurrent immune stimulation (**Figure 1.2**).

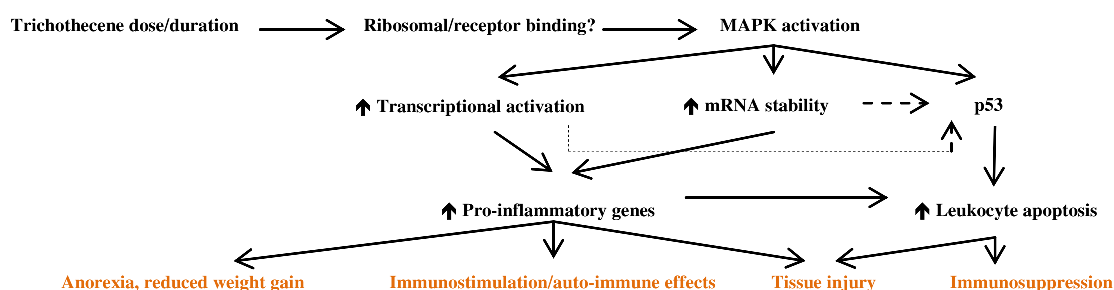


Figure 1.2 Cascade of trichothecenes in animals: trichothecenes enter mammalian cells via diffusion and bind to the ribosomes, which activates mitogen-activate protein kinases (MAPKs) by phosphorylation. The activation leads to transcriptional activation, mRNA stability and activation of caspases with subsequent chronic and immunotoxic effects (Pestka *et al.*, 2004).

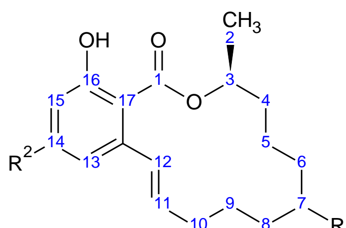
Trichothecenes type A and B inhibit the initiation of protein synthesis by the binding suppression of an amino acid in the amino acid sequence, synthesis of RNA and interference with metabolism of membrane phospholipids (Schiefer and Beasley, 1989).

Absorption of type A trichothecenes (*e.g.* T-2) occurs in the small intestine. In swine, T-2 was detected in the blood in less than 30 minutes after oral administration. Type B (*e.g.* DON) has an exalted and expeditious absorption rate (15 to 30 min) in the stomach and small intestine (Eriksen and Petterson, 2004). The main metabolite is deepoxy-deoxynivalenol-1 (DOM-1), however, only 5% of total DON is metabolized (Dänicke *et al.*, 2004). To emphasize, the intestinal epithelium is the first target tissue of food contaminants, consequently clinical gastro-intestinal outcomes are in most cases observed (Pinton *et al.*, 2012). Trichothecenes showed to be toxic to all species. However sensitivity varied considerably between toxins and species with swine being the most sensitive farm animal.

1.2.2 *Fusarium* mycotoxins: myco-estrogens

1.2.2.1 Chemistry

Zearalenone (ZEN) is the principal representative of the group of non steroidal myco-estrogens. The structure consists of a phenolic resorcyclic acid lactone, chemically described as 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-2-2-benzoxacyclotetradecin,1,7-dione (**Figure 1.3**). Main production by *F. graminearum*, *F. crookwellense*, *F. sporotrichioides* and *F. culmorum* is described, consequently co-occurrence with DON and other trichothecenes is contingent (Pittet, 1998).



Myco-estrogens		
	R1	**R2**
Zearalenone (ZEN)	O	OH
α-zearalenol (α-ZEL)	OH (α)	OH
β-zearalenol (β-ZEL)	OH (β)	OH

Figure 1.3 Basic structure of zearalenone, α-zearalenol and β-zearalenol

The production, mainly in corn, as well as in other crops such as wheat, barley and oats, depends on environmental conditions and is favoured by high humidity and low temperature resulting in a wide geographical spread (Peraica *et al.*, 1999; Richard *et al.*, 2007; Yazar and Omurtag, 2008), but also agricultural measures such as soil tillage and fungicide use can influence the presence of fusariotoxins (Audenaert *et al.*, 2010; Landschoot *et al.*, 2012). Most abundant derivatives of ZEN are α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (**Figure 1.3**).

1.2.2.2 Toxicity

ZEN and its derivatives constitute an important class of endocrine disrupters, which causes strong estrogenic outcomes, alters consequently the reproductive tract and is associated with hyperestrogenism although haematotoxic, cytotoxic and genotoxic properties were also described (Ostry, 1998; Minervini *et al.*, 2005; Turcotte *et al.*, 2005). Hyperestrogenism involves vulval edema, enlargement of uterus, testicular and ovarian atrophy, stillbirths in females and abortion. In humans, the occurrence of ZEN in plasma was associated with precocious puberty, endometrial adenocarcinomas and hyperplasia (Saenz de Rodriguez *et al.*, 1985; Tomaszewski *et al.*, 1998).

There is limited evidence in experimental animals for the carcinogenicity of ZEN. Therefore, ZEN is not classifiable as to its carcinogenicity to humans (*Group 3*) (IARC, 2002).

ZEN is rapidly absorbed after oral administration and metabolized in the intestinal cells to α-ZEL and β-ZEL (ratio 3/1). Recently, Ayed *et al.* (2011) argued that ZEN and α-ZEL exhibited the same range of cytotoxicity and genotoxicity, and both were more cyto- and genotoxic than β-ZEL (Ayed *et al.*, 2011).

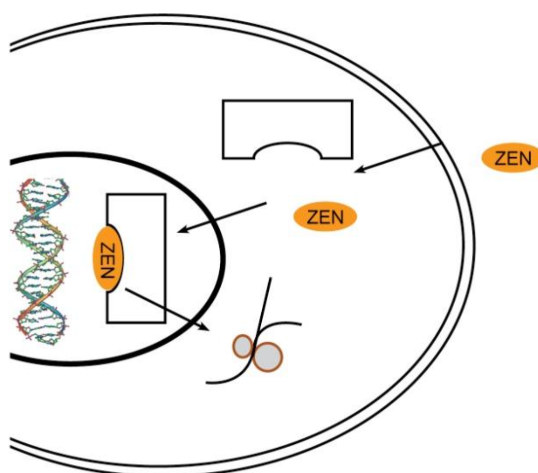


Figure 1.4 Cascade of zearalenone in animals: zearalenone interacts with the cytosolic estrogen receptor. The mycotoxin passively crosses the cell membrane and binds to the receptor. The formed complex is transferred to the nucleus where it binds to specific nuclear receptors and generates estrogenic responses via gene activation, resulting in the production of mRNA that codes for proteins, normally expressed by the receptor-estrogen complex-binding (Cousin *et al.*, 2005).

The structure of these xeno-estrogens resembles 17β-estradiol, exerting its toxic action by competitively binding to estrogen receptors (α and β) (Creppy, 2002). The mycotoxin passively crosses the cell membrane and binds to the estrogen-receptor. The formed ligand-receptor-complex is transferred to the nucleus where it binds to specific nuclear receptors, and generates estrogenic responses via gene activation. Production of mRNA that codes for

proteins is accomplished, normally expressed by the receptor-estrogen complex-binding (Cousin *et al.*, 2005) (**Figure 1.4**). The binding varied in binding affinity in descending order from α-ZEL to β-ZEL and ZEN, still 17β-estradiol has a 100 to 1000-fold higher affinity (Kelner *et al.*, 1982; Minervini *et al.*, 2005; Turcotte *et al.*, 2005). Likewise, the central nervous system consists amongst others of α- and β-estrogen receptors and effects on this level are reported as ZEN is able to pass the blood-brain-barrier, still the ovaria and uterus are more sensitive (Kelner *et al.*, 1982).

1.2.3 Fungal diseases related to *Fusarium*

The ascomycetous fungus *F. graminearum* (*Gibberella zeae*) is the most prominent causal agent of *Fusarium* head blight (FHB) on wheat, barley and other small grain cereals, and of ear rot on maize in the United States, Canada, and Europe (Desjardins *et al.*, 1996; Goswami and Kistler, 2004).

1.2.3.1 *Fusarium* head blight

FHB is a fungal disease, caused by a complex of 17 species, and is of economical importance for wheat and other small grains (Leonard and Bushnell, 2003). Main causal agents associated with FHB symptoms in Europe are *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium* (*M.*) *nivale*. Mycotoxin production is described for all these species, except for *M. nivale*. A large geographic dispersion is described as species react differently to temperature and exhibit different moisture requirements (Champeil *et al*., 2004). FHB intensity and mycotoxin contamination vary year per year and are influenced by environmental growing conditions, local agronomic systems as well as interaction between both factors (Landschoot, 2012).

Yield and quality losses of the host plant are described and include flower abortion, decrease in weight, highly damaged grains, negative affection of grain processing qualities and most importantly production of mycotoxins (Champeil *et al.*, 2004; Landschoot, 2012).

The life cycle of *Fusarium* in wheat is represented in **Figure 1.5**. In detail, *Fusarium* species reside and multiply on infected crop residues of small grains, maize and other hosts. During moderate periods sporulation occurs whereas two types of spores are formed: the asexual macronidia or micronidia, and the sexual ascospores. Asexual conidia are formed by *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *M. nivale*, while sexual spores are composed by *Gibberella* (*G.*) *avenacea*, *G. zeae* and *Monographella nivalis*. Conidia are sequestered by rain drops and are locally dispersed by splashes, while ascospores are forcibly

discharged into the air over long distances. Bluish-black perithecia on the surface of the crop residues are the source of these sexual spores. Infection occurs when spores land on susceptible wheat heads. The fungal spread is effected through vascular bundles in rachis and rachilla, resulting in the dysfunctionality of xylem and phloem and subsequently into death of the spikelet. Related symptoms of FHB include brown to black discoloration at the base of the floret, progressively causing a light tan or bleached spikelets.

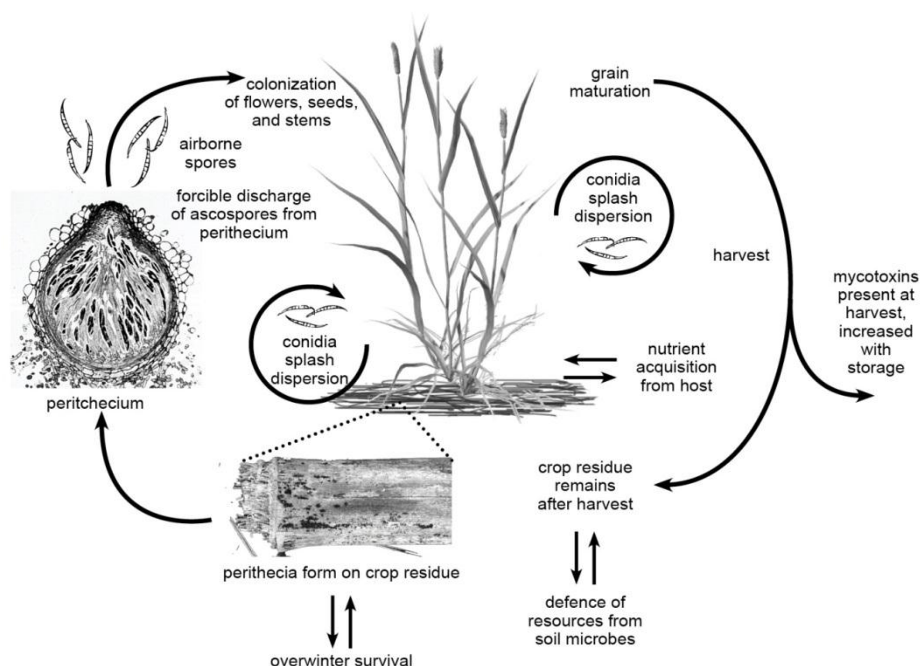


Figure 1.5 The life cycle of *Fusarium graminearum* (sexual phase, *Gibberella zeae*), causal agent of *Fusarium* head blight on wheat

1.2.3.2 *Fusarium* ear rot

Ear rot is a major disease in all maize-growing areas all over the world. Diseases resulting from *Fusarium* are seed rot, root rot, stalk rot and ear rot. Maize is an important staple crop cultivated worldwide (Schollenberger *et al.*, 2012). In Belgium, 9.2 million tons of maize plants intended for livestock feed are annually harvested from 247,000 hectares of farm land. However, only limited information is available concerning the *Fusarium spp.* ecology and mycotoxin pattern in Belgian maize fields. Insight into the relative importance of the various mycotoxins and within-field distribution for different maize varieties will help to develop prevention strategies for ear rot, and related mycotoxin contaminations (Desjardins *et al.*, 1993).

Crop quality in maize is often reduced by ear rot caused by a complex of *Fusarium* species. Two distinct forms of maize ear rot can be distinguished: maize red ear rot (*Gibberella* ear rot) and maize pink ear rot (*Fusarium* ear rot) (Scauflaire *et al.*, 2011). Maize red ear rot is mainly

caused by *F. graminearum*, *F. subglutinans* and *F. avenaceum*, whereas pink ear rot is caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (Desjardins *et al.*, 1996; Goswami and Kistler, 2004). The outcome of infection varies from minor damage to total destruction of the host plant. Hence, maize ear rot leads to dramatic yield losses and accumulation of mycotoxins. The occurrence of DON, its acetylated forms and ZEN are found by maize red ear rot (*F. graminearum* and *F. culmorum*), while epidemics of *F. sporotrichioides* lead to a higher incidence of T-2 and HT-2 (Logrieco *et al.*, 2002).

Correlations between rot diseases and *Fusarium* infection are not always clear, suggesting that final ear and stalk rots are not only dependent on the level of resistance to *Fusarium spp.*, but also on stress subsequently imposed on the plant (Munkvold, 2003).

Several routes are described for the entry of the fungus into the plant, comparable to those of FHB (**Figure 1.5**). Primary infection of the stalk is probably due to stubbles on the field during winter; secondary ear rot infection might be induced during flowering (Papst *et al.*, 2007). The spore entry into maize ears occurs through wounds by lepidopteran insect vectors or birds, wind dispersal, seed transmission, and most importantly the growth of mycelium via silks to kernels and from spores germinating on the silk (Munkvold, 2003; Reid *et al.*, 2006; Miller *et al.*, 2007; Parsons and Munkvold, 2010). Infection usually starts from the top of the ear and develops a reddish mold covering the ear extensively (Munkvold, 2003).

1.2.3.3 *Fusarium* crown rot

F. graminearum, *F.* culmorum and *F.* pseudograminearum are also able to cause *Fusarium* crown rot on different small-grain cereals, in particular wheat and barley. Like FHB and *Fusarium* ear rot, yield and quality losses are described resulting in contamination of the grains with mycotoxins.

Figure 1.6 Symptomatic outcome of *Fusarium* head blight, *Fusarium* ear rot and *Fusarium* crown rot

Crown rot, also known as root and foot rot, causes a brown discoloration on roots and on the subcrown internodes of the main stem. *Fusarium* crown rot symptoms vary depending on the time of infection. If the fungus attacks the plant in the early stage, pre- and post-emergence

seedling death occurs; if infection occurs in a later stage of the season brown leasions appear on the internodes of the main stem (Scherm *et al.*, 2013). **Figure 1.6** illustrates the symptomatic outcome of FHB, *Fusarium* ear rot and *Fusarium* crown rot, respectively.

1.2.4 Role of mycotoxins in the infection process

The key question which involves every researcher is *what induces mycotoxin biosynthesis in Fusarium*? *Fusarium* fungi produce effectors, cell-wall degrading enzymes and mycotoxins to colonize plants. These plants respond to infections by producing defence-related hormones, pathogenis-related proteins, reactive oxygen and proteins involved in cellular detoxification.

DON is the most thoroughly studied mycotoxin in this respect. The mycotoxin is known to be a virulence factor, therefore the regulation of its biosynthesis has conceived considerable attention. Several signals *in planta* may play an important role trigerring the DON biosynthesis. Certain compounds regulate the *TRI* gene expression and subsequent DON biosynthesis.

The acidity of the medium is an important factor promoting DON biosynthesis (Gardiner *et al.*, 2009). A low pH will activate *TRI* genes expression and subsequent mycotoxin biosynthesis. Reactive oxygen species such as H_2O_2 have also been identified as one of the inducers of DON biosynthesis (Ponts *et al.*, 2006). Several H_2O_2-generating fungicides report to induce *TRI* gene expression and accumulation of mycotoxins (Audenaert *et al.*, 2010). Also phenolic acids prove to enhance mycotoxin production (Kazan *et al.*, 2012).

1.3 MASKED MYCOTOXINS

1.3.1 Introduction

In the past decades, it has become clear that in mycotoxin-contaminated commodities, many structurally related compounds generated by plant metabolism, fungi or food processing coexist with their parent mycotoxins. In the mid 80ies the topic of masked mycotoxins received attention because in some cases of mycotoxicosis clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed (Gareis *et al.*, 1990). The unexpected high toxicity was attributed to undetected conjugated forms of mycotoxins that possibly are hydrolyzed into the parent toxins in the digestive tract of animals.

Young *et al.* (1984) showed that the DON content of yeast-fermented food products was higher than that of the contaminated flour used for their production, so it has been speculated that a DON conjugate of some form arising from plant metabolism might exist. Savard (1991) was the first to chemically synthesize glucose and fatty-acid conjugates of DON, while Sewald *et al.* (1992) could identify deoxynivalenol-3-glucoside (DON-3G) as a DON metabolite in maize cell suspension cultures. The glucosylated form of ZEN, zearalenone-14-glucoside (ZEN-14G) is certainly the best studied conjugated form. It was originally found as a fungal metabolite (Kamimura, 1986) but Engelhardt *et al.* (1988) reported that ZEN was also transformed to ZEN-14G by plant cultures. Gareis *et al.* (1990) hypothesized that ZEN-14G was cleft during digestion in swine, releasing the estrogenic aglucone, ZEN. Sulfate and glucuronide conjugates of ZEN were shown to occur in urine of ZEN-fed animals (Mirocha, 1981), although their identity could not be confirmed. Zearalenone-14-sulfate (ZEN-14S) was isolated by Plasencia and Mirocha (1991) as a fungal metabolite from *Fusarium*.

Two types of masked mycotoxins can be distinguished: extractable, conjugated and non-extractable, bound varieties. Extractable, conjugated mycotoxins can be detected by appropriate analytical methods when their structure is known and analytical standards are available. Non-extractable, bound mycotoxins are covalently or non-covalently attached to polymeric carbohydrate or protein matrices. Bound mycotoxins are not directly accessible and have to be liberated from the matrix by chemical or enzymatic treatment prior to chemical analysis.

The term masked mycotoxins refers to the mycotoxin derivatives that are undetectable by conventional techniques because their structure has been altered in the plant or through food processing. The term conventional applies to the analytical detection methods that have

initially been developed for specific mycotoxins only. This can lead to a failure of detecting these specific compounds, which underlines a significant underestimation of the mycotoxin content.

Up to now, only glucoside and sulfate conjugates of ZEN, DON, T-2 and HT-2 have been proven to occur in naturally-infected cereals such as wheat, barley and maize, while fusaric acid methylamide was shown to occur in infected vegetables. To date, structures of these metabolites of plants have been elucidated for ZEN, DON, NIV, FUS-X, T-2, HT-2, ochratoxin A (OTA), destruxins and fusaric acid (**Figure 1.7**).

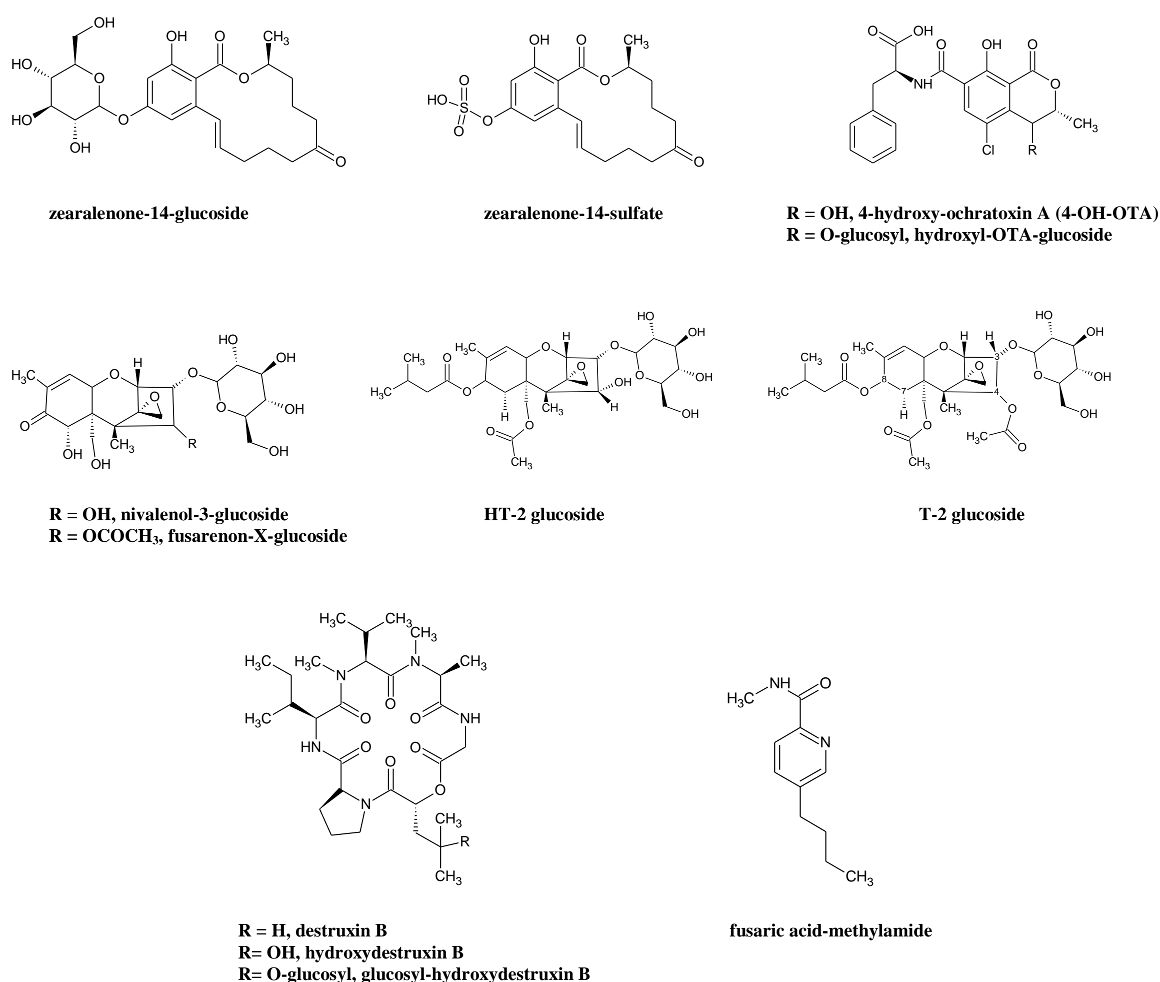


Figure 1.7 Structurally elucidated masked mycotoxins

1.3.2 Formation of masked mycotoxins

1.3.2.1 Introduction to the conjugation of xenobiotics

Plants can decrease the toxicity of phytotoxins either by chemical modification and/or inclusion into the plant matrix. Xenobiotic conjugation in higher plants has been an area of intense research. The mechanisms applied by plants and mammals in metabolism of xenobiotics are remarkably analogous. Similar classes of compounds or functional groups are frequently metabolized by comparable mechanisms. *In planta*, oxidation, reduction and hydrolysis reactions occur with similar frequency and are referred to as phase I-reactions. Hydrolysis, glucose and glutathione conjugation (*i.e.* phase II-reactions) appear rapidly in plants.

In the next section, possible conjugation patterns and potential target molecules *in planta* are detailed (Lamoureux and Rusness, 1986). ***Glucoside conjugation*** is the most common metabolization process in plants. Functional groups involved in glucose conjugation are X-OH, X-COOH, X-NH_2, X=NH, along with cases of X-SH and X-NOH. Many xenobiotics do not contain these functional groups, nevertheless these links are introduced by phase I-reactions and can thereafter be metabolized to glucose conjugates. **Table 1.1** indicates the possible phase I-reactions producing metabolites susceptible to glucose conjugation in higher plants.

Table 1.1 Phase I reactions producing metabolites susceptible to glucose conjugation in higher plants (Lamoureux and Rusness, 1986)

Phase I reaction	Class of xenobiotic	Metabolites susceptible to glucoside conjugation
Hydrolysis	Carbamate	Phenols
	Anilide	Anilines, carboxylic acids
	Phosphorothioate	Phenols
	Ester	Carboxylic acids, alcohols
	N-hydroxyl derivates	N-hydroxyls
Glutathione conjugation	Diphenyl ether	Phenols
Reduction	Nitroaromatic	Anilines
Oxidation	Alkyl	Alcohols
	Aryl	Phenols
Isomerization	Cyclic amides	Alcohols, amines

Phenols, alcohols or xenobiotics that are metabolized through these forms as intermediates are most commonly metabolized to ***β-O-glucosides***. Generally, O-glucosides have been characterized as β-stereochemic by hydrolysis with β-glucosidase. Plants are capable of coupling with galactose and endogenous substrates such as flavones, *i.e.* mono-saccharides of xenobiotics involving carbohydrate moieties other than glucose, however these transformations are extremely rare. ***N-glucosides*** can be formed out of aromatic and

heterocyclic xenobiotics that contain a primary or secondary amino group. In some cases resistance or susceptibility to an herbicide has been correlated to the ability of a plant species or cultivar to detoxify an herbicide by the formation of an N-glucoside. Xenobiotic and endogenous substrates that contain free carboxyl groups are commonly metabolized in plants to ***glucose ester-conjugates***. Harvey *et al.* (1978) and Suzuki *et al.* (1981) proved the formation of ***N-O-glucosides*** by the conversion of oxamyl insecticides in tobacco, alfalfa, tomato and orange fruit. ***S-glucosides*** rarely occur in higher plants, however glucosinolates represent a major class of natural products found in cruciferous plants such as black radish (*Raphanus sativus L. niger*) (Njumbe Ediage *et al.*, 2011). The first ***complex glucose-conjugates*** identified in plants were β-gentiobiosides (β-(1-6)-glucosyl-β-(1-0)-glucosides). Polyglucosides as well as heterosaccharide conjugates occur *in planta*.

Glutathione conjugation (γ-L-glutamyl-L-cysteinylglycine, *i.e.* nucleophilic thiol) involves glutathione-S-transferase and takes place on electrophilic sites of hydrophilic xenobiotics. Mycotoxins have different electrophilic centres which can be catalyzed by gluthation-S-transferases. Conjugation with gluthation attaches a side group containing two carboxyls, amino, peptide bonds and a thiol-group which gives rise to a highly polar and hydrophilic molecule. Glutathione conjugates are rapidly metabolized *in planta* to cysteine conjugates by a complex network of processing reactions. These conjugates are often malonated into N-malonylcysteineconjugates (Marrs, 1996). Acetylated xenobiotics conjugates are not commonly formed in higher plants, however direct or indirect conjugation with malonic acid is frequently occurring. D-amino acids, aromatic and heterocyclic compounds containing a primary amino group can be metabolized in plants via amide bond formation to ***N-malonyl*** and ***O-malonyl conjugates***.

Amino acid conjugation occurs when xenobiotics contain a free carboxyl group, usually in completion with reactions such as glucose-ester formation or aromatic ring hydroxylation/glucoside formation (Mumma and Hamilton, 1979). Amino acid conjugation is however not common in plants, only structures with similar amino acid features are involved.

Most plants consist of lignified cells, so the occurrence of ***bound residues*** tends to be much more common in xenobiotic plant metabolism. Many heterocyclic and aromatic xenobiotics that contain or can be metabolized to yield functional groups (X-OH, X-COOH, X-NH_2 and X-SH) form bound residues. These conjugates are incorporated or associated with most of the biological polymers of plants including lignin, carbohydrate polymers, and proteins. These bound residues are referred to as insoluble and non-extractable.

Most conjugation products lead initially to the formation of more polar products, however reports clearly indicate the presence of ***lipophilic conjugates*** (*e.g.* ester conjugates formed primarily from palmitic and linoleic acids) (Frear and Swanson, 1977).

1.3.2.2 Conjugation of mycotoxins

Mycotoxins can, like other xenobiotics, partially be metabolized. Metabolization occurring in living plants and fungi can play an important role in food and feed safety as the presence of a wide variety of conjugation products is described (Berthiller *et al.*, 2009a). In the next paragraph currently known conjugated mycotoxins are described in a detailed overview (**Table 1.2**). Berthiller *et al.* (2009a) stated that the origin of conjugated mycotoxins can be divided into four main sources: fungal, plant, food processing and mammalian conjugates.

Fungal conjugates are directly excreted by fungi, however, only few examples of these forms are described. 3-ADON and 15-ADON, which are frequently found in *Fusarium*-contaminated food and feed, are the most well-known substances in this respect. It is important to emphasize that 3-ADON and 15-ADON arise from a common precursor 3,15-diacetyl-deoxynivalenol (3,15-diADON), and are subsequently biosynthetic precursors of DON in molds (Kimura *et al.*, 2007). As stated in **Table 1.2**, compounds such as 4-propionyl-HT-2 toxin, 8-hexanoyl-neosolaniol, 8-butyryl-neosolaniol, 8-isobutyryl-neosolaniol, and 8-pentenoyl-neosolaniol are found in *F. sambucinum* and *F. sporotrichioides*. The incidence of 3-lactyl-deoxynivalenol was found in *Fusarium*-infected barley, however it remains unclear whether it is a fungal or a plant conjugate. Conjugates of the myco-estrogen ZEN, ZEN-14S, ZEN-14G and zearalenone-14,16-β-diglucoside have been detected in fungal cultures (*e.g. Rhizopus arrhizus, Rhizopus spp.* and *Thamnidium elegans*). A trichothecene α-glucoside, 15-monoacetoxyscirpenol-4-α-glucoside, was isolated from a *F. sambucinum* strain, and seems to be the sole glucoside of a type A-trichothecene produced by the fungus itself (Gorst-Allman *et al.*, 1986).

As earlier described, plants protect themselves from xenobiotic compounds (*e.g.* pesticides, mycotoxins) by converting them to more polar metabolites. ***Plant conjugates*** are the main part of this thesis. The great capability of plants to metabolize mycotoxins is pointed out in **Figure 1.8** and **Table 1.2**. Seventeen different metabolites of ZEN have been identified to occur in ZEN-treated *Arabidopsis thaliana* plants. Known transformation products include ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-zearalenol-14-glucoside (α-ZEL-14G) and β-zearalenol-14-glucoside (β-ZEL-14G). Di-hexosides, hexose-pentosides and malonylglucosides of ZEN, α-ZEL and β-ZEL were also described (Berthiller *et al.*, 2006).

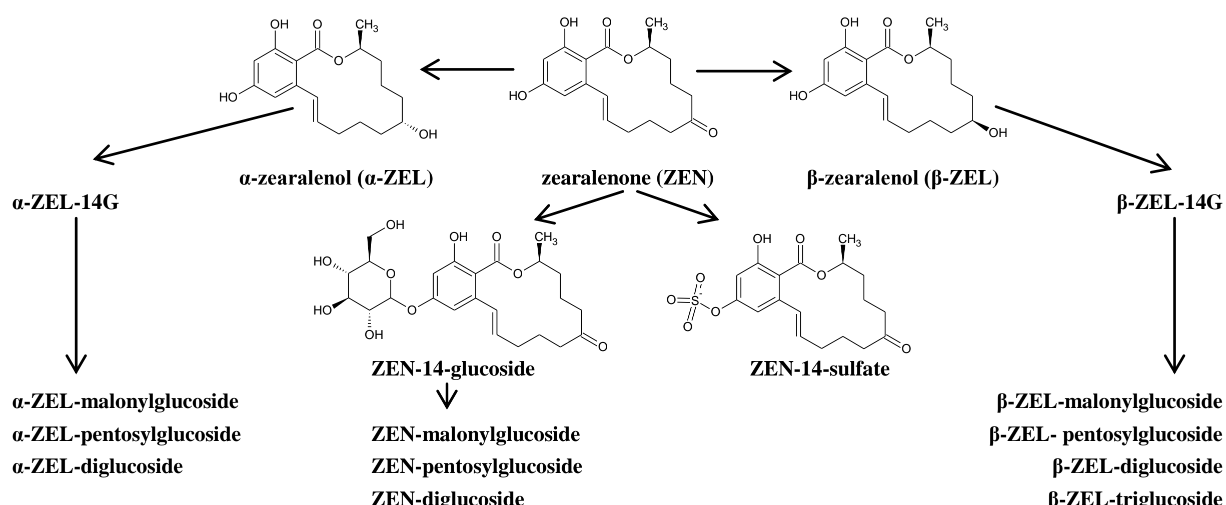


Figure 1.8 Metabolization products of zearalenone in *Arabidopsis thaliana* (Berthiller *et al.*, 2006)

Acyl conjugation, catalyzed by acyltransferases, is described for several mycotoxins *in planta*: palmitoyl-trichothecolone, palmitoyl-scirpentriol, palmitoyl-T-2 tetraol, palmitoyl-zearalenone in *F. moniliforme*-infected banana and the cinnamic acid ester of trichothecolone in *Trichothecium roseum*-infected anise seeds.

Miller *et al.* (1983) and Young *et al.* (1984) speculated that an increased amount of DON in feed was due to the enzymatic conversion of an unknown conjugate into DON. The formation of DON-3G from DON in maize cell suspension cultures and *Arabidopsis thaliana* was proven. In the latter plant, a gene encoding an uridine difosfate (UDP) glucosyltransferase with the capability to convert DON into DON-3G was identified by Poppenberger *et al.* (2003).

Plant conjugates of type C and D-trichothecenes (*e.g.* verrucarin A-glucoside, and roridine A, D and E-glucosides) were detected in *Baccharis coridifolia*. It remains unclear, however, whether the formed glucosides are less toxic storage forms or part of the mechanism of self-protection of the plant. The release of the toxic aglucons by glucosidases, set free by herbivores, might be required for animal toxicity.

Ochratoxin A, formed by *Aspergillus* and *Penicillium* species, was transformed to ochratoxin-α, ochratoxin A-methyl ester, two isomers of hydroxy-ochratoxin A as well as the glucosides and methyl esters of both of the isomers in wheat and maize cell suspension cultures. To date, the natural occurrence of these metabolites has never been described in foods. Dall'Asta *et al.* (2008) described the occurrence of fumonisin conjugates in unprocessed maize. However, the chemical composition of these naturally occurring hidden forms is still unknown.

During food processing (*i.e.* heating or fermentation steps), mycotoxins can be altered through conjugation and degradation. ***Food-processing conjugates*** are described for fumonisins and trichothecenes. Concerning fumonisins, conjugates with sugars, amino acids and proteins are known to occur. The heating of fumonisin B_1 (FB_1) with reducing sugars can yield N-(carboxymethyl)-fumonisin B_1 (Howard *et al.*, 1998). N-(1-deoxy- fructos-1-yl)-fumonisin B_1 also occurred in corn products (Poling *et al.*, 2002), and a diester of FB_1 with methyl glucose upon heating was observed. As hydrolyzed FB_1 did not yield any reaction products, the two tricarballylic acid (TCA) groups were identified as the reactive groups of fumonisins. Regarding trichothecenes, DON-3G and the acetylated DON-forms were detected in malt and beer made from naturally *Fusarium*-contaminated barley (*i.e.* fermentation). Zachariasova *et al.* (2012) reported presumable diglucosides and triglucosides of DON in beer. A possible explanation of the increase of conjugated forms in beer is twofold. A first possibility is the production of additional conjugated mycotoxins by the metabolically highly active germlings during the malting process; a second eventuality is the enzymatic release of bound mycotoxins, originally present in the cell wall polymer fraction. Although mycotoxins are generally very stable, thermal degradation (*i.e.* excessive heating) products for NIV and DON (less toxic de-epoxy metabolites) have been observed.

Mycotoxin conjugates also arise during ***mammalian metabolization***. They are not regarded as masked mycotoxins as they do not play any role in food and feed safety. During mammalian metabolization, mycotoxins are metabolized in the liver and excreted in urine (Galtier, 1998). Formation of albumin adducts, and reaction with amino acids (*e.g.* cysteine) and glutathione leads to detoxification of several mycotoxins (**Table 1.2**). However, most common conjugation products certainly are glucuronides and sulfates. These biomarkers are nowadays an emerging topic as total exposure of individuals to certain mycotoxins, regardless of their source (*e.g.* inhalation) and form of intake (*e.g.* all types of food/feed) is monitored. Recent developments of DON-3-glucuronide as a urinary biomarker of human exposure to DON should facilitate epidemiological studies of disease associations with DON (Meky *et al.*, 2003). Turner *et al.* (2008) described a method for the detection of the biomarker DON-3-glucuronide in urine to estimate the total intake of DON-derivatives in food. At present, a survey on several biomarkers is executed to determine the exposure to mycotoxins in the Belgian population (Heyndrickx *et al.*, 2013). Concurrent with the evolution and application of these techniques to measure biomarkers of exposure, the development of critical tools for molecular epidemiology can better address risk assessment questions.

1.3.2.3 Detoxification process of mycotoxins *in planta*

A detoxification process will be initiated when plants are affected with phytotoxic compounds. Mycotoxins interact with the vital cell functions of the plants, so they are assumed to be a target of the plants' detoxification metabolic processes (Berthiller *et al.*, 2012). Two major detoxification mechanisms can be differentiated in plants: chemical modification and compartmentation.

Three phases of chemical modifications of mycotoxins are distinguished during plant metabolism depending on the structure of the parent form. ***Phase I***-process includes the reduction, oxidation or hydrolysis of the parent mycotoxin, and generates reactive groups on the molecular structure of the mycotoxins by oxidation or hydrolysis. Lipophilic compounds (*e.g.* ZEN) are typically involved in this phase, while hydrophilic compounds (*e.g.* DON) are not affected.

Oxidation reactions are catalyzed by the cytochrome P-450 system while hydrolysis is catalyzed by esterases and amidases (Coleman *et al.*, 1997). These phase I-modified xenobiotics can obtain an increased toxicity in proportion to the parent form, and leads to bioactivation (*e.g.* α-ZEL) (Ayed *et al.*, 2011), however toxicity levels that are lower or even comparable, are also described (Coleman *et al.*, 1997).

Phase II consists of the enzymatic transformation of the reactive groups through conjugation, glucosidation and sulfatation (*e.g.* DON-3G). The phase II or conjugation phase results in conjugated mycotoxins through the covalent binding of hydrophilic metabolites (Poppenberger *et al.*, 2003; Vendl *et al.*, 2009). As earlier mentioned, plants consist of an amalgam of polar conjugation products such as glucose, malonic acid, glutathione and sulfate. These substances can easily bind through the involvement of enzymes (glucosyl-, malonyl - and gluthation-S-transferase, respectively) in the functional groups of xenobiotics. There is an increased evidence that an UDP-glucosyltransferase (*e.g.* UGT73C5) is involved in the glucose conjugation *in planta* (Pflugmacher and Sandermann, 1998; Brazier *et al.*, 2002; Brazier *et al.*, 2003). These glucosyltransferases transfer a sugar from an activated sugar donor (*e.g.* UDP-glucose) to an acceptor molecule (*i.e.* mycotoxin) and as such are involved in synthesis and modification of the multitude of glucoconjugates in existence in the biosphere.

Table 1.2 Several known mycotoxin conjugates and their origin

Origin	Conjugated mycotoxin	Matrix	References
Fungal conjugates	Zearalenone-14-sulfate	*F. graminearum, Rhizopus arrhizus*	Plasencia and Mirocha, 1991; El-Sharkawy *et al.*, 1991
	Zearalenone-14-glucoside	*Rhizopus spp.*	Kamimura, 1986
	Zearalenone-14,16-diglucoside	*Thamnidium elegans*	El-Sharkawy *et al.*, 1991
	15-Monoacetoxyscirpenol-4-glucoside	*F. sambucinum*	Gorst-Allman *et al.*, 1986
	4-Propionyl-HT-2-toxin	*F. sambucinum, F. sporotrichioides*	Grovey, 2007
	8-Hexanoyl-neosolaniol	*F. sambucinum, F. sporotrichioides*	Grovey, 2007
	8-Butyryl-neosolaniol	*F. sambucinum, F. sporotrichioides*	Grovey, 2007
	8-Isobuturyl-neosolaniol	*F. sambucinum, F. sporotrichioides*	Grovey, 2007
	8-Pentenoyl-neosolaniol	*F. sambucinum, F. sporotrichioides*	Grovey, 2007
	3-Lactyl-deoxynivalenol	*Fusarium*-infected barley	Rodrigues-Fo *et al.*, 2002
	3-Acetyl-deoxynivalenol	*Fusarium*-infected cereals	Scoop (2003)
	15-Acetyl-deoxynivalenol	*Fusarium*-infected cereals	Scoop (2003)
	Fumonisin fatty acid-esters	*F. verticilloides*-infected rice	Bartók *et al.*, 2010
Plant conjugates	α-Zearalenol-14-glucoside	Maize cells	Pons *et al.*, 2008
	β-Zearalenol-14-glucoside	Maize cells	Pons *et al.*, 2008
	Zearalenone-14-glucoside	Maize cells, wheat	Engelhardt *et al.*, 1988; Schneweis *et al.*, 2002
	Zearalenone-dihexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	α-Zearalenol-dihexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	β-Zearalenol-dihexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	Zearalenone-malonylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	α-Zearalenol-malonylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	β-Zearalenol-malonylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	Zearalenone-pentosylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	α-Zearalenol-pentosylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	β-Zearalenol-pentosylhexoside	*Arabidopsis thaliana*	Berthiller *et al.*, 2006
	Palmitoyl-trichothecolone	*Fusarium*-infected banana	Chakrabarti *et al.*, 1986
	Palmitoyl-scirpentriol	*Fusarium*-infected banana	Chakrabarti *et al.*, 1986
	Palmitoyl-T-2-tetraol	*Fusarium*-infected banana	Chakrabarti *et al.*, 1986
	Palmitoyl-zearalenone	*Fusarium*-infected banana	Chakrabarti *et al.*, 1986
	Trichothecolone-cinnamic-acid ester	*Trichothecium roseum*	Ghosal *et al.*, 1982
	Deoxynivalenol-3-glucoside	Maize cells, wheat, maize, barley, beer	Sewald *et al.*, 1992; Berthiller *et al.*, 2005, 2009b; Lancova *et al.*, 2008a; Kostelanska *et al.*, 2009; De Boevre *et al*., 2012a
	Deoxynivalenol-3-glutathione	wheat	Kluger *et al.*, 2013
	Verrucarin A-glucoside	*Baccharis coridifolia*	Jarvis *et al.*, 1996; Rosso *et al.*, 2000
	Roridine A, D and E-glucosides	*Baccharis coridifolia*	Jarvis *et al.*, 1996; Rosso *et al.*, 2000
	Hydroxyochratoxin A	Wheat cells, maize cells	Ruhland *et al.*, 1996
	Ochratoxin A-methyl ester	Wheat cells, maize cells	Ruhland *et al.*, 1996
	Hydroxyochratoxin A-methyl ester	Wheat cells, maize cells	Ruhland *et al.*, 1996
	Hydroxyochratoxin A-glucoside	Wheat cells, maize cells	Ruhland *et al.*, 1996
Food-processing conjugates	N-(Carboxymethyl)-fumonisin B_1	Heated glucose, corn products	Howard *et al.*, 1998; Seefelder *et al.*, 2001
	N-(1-Deoxy-fructos-1-yl)-fumonisin B_1	Heated glucose	Poling *et al.*, 2002
	Hidden fumonisin	Corn, corn products, gluten-free products	Dall'Asta *et al.*, 2008; Kim *et al.*, 2003; Park *et al.*, 2004; Dall'Asta *et al.*, 2009b
	Deoxynivalenol-diglucoside	Beer	Zachariasova *et al.*, 2012
	Deoxynivalenol-triglucoside	Beer	Zachariasova *et al.*, 2012
Mammalian conjugates	Aflatoxin-albumin	Blood	Sabbioni *et al.*, 1987
	Satratoxin-albumin	Blood	Yike *et al.*, 2006
	Ochratoxin-albumin	Blood, urine	Scott, 2005
	Patulin-cysteine	*In vitro*	Fliege and Metzler, 1999
	Patulin-histidine	*In vitro*	Fliege and Metzler, 1999
	Patulin-lysine	*In vitro*	Fliege and Metzler, 1999
	Patulin-glutathione	*In vitro*	Fliege and Metzler, 1999
	Ochratoxin A-cysteine	*In vitro*	Dai *et al.*, 2002
	Ochratoxin A-glutathione	*In vitro*	Dai *et al.*, 2002
	Deoxynivalenol-3-glucuronide	Urine	EFSA, 2004
	Zearalenone-16-glucuronide	Urine	EFSA, 2004
	Deoxynivalenol-4-sulfate	Urine	Prelusky *et al.*, 1987
	Zearalenone-14-sulfate	Urine	Mirocha, 1981; Olsen *et al.*, 1986

To date, none of the plant glucosyltransferases identified have any clear membrane-spanning or targeting signals. This statement suggests that enzymes function in the cytosol, although within that compartment, the proteins may associate as peripheral components of the endomembrane system (Lim *et al.*, 2004). Besides glucosyltransferases, also gluthation-S-transferase is noticed. The activity of gluthation-S-transferase is induced by stress (*e.g.* heavy metals) (Lyubenova *et al.*, 2007). Herbicides also induce the gluthation-S-transferase activity in crops (Holt *et al.*, 1995). Conjugation with glutathion is described for aflatoxins (Hayes *et al.*, 1991) and trichothecens (Foster *et al.*, 1975; Subramanian, 2002), however there are large quantitative differences which have not been examined to date. Recently, Kluger *et al.* (2013) proved the presence of DON-3-glutathione and seven other biotransformation products in wheat.

Through conjugation, an increased polarity and water solubility is accomplished, resulting in the inactivation and detoxification of the mycotoxins in the organism. ***Phase III*** or the compartmentation phase includes the transportation and elimination of the conjugated mycotoxins through membrane transporter systems from the cytosol to the vacuolar lumen or the apoplastic space outside the cell (Berthiller *et al.*, 2007). The carriers used for this transport are different for glucosylated, malonylated and gluthationylated xenobiotics, respectively (Berthiller *et al.*, 2012). Glucosylconjugates use the transmembrane H^+-gradient (P type ATP-ase), while gluthationylated forms are transported by ABC transporters fuelled by adenosine-5'-triphosphate (ATP) (Bartholomew *et al.*, 2002). The conjugation of highly polar glutathion conjugates assumes that biological membranes can not be transferred and are freely moved among the compartiments. Specific transporters are needed and it is assumed that conjugation is irreversible. *In casu*, the irreversible conjugation of mycotoxins which have a double bound accompanied by an electron withdrawing group, such as epoxides, lactons and aldehydes (*e.g.* trichothecenes) is observed. If degradation occurs, then the obtained degradation products do not resemble the parent form in contrast to the glucosidase activity.

Metabolism of mycotoxins in plants and animals is relatively analoguous. The main difference between these two organisms lies within distribution and excretion (*cf.* Absorption, Distribution, Metabolism and Excretion, ADME). Phase I and II-processes are quite similar to enzymatic transformations such as reduction, oxidation or hydrolysis and the conjugation with glucose, sulfates and glucuronides. *In planta* compartmentation plays an important role, while there is an active elimination or excretion via the kidney or liver *in animalia*. Detoxification

products are constantly stored in the plant cell, rather than excreted. The only exception to the rule is the excretion via root exudation. Transport of glutathion-conjugates has been described into the roots where secretion occurs (*e.g. Hordeum vulgare*) (Schröder *et al.*, 2007).

1.3.3 Occurrence of masked mycotoxins in food and feed

Knowledge on the occurrence of mycotoxin derivatives is imperative to unequivocally determine the mycotoxicological load of food or feed products. An increasing amount of data have been accumulated in the last years, showing that mycotoxins may also occur in modified forms originating from plant, fungi or animal metabolism or from food processing. As earlier mentioned, plant metabolites have been identified for DON, ZEN, T-2, HT-2, FUS-X, NIV, OTA, destruxins and fusaric acid (Cirlini *et al.*, 2012). To date, only DON-3G and some metabolites of ZEN, T-2 and HT-2 have been proven to occur in cereals and cereal-derived food and feed. Occurrence studies revealed the presence of many conjugated forms of DON, ZEN, T-2 and HT-2, however, in most cases, at lower concentrations than the parent toxin. The occurrence data available for masked trichothecenes and myco-estrogens are described in the next paragraphs.

1.3.3.1 Masked trichothecenes

One of the most widely investigated masked forms of trichothecenes are the glucoside derivatives. The availability of the commercial reference standard of DON-3G recently enabled the collection of occurrence data, albeit for this specific masked form only, whereas no quantitative information is available for other type B trichothecene glucosides (Lattanzio *et al.*, 2012b).

DON-3G was proven to occur in wheat (grains, semolina and flour) (Berthiller *et al.*, 2005; Sasanya *et al.*, 2008; Skrbic *et al.*, 2012), maize (Berthiller *et al.*, 2009b; De Boevre *et al.*, 2013b), oats (Desmarchelier *et al.*, 2011), barley, malt and beer (Lancova *et al.,* 2008; Kostelanska *et al.,* 2009; Malachova *et al.,* 2011; Varga *et al.*, 2012), and cereal-based foods (Berthiller *et al.*, 2009b; Desmarchelier *et al.*, 2011; De Boevre *et al.*, 2012a).

In 2010 durum wheat samples (n=150), were investigated on the occurrence of DON and DON-3G by Dall'Asta *et al.* (2013), revealing an ubiquitous incidence of DON-3G. 85% of the analyzed samples were contaminated in a range of 46 μg kg^{-1} to 842 μg kg^{-1}. Acetylated forms of DON (3-ADON and 15-ADON) were also observed in most of the durum wheat samples. In China the mold and (masked) mycotoxin impact on wheat is detectable with an average of 10% to 40% reduction in the crop yield, which correlates with 25,000 to 50,000

tons of moldy inedible grains per year (Wei *et al.*, 2012). Li *et al.* (2012) executed a survey of 697 wheat samples as to the occurrence of DON, DON-3G, 3-ADON and 15-ADON. Moderate concentrations of DON-3G (4 μg kg^{-1} to 238 μg kg^{-1}, mean 52 μg kg^{-1}) were found, apparently with lower amounts of 3-ADON and 15-ADON in the presence of high DON-levels. The average relative ratio of DON-3G to DON was 33% in wheat kernels and 10% in wheat flour. Natural occurrence of DON-3G was proven to be positively correlated with DON. The same authors reported median levels of 21 μg kg^{-1} DON-3G in wheat (52%, 2 μg kg^{-1} - 238 μg kg^{-1}) and 35 μg kg^{-1} DON-3G for corn (33%, 2 μg kg^{-1} - 499 μg kg^{-1}) in 2007-2008. The median levels for DON in the same survey were 31 μg kg^{-1} for wheat (88%, 2 μg kg^{-1} - 591 μg kg^{-1}) and 95 μg kg^{-1} for corn (50%, 2 μg kg^{-1} - 4374 μg kg^{-1}), respectively (Li *et al.*, 2012).

In studies concerning cereal-based foods (*e.g.* bread, snacks, biscuits, pasta and infant food) DON-3G was only detected in two of the 84 samples (<LOQ, 100 μg kg^{-1}) (Vendl *et al.*, 2010). Malachova *et al.* (2011) observed a contamination level of up to 80% (5 μg kg^{-1} to 72 μg kg^{-1}) using a more sensitive method. Sasanya *et al.* (2008) reported that some wheat-based samples contained significantly higher values of DON-3G compared to DON.

Kostelanska *et al.* (2009) studied 176 beers for DON-3G revealing a maximum level of 37 μg L^{-1}. Remarkable was the fact that DON-3G levels in beer exceeded the DON concentration, however, based on consumption data no toxicological concerns could be concluded. Another study, on the contrary, revealed no contamination of DON-3G in beer (Vendl *et al.*, 2010). Varga *et al.* (2012) performed a large beer-survey (n=374) where, in contrast to previous reports, 93% and 77% of the samples were contaminated with DON and DON-3G, respectively. A risk analysis confirmed possible excesses of the total daily intake (TDI) of DON in the Austrian adult population. Zachariasova *et al.* (2012) suggested the occurrence of di- and tri-glucosylated forms of DON in beer, however, these findings have not yet been elucidated, and therefore its contribution to the overall DON content is unclear. All data as presented allow the conclusion that for some samples DON-3G amounts have exceeded the amounts of DON.

The relative proportion of DON-3G to DON varied in relation to years and genotypes ranging in level from 30% (Dall'Asta *et al.*, 2013), 46% (Berthiller *et al.*, 2009b) to even 70% (De Boevre *et al.*, 2012a). These results suggest that it is almost impossible to predict the concentration of DON-3G from the concentration of DON alone.

Berthiller *et al.* (2012) stated that preliminary data were collected after quantification of masked DON in naturally-infected barley samples by applying a hydrolysis procedure with trifluoroacetic acid (Zhou *et al.*, 2008). Applying this procedure an increase of 7% to 75% of DON was observed in corn and wheat. The latter could not be explained by the presence of the acetylated forms, and the average increase of DON after trifluoroacetic acid hydrolysis was 14%.

Nakagawa *et al.* (2011) were the first to describe two new *Fusarium* masked mycotoxins in artificially-contaminated wheat with *F. graminearum*, fusarenon-X-glucoside and nivalenol-glucoside. Quantification was unfortunately not executable, however, 15% of the parent form was estimated to be converted into the masked forms. The presence of these conjugates clearly indicated possible natural occurrence.

Recently, Busman *et al.* (2011) reported the presence of T-2-3-*O*-glucoside (T-2-3G) and HT-2-3-*O*-glucoside (HT-2-3G) in articificially-contaminated *F. sporotrichioides* cultures. The natural occurrence of these two glucoside derivatives in wheat and oats was clearly documented by Lattanzio *et al.* (2012b). Also, an additional masked form, HT-2-4-*O*-glucoside, was found to be present. A conversion of 24% and 27% for T-2 and HT-2 glucosides, respectively, was proven. Similar results were found by our research group (*Chapter 6*). Veprikova *et al.* (2012) observed that T-2-3G and HT-2-3G occurred in 80% and 75% of the wheat samples with a conversion of 16% to 17%, respectively. Natural occurrence of HT-2-diglucoside was only proven in two samples. These quantitative differences are probably due to low recoveries of the analytes by the use of specific Mycosep® columns. Nakagawa *et al.* (2012) found similar results and hypothesized that mean contamination levels of T-2-3G and HT-2-3G were 24 $\mu g\ kg^{-1}$ and 41 $\mu g\ kg^{-1}$, respectively, assuming that T-2-3G/T-2 and HT-2-3G/HT-2 was equal to the DON-3G/DON-ratio.

As described, these trichothecene glucosides were isolated from *Fusarium*-infected plant material. Mc Cormick *et al.* (2012) investigated an efficient microbial whole-cell catalytic method for the preparation of T-2 glucosides with a potential large-scale availability of masked forms. Yeast species assigned to the *Trichomonascus clade* (*Saccharomycotina, Ascomycota*), classified in *Blastobotrys*, were tested for their ability to convert T-2 to possible metabolization products. The occurrence of 3-acetyl-T-2, T-2-3G and the removal of the isovaleryl group to form neosolaniol was proven.

Plant conjugates of type C and D-trichothecenes (*e.g.* verrucarin A-glucoside, and roridine A, D and E-glucosides) were detected in *Baccharis coridifolia*. As previously described, it

remains unclear whether these masked forms are less toxic storage forms or part of the mechanism of self-protection of the plant. The release of the toxic aglucons by glucosidases after exposure to herbivores might be required for animal toxicity (Berthiller *et al.*, 2009a).

1.3.3.2 Masked myco-estrogens

To date, only three research groups investigated the occurrence of masked myco-estrogens in food and feed, probably due to the unavailability of commercial reference standards (Schneweis *et al.*, 2002; Vendl *et al.*, 2010; De Boevre *et al.*, 2012a).

Schneweis *et al.* (2002) demonstrated that out of a batch of 24 wheat samples, ZEN-14G was found in 10 of the ZEN positive samples (42%) at levels ranging from 17 µg kg^{-1} to 104 µg kg^{-1}. The amounts of ZEN-14G were correlated to those of ZEN, for which a correlation of 0.86 was found. Another research group (Vendl *et al.*, 2010) analyzed 84 cereal-based products (wheat flour, whole-meal wheat bread, maize meal, biscuits, wheat flakes, bran flakes, muesli, crackers, cereal snack bars and polenta), but none of the samples contained ZEN-14G, α-ZEL, β-ZEL, α-ZEL-14G and β-ZEL-14G, notwithstanding ZEN-14S being observed in low concentrations (6.1 µg kg^{-1}). De Boevre *et al.* (2012a) analyzed 175 cereal-based foods, where 30 samples contained ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S. The incidence of ZEN in food and feed matrices was 80%. α-ZEL and β-ZEL, respectively, occurred in 53% and 63% of the samples. ZEN-14G (5%) was detected from trace levels up to 274 µg kg^{-1}. One maize sample proved the co-occurrence of ZEN-14G (274 µg kg^{-1}), ZEN-14S (51 µg kg^{-1}), β-ZEL-14G (92 µg kg^{-1}) and a relatively low amount of ZEN (59 µg kg^{-1}), suggesting that approximately 90% of the original ZEN had been metabolized (De Boevre *et al.*, 2012a).

1.3.3.3 Other masked mycotoxins

As shown in **Table 1.2**, other masked mycotoxins than conjugates of trichothecenes and myco-estrogens are prevalent; the most important groups are mentioned in the following section.

Fumonisins are a group of structurally-related *Fusarium* mycotoxins produced mainly by *F. verticilloides* and *F. proliferatum*. The problem of fumonisin contamination is further complicated by the fact that hydrolyzed forms and degradation products such as N-carboxymethyl-fumonisins and N-(1-deoxy- fructos-1-yl)-fumonisin B1 (N-FB1) were found, although the latter was detected in small amounts only, in thermally treated products. More recently, other unidentified bound forms were detected in thermally treated food products

such as cornflakes (Kim *et al.*, 2003; Park *et al.*, 2004; Dall'Asta *et al.*, 2008; Dall'Asta *et al.*, 2009a, 2009b).

To date, occurrence data are still very poor (Falavigna *et al.*, 2012). Dall'Asta *et al.* (2009b) reported the occurrence of bound fumonisins in raw maize and suggested that non-covalent interactions were responsible for the masking phenomenon. For this reason, a clear distinction needs to be addressed to covalently bound fumonisins (bound to starch, proteins and reducing sugars) and non-covalently bound or hidden fumonisins (associative interaction between toxins and matrix macro-constituents). Falavigna *et al.* (2012) were the first to describe co-occurrence of these fumonisin forms.

Kim *et al.* (2003) reported the occurrence of bound fumonisins, while pointing out that a 2.6 times higher content of FB_1 was found after hydrolysis. They observed that after performing alkaline hydrolysis of contaminated corn products (*e.g.* extruded products such as corn flakes) the amount of fumonisins released was often higher than the stoichiometrically expected value. Park *et al.* (2004) revealed in all 30 samples bound FB_1 at significant levels. Dall'Asta *et al.* (2009b) analyzed 21 gluten-free products at concentration levels comparable or higher than those found for the parent forms. The same research group observed in all 97 maize samples 50 µg kg^{-1} to 4,000 µg kg^{-1} (median, 3,520 µg kg^{-1}) free fumonisins, and 50 µg kg^{-1} to 6,900 µg kg^{-1} for total fumonisins (after alkaline hydrolysis). The amount of the latter proved to be significantly higher than fumonisins in 84% of the samples (Dall'Asta *et al.*, 2009b). As shown in **Table 1.2**, fungal metabolites in the form of fumonisin fatty-acid esters were identified in *F. verticillioides*-inoculated rice (Bartók *et al.*, 2010). In plants they play an important role in the formation of hidden fumonisins (Dall'Asta *et al.*, 2012). Also, a relationship between the amount of masked fumonisins and the ratio of oleic/linoleic acid in maize was suggested.

No studies have been published concerning main metabolites of ochratoxin A, ochratoxin α, and 4-hydroxy-ochratoxin A to determine whether or not these derivatives also occur in naturally-contaminated food. Destruxins are cyclic hexadepsipeptides produced by species of *Metarrhizium*, *Alternaria* and *Trichothecium,* which are toxic to a wide range of invertebrates and plants (Soledade *et al.*, 2002). Cruciferous crops hydroxylate destruxins and conjugate the hydroxylated derivatives to glucose, however, no occurrence data are currently available. Fusaric acid is one of the oldest-known mycotoxins. Transformation of fusaric acid to its masked N-methylamide derivative by many plant species was documented extensively half a century ago (Karlovsky, 1999).

1.3.3.4 The fate of processing on mycotoxin conjugates

Agricultural products in the food chain are hardly ever used as such because prior to consumption processing steps including fermentation, chemical hydrolysis or germination are involved. Food processing can potentially influence the mycotoxin levels as such. The use of living cells, and enzymes as well as alkaline or acidic hydrolytic conditions during processing can lead to the liberation or generation of masked mycotoxins (Berthiller *et al.*, 2012).

In food industry, two fermentation processes are relevant: production of microbial cells and transformation of the source material (*e.g.* production of beer, wine or bread) (Stanbury *et al.*, 1999) and production of microbial cells for food production that is limited to a myco-protein made from *F. venenatum* (Quorn®) (Wiebe *et al.*, 2002). During these processes, a variety of enzymes are involved, which are described in **Table 1.3**

Table 1.3 Enzymes involved during food processing

Enzyme	Food processing
amylases	baking, brewing, coffee fermentation, and corn syrup production
proteases	baking and brewing
pectinases	production of bread and coffee
β-glucanases	brewing
amyloglucosidase	corn syrups
pullulanase	corn syrups

The production of beer, glucose syrups, bread, coffee and chocolate and the formation of possible conjugates are discussed in the next paragraph.

As earlier mentioned, DON-3G is frequently occurring in beer (Kostelanska *et al.*, 2009; Varga *et al.*, 2012). Beer production involves two crucial steps: germination and yeast fermentation. Lancova *et al.* (2008) and Kostelanska *et al.* (2009) proved that DON-3G levels increase after malting of barley grains and the contaminant is subsequently transferred into beer. Whereas levels of DON-3G were below the limit of detection (LOD) in the grains, their levels increased during the germination process leading to an accumulation in the germ bud. Indeed, Maul *et al.* (2012) proved that germination induces the glucosylation of DON in various grains. The latter is a waste product of the malting process, not directly consumable for humans, and is used by feed industry and food supplement providers because of its high protein content (Lancova *et al.*, 2008). Kostelanska *et al.* (2009) and Papadopoulou-Bouraoui *et al.* (2004) reported a positive correlation between the mycotoxin levels and the alcohol content. Varga *et al.* (2012) also proved that non-alcoholic beers showed the lowest percentage of contamination (47% and 26% positive samples for DON-3G and DON, respectively). This outcome is probably a consequence of the different technological

processes involved in the production of non-alcoholic beer compared to alcoholic beers. These involve earlier stopped fermentation, the use of specific yeasts and dilution of the beer.

In Asian culture, the daily use of soy sauce is commonplace. The production includes a two-step fermentation process, where *Aspergillus* fermentation is followed by yeast fermentation (Hui, 1999), however, mycotoxin conjugates in soy products have not been described so far.

Glucose syrups (> 90% glucose, industrial use; 10% - 43% confectionery manufacture) are liquids resulting from the hydrolysis of starch with the involvement of bacterial and fungal enzymes. Maize is commonly used as a starch source, although other crops such as potatoes, wheat, barley, rice and cassava can be applied as well. Based on the knowledge of occurrence of masked forms in these crops, no data are currently available.

During the baking process, cereal flour needs to be fermented with yeast or fermenting agents. The production of flour from raw commodities has assured a decrease of the contamination level of both DON-3G and DON (Kostelanska *et al.*, 2011a; Simsek *et al.*, 2012). However, an increase of 145% of DON-3G was observed by adding bakery improvers (*i.e.* enzymes: protease, 16% and xylanase, 39%). The heating process slightly decreased the levels of DON-3G and DON, whereas thermal-degradation products of DON were found. In paragraph 3.3.1.7, an experimental trial concerning the fate of DON and DON-3G under baking conditions is illustrated.

OTA is a prevalent mycotoxin in cocoa and coffee beans. As the production of chocolate and coffee needs to bear roasting, acid fermentation and autoclavation, conjugates of this mycotoxin are presumably formed (Suarez-Quiroz *et al.*, 2004; Mounjouenpou *et al.*, 2008; Abrunhosa *et al.*, 2010). Besides roasting, fermentation and extensive heating, the rinsing of commodities with alkaline liquids (*e.g.* limewater) is an important process to get rid of parent toxins such as DON, ZEN, aflatoxins and fumonisins. Nixtamalisation of maize assures the improvement of maize's nutritional value by the release of niacin from a bound to a parent form. Masked forms have not been described.

In general, food processing implies a reduction of the content of parent mycotoxins. Food processing can however lead to the liberation or generation of masked mycotoxins. So, a crucial concept is the need to minimize the total mycotoxin load in the raw commodity prior to processing. To date, information is still lacking on many state-of-the-art processing steps and the occurrence of masked mycotoxins.

1.3.4 Analytical tools for the detection of masked mycotoxins

Along with the discovery of mycotoxins came an ever increasing need for critical analytical procedures. Thin layer chromatography (TLC) was the most often used procedure for analysis, however rapid expansion of methods became available and high performance liquid chromatography (HPLC) has become the reference method of choice. As mycotoxins were discovered in a variety of matrices, the need for clean up procedures to eliminate interfering substances prior to analysis became more necessary for the variety of expanding technologies for (masked) mycotoxins (Richard, 2012).

Masked mycotoxins may elude analysis because of changed physicochemical properties of their molecules leading to a modified chromatographic behaviour, because of modification of an epitope recognized by antibodies used for the detection, or because of impaired extraction efficiency caused by increased polarity when a less polar solvent is used for the extraction of non-modified mycotoxins. Indeed, all of these effects lead to underestimation of the total mycotoxin content of the sample.

The detection and quantification of mycotoxin concentrations in matrices destined for human and animal consumption is a prerequisite for the general food and feed safety. The objective of any analytical study includes the expeditious and accurate determination of the amount of analytes present. Method selectivity in particular plays a crucial role since the outcome of a false positive may have severe consequences, for example bilateral trade. At the legislative level, data integrity represents key decision making tools and therefore a communal law on the assessment of contaminants in food is required (96/23/EC, 1996).

The analysis for the presence of mycotoxins in matrices is rather complicated, and is a multi-stage process that consists of three distinct phases: sampling, sample preparation and analysis. In the next sub-sections a general summary concerning sampling procedures (1.3.4.1), sample preparation (extraction and clean-up procedures, 1.3.4.2) and analytical techniques (chromatographic, 1.3.4.3-1.3.4.5, and immunochemical methods, 1.3.4.6) applied for the analysis of parent and masked mycotoxins will be pointed out.

1.3.4.1 Sampling

In research, correct decisions concerning the fate of commercial lots can only be made if mycotoxin concentrations in the lot are determined with a high degree of accuracy and precision. The concentration of the mycotoxins of interest in a bulk lot is usually estimated by measuring the mycotoxin concentration in a sample taken from the lot.

The selection method of the sample from the bulk and the size of the sample are crucial. In quality assurance and research, a precise and accurate estimate of the true lot mycotoxin concentration becomes important. However, the true mycotoxin concentration cannot be determined with full certainty. Accuracy and precision are two uncertainties associated with a sampling plan (Cochran and Cox, 1957).

Accuracy or bias (Acc) is the closeness of measured values to the true value. A consistent deviation on the average of the *n* measured values (X_i) influences the true value (V_t) (**Equation 1.1**). Biases occur during the sample selection process, however controlling and reducing biases is difficult not knowing the true mycotoxin concentration in the bulk lot. Continuously testing performance is a prerequisite to reduce any form of bias.

Precision or variability is defined as the closeness of measured values (x_i) to each other. Three statistical measures of variability can be used as a measure of precision: variance (V) (**Equation 1.2**), standard deviation (stdev) (**Equation 1.3**) and variation coefficient (VC) (**Equation 1.4**).

Equation 1.1-1.4 Equations for the determination of the accuracy (1.1), variance (1.2), standard deviation (1.3) and the variation coefficient (1.4)

(1.1)† $Acc = V_t - \frac{X_i}{n}$ **(1.2)** $V = [\sum \frac{(x_i - m)^2}{(n-1)}]$ **(1.3)** $stdev = \sqrt{V}$ **(1.4)** $CV = \frac{stdev}{m}$

† V_t = true value; X_i = average of the *n* measured values; *n* = amount of values; x_i = measured value and m = average of the *n* measured x_i values

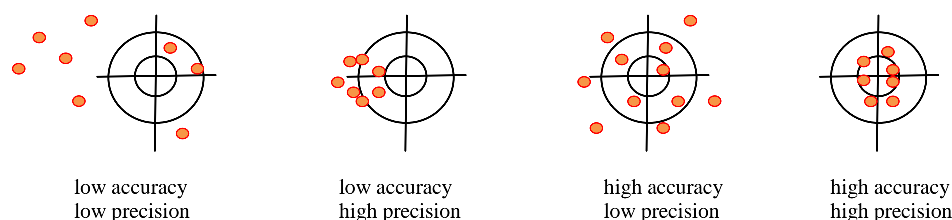


Figure 1.9 Determination of the uncertainty of a process with parameters as accuracy and precision

Describing the uncertainty of a process, various combinations of accuracy and precision can be obtained. The optimal condition of a certain process (*i.e.* sampling plan) is a high accuracy and high precision (**Figure 1.9**).

The sampling step is the largest source of variability associated with the mycotoxin test procedure (Dickens *et al.*, 1979). Even when using accepted sampling selection equipment and random procedures, the sampling error is large because of the extreme mycotoxin distribution among contaminated particles within a lot. Whitaker *et al.* (1974, 1993 and 1998) constructed equations describing the variance for several commodities and mycotoxins. The sampling variance increases in proportion to the concentration and declines with an increase in sample size.

Two types of errors are associated with a mycotoxin sampling plan: acceptable lots (concentration mycotoxin ≤ maximum legal limit or guidance value) result in positive results and will be rejected by the sampling plan (false positives or seller's risk), and bad lots (concentration > maximum legal limit or guidance value) will be accepted (false negatives or buyer's risk). An evaluation of these two risks must be considered to maintain an optimal quality control program **(Figure 1.10)**.

Procedures collecting samples from a bulk lot is of great importance, because every individual item should have an equal chance of being chosen. Contaminated particles are often not uniformly contaminated and variously distributed throughout the lot, therefore a homogenous sampling distribution is crucial. Bauwin and Ryan (1982) stated that a sample is a combination of small incremental samples taken from different locations throughout the bulk. FAO/WHO recommended for each 200 kg of bulk product, 200 g of sample should be collected (FAO, 2001). Commission Regulation No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs clearly stated the method of sampling for cereals and cereal products depending on product and lot weight (401/2006/EC, 2006). The multiple collections need to be blended and subdivided until the desired test or laboratory sample is obtained. Sizes of subsamples, taken from the laboratory samples, vary depending on the particle size; the smaller the particle size, the more homogeneous the sample, the less increasing error or uncertainty (Maestroni and Cannavan, 2011).

Sampling selection methods differ depending on whether the lot is either static or dynamic. Static lots are commodities which are stored in trucks, silos or containers. Samples from different locations in the lot should be collected, regarding the 200/200 g kg^{-1} ratio, to obtain a representative sample. Random sampling through dynamic lots (*i.e.* conveyor belt) is more nearly achievable because increments are taken across the entire length at periodic intervals of the moving stream.

To counter problems concerning variability in the sample particle size and selection methods, novel sampling methods need to be developed. Recently, Sanders *et al.* (2013) described a promising technique by collecting wheat dust instead of wheat kernels. A correlation between the mycotoxin contamination level in the wheat dust and the grain kernels was observed.

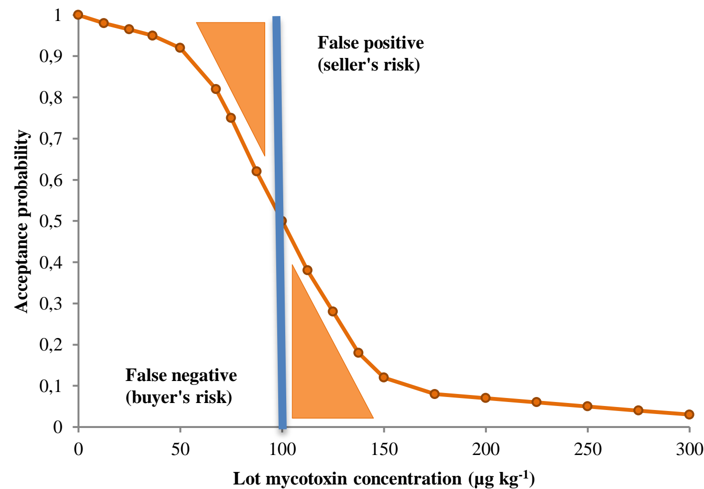


Figure 1.10 Operation Characteristics (OC) curve for the acceptance probability of mycotoxin sampling in a bulk lot (*e.g.* guidance value ZEN, 100 µg kg^{-1}), defined with possible outcomes when lots are classified as accepted or rejected.

As a conclusion, the sampling procedure accounts for up to 82% of variability. The main causes are the small contamination percentage of the kernels, and the difficulty to obtain a representative amount of contaminated kernels into the analytical sample on the basis of small sample sizes (Whitaker *et al.*, 1998). Sample preparation, described in the next paragraph, accounts for 9% of the total variability of a test procedure and can be reduced by increasing the subsample size and grinding into finer particles.

1.3.4.2 Extraction and clean-up procedures

Analytical methods based on chromatography or immunoassays require solvent extraction to liberate the mycotoxins of interest from the sample matrix, and the subsequent clean-up of the extract in order to reduce matrix interferences (Krska *et al.*, 2008). Extraction should be quantitative, preferably specific to the (group of) target analytes and compatible with the analytical method used. Combinations of solvents with the addition of modifiers are applied for extraction based on the physicochemical properties of (masked) mycotoxins, the matrix and type of the used clean-up (Zöllner and Mayer-Helm, 2006). Two types of extraction are considered: liquid-liquid extraction (LLE) and solid-liquid extraction (SLE).

LLE consists of two immiscible liquid phases where mycotoxins will migrate in the extraction solvent until equilibrium is reached (*i.e.* soluble matrices). The desired compounds are then concentrated and interferences can already partially be eliminated. SLE is used when the sample is available in solid form (*e.g.* cereal-based foods), where (masked) mycotoxins are dissolved in the extraction solvent in the solvent mixture. Mostly organic solvents/water mixtures are used such as methanol/water (v/v) and acetonitrile/water (v/v) for the extraction of (masked) mycotoxins from food and feed. Extraction mixtures for the determination of DON and ZEN and their naturally-occurring masked forms are based on acetonitrile and water in different ratios (**Table 1.4**), eventually acidified with acetic acid or formic acid. The use of dichloromethane showed minimization of matrix interferences and extraction of both polar and less polar mycotoxins, however, the use of chlorinated solvents is discouraged today. Procedures used for extraction are shaking and blending, afterwards the extracts are filtered and analyzed with or without prior solid phase extraction (SPE) clean-up.

Following extraction, the resulting raw extract is usually further processed to remove interferences and for preconcentration, and makes the identification of (masked) mycotoxins as unambiguous as possible (Rosenberg *et al.*, 1998). Matrix components such as lipids, chlorophyll, carbohydrates and peptides make an additional purification step necessary prior to the ultimate detection. Commonly-used purification methods in the determination of (masked) mycotoxins employ liquid-liquid separation (LLS), SPE, immunoaffinity columns (IAC), and one-step multifunctional clean-up columns (Mycosep®) (Trucksess, 1994).

LLS is based on the partitioning between immiscible solvents, one of which contains the analyte. The analyte or interferences migrate into the other phase until equilibrium has been reached. As LLS is a batch method, automation is not possible, therefore the method is often

replaced by SPE or IAC. An example of LLS is the addition of hexane to counter lipophilic interferences (De Boevre *et al.*, 2012b).

SPE and IAC require a sequence of three to four steps: preconditioning, retention of the extracted substances on the packing material of the column, washing of undesirable compounds and elution of analytes of interest. SPE methods have been developed for a number of mycotoxins as a convenient alternative to LLS (Langseth and Rundberget, 1998). C8 and C18-bonded silica columns are most frequently used as they are pressure resistant and show high reproducibility. Anionic compounds (*e.g.* fumonisins and OTA) can be isolated by Strong Anionic Exchange (SAX) columns via the ion exchange mechanism. The use of IAC has become increasingly popular in recent years as a high specificity is guaranteed owing to the antibody-mycotoxin interaction, minimal interferences and low detection levels (Visconti *et al.*, 1998). However, in masked mycotoxin analysis there is a lack of appropriate IAC-columns (1.3.4.6). Nevertheless, very recently, a new method was presented for the determination of DON, DON-3G and 3-ADON in beer, based on a DON-specific immunoaffinity column (DONPREP™) (Kostelanska *et al.*, 2011b).

Mycosep® columns consist of adsorbents, packed in a plastic tube. Romer (1986) developed these columns featuring a forcing of the extract through the packing material when the column is inserted into the culture tube. Mycosep® columns offer advantages of speed, simplicity, solvent efficiency and in some cases increased recovery and lower costs. This column was tested for the clean-up of DON-3G in wheat and maize obtaining a recovery of 59 ± 11% (Berthiller *et al.*, 2005), while Sasanya *et al.* (2008) observed 70% for the same analyte with a purification step on a C18 cartridge. Using Multisep® 227 clean up, Kadota *et al.* (2003) obtained good recoveries ranging from 84% to 115% for trichothecenes and DON-3G.

Another approach worth highlighting is the QuEChERS procedure (Quick, Easy, Cheap, Effective, Rugged and Safe). The key principle is the partitioning of an acetonitrile/water mixture induced by addition of organic salts (*e.g.* NaCl and $MgSO_4$). Anastassiades *et al.* (2003) described that analytes are transferred into the organic phase, where more polar matrix impurities are left in the aqueous layer. To date, only Miller and Greenhalgh (1988) proved the effectiveness of this method for the extraction and purification of several mycotoxins, unfortunately, based on other studies DON-3G showed poor recovery value, probably on account of its high polarity which does not effectively allow the transfer into the acetonitrile layer.

Extractable conjugated mycotoxins are mostly more polar than their parents, therefore SPE clean-up techniques might not be suitable in multi-mycotoxin analysis. In any case, the wide polarity range of the considered analytes did not allow the adoption of a single clean up strategy. As more sensitive LC-MS/MS equipment has become available, it is convenient to replace clean-up by diluting the sample extracts (Berthiller *et al.*, 2009b). Typically, extracts obtained from the acetonitrile/water/acetic acid (v/v/v) solvent system were suitable for LC-MS/MS analysis without further clean-up (Sulyok *et al.*, 2006).

In **Table 1.4** an overview is given as to the sample extraction and clean-up methods currently used for masked mycotoxin analysis for trichothecenes and myco-estrogens.

Concerning hidden fumonisins, extraction and clean-up are based on an alkali treatment of the sample which induces the loss of the TCA side chains of fumonisins, releasing the hydrolyzed fumonisins (HFBs). Using this approach it is possible to determine the amount of bound or hidden forms. An improvement of the procedure by adding 1% sodium dodecylsulfate (SDS) for protein dissolution and 2N KOH for hydrolysis was proposed by Park *et al.* (2004). The hydrolyzed sample was then extracted with different solvents (methanol-ethylenediamine tetracetic acid 0.01 M or methanol/acetonitrile/water (25/25/50, v/v/v) and finally cleaned up by OASIS™ HLB columns. Recently, Dall'Asta *et al.* (2011) proposed the simultaneous determination of the main fumonisins (FB1, FB2 and FB3) and their masked derivates as hydrolyzed forms (HFB1, HFB2 and HFB3) in maize and maize-based foods without any sample clean-up. The residue obtained from the determination of free forms underwent a hydrolysis step with 2 N NaOH for 60 min at 25 °C. The aqueous phase was then extracted with ethyl acetate, dried under nitrogen stream and redissolved in water/acetonitrile before LC-MS/MS analysis.

1.3.4.3 Chromatographic methods

The occurrence of masked mycotoxins leads to a general underestimation of the total mycotoxin content in the sample. Subsequent failure to recognize hazardous contamination by mycotoxin monitoring implies the potential exposure of the consumer to doses exceeding tolerable limits. A solution to this problem is to extend current analytical methods for mycotoxins by including conjugates and other relevant derivatives. For all these reasons, several methods mainly based on mass spectrometry have been developed for the determination of masked mycotoxins in food and feed (Cirlini *et al.*, 2012).

Analytical procedures have improved continuously over the past years. Within the field of mycotoxin analysis, an increasing trend was observed involving the transition from traditional TLC, gas chromatography (GC) with electron capture detection (ECD), (ultra) high performance liquid chromatography ((U)HPLC) with fluorescence and UV detection strategies, towards procedures involving the application of highly accurate quantitation tools such as mass spectrometry (MS) (Richard, 2012).

The TLC approach was popular due to its coherent features such as line principle, higher sample throughput, lower operating costs, convenience in identification by comparison of spot color and fast application, however the application remained too insensitive. Lin *et al.* (1998) reviewed several TLC applications for mycotoxin analysis; TLC for masked mycotoxin analysis is not suitable as these occur mostly in lower concentrations than their parent form.

Main focus in analysis of masked mycotoxins is liquid chromatography-tandem mass spectrometry (LC-MS/MS), however GC methods are also at hand for the quantification of trichothecenes, ZEN, OTA and fumonisins (Krska *et al.*, 2001; Turner *et al.*, 2009). As derivatization is needed to render the mycotoxins volatile, extractable glucosylated derivatives are far too much polar for derivatization. For this reason, only methods for acetylated forms, 3-ADON and 15-ADON, are described. These are derivatized by means of a trimethylsilylimidazole (TMS) reagent containing TMS-trimethylchlorosilane-ethyl acetate (10/90, v/v/v) (Tanaka *et al*., 2000). Tran and Smith (2011) were the first to record an indirect determination of masked DON using GC-MS via hydrolysis with trifluoromethanesulphonic acid. However, the application was not suitable to identify any masked forms.

LC-MS/MS is a hyphenated detection technique, and is particularly useful for the simultaneous determination of multiple (masked) mycotoxins. Moreover, as only three derivatives (DON-3G, 3-ADON and 15ADON) are nowadays commercially available as

reference standards to be used for monitoring purposes, the identification power of MS detection may be fully exploited for the identification of unknown derivatives as well (Berthiller *et al.*, 2007).

LC is a dynamic separation technique, partitioning mixtures of *in casu* mycotoxins. The basis of separation of target molecules is the difference in affinity between the mobile phase and stationary phase (Baars, 1996). The composition and nature of these two phases are crucial to achieve the desired separation of target compounds. The most frequently used stationary phase is silica gel allowing different bonded phases to be formed by reaction of the surface OH-groups of the silica gel with an appropriate reagent that chemically links an organic moiety to the silica surface (Monbaliu, 2011). The nature of organic moiety will determine the type of interaction that will take place between the solution and surface (Scott, 2003). The decrease of the particle size of the chromatographic column (*e.g.* 1.7 µm) will lead to a gain of efficiency and increased pressure (*i.e.* UHPLC).

MS provides high detection selectivities owing to the ability to separate or filter ions according to their mass-to-charge ratios (*m*/*z*). MS includes ionization which implies a conversion of sample molecules into ions in gas phase. In LC-MS applications electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are widely used. ESI involves the transfer of analytes, generally ionized in the condensed phase, into the gas phase as an isolated entity. APCI is a supplementary soft ionization technique to electrospray. This technique is used to analyze smaller, thermally stable polar and non-polar compounds because multiple charged ions are not generated in APCI, and it operates at high temperatures (350 °C - 550 °C). Two major factors determining source selection are polarity and molecular weight of the compound. Generally speaking, ESI requires the presence of pre-formed ions in solutions and is therefore better suited to the analysis of polar compounds; ESI should also be chosen to run high molecular weight analytes as these types of compounds are less likely to survive hot vaporizer temperatures of APCI. Also, this type is often employed to ionize polar mycotoxin metabolites, which is crucial for charged metabolites (*e.g.* ZEN-14S), because APCI cannot transfer these charged ions into the gas phase. APCI has been used for the ionization of DON-3G (Berthiller *et al.*, 2005; Lancova *et al.*, 2008; Kostelanska *et al.*, 2009; Zachariasova *et al.*, 2008).

Before the ions are amplified and recorded according to the current intensities at each mass in a mass spectrum, the ions are separated according to their *m*/*z*-ratio. During this research, a Quattro Premier XE® MS/MS was applied, consisting of two mass analyzers or quadrupoles

(Q) and a T-wave collision cell. Collision cells differ according to the type of instrument. T-wave technology minimizes ion transit times and provides optimum performance for fast analyses, high speed and sensitivity analyses (Baars, 2001). In combination with selected ion monitoring (SIM) or selected reaction monitoring (SRM), highly structure-specific detection of (masked) mycotoxins can be achieved, even with complex matrices (Berthiller *et al.*, 2006). During SRM a specific ion (precursor ion) is selected in the first quadrupole (Q1) from among various ions ionized by the ionization probe, subsequently the ion is destructed (collision-induced dissociation, *in casu* T-wave) in the collision cell, and a specific ion is detected from among the fragmented ions (product ions) in Q2. An illustration of the sequence of ionized ions in the Quattro Premier XE® MS/MS is shown in **Figure 1.11**.

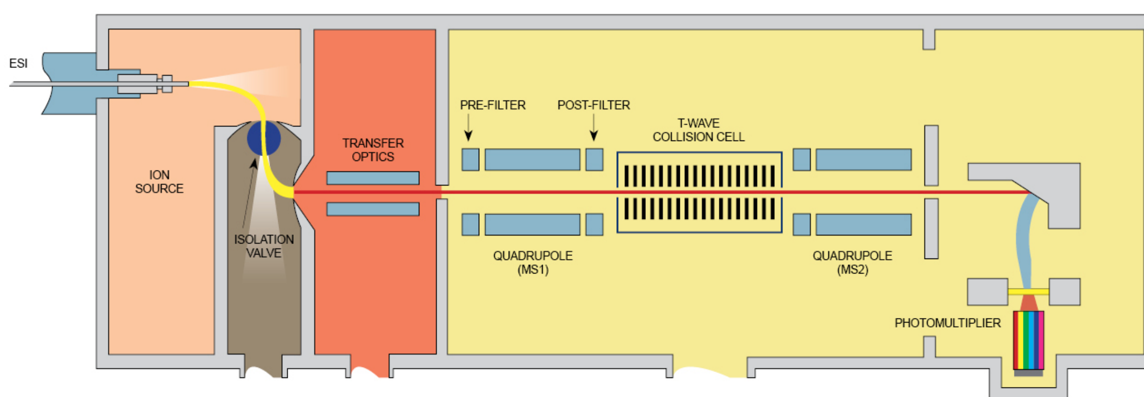


Figure 1.11 The sequence of ionized ions in the Quattro Premier XE® MS/MS

The above-mentioned selectivity, in addition to the resolution of chromatographic separation, adds great value to this technique. To confirm the presence of mycotoxins, using LC-MS/MS, two transitions between the precursor and fragments should be monitored. According to the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, a system of identification points should be applied to interpret the data (2002/657/EC, 2002). For the confirmation of substances listed in Group B of Annex I of Directive 96/23/EC (*i.e.* mycotoxins), a minimum of 3 identification points are required. These include the incorporation of 1 precursor ion and 2 fragment ions. In terms of identification and confirmation, the first criterion is that the relative retention time, relative to an internal standard (IS), should not exceed 2.5%. An IS implies a substance not contained in the original sample with physical-chemical properties as similar as possible to those of the analyte that has to be identified, and which is added to each sample as well as to each calibration standard. The addition of an IS is done to correct for the loss of analyte during sample preparation or

sample inlet. The second identification point involves that the relative abundance of both transitions should not exceed the range of 20% to 50%, depending on the relative intensity between the transitions. Also, all SRM-transitions should possess a signal-to-noise (*s/n*) ratio higher than 3:1 (2002/657/EC, 2002).

In conclusion, LC-MS/MS offers a powerful tool for the identification and characterization of polar non-volatile mycotoxins and their conjugates. In **Table 1.5** an overview is pointed out of current LC columns and MS methodologies used for masked mycotoxin analysis (Cirlini *et al.*, 2012; Berthiller *et al.*, 2012).

A wide series of MS-based multi-mycotoxin methods were recently proposed as described in **Table 1.5**. Berthiller *et al.* (2005) were the first to describe the occurrence of DON and its masked forms using QTrap® LC–MS/MS and APCI ionization (structural information) and UV detection (quantification). Same authors (2006) used the same instrumentation for the analysis of masked ZEN forms, however, SRM was selected for the analysis, while for identification and characterization of the derivatives enhanced product ion (MS/MS) and multiple mass spectrometry (MS^3) modalities were chosen.

For most masked mycotoxins the majority of LC-MS methods relies on simple dilute and shoot strategies and reversed-phase chromatography (Berthiller *et al.*, 2012). The use of hydrophilic interaction liquid chromatography (HILIC) was proposed as the applicability for the retention of polar compounds was oftentimes proven (Liu *et al.*, 2010; Liu *et al.*, 2011). However, De Boevre *et al.* (2012b) tested three columns with HILIC properties (XBridge® HILIC, Discovery® HS F5 and TSKgel® Amide-80), and no satisfactory retention of polar compounds such as DON-3G was obtained.

Sulyok *et al.* (2006) described a method for the determination of 39 parent and masked mycotoxins in 2 consecutive chromatographic runs (ESI^- and ESI^+), while Vendl *et al.* (2009) delineated an LC-MS/MS method using both ESI^- and APCI probes for the simultaneous determination of 8 masked forms in one ESI^--run.

The methods were performed on a Qtrap® LC–MS/MS, fully exploiting the selectivity of MS detection and avoiding any clean-up steps in an oversimplified extract.

Table 1.4 Variety of extraction and clean-up used in masked mycotoxin analysis

Matrix	Masked analyte	Solvent	Clean-up	Reference
Wheat and maize kernels	DON-3G	ACN[†]/H_2O (84/16, v/v)	Mycosep® 230	Schneweis *et al.*, 2002
Arabidopsis thaliana	ZEN-14G and ZEN-14S	ACN/H_2O (75/25, v/v)	None	JEFCA, 2010
Cereals	DON-3G, ZEN-14G and ZEN-14S	ACN/H_2O (HOAc[‡]) (v/v)	None	Dall'Asta *et al.*, 2009b
Beer, porridge, pasta and corn flour	DON-3G, ZEN-14G and ZEN-14S	ACN/H_2O (1% HOAc) (v/v/v)	C18-SPE, IAC and Mycosep® 226 and 230	Kostelanska *et al.*, 2010
Cereal based food	DON-3G, ZEN-14G and ZEN-14S	ACN/H_2O/HOAc (v/v/v)	None	Scoop, 2003
Certified matrix, wheat and barley	DON-3G	ACN/H_2O/0.1% FA[§] (v/v/v)	None	Kostelanska *et al.*, 2011
Beer and malt	DON-3G	Deionized H_2O, ACN/H_2O (84/16, v/v)	IAC DONPREP™	Sasanya *et al.*, 2008; Zachariasova *et al.*, 2010
Beer	DON-3G	ACN	None	Varga *et al.*, 2012
Wheat and maize	DON-3G	ACN/H_2O (84/16, v/v)	Mycosep® 230	Berthiller *et al.*, 2005
Wheat	DON-3G	MeOH/dichloromethane (50/50, v/v)	Strata-X®	Sasanya *et al.*, 2008
Malts (beer)	DON-3G	ACN/H_2O (84/16, v/v)	Mycosep® 226	Lancova *et al.*, 2008
Malts (beer)	DON-3G	ACN/H_2O (84/16, v/v)	None	Kostelanska *et al.*, 2010
Maize	DON-3G	ACN/H_2O/HOAc (various) (v/v/v)	None	Vendl *et al.*, 2009
Maize and maize products	DON-3G	ACN/H_2O (84/16, v/v)	None	Wei *et al.*, 2012
Wheat	DON-3G	ACN/H_2O (84/16, v/v)	Various ELISA	Zachariasova *et al.*, 2008
Wheat	ZEN-14G	ACN/H_2O (84/16, v/v)	Florisil® Bond Elut	Schneweis *et al.*, 2002
Maize	ZEN-14S	ACN/H_2O/HOAc (various) (v/v/v)	None	Vendl *et al.*, 2009
Maize, wheat, oats, bread and breakfast cereals	DON-3G and masked ZEN-forms	ACN/H_2O/HOAc (79/20/1, v/v/v)	Hexane defatting	De Boevre *et al.*, 2012b (*Chapter 2*)

Table 1.5 Overview of LC columns, solvents and MS mode used in masked mycotoxin analysis

Masked analyte	Column	Solvent	MS	Mode	Reference
DON-3G	Aquasil® RP18, 100 x 4.6 x 3	MeOH/H_2O (85/15) (v/v)	QTrap® MS/MS	APCI$^-$	Berthiller *et al*, 2005
DON-3G	Synergy® fusion, 150 x 4.6 x 4	MeOH/H_2O (70/30) (v/v)	QP8000® MS/MS	Full scan$^+$	Sasanya *et al.*, 2008
DON-3G	Gemini® C18, 100 x 4.6 x 5	MeOH/HOAc (v/v)	QTrap® MS/MS	ESI$^-$	Berthiller *et al.*, 2009
DON-3G	Gemini® C18, 100 x 4.6 x 5	MeOH/H_2O/HOAc + 5 mM ammonium acetate (v/v/v)	QTrap® MS/MS	ESI$^-$	Varga *et al.*, 2012
DON-3G	Synergy® hydro RP, 100 x 3 x 4	MeOH/H_2O/HOAc (v/v/v)	LCQ® MS/MS	APCI$^\pm$	Lancova *et al.*, 2008
DON-3G	Synergy® hydro RP, 100 x 3 x 4	MeOH/H_2O/HOAc (v/v/v)	LCQ® MS/MS	APCI$^\pm$	Kostelanska *et al.*, 2009
DON-3G	Synergy® hydro RP, 150 x 3 x 4	MeOH/H_2O/HOAc (v/v/v)	LCQ® MS/MS	APCI$^-$	Zachariasova *et al.*, 2008
DON-3G	Ultrasphere® ODS, 150 x 4.6 x 5	MeOH/H_2O (15/85, v/v))	UV- QTrap® MS/MS	APCI$^-$/ESI$^-$	Schneweis et al., 2002
DON-3G	Acquity UPLC® HSS T3, 100 x 2.1 x 1.8	Ammonium formate/MeOH (v/v)	TOF-MS, Orbitrap™-MS	ESI$^\pm$	Kostelanska *et al.*, 2011
DON-3G	Acquity UPLC® HSS T3, 100 x 2.1 x 1.8	Ammonium formate/MeOH (v/v)	TOF-MS, Orbitrap™-MS	ESI$^-$	Sasanya *et al.*, 2008; Zachariasova *et al.*, 2010
ZEN conjugates	Aquasil® RP18, 100 x 4.6 x 3	MeOH/HOAc (v/v)	QTrap® MS/MS	ESI$^-$	Berthiller *et al.*, 2006
ZEN-14G	Nucleosil® C18, 120 x 125 x 2	ACN/FA (v/v)	VG® Quadrupole	ESI$^+$	Schneweis *et al.*, 2002
Multi-masked	Synergy® Polar RP18 150 x 3 x 4	ACN/H_2O + 5 mM ammonium acetate (v/v)	QTrap® MS/MS	ESI$^-$	Vendl *et al.*, 2010
Multi-masked	Synergy® Polar RP18, 150 x 4.6 x 5	ACN/HOAc (v/v)	QTrap® MS/MS	ESI$^-$	Vendl *et al*, 2009
Multi-masked	Gemini® C18, 150 x 4.6 x 5	MeOH/HOAc (v/v)	QTrap® MS/MS	ESI$^\pm$	Sulyok *et al.*, 2006
Multi-masked	Zorbax® XDB C18, 100 x 4.6 x 3.5	MeOH/H_2O/HOAC (v/v/v)	Quattro Premier XE® MS/MS	ESI$^+$	De Boevre *et al.*, 2012b (*Chapter 2*)
Multi-masked	XTerra® Waters C18, 250 x 2.1 x 5	MeOH/H_2O/HOAc + 5 mM ammonium acetate (v/v/v)	QTrap® MS/MS	ESI$^\pm$	Dall'Asta *et al.*, 2009b
Multi-masked	Synergy Hydro RP, 150 x 3 x 4	ACN/H_2O + 5 mM ammonium acetate (v/v)	QTrap® MS/MS	APCI$^-$/ESI$^-$	Kostelanska *et al.*, 2010

[†]ACN = acetonitrile; [‡]HOAc = acetic acid; [§]FA = formic acid

Zachariasova *et al.* (2010) used a novel approach using both UHPLC/Time of flight (TOF)-MS and UHPLC/Orbitrap-MS systems. The chromatographic conditions were tested in order to obtain an optimal peak separation with a ternary mobile phase system composed of 5 mM ammonium formate (pH 5.6), 5 mM ammonium formate containing 0.1% of formic acid (pH 2.7) and methanol. TOF-MS has a high resolving power and is sensitive in full scan modes. This can target analytes but also provide empirical formulae of unknown compounds, while tandem MS can help identifying mycotoxin conjugates through precursor ion and neutral loss scans (Berthiller *et al.*, 2012).

Kostelanska *et al.* (2009) delineated a short UPLC method for the determination of masked DON forms in beer, using TOF-MS (ESI^-). Same authors developed a comparable method using UPLC/Orbitrap-MS for the same type of analysis in wheat.

De Boevre *et al.* (2012b) developed a single chromatographic run in ESI^+-mode for *Fusarium* mycotoxins DON, ZEN, T-2 and 10 derivatives thereof using a Quattro Premier XE® MS/MS (*Chapter 2*).

All the described methods were applicable for either masked ZEN or DON forms or both. Concerning hidden fumonisins the same detection approaches were applied. Dall'Asta *et al.* (2009b) described an LC-MS/MS system in maize and gluten-free foods with an ESI^+ interface. The chromatographic conditions used water and methanol as eluents, both having been added with 0.1% of formic acid. The method allowed determining parent and hidden fumonisins without any clean-up.

1.3.4.4 MS fragmentation of trichothecenes and myco-estrogens

A literature overview of possible MS fragmentation patterns of trichothecenes and myco-estrogens, mycotoxins included in this PhD-dissertation, is presented in the following section.

Trichothecenes

Possible fragmentation patterns of trichothecenes (*in casu*, DON (1) and T-2 (2)) are presented in **Figure 1.12**. Collision-induced dissociation (CID) of extractable conjugated mycotoxins in MS/MS often yields the parent toxin ion or fragments thereof. The neutral loss of the polar conjugate (*e.g.* glucose-function, *m/z* 162) is considered for DON-3G and glucosylated masked forms of other trichothecenes. Possible fragment pathways are proposed according to the parent ion. The possible neutral losses by CID found both for type A and B trichothecenes are pointed out in **Table 1.6**.

Table 1.6 Overview of the loss of molecules by CID of trichothecenes (Berger *et al.*, 1999)

Mass unit	Neutral molecule	Possible interpretations	Mass shift	Trichothecene
-18	H_2O	OH-function	*m/z* 319 to 301	*type B*
-28	CO	Epoxide-function or formaldehyde (type B)	*m/z* 231 to 203	*type A and B*
-36	H_2O, H_2O	OH-function	*m/z* 297 to 261	*type B*
-42	CH_2CO	Acetyl-function	*m/z* 125 to 83	*type A and B*
-48	H_2O, CH_2CO	OH-function	*m/z* 297 to 249	*type B*
-60	CH_3COOH	Acetyl-function	*m/z* 400 to 339	*type A*
-66	CH_2O, H_2O, H_2O	OH-function and acetyl-function	*m/z* 297 to 231	*type B*
-102	$(CH_3)_2CHCH_2COOH$	Iso-valeryl-function	*m/z* 467 to 365	*type A*
-120	CH_3COOH, CH_3COOH	Acetyl-function	*m/z* 365 to 245	*type A*
-162	$C_6H_{12}O_6$	Glucose-function	*m/z* 459 to 297	*type A and B*

Myco-estrogens

An overview of loss of neutral molecules by CID of myco-estrogens (*e.g.* ZEN) is presented in **Table 1.7**. Possible fragmentation patterns of myco-estrogens are presented in **Figure 1.13**. As already described for masked trichothecenes, neutral losses of the polar conjugates for ZEN-14G and ZEN-14S (*e.g.* glucose or sulfate, *m/z* 162 and *m/z* 81, respectively) are considered. The structure of the parent form then provides possible fragmentation pathways. The protonation of ZEN occurs in two different ways, leading to diverse fragments (**Figure 1.13, 1** and **2**) (Filho *et al.*, 1997).

Table 1.7 Overview of the loss of molecules by CID of myco-estrogens (Filho *et al.*, 1997)

Mass unit	Neutral molecule	Possible interpretations	Mass shift
-18	H_2O	OH-function	*m/z* 319 to 301
-28	CO	Keto-function	*m/z* 231 to 203
-42	CH_2CO	Acetyl-function	*m/z* 203 to 161
-70	$CH_2COC_2H_4$	Acetyl-function	*m/z* 125 to 55
- 81	OSOOH	Sulfate-function	*m/z* 400 to 319
-112	$C_5H_{10}COCH_2$	Acetyl-function	*m/z* 301 to 189
- 162	$C_6H_{12}O_6$	Glucose-function	*m/z* 481 to 319

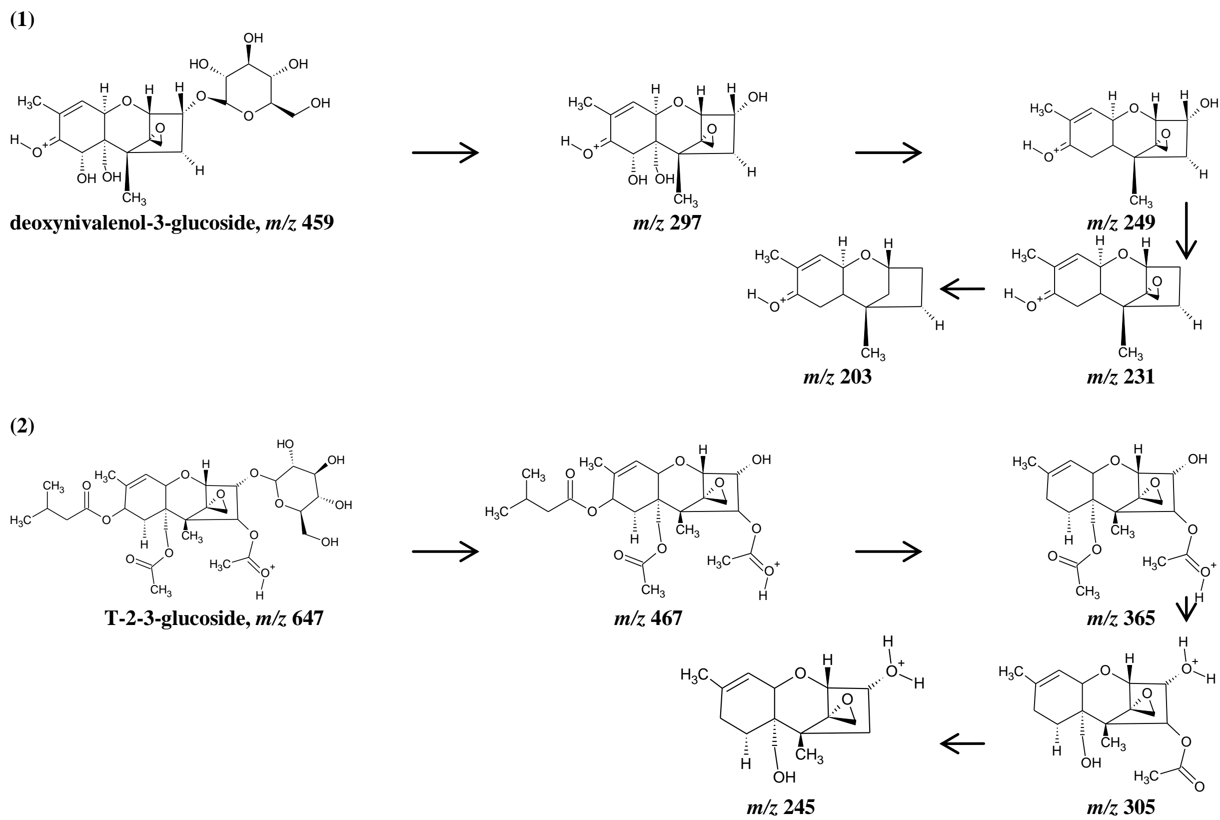


Figure 1.12 Fragmentation patterns of trichothecenes, example of deoxynivalenol (1) (Berger *et al.*, 1999) and T-2-toxin (2)

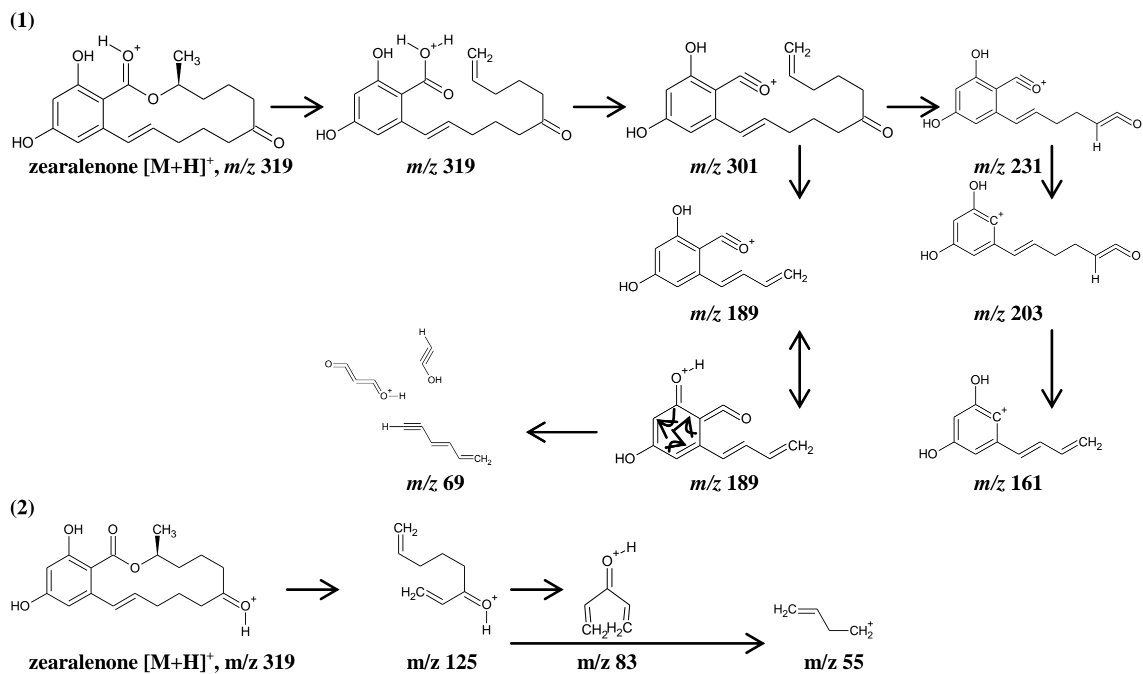


Figure 1.13 Fragmentation patterns of myco-estrogens (Filho *et al.*, 1997)

1.3.4.5 Strategies to detect unknown conjugated mycotoxins

The unavailability of reference standards of masked conjugated mycotoxins and the possible occurrence of unknown masked forms involve two major complications. The importance of detection of these unknowns is crucial as occurrence in food and feed implies a possible safety risk.

Currently, more than 400 mycotoxins are known with numbers increasing with the discovery of masked mycotoxins. Due to the complexity of different chemical structures of secondary metabolites, detection of these compounds remains a challenge. As already described, multi-mycotoxin methods nowadays are based on the separation by LC-MS/MS either by analysis of preselected compounds with triple quadrupole MS or by the untargeted detection in full scan mode with HRMS.

Compared to LC-MS/MS, the preselection of targeted compounds is no prerequisite and compound detection depends on the predefined full scan range. Another advantage of HRMS is that acquired data can even be evaluated retrospectively for additional compounds. Also, parallel to full scan detection, fragmentation experiments using higher energy collision dissociation (HCD) enable detection of fragment ions for compound confirmation where analytical standards are available. Recently, Lattanzio *et al.* (2012b) proved comparable performance of HRMS and MS/MS with the additional advantage of retrospective data evaluation with HRMS. In spite of the limited structural information obtained by mass spectrometry, this technique is successfully used in the structure elucidation of metabolites, since additional information such as the structure of the parent compound and its general fragmentation pathway are usually available.

HRMS followed by nuclear magnetic resonance (NMR) detection and/or other spectroscopic measurements (*e.g.* UV or IR) is the method of choice for the isolation of unidentified conjugated compounds with one-dimensional (1D)- or two-dimensional (2D)-NMR measurements, required for the unambiguous structure elucidation (Cirlini *et al.*, 2012). However, when a conjugate can only be identified by means of MS due to the small amount available, the compound should be synthesized using a chemical and/or a biochemical strategy. After full characterization of the natural-identical molecule, its spectral behaviour should be compared with that recorded for the natural conjugate to univocally assign the chemical structure of the novel conjugate. Moreover, the application of multiple tandem mass spectrometric experiments (MS^n) is well suited for structure elucidation of conjugates since they permit the differentiation between the fragments of the conjugate and of the precursor, by

investigation of the CID in MS^n spectra. In paragraph 1.3.4.4 possible fragmentation patterns are described for several mycotoxins. The acquired fragmentation pattern can be a useful tool to confirm the existence of the parent mycotoxin. In detail, precursor ion scans allow to find out higher molecular weight conjugates when the mass of the parent mycotoxin is fixed in the third quadrupole. Moreover, neutral loss scan experiments identify the loss of a well-known group from the conjugate, such as glucose or sulfate (*m/z* 162 and 81, respectively). However, low sensitivity over a wide mass range is frequently observed so a better sensitivity can be achieved by performing product ion scans which requires the presumption of the mono-isotopic mass of the unknown conjugated ions ($[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$) (Berthiller *et al.*, 2012).

Hence, the method of choice for screening unknown conjugates is the recording of full scans over a wide mass range (*m/z* 200 to 1,000). Instruments which can offer accurate masses are Time-of-Flight (TOF) and Orbitrap technology. The latter techniques provide high-resolution data of the molecular and fragment ions, being thus particularly powerful for structure elucidation of unknown compounds. During TOF, ions are accelerated by an electrical field to equal kinetic energy with the velocity of the ion depending on *m/z*. Inside the Orbitrap ions are electrostatically trapped while rotating around the central electrode and performing axial oscillation. The frequency of oscillation and hence of the induced current is specific, given the *m/z*. The frequency and amplitude of the induced current can be then converted to a spectrum. These instruments are superior to quadrupole mass spectrometers in terms of full-scan sensitivity, mass accuracy, parallel detection and resolving power (Perry *et al.*, 2008; Berthiller *et al.*, 2012).

To date, this technology has rarely been applied for novel masked mycotoxin detection because other additional post-data acquisition mathematical tools need to be used to perform molecular identification. Nakagawa *et al.* (2012) applied this technology for the identification of two new *Fusarium* mycotoxin conjugates, namely fusarenon X-glucoside and nivalenol-3-glucoside. Identification was based on accurate mass measurement of characteristic ions and MS/MS fragmentation patterns, although the absolute structure could not be clarified.

In contrast to Orbitrap, TOF instruments have frequently been used to confirm postulated chemical formulas of mycotoxin conjugates such as T-2-3-glucoside (T-2-3G) and HT-2-3-glucoside (HT-2-3G) (Busman *et al.*, 2011), verrucarin A-glucoside, roridine-A, D and E-glucosides (Jarvis *et al.*, 1991), and palmitoyl-conjugates of ZEN, DON and T-2 (Chakrabarti *et al.*, 1986)).

Cirlini *et al.* (2012) mentioned the problems of detection related to associative binding between mycotoxins and macromolecules such as starch or proteins. The study of this interaction is usually quite troublesome, since indirect methods should be used. When proteins of known tridimensional structure are involved, the application of an *in silico* docking method is probably helpful for supporting the interaction hypothesis. *In silico* docking techniques are typically being used to investigate the complementarity at the molecular level of a ligand (*i.e.* mycotoxin) and a protein target. Docking is a term used for computational schemes that attempt to predict the structure of the intermolecular complex formed between two or more constituent molecules: a receptor and a ligand (Sousa *et al.*, 2006). The receptor is most of the time a protein, while the ligand can be another protein or a small molecule (*i.e.* mycotoxin). The problem that molecular docking has to solve can be defined as follows: to give the atomic coordinates of two molecules, and predict their correct bound association. As such, docking studies can be used to understand and to identify the structural features that are important for binding and for *in silico* screening efforts in which suitable binding partners can be identified.

1.3.4.6 Immunochemical methods

In the last decade, immunochemical methods have been increasingly used in the food and feed analysis sector. Tests for mycotoxins which allow for the screening of agricultural commodities with results within less than 20 minutes are gaining acceptance, and are progressively being integrated into routine quality monitoring procedures (Delmulle *et al.*, 2005). Immunochromatographic assays, lateral flow tests or strip tests have received increasing market attention (Zheng *et al.*, 2006).

Enzyme-linked immunosorbent assays (ELISAs) have become a standard tool for the rapid monitoring of mycotoxins (Molinelli *et al.*, 2009). They offer the advantage of speed, ease of operation, on-site application, sensitivity and high sample throughput. Lateral-flow devices are based on a test format which includes sample flow along an analytical nitrocellulose membrane due to capillary forces and allows performing fast and easy to handle immunoassays. Strip tests are based on a competitive immunoassay format using a labeled antibody as signal reagent. Most are qualitative assays, but an increasing trend is observed towards semi-quantitative as well as multi-mycotoxin approaches. Recently, Lattanzio *et al*. (2012a) developed a dipstick immunoassay for the semi-quantitative determination of ZEN, T-2, HT-2, DON and fumonisins in wheat, oats and maize. Also, Njumbe Ediage *et al*. (2012) constructed a membrane- and gel-based flow through immuno assay for OTA, FB1, DON and ZEN in peanut cake, maize and cassava.

As already described, mainly chromatographic methods were developed for masked mycotoxin determination and quantification. However, for fast screening, it is important to develop on-site methods for the detection of masked and parent forms. To date, the use of immunochemical methods for masked forms is only at the start of development (Goryacheva and De Saeger, 2012).

The main driving force of immunochemical methods is the use of an antibody, which defines the specificity and sensitivity of the method. Mycotoxins are not immunogenic, due to their low molecular weight, so an immunogen needs to be synthesized by coupling the hapten (*i.e*. target mycotoxin) to a carrier protein (Goryacheva and De Saeger, 2012). No antibodies were specifically developed for masked mycotoxins so the key point for antibody-based methods for masked mycotoxin determination is cross reactivity (CR) of the specific antibody towards masked mycotoxins. Knowledge on CR when applying immunochemical methods towards masked forms is crucial for the interpretation of the data. A misinterpretation of the results can lead to an overestimation of the effective parent mycotoxin contamination. If the antibody

shows affinity for masked forms, the sum of parent and masked forms will be determined, meaning there is high CR both for parent and masked forms. If for immunogen synthesis the hapten was conjugated to a protein through parts of the molecule as the one conjugated in the masked form, high binding of this masked form with the antibody will be assumed. Only the parent form will be recognizable. In **Table 1.8** a résumé of the different commercial immunochemical kits with associated CRs is presented. As DON-3G is a commercial available reference standard, this is the most frequently studied masked form (Goryacheva and De Saeger, 2012).

As for ELISA, two approaches can be distinguished: direct competitive and indirect competitive ELISA. During direct competitive ELISA, specific antibodies are bound and the analyte of interest competes with horseradish peroxidase labeled analytes for binding with a restricted amount of antibodies bound on the well. After incubation, the non-bounds are removed by a washing step and chromogenic substrate is added. Furthermore, enzymatic activity is inversely proportional to the mycotoxin concentration. The indirect approach implies that the mycotoxin or its analogue conjugated to a protein is bound. A mixture of analyte and specific antibody is added to each well, and analytes and the bound mycotoxin are competing to be able to bind with the antibody. After a washing step, the bound specific antibody is detected by a secondary antibody labeled with horseradish peroxidase. Enzymatic activity in each well is also inversely proportional to the mycotoxin concentration (Goryacheva and De Saeger, 2012). There are still no antibodies with a high CR for DON-3G and a low CR for the parent form DON (**Table 1.8**). Only indirect approaches can be used for the determination of the masked form via hydrolysis. A hydrolysis step with trifluoromethanesulfonic acid (TFMSA) before ELISA was introduced by Tran and Smith (2011), and showed acceptable results. The use of 0.5 M TFMSA, incubated at 22°C for 20 min, showed to be the optimal condition in corn, while 40°C and 40 min in wheat proved to be the most optimal.

IACs are used for mycotoxin pre-concentration (*i.e.* clean-up, paragraph 1.3.4.2) before chromatographic analysis. Vendl *et al.* (2009) tested 5 IACs, however, no CR was observed, meaning that these are not suitable for direct DON-3G determination. Easi-extract ZEN®, Zearatest® and Zearastar® were tested, and both α-ZEL and β-ZEL showed cross reactivities close to ZEN. Versilovskis *et al.* (2011) proved that DON-Prep®, Neocolumn DON® and DZT® MS-prep showed higher cross reactivity to DON-3G, while no or very small cross

reactivity was observed for ImmunoClean C DON®, Easi-extract ZEN®, Neocolumn ZEN® and ImmunoClean C ZEN®.

Despite great efforts in optimization of robust and simple assays, it is still an unresolved issue for multi-(masked) mycotoxin analysis. An emerging technique in academic research is the development of biosensors. Biosensors are analytical devices that combine a biological receptor with a physicochemical detector. Many optical biosensors are based on the surface plasmon resonance (SPR) technique. A thin layer of gold on a high refractive index glass surface absorbs laser light, producing electromagnetic waves (surface plasmons) on the gold surface. Only when a specific angle and wavelength of incident light is obtained, a measurable signal (*i.e.* binding of a target analyte to a receptor on the gold surface) will be detected, which is highly dependent on the surface of the gold. Dorokhin *et al.* (2011) determined the CR of a multiplex micro assay sensor based on a SPR biosensor (IBIS I SPR instruments, IBIS Technologies) for both DON and ZEN masked forms. Kadota *et al.* (2010) described an SPR sensor with an anti-DON monoclonal antibody, determining the CR of 3-ADON, 15-ADON and DON-3G (**Table 1.8**).

Recently, Beloglazova *et al.* (2013) developed new immunochemical techniques for the determination of ZEN-14G based on its hydrolysis to the parent ZEN. The methods were based on the combination of acidic or enzymatic hydrolysis of the masked mycotoxin to the parent form (*i.e.* ZEN), taking ZEN-14G concentration as the difference between the ZEN concentration after and before cleavage of the glucosidic bond. The developed technique proved to be suitable for the simultaneous immunochemical determination of both mycotoxins. The LOD for ZEN-14G, achieved for the enzyme-linked immunoassay was 3 µg kg^{-1}, while the cut-off level (CO) for the qualitative gel-based immunoassay, designated as the lowest analyte concentration characterized by the absence of color development at a fixed detection time, was 75 µg kg^{-1}. TFMSA was checked for acidic hydrolysis and resulted in approximately 70% of glucosidic bond cleavage in optimal conditions. Seven different glucoside hydrolases were tested during the design of the enzymatic hydrolysis technique whilst the most suitable enzyme (glucosidase from *Aspergillus niger*) allowed cleaving 102% of ZEN-14G in standard

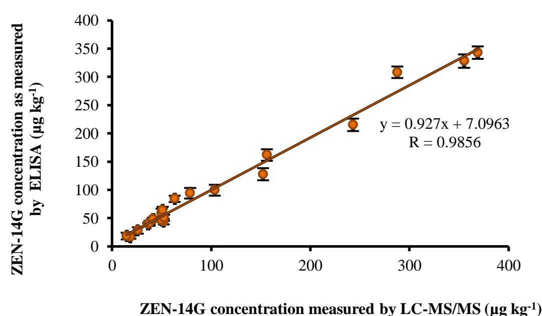


Figure 1.14 Linear regression equation derived using ELISA and LC–MS/MS data for ZEN-14G screening in maize samples (*n=5*)

solutions and 85% in cereal samples. Summarizing, for the first time an immunochemical approach was used for ZEN-14G determination in real samples, and an enzymatic hydrolysis procedure was successfully applied for cereal screening. The efficient analytical performance of the test was proven by using LC-MS/MS as a confirmatory method. This immunochemical technique for ZEN-14G determination demonstrated a good correlation with LC-MS/MS (**Figure 1.14**). The developed ELISA technique was able to screen large numbers of real samples in a relatively short time.

As a conclusion, ELISA, IACs and developed biosensors should be used as a screening technique, whereas chromatographic methods like LC-MS/MS are preferred to confirm any positive results.

Table 1.8 Commercial kits and cross reactivity of ELISA, IACs and sensors applied for masked mycotoxin analysis

Commercial kit	Supplier	Studied cross-reacting mycotoxin	Cross reactivity (%)	Reference
ELISA				
Ridascreen® DON	R-Biopharm	DON-3G	82-98	Ruprich and Ostry, 2008
Agraquant®	Romerlabs	DON-3G	52	Tangni *et al.*, 2012
Agraquant®	Romerlabs	DON-3G	4.8 (no specifications)	Tran and Smith, 2011
DON EIA®	Europroxima	DON-3G	115	Tangni *et al.*, 2012
Veratox®	Neogen Corp.	DON-3G	157	Tangni *et al.*, 2012
Rosa LF-DONQ®	Charm	DON-3G	8	Tangni *et al.*, 2012
MycontrolDON®	Aokin	DON-3G	22	Tangni *et al.*, 2012
Ridascreen® fumonisin	R-Biopharm	Hydrolyzed FB1, N-deoxy-fructosyl-FB1, FB1-starchadducts	0, other CRs not quantified	Dall'Asta *et al.*, 2008
IAC				
DON-Prep®	R-Biopharm	DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S	0	Vendl *et al.*, 2009
DONtest®	Vicam	DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S	0	Vendl *et al.*, 2009
Easi-extract ZEN®	R-Biopharm	DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S	0	Vendl *et al.*, 2009
Zearatest®	Vicam	DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S	0	Vendl *et al.*, 2009
Zearastar®	Romerlabs	DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S	0	Vendl *et al.*, 2009
DON-Prep®	R-Biopharm	DON-3G, ZEN-14G†	58, 0	Versilovskis *et al.*, 2011
Neocolumn DON®	Neogen Corp.	DON-3G, ZEN-14G†	48, 0	Versilovskis *et al.*, 2011
ImmunoClean C DON®	Aokin	DON-3G, ZEN-14G†	< 1	Versilovskis *et al.*, 2011
Easi-extract ZEN®	R-Biopharm	DON-3G, ZEN-14G†	0	Versilovskis *et al.*, 2011
Neocolumn ZEN®	Neogen Corp.	DON-3G, ZEN-14G†	0	Versilovskis *et al.*, 2011
ImmunoClean C ZEN®	Aokin	DON-3G, ZEN-14G†	0	Versilovskis *et al.*, 2011
DZT® MS-prep	R-Biopharm	DON-3G, ZEN-14G†	41,0	Versilovskis *et al.*, 2011
Fumonitest®	Vicam	Hydrolyzed FB1	CR not quantified	Kim *et al.*, 2003
SENSOR				
Micro assay Sensor based on surface plasmon resonance (SPR)	IBIS Technologies	*Anti-DON monoclonal antibody:* 3-ADON, 15-ADON, DON-3G *Anti-ZEN monoclonal antibody:* α-ZEL, β-ZEL	 71, 66, 36 59, 137	Dorokhin *et al.*, 2011
Surface plasmon resonance sensor (SPR)	Biacore T100	*Anti-DON monoclonal antibody:* 3-ADON, 15-ADON, DON-3G	276, < 1, 60	Kadota *et al.*, 2010

† Other tested mycotoxins were 3-ADON, 15-ADON, DON-3-glucuronide, DOM-1, nivalenol, ZEN-14-glucuronide, α-ZEL and β-ZEL

1.4 LEGISLATION AND TOXICOLOGICAL CONSIDERATIONS

Legislation clearly intends to increase food and feed safety. In 2002, a regulation was set up laying down the general principles and requirements of the food law, while establishing the European Food Safety Authority (EFSA) and laying down procedures in matters of food safety. It provided the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food, taking into account in particular the diversity in the supply of food including traditional products, whilst ensuring an efficient functioning of the internal market. Also, it applied to all stages of production, processing and distribution of food and feed (178/2002/EC, 2002). Current legal limits and control strategies only focus on the parent mycotoxins.

Cereal-based products are very important in the human diet and their quality and safety should be controlled during processing throughout the entire food chain (Yazar & Omurtag, 2008). To avoid consumer health risks, the European Union (EU) has set maximum levels as a risk management strategy so as to achieve a high level of public health protection for DON and ZEN in foodstuffs (1881/2006/EC, 2006; 1126/2007/EC, 2007), and guidance values for DON and ZEN in products intended for animal feeding (2006/576/EC, 2006) (**Table 1.9**). To date, no EU maximum limits for the sum of T-2 and HT-2 were registered. However, recently indicative values were introduced according to the Commission Recommendation of 27/03/2013 regarding the occurrence of T-2 and HT-2 in cereals and cereal products in food and feed (2013/165/EU, 2013).

The EFSA has centralized opinions on this matter, laying down a tolerable daily intake (TDI) for several toxins. It has established a TDI for DON of 1.00 µg kg^{-1} body weight (bw) day^{-1}, a provisional TDI of 0.25 µg kg^{-1} bw day^{-1} for ZEN and a combined provisional TDI of 0.06 µg kg^{-1} bw day^{-1} for the sum of T-2 and HT-2. However, recently the EFSA has established a TDI of 0.10 µg kg^{-1} bw day^{-1} for the sum of T-2 and HT-2 (EFSA, 2011a, 2011c).

Most regulations are concerned with controlling aflatoxins because they are considered the most toxic and carcinogenic of the naturally occurring mycotoxins (FAO, 1995). Approximately a hundred countries have developed specific limits for mycotoxins in food and feed with the population in these countries representing 87% of the world's inhabitants.

Nevertheless, maximum levels differ widely on national levels because of a lack of agreement on what constitutes a safe maximum level for humans. Agreeing on and setting international regulatory standards is very complicated and potential health benefits as well as political and economical issues have to be considered.

Table 1.9 Current maximum limits for DON and ZEN in foodstuffs according to No 1881/2006 (EC), guidance values for DON and ZEN in feed according to Commission Recommendation No 2006/576 (EC) and indicative values for the sum of T-2 and HT-2 in food and feed according to Commission Recommendation No 2013/165 (EU)

Mycotoxin	Foodstuffs: maximum levels	µg kg^{-1}
Deoxynivalenol	- Unprocessed cereals other than durum wheat, oats and maize	1,250
	- Unprocessed durum wheat and oats	1,750
	- Unprocessed maize	1,750
	- Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits), bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in [†]	750
	- Pasta (dry)	750
	- Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
	- [†] Processed cereal-based foods and baby foods for infants and young children	200
Zearalenone	- Unprocessed cereals other than maize	100
	- Unprocessed maize	200
	- Cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs listed in [†, ‡, §]	75
	- [†] Maize intended for direct human consumption, maize flour, maize meal, maize grits, maize germ and refined maize oil	200
	- Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize based breakfast cereals	50
	- Maize snacks and maize based breakfast cereals	50
	- [‡] Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children	20
	- [§] Processed maize-based foods for infants and young children	20
	Foodstuffs: indicative values	**µg kg^{-1}**
Sum of T-2 and HT-2	- Unprocessed cereals	
	- Barley (including malt) and maize	200
	- Oats (not shelled)	1,000
	- Wheat, rye and other cereals	100
	- Cereals for direct human consumption	
	- Oats	200
	- Maize	100
	- Other cereals	50
	- Cereal products for human consumption	
	- Oats bran and oats flakes	200
	- Bran of cereals with the exception of oats bran and oats flakes, and milling products of maize	100
	- Other milling products of cereals	50
	- Breakfast cereals with the inclusion of formed cereal flakes	75
	- Bread (with the inclusion of small bakery products), pastry, cookies, cereal snacks and pasta	25
	- Processed cereal-based foods for infants and young children	15

Feed	Feed: guidance values relative to a feeding stuff with a moisture content of 12 %	µg kg^{-1}
Deoxynivalenol	- Feed materials	
	- Cereals and cereal products with the exception of maize by-products	8,000
	- Maize by-products	12,000
	- Complementary and complete feeding stuffs with the exception of:	5,000
	- Complementary and complete feeding stuffs for pigs	900
	- Complementary and complete feeding stuffs for calves (< 4 months), lambs and kids	2,000
Zearalenone	- Feed materials	
	- Cereals and cereal products with the exception of maize by-products	2,000
	- Maize by-products	3,000
	- Complementary and complete feeding stuffs	
	- Complementary and complete feeding stuffs for piglets and gilts (young sows)	100
	- Complementary and complete feeding stuffs for sows and fattening pigs	250
	- Complementary and complete feeding stuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	500
	Feed: indicative values	**µg kg^{-1}**
Sum of T-2 and HT-2	- Cereal products for feed and compound feed	
	- Milling products of oats (chaff)	2,000
	- Other cereals products	500
	- Compound feed with the exception of cat feed	250

Establishment of mycotoxin limits and regulations may be influenced by several factors both scientific and socio-economic in nature, including: availability of toxicological data, availability of data on occurrence in different commodities, knowledge of mycotoxin distribution concentration within lots, availability of analytical methods, national legislation and need for sufficient food supply (Egmond and Jonker, 2004).

The EFSA requested the evaluation of mycotoxins such as *Alternaria* toxins and ZEN, however no explicit plan was considered for masked forms (EFSA, 2011b). The evaluation concerning masked mycotoxins is not (yet) available due to the lack of occurrence, bioavailability and toxicological data, however the Joint FAO/WHO Expert Committee considered DON-3G and the acetylated forms 3-ADON and 15-ADON as an additional contributing factor for dietary exposure to DON (JECFA, 2010). Until now, DON and its derivatives are considered equally toxic by health authorities, however the proved high toxicity of 15-ADON in comparison with 3-ADON and DON should be taken into account to determine the maximum safe levels of the different trichothecenes in food and feed (Pinton *et al.*, 2012). Masked forms of ZEN were not considered. Making these statements it encouraged research and industry to further investigate masked mycotoxins.

Up to now, data regarding the *in vivo* toxicological relevance of masked mycotoxins were lacking. As masked toxins present an emerging issue it is not a surprise that for most toxicological study types such as genotoxicity, short-term and long-term toxicity including carcinogenicity, reproduction and developmental studies, no data have been made available. As conjugation is known to be a detoxification process, it seems likely that conjugated mycotoxins exhibit a lower acute toxicity compared to their parent compounds. Poppenberger *et al.* (2003) already proved that DON-3G dramatically reduces the ability to inhibit protein synthesis of wheat ribosomes *in vitro*. Same authors reported that ZEN-14G yields lower estrogenic activity compared to ZEN (Poppenberger *et al.*, 2006).

Recently, Berthiller *et al.* demonstrated the toxicological relevance of DON-3G by demonstrating that several lactic acid bacteria can hydrolyse DON-3G during digestion *in vitro* (Berthiller *et al.*, 2011). Acidic conditions, hydrolytic enzymes and intestinal bacteria, mimicking different stages of digestion were included. DON-3G was found resistant to 0.2 M HCl for at least 24 h at 37 °C (pH 1.7), suggesting that it will not be hydrolyzed in the stomach of mammals. While human cytosolic β-glucosidase also had no effect, fungal cellulase and cellobiase preparations could cleave a significant portion of DON-3G. Most

importantly, several lactic acid bacteria such as *Enterococcus durans*, *Enterococcus mundtii* or *Lactobacillus plantarum* showed a high capability to hydrolyze DON-3G.

De Nijs *et al.* (2012) confirmed the statements made. DON-3G was investigated on its potential transformation to DON in a digestion model representing the upper gastro-intestinal tract, as well as the absorption of DON-3G by intestinal epithelium using an *in vitro* model. Less than 5% of DON-3G proved to be hydrolyzed in the upper gastro-intestinal tract. Using Caco-2 cells in Transwell chambers, absorption was calculated on the apical and basolateral side after 0h, 4h and 24h. No absorption of DON-3G was proved with a full recovery of 106% DON-3G. In conclusion, the findings were in accordance with Berthiller *et al.* (2011) proving that DON-3G was resistant to conditions of the mammalian stomach and human cytosolic β-glucosidase.

The first *in vivo* experiment was executed by Nagl *et al.* demonstrating that DON-3G is partly bioavailable in rats. The majority of the administered DON-3G was cleft during digestion and subsequently excreted in feces. Thus, DON-3G present in food and feed seems to have a significantly lower toxic equivalency compared to DON. However, due to the differences regarding the anatomy and gut microbiota, the bioavailability and metabolization may be species dependent and should be experimentally determined. Unfortunately, the limited availability of pure DON-3G standards precludes testing of larger animals such as swine (Nagl *et al.*, 2012).

Previous report stated the *in vivo* metabolic fate in rats, while there are no current data concerning the catabolic fate of DON-3G, ZEN-14G and ZEN-14S in humans. Berthiller *et al.* (2011) applied a single-strain bacterial exposure, while human microbiota were not considered. Recently, Dall'Erta *et al.* (2013) demonstrated that these masked forms were effectively deconjugated by human colonic microbiota releasing their toxic aglucones and generating unidentified catabolites. The tremendous amount of different bacterial strains and associated enzymes in the human colon makes the gastrointestinal tract a remarkable bioreactor, able to chemically transform most of compounds ingested by humans. An *in vitro* digestion assay was executed for DON-3G, ZEN-14G and ZEN-14S by the use of four synthetic juices, namely saliva, gastric juice, duodenal juice and bile juice. After incubation the masked forms were fully recovered with amounts of 99.5%, 97.3% and 98.6%, respectively. Concurrently, an *in vitro* fecal fermentation assay with incubation of 30 min and 24h was executed. ZEN-14G and ZEN-14S were completely cleft at 30 min of incubation. ZEN, however, was only partially recovered in the fecal slurry which implied possible

catabolites of ZEN. After 30 min DON-3G was not deglucosylated, nevertheless complete degradation (90% DON) was observed in the fecal slurry. Discrepancies in the anatomy and gut microbiota were proven in this study as only traces of DOM-1 were found in humans, whereas in rats this compound clearly showed to be a major metabolite (Nagl *et al.*, 2012).

Concerning ZEN-forms, Ayed *et al.* argued that ZEN and α-ZEL exhibited the same range of cytotoxicity and genotoxicity, and both were more cyto- and genotoxic than β-ZEL (Ayed *et al.*, 2011). Recently, a study was executed on the amount of masked ZEN-forms in rats (Versilovkis *et al.*, 2012). After administration of ZEN-14G, ZEN was found in the stomach suggesting the possibility of hydrolysis as was previously concluded for the acetylated DON-forms. Small amounts of ZEN-14G were detectable in the small and large intestines suggesting that they were not fully hydrolyzed. Notwithstanding, the large occurrence of α-ZEL (Videmann *et al.*, 2012) and a sharp decrease of ZEN-14G in the small intestine proved hydrolysis (Versilovkis *et al.*, 2012), as a consequence, the total human exposure and risk assessment to mycotoxins might be underestimated. As already mentioned, ZEN is degraded into α-ZEL and β-ZEL, which are subsequently conjugated with glucuronic acid and excreted in the urine in mammals (Zinedine *et al.*, 2007; JECFA, 2010). It appears likely that the toxic (estrogenic) effects of ZEN-14G equal that of ZEN in mammals.

To date, no toxicokinetic and toxicodynamic investigations were executed, which would enable risk assessment of masked mycotoxins in comparison with those of parent mycotoxins. The few investigated masked forms show a low general toxicity, but do have an increased bioavailability. The partial reactivation of masked mycotoxins during digestion in mammals remains a general health threat. To ascertain the statements regarding masked forms, a full metabolism study should be carried out, preferably by the incorporation of mycotoxins in feed of species reliable as to extrapolation to humans.

1.5 RISK ANALYSIS

1.5.1 Introduction

While there are many definitions of the word *risk*, the Environmental Protection Agency (EPA) more specifically considers risk to be the chance of harmful effects to human health or to ecological systems resulting from exposure to an environmental stressor. A stressor is any physical, chemical, or biological entity that can induce an adverse response. Stressors may adversely affect specific natural resources or entire ecosystems, including plants and animals, as well as the environment with which they interact (EPA, 2013).

From the first stages of food production, chemical hazards are present in food and feed. A division into 4 typical groups is proposed: *environmental contaminants* which occur via soil, air or water (*e.g.* dioxins and heavy metals), *agrochemical residues* (*e.g.* fungicides, pesticides and veterinary drugs), *microbiological contaminants and endogenous toxicants* due to natural contamination (*e.g.* toxins produced by bacteria and molds, mycotoxins, marine biotoxins and plant originated structures like caffeine and solanine) and *contaminants* related to *industrial food* and *feed processing* (*e.g.* additives, disinfectantia, acrylamide, benzene, furan and food contact materials). These threats can be present or are formed *from farm to fork*. Chemical hazards in the agricultural food chain are illustrated in **Figure 1.15**.

Figure 1.15 Overview of possible sources of chemical hazards in the agricultural food chain

A quantitative risk assessment of chemical hazards is essential to provide risk managers and consumers with health advice. Three footholds are charged in the risk analysis framework: risk management, risk assessment and risk communication. The schematic framework according to the Codex is pointed out in **Figure 1.16**.

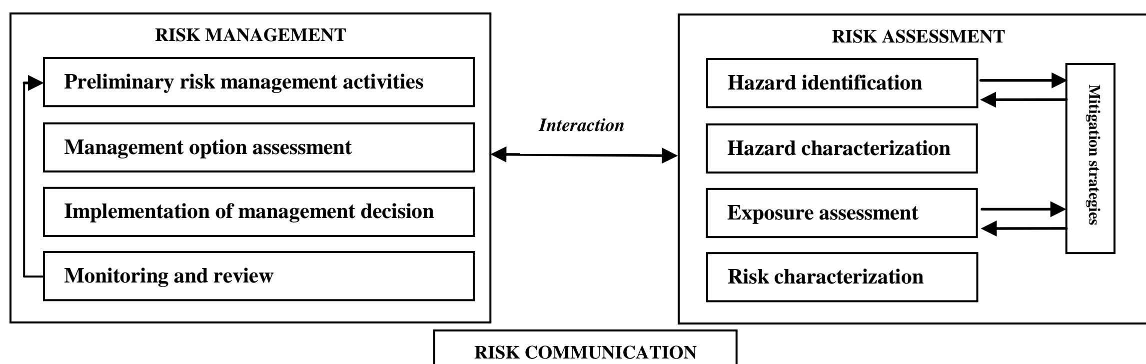


Figure 1.16 Schematic framework of the Codex for risk analysis (Codex, 2013)

1.5.2 Risk management

Risk management is the process of weighing policy alternatives to accept, minimize or reduce assessed risks, to select and implement appropriate options and to evaluate the protection of public health (WHO, 2013). Examples of risk management actions include establishing national quality standards or determining allowable levels of contamination in food and feed.

Risk assessment is determined on a scientific level and provides the information on potential health risks, while risk management is policy-based and implies the action taken to be based on consideration of that and other information. Risk assessors are the official instances responsible for the protection of the consumer (*e.g.* Federal Agency for the Safety of the Food Chain (FASFC, Belgium) and EFSA), business company leaders whose products can be susceptible to risks, or private citizens who are making decisions regarding risk. Risk managers, however, are the national governments and the EU. Four components in the risk management framework are summarized.

1.5.2.1 Preliminary risk management activities

Preliminary risk management activities include the establishment of a risk profile to facilitate consideration of the issue within a particular context, and provide as much information as possible to guide further action. As a result of this process, the risk manager may commission a risk assessment as an independent scientific process to inform decision-making.

1.5.2.2 Evaluation of risk management options

The evaluation of risk management options comprises the weighing of available options for managing a food safety issue in the light of scientific information on risks and other factors, and may include reaching a decision on an appropriate level of consumer protection. Optimization of food control measures in terms of their efficiency, effectiveness, technological feasibility and practicality at selected points throughout the food chain is an important goal. A cost-benefit analysis could be performed at this stage.

1.5.2.3 Implementation of the risk management decision

Regulatory food safety measures are involved within this context, which may include the use of Hazard Analysis Critical Control Points (HACCP). Flexibility in the choice of individual measures applied by the food industry is a desirable element, as long as the overall program can be objectively shown to achieve the stated goals. Ongoing verification of the application of food safety measures is essential.

1.5.2.4 Monitoring and review

Monitoring and review include the gathering and analysis of data so as to give an overview of food safety and consumer health. Monitoring of contaminants in food and food borne disease surveillance should identify new food safety problems as they emerge. Where there is evidence that required public health goals are not being achieved, a redesign of food safety measures will be needed (WHO, 2013).

As described above, various factors have to be taken into account to set up a straight risk management plan (**Table 1.10**) (EPA, 2013).

Table 1.10 Factors influencing the risk management strategy

Factors	Detailed description of factors
Scientific factors	The basis for the risk assessment, including information drawn from toxicology, chemistry, epidemiology, ecology, and statistics.
Economic factors	Information for the risk manager on the cost of risks and the benefits of reducing them, the costs of risk mitigation or remediation options and the distributional effects.
Laws and legal decisions	Factors that define the basis for risk assessments, management decisions, and, in some instances, the schedule, level or methods for risk reduction.
Social factors	Factors like income level, ethnic background, community values, land use, zoning, availability of health care, life style, and psychological condition of the affected populations, may affect the susceptibility of an individual or a definable group to risks from a particular stressor.
Technological factors	The feasibility, impacts, and range of risk management options.
Political factors	Factors based on the interactions among branches of the government, with other local government entities, and even with foreign governments; these may range from practices defined by policy and political administrations through inquiries from special interest groups, or concerned citizens.
Public values	The values reflect the broad attitudes of society about environmental risks and risk management.

1.5.3 Risk Assessment

Risk assessment contributes to improving food safety and to building public confidence in the way risk is assessed. It is a specialized field of applied science that involves reviewing scientific data and studies in order to evaluate risks associated with certain hazards (EFSA, 2013).

Risk depends on several factors like: the quantity of the chemical present in the environmental medium, the exposure of the organisms to the contamination and the inherent toxicity of the chemical. The risk assessor evaluates the frequency and magnitude of human and/or ecological exposures that may occur as a consequence of contact with the contaminated medium. The evaluation of the exposure is then combined with information on the inherent toxicity of the chemical (*i.e.* the expected response to a given level of exposure) to predict the probability, nature, and magnitude of the adverse health effects that may occur. In an ideal world, all risk assessments would be based on a very strong knowledge base, *i.e.* reliable and complete data on the nature and extent of contamination, the magnitude and frequency of human and ecological exposure, and the inherent toxicity of all of the chemicals. However, in real life, information is usually limited to one or more of these key data needed for risk assessment calculations. This means that risk assessors often have to make estimates and rely on judgment when performing risk calculations, and consequently all risk estimates are uncertain to some degree. For this reason, a key part of a good risk assessment is a fair and open presentation of the uncertainties in the calculations and a characterization of how reliable (or how unreliable) the resulting risk estimates really are (EPA, 2013).

Risk assessment consists of 4 main stages: hazard identification, hazard characterization, exposure assessment and risk characterization.

1.5.3.1 Hazard identification

The general question regarding hazard identification is: *What health problems are caused by the stressor*? Hazard identification is the process of determining whether exposure to the analyzed stressor causes an increase in the incidence of specific adverse health effect (*e.g.* mucosal lesions and stillbirths) and whether these adverse health effects are likely to occur in humans. The process examines the available scientific data for a given group of stressors and develops an evidence weight for the characterization of the link between the adverse outcomes and the stressor. Clinical trials on humans provide the optimum evidence of the link between a stressor and the effect, however ethical limitations are often imposed.

Epidemiological studies involve a statistical evaluation of populations to examine whether there is an association between exposure to a stressor and a human health effect. The advantage is that humans are involved while the weakness of such studies results from generally not having accurate exposure information and the difficulty of teasing out the effects of multiple stressors. When clinical human trials are unavailable, data from *in vitro* and *in vivo* animal studies (*e.g.* rats, mice, rabbits, dogs, ...) are relied on to draw inference about the potential hazard to humans. However, extrapolating results to humans still involves uncertainties.

Besides evaluation of the toxicity of chemical stressors, hazard identification comprises the investigation of sources of occurrence, mechanisms of formation and quantification of the chemical substances in several matrices. Once the factors influencing the occurrence of the hazard are fully understood, appropriate mitigation strategies may be formulated (Vinci Medeiros, 2011).

1.5.3.2 Hazard characterization

This parameter is closely related to hazard identification as it considers the nature of the effect and its relevance for humans, and whether there is likely to be a threshold dose below which there would be no effect (Vinci Medeiros, 2011). The characterization includes dose-response considerations and evaluation of the relevance of certain endpoints(s) in experimental systems for humans.

When possible, a health-based guidance value (HBGV, *i.e.* exposure level at where there is no appreciable health risk) such as TDI is established (EFSA, 2012). When there is absence of human toxicological data, the HBGV is based on data from repeated dose studies on experimental animals. A reference point (RP) needs to be identified based on the dose response relationship; the RP is mostly referred to as the benchmark dose lower confidence limit (BMDL). The BMDL is the estimate of the lowest dose that is 95% certain to cause no more than a specified change in response over background. However, occasionally no appropriate RPs are available and then no observed adversed effect levels (NOAELs) are estimated, which are the highest doses not causing a statistically significant adverse effect compared to the controls. HBGV is established by dividing the RP by uncertainty or safety factors (*i.e.* 100 – 1,000) to account for extrapolation from animals to humans and for variability in human sensitivity. The safety factor usually chosen is 100 in the situation where an NOAEL is derived from a long- term animal study on the assumption that humans are ten-times as sensitive as the test animal(s) and that a ten-fold range of sensitivity within the

human population may exist. When no adverse health effects are seen in long-term studies, a safety factor of 100 may be applied to the NOAEL derived from short-term studies where higher dose levels have been used and an effect has been noted. JECFA stated that on limited occasions higher safety factors (*e.g.* 200, 500 or 1,000) were employed depending on the quality and quantity of relevant data (JECFA, 2010).

For some substances, like mycotoxins, acute reference doses (ARfD) are used as HBGV, based on short-term toxicity data from experimental animals. However, these statements made are applicable for genotoxic and carcinogenic substances (*e.g.* aflatoxins), therefore EFSA proposed the margin of exposure approach (MOE) as a harmonized procedure for the risk assessment of substances that are both genotoxic and carcinogenic. The MOE takes into account that carcinogens differ in their potency and likelihood of inducing a tumor at a given dose over time. A new concept in risk analysis is the threshold of toxicological concern (TTC) which is a concept that refers to the establishment of a level of exposure for all chemicals, whether or not there are chemical-specific toxicity data, below which there would be no appreciable risk to human health (Kroes *et al.*, 2002).

1.5.3.3 Exposure assessment

The exposure of the population to stressors is assessed by the integration of the consumption and occurrence data. The exposure is evaluated individually using individual consumption data points and the body weight (bw) of each consumer according to the following formula (**Equation 1.5**).

$$E_{i,j} = \frac{\sum_{k=1}^{n} C_{i.k}}{bw_i} \times L_{k,j}$$

where $E_{i,j}$ is the exposure of the contaminant *j* for the subject *i* of food *k*; $C_{i,k}$ is the consumption level of the food *k* by the subject *i* (*k*=1 to N); $L_{k,j}$ is the level of contaminant *j* in the food *k*, and bw_i is the body weight of subject *i*.

Equation 1.5 Calculation of exposure assessment

Traditionally in food and feed area, exposure assessment is based on deterministic endpoints, and the mean or maximum level of exposure. Increasingly more probabilistic methods are included to characterize the hazards as well as the exposure. The mentioned approach allows for more description of variability in the population as well as uncertainty in the risk estimates (Codex, 2013). Many outcomes are possible from exposure assessment ranging from a qualitative description, over prioritization of risks to a quantitative estimation of the risk at specified exposure levels.

Deterministic approaches are usually based on single value in- and outputs and provide a relatively straightforward means of using a risk assessment to develop risk management

options (*e.g.* setting of maximum levels). However, the approach comes at a cost in providing less accurate information *e.g.* limited insights into uncertainty and a tendency to focus on extreme situations such as worst-case scenarios. Probabilistic approaches provide the means to overcome some of these deterministic disadvantages. The in- and outputs are distributions of values and can incorporate both uncertainty and variability (Codex, 2013). As a result of the complexity of assessing exposures and providing better guidance to risk managers, probabilistic approaches may need to be applied. These will require more complete data sets and not just the pooled or mean data that have usually been submitted. The setting of HBGVs, such as acceptable daily intakes (ADI) and TDIs provides quantitative information from a risk assessment enabling them to make decisions concerning the protection of human health.

Maximum levels are established by the Codex Committee on Contaminants in Foods with advice from JECFA to be compatible with TDI, and are based on the lowest level of contamination that can be reasonably achieved without removing the food from the food supply. These maximum levels are only endorsed for which JECFA has completed a safety assessment or has performed a quantitative risk assessment (Codex, 2013).

Biomarker based methodologies are often employed to estimate human exposure to chemical contaminants in food. Measurements of the biomarkers (*e.g.* deoxynivalenol-3-glucuronide) are executed on urine, blood, breast milk, hair, buccal swaps, exhaled hair or feces (Kroes *et al.*, 2002). This approach, besides taking different exposure pathways into consideration, also includes bioavailability from food matrices (O'Brien *et al.*, 2006). Biomonitoring is not applicable for all chemicals because of interferences, the reactive nature or the half-life of the agent being too short (Paustenbach, 2000)

1.5.3.4 Risk characterization

Risk characterization integrates the information obtained from hazard characterization and exposure assessment into statements or opinions, suitable for risk management decisions. These can be quantitative estimates of health risk at certain exposure levels, or can be in form of a level of exposure without significant adverse health effect, such as ADI or TDI (including provisional (P) and temporary (t) qualifying designations). ADIs are generally designated for substances intentionally added or present as a result of other approved intentional uses (*e.g.* pesticide residues). TDIs are used for unavoidable contaminants such as mycotoxins. Comparison of these values with the estimated exposure helps in the identification of critical foods or critical target populations in case the ADI or TDI is approached or even exceeded. Directing the risk management measures is obtained in this way (Codex, 2013).

1.5.4 Risk communication

Risk communication is defined as the interactive exchange of information and opinions throughout the risk analysis process about risk to the related instances and consumers.

All communities need a way to communicate about present, emerging, and evolving risks. There is a general consensus that risk communication is a two-way process between the communicator(s) and the recipients of the messages, but beyond that, different definitions often include unique variables and understandings. Risk communication definitions are often similar to the definition offered by Covello (1992), *i.e.* “a process of exchanging information among interested parties about the nature, magnitude, significance, or control of a risk”. Other definitions emphasize the importance of risk management (McComas, 2006), the need for dialogue between communicators and stakeholders, and the necessity of ongoing risk monitoring (Palenchar, 2005).

Organized and centralized risk communication efforts grew out of legal and regulatory mechanisms regarding community right-to-know, that required organizations or institutions (specifically in chemical and manufacturing fields) to inform communities of any potential consequences of their existence (Palenchar, 2008).

As official risk communicators engaged with publics, interest increased in how such communication could be most effective, with initial questions focused on message creation, but expanding to query how audiences process and act on messages, leading to a deep body of risk communication research.

Fischhoff was one of the leading pioneers of risk communication research, and worked in this area starting in the late 1970s, culminating in the identification of seven evolutionary stages of risk communication and best practices (Fischhoff, 1970; EFSA, 2013) (**Table 1.11**).

Table 1.11 Seven stages of risk communication and best practices

Seven bullet points of risk communication
1. Get the numbers right
2. Tell key publics what the numbers mean
3. Explain what the numbers mean
4. Show publics they have accepted similar risks before
5. Explain how risk benefits outweigh the costs
6. Treat publics with respect
7. Make publics partners of risk communicators

Other researchers believe that effective communication must take into account how various publics perceive risk influenced by societal and cultural factors rather than just focusing on science (Campbell, 1996). However, since Fischhoff’s seminal work, additional factors have

been identified that contribute to effective public warnings. This includes information on how publics respond differently to risks and the role of media in educating the public about risks (Sheppard, 2012).

Scientific results cannot always be easily converted into simple guidelines and advice that non-scientists like the public or the media can easily understand or follow.

The responsibilities of a risk communicator is to communicate food and feed safety advice to its principal partners, stakeholders and the public at large in a timely, clear and helpful way, to help bridge the gap between science and the consumer (EFSA, 2013). The Communications Strategy of official instances like EFSA sets out the following key priorities (**Table 1.12**).

In support of these priorities, EFSA is implementing its communications activities with respect to the people's opinion. In this respect, the EUROBAROMETER was established, which is a survey to analyze the impact of food and feed safety in Europe.

Table 1.12 Key priorities of a communication strategy according to EFSA

Key priorities of communications strategy	
Simplicity and transparency	Increase relevance and understanding of communications for key target audiences and informed lay audiences, in co-operation with Member States.
Independence	Augment proactive communications on the independence of EFSA's risk assessment advice.
Visibility and outreach	Enhance outreach, in the EU and beyond, by increasing awareness and recognition of EFSA and its role and work as risk assessor.
Coherence	Further increase the coherence of risk communications across the EU and beyond.
Dialog	Enhance dialogue with stakeholders and increase audience interactivity.

General conclusions of the survey were that the majority of the European population associate food and eating with enjoyment, those who are concerned about possible food-related risks tend to worry more about chemical contamination of food (*e.g.* pesticides and antibiotics) rather than bacterial contamination or health (*e.g. Salmonella*) and nutrition issues. Most Europeans have confidence in national and European food safety agencies as information sources on possible risks associated with food, however accurate information will mainly be consulted at the doctor's surgery.

1.5.5 Risk management and risk assessment of mycotoxin contamination

Mycotoxin contamination impairs farm operations as well as food and feed production in various ways because of the invisible, odorless and tasteless mycotoxins. A risk management concept has to be adopted in order to reduce the risk to a defined and acceptable level (Binder, 2007).

The implementation of an excessive risk management practice protocol for the maximization of plant performance and decrease of plant stress diminishes the mycotoxin contamination substantially. Prevention and control are the key, however not considerably enough to manage the mycotoxin risk. Edwards (2004) proposed planting of adapted varieties, a proper fertilization and tillage regime, the use of prediction models, weed control, necessary irrigation and proper crop rotation (**Figure 1.17**).

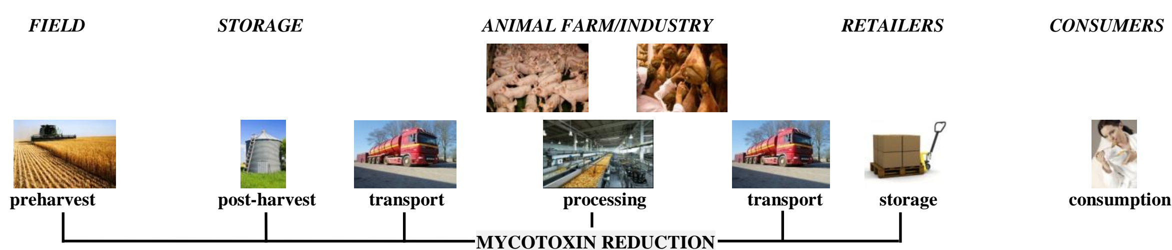


Figure 1.17 Possible mycotoxin-reduction key-points ***from farm to fork***

1.5.5.1 Planting

A crop rotation schedule should be inventoried to avoid planting the same commodity in a field in two consecutive years. Crops that are no hosts for *Fusarium* species are potato, clover and alfalfa. Wheat stubble, maize stalks and rice stubble can be major sources of molds and act as powerful inocula as temperatures increase during spring. Airborne release of spores proved to have a peak during and after rainy periods. So, tilling or ploughing the field by destroying or removing old seed heads, stalks and other debris is an effective measure.

The soil might be tested for the need of a fertilizer and for pH to avoid plant stress in development of the seed. Seed varieties resistant to seed-infecting fungi and insect pests should be grown. Planting of the crop needs to be timed to avoid high temperatures and drought stress during the period of seed development and maturation. Also, the maintenance of the recommended row and intra-plant spacing for species varieties should be taken into account to avoid overcrowding.

1.5.5.2 Pre-harvest measures

Before harvest, bio-control based methodologies such as the use of antagonistic micro-organisms, non-toxigenic strains and competitive colonization of stubbles need to be addressed. A key factor in mycotoxin management at this stage is the use of plant protection products (PPP). However, due to Directive 91/414, many substances have been excluded from market use, leading to a lack of efficient solutions and the development of resistance (91/414/EG, 1991). Hence, the milestone is to obtain a sufficient and diverse PPP-toolbox. The use of azoles (triazoles) contributes to the *Fusarium* control, and ban of azoles would negatively affect both quantity and quality parameters.

The proper use of insecticides and fungicides within an integrated pest management program is appropriate. Other control strategies are controlling weeds, minimizing mechanical damage to plants during cultivation and avoiding excessive irrigation during anthesis and ripening of the crops. The application of prediction models before PPP is useful for the management of treatments and to support farmers (Landschoot *et al.*, 2012). It is advisable to plan the date of harvest when a low moisture content and full maturity is assured as a delayed harvest shows a significant increase of mycotoxin content in crops.

1.5.5.3 (Post)-harvest measures

Each load should be checked on moisture content (<15%), whereas the storage devices need to be clean, dry, and free of insects and visible fungal growth. A newly developed controlling mechanism is a wireless sensor network that monitors temperature, humidity and CO_2 in grain silos in different strategic spaces (Shapira and Paster, 2004).

1.5.5.4 Storage

The stored commodities need to be continuously aerated by forces as air circulation. Fungal growth is favored during sun drying in high humidity (*i.e.* tropical climates). An increase of 2 °C to 3 °C indicates a microbial growth and/or insect infestation, therefore temperature should be controlled continuously. The sole possibility to assess the quality of ingredients is monitoring the mycotoxin levels using the appropriate sampling and testing programs (Binder, 2007; CAST, 2003).

1.5.5.5 Control and elimination strategies for mycotoxins

The COCERAL Mycotoxin Management Survey of 2009 stated: *How do you limit mycotoxin risks*? The report noticed that 100% of the risk was eliminated by advising on Good

Agricultural Practices (GAP), 83% by the recommendation of *Fusarium* resistant seeds and 94% by adapted fungicide treatments. Also, the question was raised: *When do you test for mycotoxins*? Only 7% indicated before harvest, 20% at harvest and 81% at delivery (= first processing industry), whereas the method of choice was quantitative ELISA (93%) (COCERAL, 2009). Results created by rapid tests can be satisfactory, while under certain conditions validated chromatographic methods might be necessary. Testing of mycotoxins involves three different steps: sampling, sample preparation and analysis (1.3.4). Quality assurance in mycotoxin analysis has become an important and critical issue. The purity of calibrants, sampling programs, proficiency testing by means of comparative laboratory tests and the use of certified reference materials are the basis of a proper quality management in the laboratory (Krska *et al.*, 2001; Egmond and Jonger, 2004).

The best strategies cannot eliminate mycotoxin contamination in years favorable to disease development. Some *Fusarium* species are widespread colonizers of crop residues where the pathogen survives during winter (COCERAL, 2009). To date, the most practical approach to an evident mycotoxin manifestation is its redirection into feed for less susceptible animal species (*i.e.* ruminants) or the blending of contaminated and non-contaminated commodities so that average contamination levels will meet the accepted standards. However, these described methods are prohibited in the EU, consequently other lines of attack need to be applied (2002/32/EC, 2002).

Some treatments have been found for the reduction of specific mycotoxin levels, however so far no single method has been developed equally effective against a wide variety of mycotoxins (Shapira and Paster, 2004). Chemical decontamination processes include radiation, oxidation, reduction, ammonization, alkalization, acidification and deamination (Kabak *et al.*, 2006). However, these methods are not allowed in the EU as toxic derivatives can occur. Decreasing the total bioavailability by introducing mycotoxin binding non-nutritive agents or adsorbants, leading to the reduction of the mycotoxin uptake and distribution into the blood and the target organs is the most commonly used strategy for reducing the exposure to mycotoxins (Binder, 2007). A large stability of the sorbent-toxin bond within a broad pH-level (*i.e.* gastro-intestinal tract) is a prerequisite in order to prevent desorption of the toxin. EFSA (2009) summarized existing commercial mycotoxin elimination strategies in a review: **Table 1.13** points the general classes.

Table 1.13 Mycotoxin detoxifying agents according to EFSA (2009)

Mycotoxin adsorbing agents	
Aluminosilicates	*Bentonite, montmorillonite, zeolite and hydrated sodium calcium aluminosilicate (HSCAS)*
Yeast cell walls	*Saccharomyces cerevisiae (glucomannan)*
Micronized fibres	*Dietary fibre*
Bacteria	*Lactic acid bacteria like Lactococcus, Lactobacillus, Pediococcus and Leuconostoc*
Polymers	*Cholestyramine and polyvinylpyrrolidone*
Activated charcoal	
Mycotoxin-biotransforming agents	
Bacteria	*Gram$^+$ and anaerobic bacteria: Eubacteria* *Gram$^+$ and anaerobic bacteria: Corynebacterium and Mycobacterium* *Gram$^-$ and aerobic bacteria: Flavobacterium, Pseudomonas and Alcaligenes*
Fungi	*Aspergillus, Eurotium herbariorum, Rhizopus spp., Penicillium raistricki and Rhinocladiella atrovirens*
Yeast	*Trichosporon mycotoxinivorans, Phaffia rhodozyma and Xanthophyllomyces dendrorhous isolates*
Enzymes	*Protease A, pancreatine, carboxypeptidase A, epoxidase and lactonohydrolase*

Many applications have been described for aflatoxins (Novasil®, HSCAS), however for trichothecenes and ZEN controversial results were recorded. The addition of glucomannan (*i.e.* polysaccharide as dietary fibre) or clays resulted in efficacy (Swamy *et al.*, 2004; Chowdhury and Smith, 2005), while no reduction of toxic effects was observed by others (Diaz *et al.*, 2005; Döll *et al.*, 2005; Galvano *et al.*, 2013). Cholestyramine (*i.e.* a bile acid sequestrant) showed to be effective for the binding of fumonisins and ZEN. Concerning trichothecenes, only activated charcoal showed to have an ability to bind DON and NIV.

The inclusion of bacterial isolates or enzymes (*i.e.* enzymatic and microbial detoxification) is another research focus as specific targeted biotransformation can be established. New species of *Eubacterium* (Mycofix® Plus 3.0) showed to have the ability to transform the epoxide-ring of trichothecenes (He, 1992; Hanelt *et al.*, 1994). These methods are nowadays the most prominent approaches for the eradication of mycotoxins, however this research field is still in full progress.

1.5.5.6 Application of an HACCP system in mycotoxin control

A food and feed safety management system for the identification and control of hazards within the product and processing system needs to be considered. When properly implemented, this system should result in a reduction of mycotoxin levels in many commodities. However, at farm level there are a few drawbacks as many factors influencing mycotoxin contamination are environmentally related (*e.g.* weather and insects), and difficult or even impossible to control. For this reason Critical Control Points (CCP's) do not exist in the field, nevertheless post-harvest CCP's can be identified.

The implementation of an HACCP concept has been outlined in 7 points or steps as described in **Table 1.14** (FAO, 2001; Binder *et al*., 2007).

Table 1.14 A hazard analysis critical control point (HACCP) concept with focus on fungal toxins

Principles of an HACCP plan	Exemplary measures
1. Hazard analysis	Identification of potential hazards, *i.e.* points where mycotoxin or mold infestations could occur, assessment of risks associated and description of preventive measures.
2. Critical Control Points (CCPs)	Definition of materials and processes, that have to be monitored for fungal contaminants.
3. Critical limits	Determination of maximum tolerable toxin levels, that are acceptable within an operation.
4. Monitoring procedures	Establishment of procedures for monitoring for CCPs *e.g.* sampling, sample preparation and analytical testing.
5. Corrective actions	Establishment of a procedure for corrective actions, when monitoring at a CCP indicates a deviation from an established critical limit *e.g.* plan measures to prevent fungal infestation, introduction of proper maintenance and sanitation procedures and development strategies for detoxification (if applicable).
6. Verification procedures	Establishment of procedures for verification to confirm the effectiveness of an HACCP plan *e.g.* audit plan, sampling and testing plans.
7. Documentation and record keeping	Set up documentation of all procedures and records, appropriate to these principles and their application.

Up to now, the complete elimination of mycotoxin contaminated commodities is not achievable. Uniform guidance strategies worldwide are a prerequisite to consider whilst attempting to control and manage contamination by various mycotoxins.

As a conclusion, the primary line of defense is GAP followed by the implementation of Good Manufacturing Practices (GMP) during handling, storage, processing and distribution of cereals for food and feed. These parameters should be included in a complementary management system or HACCP-manual for the control of mycotoxins.

CHAPTER 2

IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

Redrafted after:

De Boevre, M. , Diana Di Mavungu, J., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut, A., Berthiller, F., Van Peteghem, C. and De Saeger, S. Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food.

Food Additives and Contaminants (2012), 29, 5, 819-835

CHAPTER 2 IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE SIMULTANEOUS DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

SUMMARY

A liquid chromatography tandem-mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin, HT-2-toxin, and their fungal and plant conjugates thereof, including 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol-3-glucoside, α-zearalenol, β-zearalenol, zearalenone-14-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside and zearalenone-14-sulfate in maize, wheat, oats, breakfast cereals and bread.

Extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. After filtration, the extract was evaporated and the residue was redissolved in mobile phase for injection. The mobile phase that consisted of a mixture of methanol and water with 10 mM ammonium acetate, was adjusted to pH 4 with glacial acetic acid. A sample clean-up procedure was not included, because of the low recoveries of parent and masked mycotoxins and their differences in polarity. The developed method allowed the simultaneous determination of thirteen *Fusarium* mycotoxins in a one-step chromatographic run using a Waters Acquity UPLC system coupled to a Quattro Premier XE® mass spectrometer. The method was validated for several parameters such as linearity, apparent recovery, limit of detection, limit of quantification, precision, expanded measurement uncertainty and specificity. The values for the limit of detection varied from 5 µg kg^{-1} to 13 µg kg^{-1}, those for the limit of quantification from 10 µg kg^{-1} to 26 µg kg^{-1}. The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in the Commission Regulation (EC) No 401/2006 and Commission Decision (EC) No 2002/657.

Keywords: masked mycotoxins, *Fusarium*, food and feed, HPLC-MS/MS

2.1 INTRODUCTION

Mycotoxins are a major public health concern because they are the most hazardous of all food contaminants in terms of chronic toxicity (Sulyok *et al.*, 2006). Research has mainly focused on the parent mycotoxins, while as to mycotoxin conjugates, the so called *masked mycotoxins*, very limited data are available on the occurrence in food and feed, as well as their toxicity. They are referred to as masked mycotoxins because they have the ability to escape established analytical methods, applied in monitoring programs.

Ensuring the safety of agricultural products, the development of reliable, sensitive and selective analytical methods is required for the simultaneous determination of parent and masked mycotoxins. Due to the chemical and thermal stability of mycotoxins, they survive several processing steps and therefore even appear in processed cereal products. As a consequence, to be able to conduct quantitative exposure assessments, it is necessary to develop and validate methods for the determination of mycotoxins in cereals and highly-consumed cereal-derived processed food such as bread and breakfast cereals (Schneweis *et al.*, 2002; Lancova *et al.*, 2008; Zachariasova *et al.*, 2008; Berthiller *et al.*, 2009b; Kostelanska *et al.*, 2009; Koppen *et al.*, 2010; Vendl *et al.*, 2010).

In *Chapter 2* different chromatographic columns, extraction solvents and clean-up methods were compared for the determination of the *Fusarium* mycotoxins deoxynivalenol (DON), zearalenone (ZEN), T-2-toxin (T-2) and 10 derivatives thereof using an LC-MS/MS-method. The procedure was validated according to the EC criteria and applied to five frequently-consumed food matrices such as maize, wheat, oats, bread and breakfast cereals.

2.2 MATERIALS AND METHODS

2.2.1 Reagents and chemicals

Methanol (LC-MS grade) was purchased from BioSolve BV (Valkenswaard, The Netherlands), while acetonitrile (Analar Normapur), n-hexane (Hipersolv Chromanorm) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%), formic acid and tris (hydroxyl methyl) amino methane (Tris) were supplied by Merck (Darmstadt, Germany). Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Ethyl acetate was purchased from Across Organics (Geel, Belgium).

Individual mycotoxin solid standards (1 mg) of DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), deepoxydeoxynivalenol (DOM), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN) and HT-2 toxin (HT-2) were purchased from Sigma Aldrich NV/SA (Bornem, Belgium). T-2 solid standard (1 mg), and deoxynivalenol-3-glucoside (DON-3G) (50.2 ng μL^{-1} in acetonitrile) were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). ZEN (5 mg) was supplied by Fermentek (Jerusalem, Israel). All mycotoxin solid standards were dissolved in methanol (1 mg mL^{-1}) and were storable for a minimum of 1 year at -18 °C (Spanjer *et al*., 2008), while the DON-3G-solution was kept at 4 °C. The mycotoxin standards zearalenone-14-glucoside (ZEN-14G), α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G) and zearalenone-14-sulfate (ZEN-14S) were prepared according to an in-house developed procedure (*Chapter 6*).

Working solutions of DON, 3-ADON, 15-ADON, DOM, ZEN, α-ZEL, β-ZEL, ZAN, HT-2 and T-2 (10 ng μL^{-1}) were prepared in methanol and stored at -18 °C, while DON-3G was dissolved in acetonitrile and stored at 4 °C. The DON-mixture (including DON, 3-ADON, 15-ADON and DON-3G), the ZEN-mixture (including ZEN, α-ZEL, β-ZEL, T-2 and HT-2) and the internal standard-mixture (including DOM and ZAN) were prepared in methanol, stored at -18 °C and renewed monthly.

The standard solutions of the masked derivatives ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S (20 ng μL^{-1}) were prepared in methanol, keeped at 4 °C and renewed monthly. Tris, functioning as a counter ion, was added to ZEN-14S for increasing the stability of the mycotoxin standard in solution.

2.2.2 LC-MS/MS conditions

LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE® mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface by injecting a volume of 10 µL. Chromatographic separation was performed, applying a ZORBAX Eclipse XDB C18-column (3.5 µm, 100 mm x 4.6 mm) (Agilent Technologies, Diegem, Belgium). The column was kept at room temperature (21.5 °C; range [21 °C-22.4 °C]), while the autosampler was set at 10 °C. A mobile phase consisting 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 to pH 4 with glacial acetic acid was used at a flow rate of 0.2 mL min^{-1}. The gradient elution program started at 50% mobile phase A for 2 min with a linear increase to 100% mobile phase B in 8 min. An isocratic gradient of 100% mobile phase B initiated at 10 min for 5 min. The duration of each HPLC run was 20 minutes, including reequilibration. The ESI interface was used in the positive electrospray ionization mode (ESI$^+$).

Table 2.1 Optimized ESI$^+$ MS/MS-parameters

Analyte	Precursor ion (*m/z*)	Molecular ion	Cone Voltage (V)	Product ions (*m/z*)	Collision energy (eV)	Ion ratio‡	Retention time (min)	Retention factor(k')§
DON	297.4	$[M+H]^+$	37	203.4† 249.3	14 12	1.696	6.0	3.5
DON-3G	476.1	$[M+NH_4]^+$	15	248.6† 296.9	18 12	0.132	5.5	3.2
3-ADON	356.1	$[M+NH_4]^+$	25	203.1† 339.2	16 15	0.506	8.5	5.4
15-ADON	356.1	$[M+NH_4]^+$	18	137.4† 339.2	25 8	4.173	8.5	5.4
HT-2	442.2	$[M+NH_4]^+$	27	215.3† 263.3	15 15	1.024	12.2	8.2
T-2	484.1	$[M+NH_4]^+$	31	245.4† 305.4	14 15	1.280	13.0	8.8
ZEN	319.2	$[M+H]^+$	37	283.3† 301.4	15 10	1.184	14.2	9.7
α-ZEL	321.3	$[M+H]^+$	30	285.4† 303.3	12 8	0.143	12.9	8.8
β-ZEL	321.0	$[M+H]^+$	25	285.1† 303.3	10 8	0.879	12.9	8.8
ZEN-14G	481.2	$[M+H]^+$	37	283.4† 319.2	15 15	3.695	11.3	7.5
α-ZEL-14G	483.3	$[M+H]^+$	30	321.2† 283.9	22 20	0.156	10.8	7.2
β-ZEL-14G	483.0	$[M+H]^+$	30	321.1† 283.7	18 22	0.954	10.8	7.2
ZEN-14S	399.0	$[M+H]^+$	30	133.2† 319.3	18 18	0.876	10.2	6.7
DOM (IS)	281.1	$[M+H]^+$	30	108.8† 233.3	20 12	0.943	6.9	4.2
ZAN (IS)	321.2	$[M+H]^+$	35	189.1† 303.3	22 14	0.186	14.0	9.6

† quantifier ion
‡ mean of the ion ratio
§ $k' = (t_R - t_M)/t_M$, where t_R = retention time of the analyte and t_M = time taken for the mobile phase to pass through the column

The MS parameters include the following settings: ESI source block temperature 150 °C; desolvation temperature 300 °C; capillary voltage 4 kV; argon collision gas 9.10^{-6} bar; cone gas flow 50 L h^{-1} and desolvation gas flow 800 L h^{-1}. The acquisition of data was performed, applying selected reaction monitoring (SRM) with a dwell time ranging from 0.017 to 0.100 s, in order to increase the sensitivity of the mass spectrometric conditions. The cone voltage and the collision energy were optimized and selected for the most suitable precursor ion of each analyte, implemented with 2 product ions. These MS-parameters, as well as the retention time and the retention factors are shown in **Table 2.1**. MassLynx™ version 4.1. and QuanLynx® version 4.1. software (Micromass, Manchester, UK) were used for data acquisition and processing.

2.2.3 Sample preparation

Oats, wheat and maize kernels were ground using the M20-grinder (Ika Werke, Staufen, Germany), while bread and breakfast cereals were pulverized with the Moulinette 320-grinder (Moulinex, Barcelona, Spain). Before weighing, the ground material (particle size ≤ 350 µm) was vigorously homogenized with a spatula.

Two and a half grams of the sample were weighed in a Gosselin® extraction tube. Twelve and a half µL of the stock solution of the internal standard mix (50 µg kg^{-1}) was added. The standards were allowed to soak for 15 minutes, preceded by 2 minutes of vortex mixing (Labinco BV, Breda, The Netherlands). An extraction with 10 mL acetonitrile/water/acetic acid (79/20/1, v/v/v), combined with a hexane defatting (5 mL) was performed using the Agitator decanter overheadshaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 60 minutes for the whole procedure.

The sample extract was centrifuged at 3,000 g for 15 minutes, afterwards, the supernatant (hexane layer) was removed using a glass pasteur pipette with a bulb, while the aqueous layer was filtered through an Ederol-filter paper (12.5 cm, quality 15, VWR, Belgium). The filtrate was evaporated to dryness under a gentle N_2-stream at 40 °C using an evaporator module (Grant Instruments Ltd, Cambridge, United Kingdom) and was redissolved in 100 µL of injection solvent, consisting of methanol/water (50/50, v/v) and 10 mM ammonium acetate, adjusted to pH 4 with glacial acetic acid. Finally, the redissolved sample was vigorously vortexed for 3 minutes, collected in an Ultrafree-MC centrifugal device (Millipore, Bedford, USA) and centrifuged for 10 minutes at 10,000 g.

Solid phase extraction (SPE) protocols for Florisil® Bond Elut, Oasis® HLB, Multisep® 225 and Mycosep® 229 were investigated and are detailed here. Florisil® Bond Elut (Varian, California, USA), a magnesium silicate based cartridge, is considered to separate esters, ketons and carbohydrates from matrix substances. Five mL of the primary extract (no inclusion of the hexane defatting step) were evaporated to dryness and redissolved in 5 mL of methanol. The columns were conditioned with 10 mL dichloromethane/hexane (30/70, v/v). Then, the extract was applied on the column, followed by a washing step with 2 x 5 mL dichloromethane/hexane (30/70, v/v). Finally, elution was performed using 10 mL of methanol.

The Oasis® HLB sorbent (Saint-Quentin, France) is a macroporous polymer based on a balanced ratio of hydrophilic and lipophilic monomers, with the ability to retain polar and non polar compounds. An activation and conditioning step was applied using 2 x 5 mL of methanol/acetic acid (99/1, v/v) and 2 x 5 mL of water, respectively. The primary sample extract, additionally diluted with 10 mL of water, was allowed to pass through the cartridge. Subsequently, a washing step with 5 mL of water was performed, followed by elution with 5 mL of methanol/acetic acid (99/1, v/v).

Multisep® 225 column (Romer Labs, Tulln, Austria) is a basic SPE column in a syringe format, and contains a proprietary mixture of various adsorbents, specifically designed for trichothecenes. After collection of 5 mL of the primary extract, 7.5 mL of the extraction solvent acetonitrile/water/acetic acid (79/20/1, v/v/v) and 27.5 mL acetonitrile/acetic acid (99/1, v/v) were added. Thirty mL of each sample were pushed through the column and immediately collected in a Gosselin® extraction tube. Afterwards, the column was washed with 5 mL of acetonitrile/acetic acid (99/1, v/v), collected in the same tube and dried under pressure applying a syringe.

Mycosep® 229 (Romer Labs, Tulln, Austria) is a push-through cartridge that filters the extract when passing upwards through the filter, while interferences and impurities are retained on the column packing. Finally, the purified extract, containing the mycotoxins, is ended in the collector sheath of the column. Seven and a half mL of the primary extract was transferred into the glass tube and collected after the filtering.

All the extracts were evaporated to dryness under a gentle N_2-stream and, as described above, prepared for injection in the LC-MS/MS system.

2.2.4 Method validation

Based on the Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, the method was validated in terms of linearity, apparent recovery, limit of detection (LOD), limit of quantification (LOQ), precision, expanded measurement uncertainty and specificity using blank matrices of wheat, maize, oats, breakfast cereals and bread (401/2006/EC, 2006).

Five blank samples of each matrix were spiked in triplicate during 3 consecutive days with the different mycotoxins at 5 concentration levels of 25, 50, 100, 200 and 400 µg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G, and 2.5, 10, 20, 40, 80 µg kg^{-1} for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2. The use of isotopic labelled internal standards was preferred, however due to the high cost at that time, structural analogues of the analytes were a good alternative. Therefore, DOM and ZAN were added as internal standards. DOM was applied as a structural analogue for DON, 3-ADON, 15-ADON, DON-3G, T-2 and HT-2 using the relative standard peak area, while ZAN was used for the myco-estrogens and their derivatives.

The linearity of the method was assessed for each toxin in standard solution and in food matrix, by fitting the data with a linear regression model and describing the residual analysis. The apparent recovery was determined using a matrix matched calibration plot. In detail, blank matrices were spiked with the target toxins and analyzed according to the developed method. The observed signal (expressed as the relative peak area) was plotted against the actual concentration. The measured concentration was determined using the calibration curves, and the apparent recovery was calculated by **Equation 2.1**. The ratio of the relative peak area of the spiked sample was measured and inserted in the calibration curve.

Equation 2.1 Formula apparent recovery

$$\frac{\text{measured concentration } (\mu g\ kg^{-1})}{\text{actual spiked concentration } (\mu g\ kg^{-1})} = \text{apparent recovery } (\%)$$

The LOD and LOQ were verified by the signal to noise ratio (s/n) according to the IUPAC-guidelines, which stated to be more than 3 and 10, respectively (IUPAC, 1995).

The intra-day precision of the method was determined by the analysis of three replicates at five different concentrations on the same day by calculating the relative standard deviation (RSD). The same approach was applied for the determination of the inter-day precision (*cf.* intra-laboratory reproducibility), differing in three days instead of one.

The combined standard uncertainty (u_c) is an estimated standard deviation equal to the positive square root of the total variance obtained by combining the intra-laboratory reproducibility (s_R), the uncertainty associated with the purity of the standards ($U(C_{ref})$), and the uncertainty associated with the mean recovery (s_{bias}). The expanded measurement uncertainty (U) was obtained by multiplying u_c by a coverage factor 2, based on a desired approximate level of confidence of 95%. The specificity was tested by the analysis of 20 blank samples of the different matrices. The calculations were executed and processed using Microsoft Office Excel 2007 (Redmond, WA, USA) and IBM SPSS 19 (Armonk, NY, USA).

2.3 RESULTS AND DISCUSSION

2.3.1 Synthesis of masked forms of zearalenone

The in-house developed procedure using a newly-developed detached leaf assay to synthesize masked forms of ZEN namely ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S is detailed in *Chapter 6*.

2.3.2 Optimization of the HPLC method

2.3.2.1 Optimization of the MS conditions

MS/MS conditions were optimized via direct infusion of 2.5 ng μL^{-1} of each compound in the mass spectrometer via a syringe pump at a flow rate of 10 $\mu L\ min^{-1}$. The mass spectrometer was operated in MS scan, product ion scan and SRM-mode. ESI^+ was chosen over the negative mode (ESI^-), because signal intensity in ESI^- was much lower, likewise the ionization in positive polarity was sufficient for all the compounds. **Figure 2.1** represents an illustration for DON-3G in a methanol-based mobile phase (methanol/water + 10 mM ammonium acetate, adjusted to pH 6.45 with glacial acetic acid). ESI^+ (*m/z* 476, $[M+NH_4]^+$) showed a twofold to 28-fold intensity in comparison with ESI^- (*m/z* 427, $[M-CH_2OH]^-$).

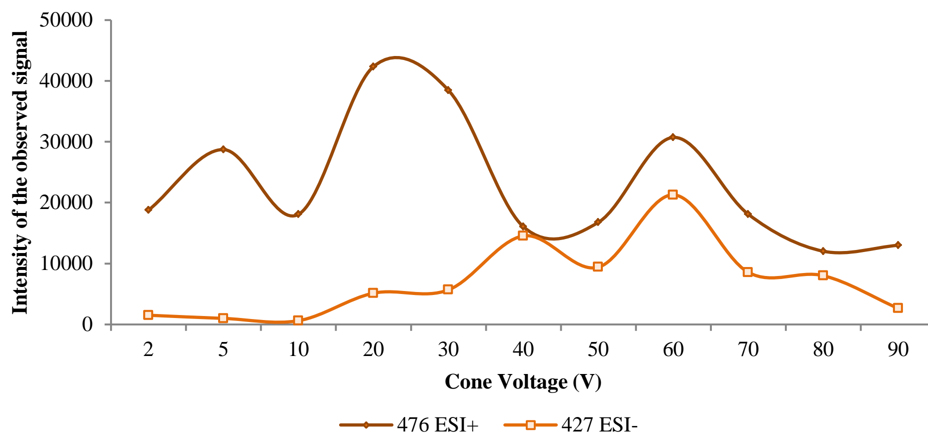


Figure 2.1 Cone voltage versus intensity (ESI^+ and ESI^-) of DON-3G in a methanol-based mobile phase

Different solvent mixtures were tested as mobile phase: methanol/water + 0.3% formic acid; acetonitrile/water + 0.3% formic acid; acetonitrile/water + 10 mM ammonium acetate, and methanol/water + 10 mM ammonium acetate, and both adjusted to pH 4 with glacial acetic acid. When optimizing the ionization parameters for DON-3G, it appeared that the use of methanol/water + 10 mM ammonium acetate, acidified with acetic acid to pH 4 in ESI^+ gave the highest intensity for that compound. According to the obtained results, an alternative for the detection of DON-3G was acetonitrile/water + 0.1% formic acid. However, as a multi-

method was developed and the other mycotoxins gave more optimal results in a methanol-based mobile phase, the latter was used. Using a mobile phase containing ammonium acetate, the formation of specific adducts might be taken into consideration. Although $[M+Na]^+$ and $[M+K]^+$ adducts exhibited the highest intensities for several analytes, these were not suitable due to lack of fragmentation in further MS² experiments. Therefore, the ammonium adducts $[M+NH_4]^+$ and the protonated $[M+H]^+$ adducts were chosen as precursor ions (**Figure 2.2** and **Figure 2.3**).

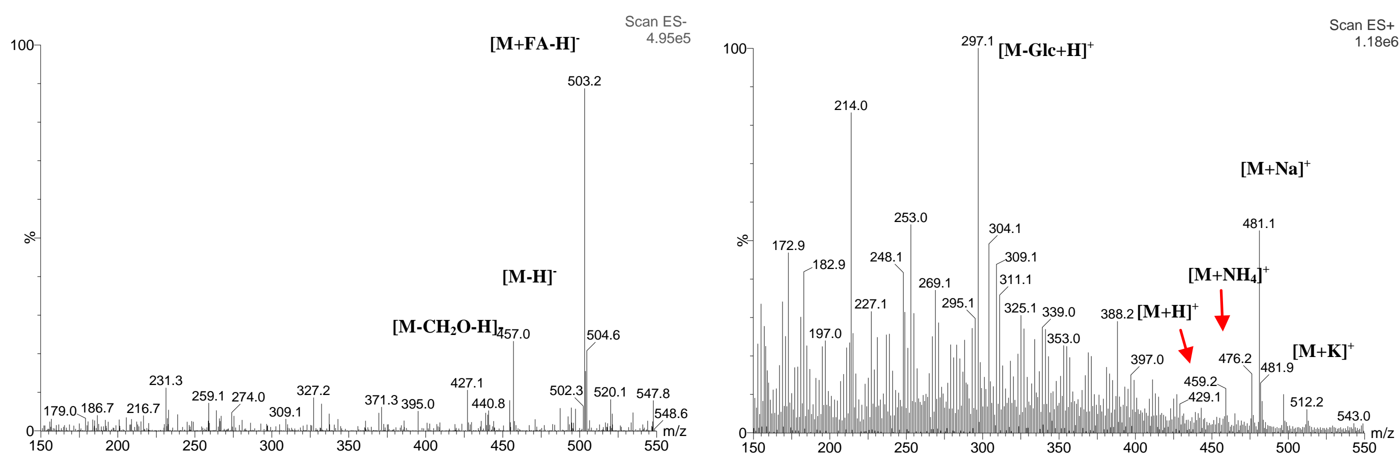


Figure 2.2 MS spectra for DON-3G in ACN + 0.1 % formic acid: ESI⁻ en ESI⁺

.

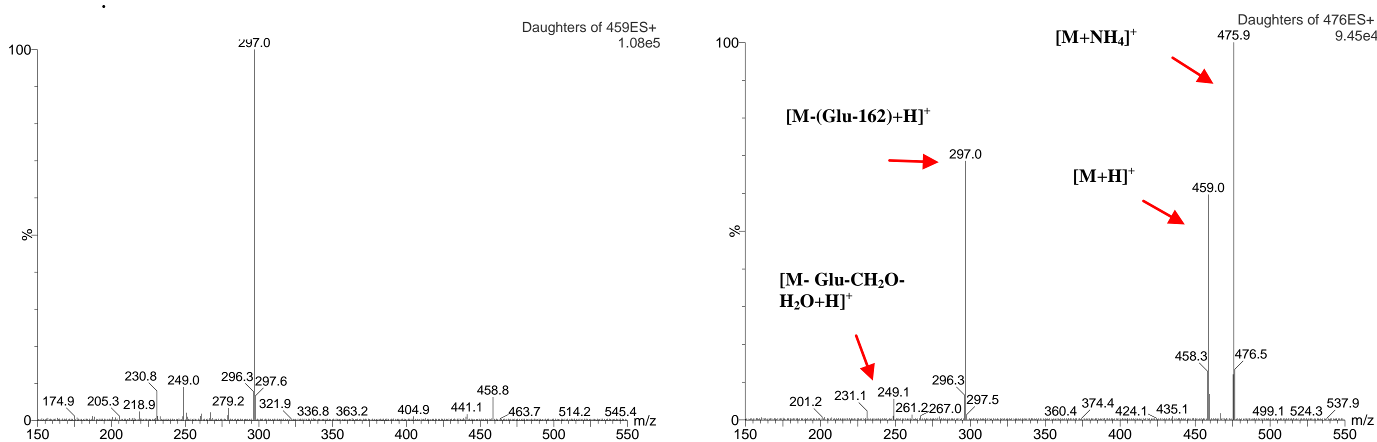


Figure 2.3 Spectra for product ions of DON-3G: protonated adduct of DON-3G and ammonium adduct of DON-3G in ESI⁺-MS/MS scan

2.3.2.2 Optimization of the LC conditions

LC conditions were optimized in order to get retention and chromatographic separation of the 13 analytes and the two internal standards in an adequate time range. Due to differences in polarity of the mycotoxins, the selection of a suitable stationary phase was of great importance. A suggestion was made to apply hydrophilic interaction chromatography (HILIC) regarding the expected retention of polar mycotoxin conjugates (Apfelthaler *et al.*, 2008;

Berthiller *et al.*, 2009a). HILIC columns are becoming quite popular (Liu *et al.*, 2010; Liu *et al.*, 2011). Three different columns with HILIC properties were investigated: XBridge™ HILIC (3.5 µm, 2.1 x 150 mm) from Waters (Millford, MA, USA), Discovery® HS F5 (3.0 µm, 2.1 x 50 mm), a combination of reversed phase and HILIC from Supelco (Bellefonte, USA), and TSKgel Amide-80 (3.0 µm, 2.0 x 150 mm), an amide-HILIC based column from Tosoh Bioscience GmbH (Stuttgart, Germany), however, no satisfactory results were obtained on retention of the most polar compounds such as DON-3G. In **Figure 2.4** the total ion chromatograms (TIC) of the three investigated HILIC columns are presented. **Figure 2.4 a** shows a peak with a retention time of 1.03 min, whereas all the 5 analytes DON, DON-3G, 15-ADON, HT-2 and ZEN co-elute in a gradient elution program. In **Figure 2.4 b** two peaks can be distinguished; however *in casu* peaks eluting before 1 min can be confounded with dead volume. The TIC in **Figure 2.4 c** shows slightly higher retention; however all analytes co-eluted. It is important to state that retention or chromatographic separation of all the analytes did not improve by adapting a gradient program.

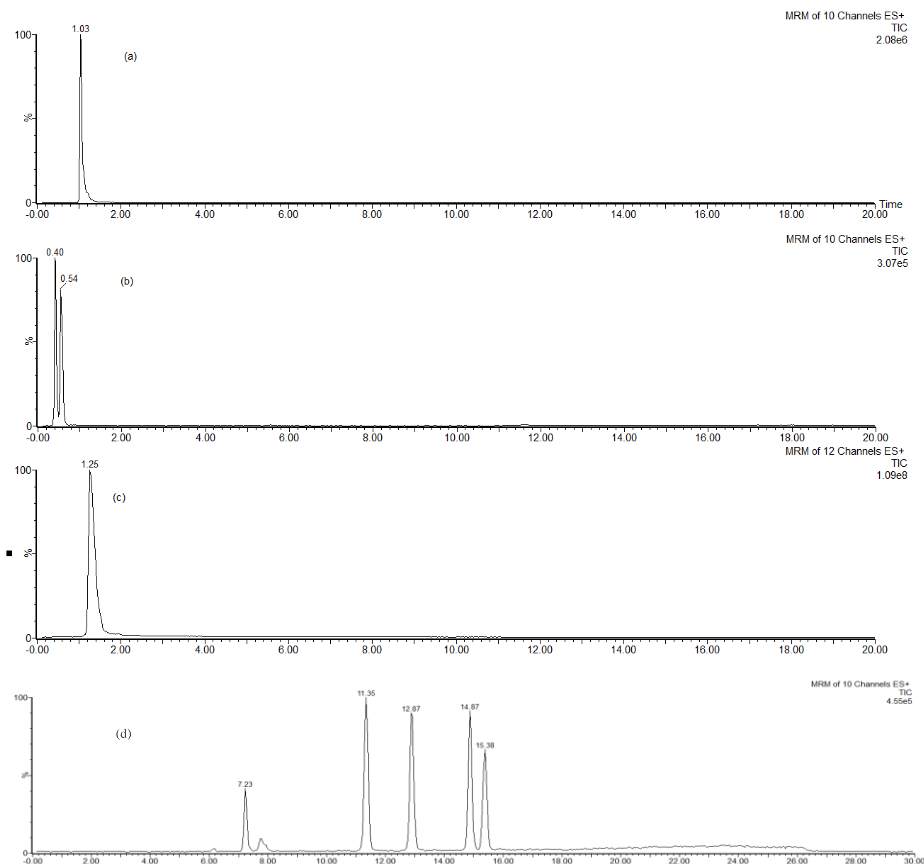
Further, a ZORBAX SB-C18 (5 µm, 4.6 x 250 mm) from Agilent Technologies (Diegem, Belgium) was used because of the experienced retention of other polar substances (Khoschsorur & Erwa, 2004). **Figure 2.4 d** represents the retention obtained for all 5 analytes with the ZORBAX SB-C18-column with the specific LC-conditions. This column enabled shortening of the LC-MS/MS run (30 min with reconditioning). By reducing the particle size, an acceptable run time of 20 minutes was obtained, applying a ZORBAX Eclipse XDB- C18 (3.5 µm, 4.6 x 100 mm) from Agilent Technologies (Diegem, Belgium). Using the UPLC-system with an UPLC-column, the chromatography could improve and eventually enhance MS-detection. However, the results obtained in this study using UPLC-columns such as the UPLC BEH C18 (1.7 µm, 2.1 x 100 mm) were not satisfactory. No retention was achieved for the polar conjugates. The ZORBAX Eclipse XDB column (3.5 µm, 100 x 4.6 mm) was available in the course of this study, proved to be suitable for the simultaneous determination of the more and less polar mycotoxins. The current method performances were satisfactory for the purpose. It is important to emphasize that, by using this column, sufficient retention was achieved of the most polar conjugate, DON-3G. The retention time of the 15 analytes ranged from 5.5 min (DON-3G) to 14.2 min (ZEN). The chromatographic retention parameters for close eluting peaks of the analyzed compounds are presented in **Table 2.1** and **Table 2.2**.

The retention factor, k', is defined as the migration rate of the analyte on a column. When rapid elution is observed, an accurate determination of the retention time is very difficult. High retention factors (>20) illustrate that elution is prolonged. In this study, retention factors of 3.2 to 9.7 were measured, consequently it can be stated that analytes are ideally retained on the column.

The selectivity factor, α, is a parameter for the chromatographic separation of two analytes on the HPLC column. The value for co-eluting compounds such as α-ZEL and β-ZEL is 1.00, as expected. Although, α describes the separation of band centers, the selectivity factor does not take the peak width into account. For this reason, the resolution factor, R_s, is also provided to show how the analytes have been separated. Baseline resolution is achieved when R_s is higher than 1.5, this is the case for the peak pairs of DON-3G and DON, DON and DOM, DOM and 3-/15-ADON, 3-/15-ADON and ZEN-14S, ZEN-14S and α-/β-ZEL-14G, α-/β-ZEL-14G and ZEN-14G, ZEN-14G and HT-2, and T-2 and ZAN.

The peak pairs T-2 and α-/β-ZEL (R_s = 0.42), and ZAN and ZEN (R_s = 0.68) were not base line separated; however these analytes can be easily distinguished because of the different SRM-transitions. In the developed method, 3-ADON - 15-ADON and α-ZEL - β-ZEL could be distinguished, although co-elution of each pair of isomers occurred. Generally, the toxins were fragmented into the same product ions with different abundance. Therefore, the co-eluting mycotoxins could be differentiated by applying the ion ratios. For 3-ADON and 15-ADON, the ion ratios (*m/z* 203) / (*m/z* 339) and (*m/z* 339) / (*m/z* 137) were determined, resulting in mean values of 0.506 (confidence interval (CI): [0.420, 0.595]) and 4.173 (CI: [3.709, 4.591]), respectively. Regarding the critical peak pair α-ZEL and β-ZEL, the ratio (*m/z* 285) / (*m/z* 303) was calculated, resulting in mean values of 0.143 (CI: [0.133, 0.153]) and 0.879 (CI: [0.854, 0.908]), respectively. According to a t-test ($p > 0.05$), means of the compounds described above, pointing no significant difference among the several food matrices. By applying different quantifier ions in the SRM transitions and bringing into consideration the difference in the ratio of the two product ions, no determination problems occurred. The ion ratios for the analytes are included in **Table 2.1**.

The LC-MS/MS chromatograms of the analytes of a spiked wheat sample (100 µg kg^{-1}) are shown in **Figure 2.5**, each in their retention window with the indicated quantifier ion (Q) and qualifier ion (q).



(a) XBridge™ HILIC (3.5 µm, 2.1 x 150 mm)
LC-conditions: Mobile Phase A (MFA) (95/5 H_2O/methanol + 10 mM NH_4-acetate adjusted to pH 4
Mobile Phase B (MFB) (95/5 Methanol/H_2O + 10 mM NH_4-acetate adjusted to pH 4
Gradient program: 100% MFA to 30% MFB in 30 min

(b) Discovery® HS F5 (3.0 µm, 2.1 x 50 mm)
LC-conditions: Mobile Phase A (95/5 H_2O/methanol + 10 mM NH_4-acetate adjusted to pH 4
Mobile Phase B (95/5 Methanol/H_2O + 10 mM NH_4-acetate adjusted to pH 4
Gradient program: 100% MFA to 30% MFB in 30 min

(c) TSKgel Amide-80 (3.0 µm,; 2.0 x 150 mm)
LC-conditions: Mobile Phase A (95/5 H_2O/methanol + 10 mM NH_4-acetate adjusted to pH 4
Mobile Phase B (95/5 Methanol/H_2O + 10 mM NH_4-acetate adjusted to pH 4
Isocratic elution: 15% MFA – 85% MFB in 20 min

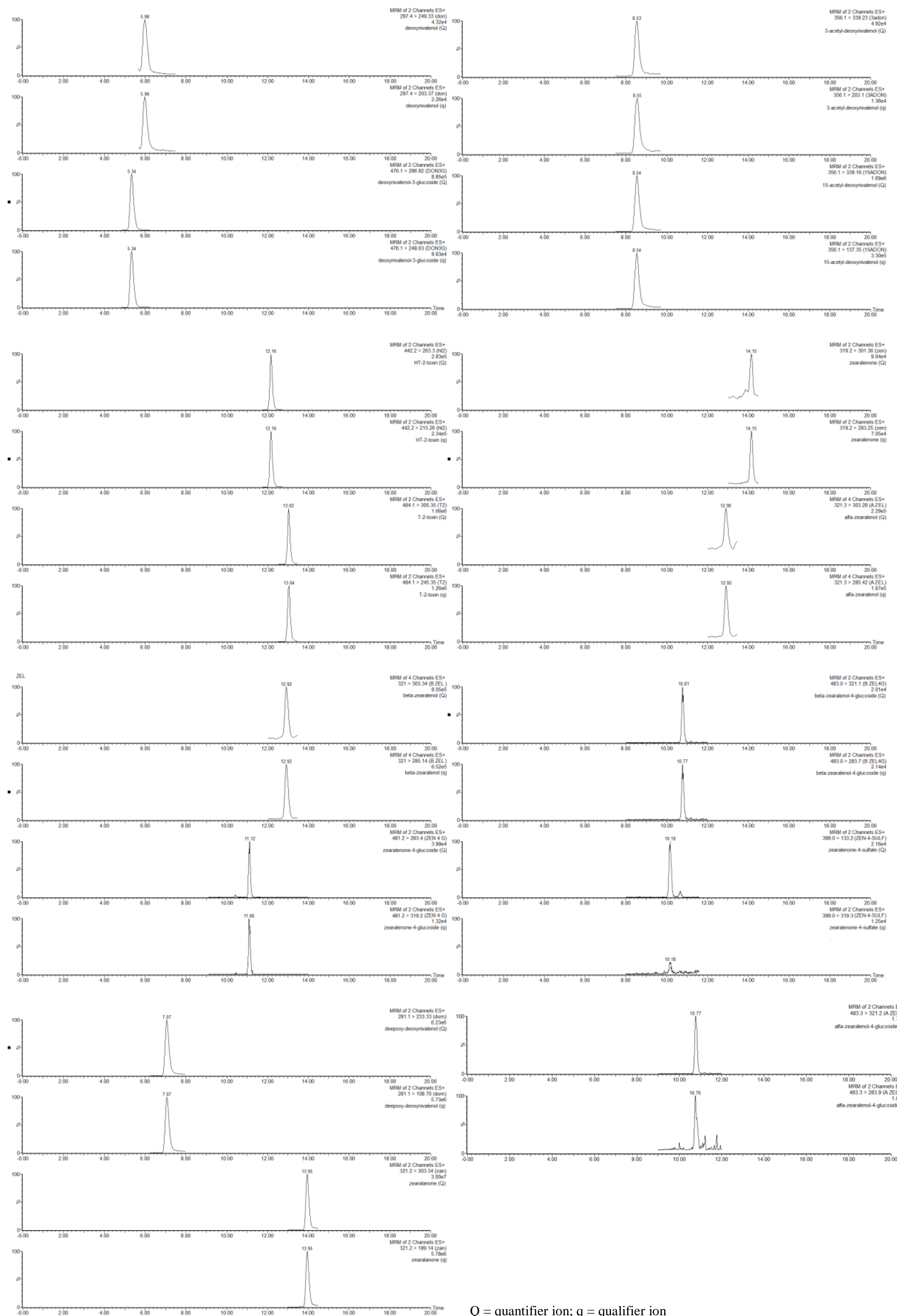
(d) ZORBAX SB-C18 (5 µm, 4.6 x 250 mm)
LC-conditions: Mobile Phase A (95/5 H_2O/methanol + 10 mM NH_4-acetate adjusted to pH 4
Mobile Phase B (95/5 Methanol/H_2O + 10 mM NH_4-acetate adjusted to pH 4
Gradient program: 50% MFA to 100% MFB in 30 min

Figure 2.4 LC-MS/MS chromatograms of the three HILIC-columns and a ZORBAX SB-C18 column

Table 2.2 Chromatographic retention parameters

Analyte pair	DON-3G-DON	DON-DOM	DOM-3-/15-ADON	3-ADON-15-ADON	3-/15-ADON-ZEN-14S	ZEN-14S-α-/β-ZEL-14G	α-ZEL-14G-β-ZEL-14G	α-/β-ZEL-14G-ZEN-14G	ZEN-14G-HT-2	HT-2-α-/β-ZEL	α-ZEL-β-ZEL	α-/β-ZEL-T-2	T-2-ZAN	ZAN-ZEN
Selectivity factor (α)†	1.12	1.19	1.29	1.00	1.24	1.07	1.00	1.05	1.09	1.06	1.00	1.01	1.09	1.02
Resolution (R_s)‡	1.56	2.40	3.73	0.00	5.88	2.75	0.00	1.87	3.50	3.21	0.00	0.42	4.44	0.68

† $\alpha = k'_B/k'_A$, where k'_B = retention factor of analyte B and k'_A = retention factor of analyte A
‡ $R_s = 2\,(t_{RB} - t_{RA})/\,(w_B + w_A)$; t_{RB} = retention time of analyte B; t_{RA} = retention time of analyte A; w_B = Gaussian curve width (peak width) of analyte B; w_A = Gaussian curve width (peak width) of analyte A



Q = quantifier ion; q = qualifier ion

Figure 2.5 LC-MS/MS chromatograms of a spiked wheat sample (100 µg kg^{-1}) in their respective retention windows

2.3.2.3 Optimization of the sample preparation and clean-up

Vendl *et al.* (Vendl *et al.*, 2009) described the use of several extraction solvents and clean-up strategies such as Mycosep® 230 and 226, and immunoaffinity columns (IAC). Justifying supplemental efforts for clean-up procedures, additional experiments were set up. The extraction efficiency was tested under acidic (acetonitrile/H_2O/CH_3COOH (79/20/1, v/v/v); methanol/H_2O/CH_3COOH (79/20/1, v/v/v)) and neutral (acetonitrile/H_2O (84/16, v/v); methanol/H_2O (84/16, v/v); methanol/H_2O/ethyl acetate (60/10/30, v/v/v)) conditions. Furthermore, the addition of a hexane defatting step was investigated. Calculating the extraction efficiency in matrix, most optimal results were obtained using 10 mL of acetonitrile/H_2O/CH_3COOH (79/20/1, v/v/v) in combination with 5 mL of hexane. Due to the lipophilicity of T-2, HT-2 and ZEN, the hexane layer was tested for these residues, though less than 2.5% occurred, which is a negligible loss. An additional advantage of the defatting step was the increased sensitivity by the acquisition of a more clean extract.

The recovery of the mycotoxins was calculated by comparing the relative peak areas of the analytes before and after the SPE procedure. Although, SPE is preferable, none of the commercially available SPE columns tested gave acceptable recoveries for all the targeted toxins (**Table 2.3**).

Table 2.3 Recovery rates (%) of the investigated SPE-columns and the dilute-and-shoot method

Mycotoxin	Florisil® Bond Elut	Multisep® 225	Mycosep® 229	Oasis® HLB	Dilute-and-shoot method
DON	30	56	53	68	92
DON-3G	66	58	55	nd	95
3-ADON	34	53	75	67	87
15-ADON	59	49	54	68	94
HT-2	12	45	60	56	92
T-2	28	42	69	55	94
ZEN	nd†	nd	29	62	106
α-ZEL	45	nd	22	66	91
β-ZEL	26	nd	41	65	88
ZEN-14G	nd	nd	63	nd	85
α-ZEL-14G	nd	nd	46	nd	86
β-ZEL-14G	nd	nd	40	nd	84
ZEN-14S	nd	nd	54	nd	85
ZAN	nd	nd	61	52	87
DOM	66	59	77	68	81

† nd = not detected

Florisil® Bond Elut (Varian,California, USA) was investigated based on experience acquired from the analysis of other polar compounds such as glucosinolates and patulin (De Smet *et al.*, 2011; Njumbe Ediage *et al.*, 2011). DON-3G gave a mean recovery of 66%, however all other target mycotoxins did not reach a value of 60%. The ZEN-equivalents were even not

retained on the Florisil®-column. Based on our laboratory experience, Multisep® 225 and Mycosep® 229 (Romer Labs, Tulln, Austria) were tested as these columns are designed to retain interferences and elute mycotoxins in a certain polarity range, while Oasis® HLB (Saint-Quentin, France) was examined for retention of both polar and non-polar compounds. Multisep® 225 showed to have a recovery lower than 60% for DON and T-2-equivalents. The Oasis® HLB-column had an overall recovery for the parent precursors lower than 70%, and in addition the masked conjugates were not retained. Among the columns tested, Mycosep® 229 showed to have an acceptable recovery as stated in **Table 2.3**, however, much higher results were obtained without the involvement of SPE, consequently, the final procedure includes the dilute-and-shoot method.

2.3.2.4 Method validation

The LC-MS/MS method was successfully validated for the mycotoxins DON, 3-ADON, 15-ADON, DON-3G, ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2 in maize, wheat, oats, breakfast cereals and bread based on the Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

By evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. In **Table 2.4** the Pearson's correlation coefficient (R) in combination with the standard error of mean (SE) is presented. R reached a value of more than 0.95 in both standard solutions and spiked food matrices. Values for SE pointed an overall small difference between the results obtained. Two results were rather high: ZEN (SE 0.019) due to two outliers and α-ZEL-14G (SE 0.021) due to 1 outlier in the batch. However, establishing the linear regression model, no parallelism of the curves occurred for the standard in neat solution and in the food matrix. Ion suppression was noted for the matrix curve, possibly due to interferences of matrix substances during ionization. Hence, matrix matched calibration curves were used for the determination of the analytes. In addition, residual plots were constructed resulting in a random distribution of the residues.

In general, the results obtained at medium level for the apparent recovery are presented in **Table 2.4**, and proved to be acceptable (range 70% - 110%). In general, the recovery rate was between 80% and 100%, however in most cases the ZEN-conjugates's recoveries were below 80% (> 70%). This was probably due to the low spiking level (10 µg kg^{-1}), because of the low amount available of the masked ZEN forms.

The developed method allowed the determination of all target mycotoxins at a low ppb-level. The LODs for maize, wheat, oats, breakfast cereals and bread varied in a range of [5-12 μg kg^{-1}], [5-11 μg kg^{-1}], [5-12 μg kg^{-1}], [7-12 μg kg^{-1}] and [8-13 μg kg^{-1}] respectively, and the LOQs were [10-24 μg kg^{-1}], [10-22 μg kg^{-1}], [10-24 μg kg^{-1}], [14-24 μg kg^{-1}] and [16-26 μg kg^{-1}] respectively.

The RSDs were determined during the precision study at the five different concentration levels. The data obtained at medium level (100 μg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G and 10 μg kg^{-1} for ZEN and the other mycotoxins) are reported in **Table 2.4**. The intra-day precision (RSD_r)- and the inter-day precision (RSD_R) data were in accordance with the described criteria (401/2006/EC, 2006). The latter criteria have only been described for all parent mycotoxins, however, they were implemented for all analyzed conjugates. The Horwitz equation, stated in 2002/657/EC (2006), was taken into account for low ppb-levels. The acetylated form 3-ADON and DON-3G represented rather high RSD_r-levels (19% and 18%, respectively) in maize; 15% (3-ADON) in oats, 16% (3-ADON) and 17% (15-ADON) in breakfast cereals, still, all in accordance with the validation criteria.

The expanded measurement uncertainty (U), calculated at a concentration level of 100 μg kg^{-1} (DON, 3-ADON, 15-ADON and DON-3G) and 10 μg kg^{-1} (ZEN and the other mycotoxins) is a criterion for the integral acceptability of the method and ranged for all the matrices from 11% to 79%. Wheat, maize, breakfast cereals and bread presented maximum expanded uncertainties of approximately 45% with outliers of 55% (T-2, maize), 60% (α-ZEL-14G, maize) and 58% (HT-2, bread). In oats, the U for the main mycotoxins were high (*e.g.* T-2 (61%), HT-2 (71%) and β-ZEL (79%)), these values were attributed to the high RSD_R-levels and therefore questionable for the specific matrix and mycotoxin.

The same batches were used for most validation parameters, however, the sample preparation procedure was completely independent, leading to completely independent data. Substantive batches of samples were not required for those parameters, however, the specificity was tested by analyzing 20 blank samples of the different matrices. The analysis of the results confirmed that no interferences occurred in the several SRM-channels.

All validation parameters were calculated using the relative peak area, with respect to the internal standards DOM (for DON, 3-ADON, 15-ADON, DON-3G, HT-2 and T-2) and ZAN (used for all other described mycotoxins) as internal standardization is required to compensate for matrix effects and to cover the losses during the clean-up procedure in this study (Monbaliu *et al.*, 2009).

The results of the performance characteristics of the LC-MS/MS method for wheat, maize, oats, breakfast cereals and bread are presented in **Table 2.4**, **2.5**, **2.6**, **2.7** and **2.8**, respectively, moreover, these were in good agreement with the criteria mentioned in the Commission Regulation (EC) No 401/2006 (401/2006/EC, 2006).

Table 2.4 Validation parameters† for the analyzed mycotoxins in wheat

WHEAT									
Analyte	Calibration range (μg kg^{-1})	R (mean)	SE	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	RSD_r (%)†	RSD_R (%)†	Apparent Recovery (%)†	U (%)†
DON	25-400	0.99	0.007	6	12	7	15	90	31
DON-3G	25-400	0.98	0.010	8	16	8	12	90	27
3-ADON	25-400	0.97	0.013	5	10	11	9	89	20
15-ADON	25-400	0.99	0.003	6	12	7	9	104	19
HT-2	2.5-80	0.98	0.015	5	10	18	17	96	36
T-2	2.5-80	0.98	0.008	9	18	12	18	81	38
ZEN	2.5-80	0.98	0.013	6	12	15	10	74	21
α-ZEL	2.5-80	0.97	0.012	5	10	18	22	77	45
β-ZEL	2.5-80	0.99	0.010	7	14	10	11	79	23
ZEN-14G	2.5-80	0.97	0.005	8	16	24	22	89	47
α-ZEL-14G	2.5-80	0.97	0.011	9	18	26	21	80	45
β-ZEL-14G	2.5-80	0.98	0.005	9	18	19	20	81	41
ZEN-14S	2.5-80	0.99	0.006	11	22	21	17	78	35

† Validation parameters are those determined at medium level: 100 μg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G, and 10 μg kg^{-1} for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2.

Table 2.5 Validation parameters† for the analyzed mycotoxins in maize

MAIZE									
Analyte	Calibration range (μg kg^{-1})	R (mean)	SE	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	RSD_r (%)†	RSD_R (%)†	Apparent Recovery (%)†	U (%)†
DON	25-400	0.99	0.003	6	12	14	12	100	26
DON-3G	25-400	0.99	0.002	8	16	18	17	85	36
3-ADON	25-400	0.98	0.003	7	14	19	21	101	45
15-ADON	25-400	0.99	0.001	6	12	11	14	100	30
HT-2	2.5-80	0.98	0.004	5	10	20	20	91	43
T-2	2.5-80	0.98	0.012	9	18	19	20	93	42
ZEN	2.5-80	0.98	0.011	7	14	11	17	71	35
α-ZEL	2.5-80	0.97	0.012	5	10	15	19	85	40
β-ZEL	2.5-80	0.98	0.007	7	14	14	16	93	35
ZEN-14G	2.5-80	0.97	0.006	7	14	21	26	85	55
α-ZEL-14G	2.5-80	0.97	0.015	10	20	15	28	73	60
β-ZEL-14G	2.5-80	0.97	0.003	10	20	19	12	72	26
ZEN-14S	2.5-80	0.95	0.012	12	24	18	15	76	31

† Validation parameters are those determined at medium level: 100 μg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G, and 10 μg kg^{-1} for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2.

Table 2.6 Validation parameters† for the analyzed mycotoxins in oats

OATS									
Analyte	Calibration range (µg kg^{-1})	R (mean)	SE	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	RSD_r (%)†	RSD_R (%)†	Apparent Recovery (%)†	U (%)†
DON	25-400	0.98	0.007	5	10	5	5	98	11
DON-3G	25-400	0.98	0.003	7	14	12	14	93	30
3-ADON	25-400	0.97	0.014	5	10	15	24	107	51
15-ADON	25-400	0.97	0.005	5	10	7	12	90	26
HT-2	2.5-80	0.97	0.011	6	12	21	29	108	61
T-2	2.5-80	0.97	0.010	9	18	27	34	95	71
ZEN	2.5-80	0.97	0.015	6	12	23	26	107	55
α-ZEL	2.5-80	0.96	0.013	6	12	23	14	104	29
β-ZEL	2.5-80	0.96	0.017	7	14	21	38	82	79
ZEN-14G	2.5-80	0.97	0.005	9	18	25	25	88	53
α-ZEL-14G	2.5-80	0.98	0.006	10	20	26	29	73	61
β-ZEL-14G	2.5-80	0.96	0.017	10	20	18	17	71	35
ZEN-14S	2.5-80	0.96	0.009	12	24	28	19	76	42

† Validation parameters are those determined at medium level: 100 µg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G, and 10 µg kg^{-1} for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2.

Table 2.7 Validation parameters† for the analyzed mycotoxins in breakfast cereals

BREAKFAST CEREALS									
Analyte	Calibration range (µg kg^{-1})	R (mean)	SE	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	RSD_r (%)†	RSD_R (%)†	Apparent Recovery (%)†	U (%)†
DON	25-400	0.97	0.012	9	18	5	6	103	13
DON-3G	25-400	0.98	0.007	12	24	5	16	103	33
3-ADON	25-400	0.99	0.006	8	16	16	12	107	26
15-ADON	25-400	0.98	0.004	7	14	17	10	91	22
HT-2	2.5-80	0.97	0.012	9	18	13	10	95	22
T-2	2.5-80	0.98	0.007	10	20	11	16	88	34
ZEN	2.5-80	0.96	0.011	9	18	15	23	78	23
α-ZEL	2.5-80	0.97	0.005	9	18	19	20	101	42
β-ZEL	2.5-80	0.97	0.012	8	16	9	17	96	35
ZEN-14G	2.5-80	0.98	0.012	10	20	26	12	83	26
α-ZEL-14G	2.5-80	0.97	0.008	11	22	27	20	76	42
β-ZEL-14G	2.5-80	0.98	0.003	11	22	19	16	70	34
ZEN-14S	2.5-80	0.97	0.009	11	22	15	22	76	45

† Validation parameters are those determined at medium level: 100 µg kg^{-1} for DON, 3-ADON, 15-ADON and DON-3G, and 10 µg kg^{-1} for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2.

Table 2.8 Validation parameters† for the analyzed mycotoxins in bread

BREAD									
Analyte	Calibration range ($\mu g\ kg^{-1}$)	R (mean)	SE	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	RSD_r (%)†	RSD_R (%)†	Apparent Recovery (%)†	U (%)†
DON	25-400	0.97	0.005	10	20	12	20	95	41
DON-3G	25-400	0.97	0.011	13	26	8	11	97	23
3-ADON	25-400	0.97	0.008	10	20	10	16	98	33
15-ADON	25-400	0.98	0.008	9	18	9	14	99	29
HT-2	2.5-80	0.97	0.004	9	18	17	27	87	58
T-2	2.5-80	0.98	0.004	11	22	12	20	84	42
ZEN	2.5-80	0.99	0.004	8	16	15	22	82	46
α-ZEL	2.5-80	0.97	0.009	9	18	10	5	95	11
β-ZEL	2.5-80	0.98	0.019	9	18	8	9	80	19
ZEN-14G	2.5-80	0.97	0.009	10	20	24	22	83	45
α-ZEL-14G	2.5-80	0.96	0.021	12	24	21	17	77	36
β-ZEL-14G	2.5-80	0.97	0.006	12	24	19	23	76	48
ZEN-14S	2.5-80	0.97	0.004	12	24	22	12	78	26

† Validation parameters are those determined at medium level: 100 $\mu g\ kg^{-1}$ for DON, 3-ADON, 15-ADON and DON-3G, and 10 $\mu g\ kg^{-1}$ for ZEN, α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G, ZEN-14S, HT-2 and T-2.

2.4 CONCLUSION

Based on the results of *Chapter 2*, it can be stated that an LC-ESI$^+$-MS/MS method was successfully developed and validated for the simultaneous determination of 13 mycotoxins, including their masked conjugates for several matrices such as maize, wheat, oats, breakfast cereals and bread. Furthermore, the additional hexane defatting step allowed increasing the sensitivity, resulting in relatively cleaner extracts.

In *Chapter 3*, *4*, *5* and *6* the developed analytical method was the basis for the accomplishment of the research on contamination levels of mycotoxins and their masked mycotoxins in different cereal-based matrices, and to measure the exposure assessment.

CHAPTER 3

NATURAL OCCURRENCE OF MYCOTOXINS AND THEIR MASKED FORMS IN FOOD, FEED AND MAIZE: THE ELABORATION OF 3 SURVEYS

Redrafted after:

De Boevre, M. , Diana Di Mavungu, J., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., and De Saeger, S. Natural occurrence of mycotoxins and their masked forms in food and feed products. World Mycotoxin Journal (2012), 5, 3, 207-219

De Boevre, M., Landschoot, S., Audenaert, K., Maene, P., Diana Di Mavungu, J., Eeckhout, M., Haesaert, G. and De Saeger, S. Within-field variability of *Fusarium* mycotoxins and their masked forms in maize crops in Belgium. World Mycotoxin Journal (submitted, 2013)

CHAPTER 3 NATURAL OCCURRENCE OF MYCOTOXINS AND THEIR MASKED FORMS IN FOOD, FEED AND MAIZE: THE ELABORATION OF 3 SURVEYS

SUMMARY

In *Chapter 3* results of three surveys in food, feed and maize are elaborated proving the natural occurrence of mycotoxins and their masked forms.

Concerning food and feed, a total of 175 cereal-based food products, 67 compound feeds and 19 feed materials were analyzed for the occurrence of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, zearalenone, α-zearalenol, β-zearalenol, and their respective masked forms, including deoxynivalenol-3-glucoside, zearalenone-14-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside and zearalenone-14-sulfate. Fibre-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal were collected in Belgian supermarkets from April 2010 to October 2011. All food samples analyzed were contaminated with an average of 2 to 6 mycotoxins, including 1 to 3 masked forms. Feed materials originating from maize and its by-products were mostly infected with deoxynivalenol, zearalenone and derivatives. Also, the glucosylated and sulfated forms occurred in substantial amounts. As well, wheat and its by-products were contaminated with α-zearalenol (wheat gluten feed and wheat bran) and zearalenone (wheat). The contamination pattern and level of feed materials were reflected in the corresponding compound feeds.

Regarding maize, a total of 288 maize samples were analyzed for the same mycotoxins. To get a better insight, the results in *Chapter 3* assessed the presence of various *Fusarium* (masked) mycotoxins in different maize varieties grown under natural infection conditions in Flanders, Belgium. The outcome showed that maize varieties were contaminated with a mixture of both parent and masked mycotoxins. Moreover, a positive correlation between the parent and masked forms was established. This indicates that a higher contamination of a particular mycotoxin is coupled with an elevated load of the other. Further, a field analysis was executed to elucidate the distribution of the various mycotoxins within the field. Except for deoxynivalenol, the maize variety did not significantly influence the mycotoxin accumulation. Subdivisions within the field with higher mycotoxin levels were observed. The characterization of these clusters is of great importance as sampling schemes for field kernels might be constructed.

Keywords: mycotoxins, masked mycotoxins, *Fusarium*, food and feed, maize, within field variability

3.1 INTRODUCTION

A quarter of the world's food crops is affected by mycotoxins (Charmley *et al.*, 1995). *Fusarium* mycotoxins in cereal-based food, animal feed and maize have been extensively investigated worldwide over recent years. Contrary to the wealth of information on the parent mycotoxins, only limited data are available for their mycotoxin derivatives in food and feed. On top of reductions in crop yield, *Fusarium* species produce, besides fumonisins, a heterogeneous blend of mycotoxins such as trichothecenes and myco-estrogens. The main sources of these fusariotoxins in human food and animal feed are wheat, barley and maize.

Maize is an important staple crop cultivated worldwide (Schollenberger *et al.*, 2012). In Belgium, 9.2 million tons of maize plants are harvested annually from 247,000 hectares of farm land, intended for livestock feed. However, only limited information is available concerning the mycotoxin pattern in Belgian maize fields. Insight into the relative importance of the various mycotoxins and spatial distribution on the field for different maize varieties will help to develop prevention strategies for ear rot, and related mycotoxin contaminations (Desjardins *et al.*, 1993).

Selection of less susceptible maize genotypes might offer some possibilities to minimize *Fusarium* infestation and the associated mycotoxin contamination. However, due to the multigenic nature of resistance, high levels of resistance have not been incorporated into high-yielding, agronomically desirable maize hybrids (Miller *et al.*, 2007). The characterization of subdivisions within the field has gained more attention and can be considered as a valuable input for sampling schemes under field conditions (Schmale *et al.*, 2005; Oerke *et al.*, 2010;).

As the majority of mycotoxins are also phytotoxic compounds, plants detoxify them through glucosylation and transport to the vacuoles. Knowledge on the occurrence of these so-called masked mycotoxins is imperative to unequivocally determine the mycotoxicological load of unprocessed grains and food or feed products (Berthiller *et al.*, 2009a).

The present *Chapter 3* describes the natural occurrence of mycotoxins DON, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), T-2, HT-2, ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and their respective masked forms including deoxynivalenol-3-glucoside (DON-3G), zearalenone-14-glucoside (ZEN-14G), zearalenone-14-sulfate (ZEN-14S), α-zearalenol-14-glucoside (α-ZEL-14G) and β-zearalenol-14-glucoside (β-ZEL-14G), in a total of 67 compound feeds, 19 feed materials, 175 cereal-based food products and 288 different commercial maize genotypes under natural infection pressure.

3.2 MATERIALS AND METHODS

3.2.1 Reagents and chemicals

All reagents and chemicals used for the analysis of mycotoxins and their masked forms are detailed in paragraph 2.2.1.

3.2.2 Collection of samples for (masked) mycotoxin analysis in food and feed

In the framework of a regular Belgian sampling program, a total of 175 cereal-based food products were collected between April 2010 and October 2011, including fibre-enriched bread (*n*=52), bran-enriched bread (*n*=36), breakfast cereals (*n*=61), popcorn (*n*=12) and oatmeal (*n*=13). The commercially available food products were purchased from different manufacturers in several supermarket chains with a good market share in Belgium. Representative samples were obtained by collecting the cereal-based retail-products with different batch numbers every three months from the same manufacturers according to a structured sampling plan. Breakfast cereals, popcorn and oatmeal were stored at room temperature in the dark until analysis, and bread was concurrently analyzed within 3 days to prevent molding of the matrix.

A total of 86 feed samples (sows, *n*=15; poultry, *n*=14; horses, *n*=14; fattening pigs, *n*=13, piglets, *n*=8; dairy cattle and young stock, *n*=3 and individual feed materials intended for compound feeds, *n*=19) were obtained in the framework of a regular Belgian monitoring program. The feed materials analyzed were beetpulp, sunflower seed meal, soy bean, soy peel, oats, barley, maize germs, maize gluten feed, maize, wheat, wheat bran pellets and wheat gluten feed, selected on their occurrence in the compound feeds.

3.2.3 Collection of samples for (masked) mycotoxin analysis in maize: experimental field layout

An experimental field trial including 36 commercial maize varieties with a differential resistance towards *Fusarium* was set up in Bottelare (Belgium) in the year 2011. The field was a completely randomized block design divided into four replications (144 elementary m² plots). The previous crop was *Triticale*, and the sowing density accounted for 111,000 seeds per hectare. N-fertilization was performed in quadruplicate according to the advice of the Soil Service of Belgium. For weed control, a herbicide containing tembotrione, flufenacet terbuthylazin and nicosulfuron was used. No fungicide against *Fusarium* head blight was applied. A total of 288 maize samples were analyzed for their mycotoxin content.

3.2.4 Determination of (masked) mycotoxin concentrations in food, feed and maize

Bread, popcorn, oatmeal and breakfast cereals were pulverized with the Moulinette 320-grinder (Moulinex, Barcelona, Spain). Halve a kg of each sample was ground. The feed samples, oats, wheat and maize kernels were milled using the M20-grinder (Ika Werke, Staufen, Germany). A cleaning and decontamination routine of the equipment was performed using water and disinfectol® after each milling practice. The ground material was vigorously homogenized with a spatula before weighing. Sample preparation and the applied LC-MS/MS-method used was performed according to the detailed approach in paragraph 2.2.

3.2.5 Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2007 (Redmond, WA, USA) and IBM SPSS 19 (Armonk, NY, USA). All statistical analysis and differences in mycotoxin levels between 2010 and 2011 were executed using the non-parametric Wilcoxon rank sum test (P) and the Kruskal Wallis test (P). The non-parametric Spearman rank correlation coefficient (ρ) was employed to determine the degree to which a monotonic relationship existed between a pair of variables.

For statistical analysis and generation of the graphics in the maize-survey, the R-statistical analysis software system (http://www.r-project.org) was used. R is an integrated environment of software for data manipulation, calculation and generation of graphics, and can be easily expanded adding new functionalities. Data are represented as boxplots and heat maps. A heat map is a graphical representation of data in which the individual values of the matrix are represented as colors. High level contamination data are represented by white/yellow rectangles while lower levels are indicated in orange/red.

Differences between groups of data were tested for significance with a non-parametric Kruskall-Wallis test and a paired non-parametric Wilcoxon signed rank test.

3.3 RESULTS AND DISCUSSION

3.3.1 Part 1: occurrence data for (masked) mycotoxins in foods from the Belgian market

Analyzed cereal-based food samples (*n*=175) were contaminated with an average of 2 to 6 different mycotoxins (median = 4) including 1 to 3 (median = 1) masked forms. DON- and ZEN-*equivalents* were taken into account, being the sum of DON, 3-ADON, 15-ADON and DON-3G, and the sum of ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G and β-ZEL-14G, respectively. The mean and maximum mycotoxin concentrations of the collected samples are shown for 2010 and 2011 in **Table 3.1**. 3-ADON, 15-ADON and DON-3G are designated as DON-*derivatives*; α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G as ZEN-*derivatives*. A detailed description of the results per food product as well as a statistical analysis of these results are provided in the following sections.

3.3.1.1 Fibre-enriched bread

DON occurred in 85% of the fibre-enriched bread samples with an average level of 34 µg kg^{-1} (10 µg kg^{-1} to 138 µg kg^{-1}), while the acetylated forms, 3-ADON and 15-ADON, were present in 38% and 33% of the samples with a mean value of 14 µg kg^{-1} (10 µg kg^{-1} to 74 µg kg^{-1}), and 9 µg kg^{-1} (9 µg kg^{-1} to 45 µg kg^{-1}), respectively.

DON-3G occurred in almost half of the samples (48%) of which one sample contained 425 µg kg^{-1}, whilst the concentration of the parent DON was 98 µg kg^{-1}. Taking into consideration the sum of DON-equivalents, a total value of 523 µg kg^{-1} was reached for that particular sample (98 µg kg^{-1} DON, 425 µg kg^{-1} DON-3G). The EU-maximum limit for DON is 500 µg kg^{-1}. Determining the DON-equivalents, and extrapolating the obtained values to the legislation of the parent mycotoxin is obviously a worst-case scenario assuming that the derivatives are totally hydrolyzed and equally toxic as their parents. The molecular masses of the derivatives are higher than the molecular parent mass. Another approach to estimate the content of the masked forms in relative form to the parent form is the determination of their ratio in mol%.

Regarding the amount of 3-ADON, 15-ADON and DON-3G formed in 2010 and 2011, there is a clear significant difference (P=0.047) according to a two-sided Wilcoxon test. In 2010 a 1.72 fold higher formation of DON-derivatives was proven in proportion to 2011. However, main difference has been due to the formation of the acetylated forms, as the occurrence of DON-3G was relatively equal (21.80% versus 21.93%).

Figure 3.1 represents the occurrence of the mycotoxin derivatives per year for the various investigated food products. There was a clear visual downward tendency among the acetylated forms for fibre-enriched bread between 2010 and 2011.

T-2 occurred in 63% of the samples with an average of 16 µg kg^{-1} (max 45 µg kg^{-1}). Fifty-eight % of the bread samples were contaminated with HT-2 in a respective average of 14 µg kg^{-1} (max 49 µg kg^{-1}). Similar results were observed, however, there was a clear difference between the absolute contamination in 2010 and 2011 (**Figure 3.2**). The indicative level for the sum of HT-2 and T-2 (25 µg kg^{-1}) was exceeded in almost half of the samples (46%), mostly contributed to matrices sampled in 2011. In 2010, concentrations of 9 µg kg^{-1} and 11 µg kg^{-1} were found (LOD), while in 2011 much higher contents were measured. **Figure 3.2** represents the mean contamination levels of T-2 and HT-2 (µg kg^{-1}) in the food commodities per year, where an inclination is observed for both mycotoxins.

A quarter of the fibre-enriched bread samples was contaminated with ZEN with an average of 29 µg kg^{-1} (max 230 µg kg^{-1}) of which 7 samples did not meet the EU-maximum limit of 50 µg kg^{-1}. In approximately same portion of samples (17% and 25%), α-ZEL and β-ZEL occurred with average values of 6 µg kg^{-1} (max 110 µg kg^{-1}) and 7 µg kg^{-1} (max 86 µg kg^{-1}), respectively. However, few samples were observed with high amounts of these toxins. Seventeen %, 4%, 10% and 21% of the analyzed bread samples were contaminated with ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G, respectively. In 2010, the observed concentrations were low, while in 2011 elevated levels with maxima of 154 µg kg^{-1}, 176 µg kg^{-1}, 153 µg kg^{-1} and 63 µg kg^{-1} for ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G, respectively were recorded.

According to a two-sided Wilcoxon test (P=0.29), there was no statistical difference between the formation of total ZEN-derivatives in the period 2010-2011. However, the occurrence of ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G was significantly higher in 2011 ($P=4\times10^{-3}$).

3.3.1.2 Bran-enriched bread

DON occurred in 75% of all the bran-enriched bread samples with an average of 25 µg kg^{-1} (max 127 µg kg^{-1}), which is low considering the maximum permissible limit of 500 µg kg^{-1}. The acetylated forms 3-ADON and 15-ADON were present in 44% of the products, also in relatively low amounts of 16 µg kg^{-1} and 7 µg kg^{-1} (maxima 59 µg $kg^{-1}$and 45 µg kg^{-1}), respectively. The statistical analysis underscores the higher incidence of acetylated forms in 2010 ($P=4\times10^{-3}$) (**Figure 3.1**).

Table 3.1 Concentrations of mycotoxins and their masked forms (mean, standard deviation and maximum) ($\mu g\ kg^{-1}$) in the different food categories analyzed (years 2010-2011)

	Fibre-enriched bread				Bran-enriched bread				Breakfast cereals				Popcorn				Oatmeal				All foods			
Occurrence data (*n*)	**52**				**36**				**62**				**12**				**13**				**175**			
Mycotoxin	#	µg kg-1	µg kg-1	µg kg-1	#	µg kg-1	µg kg-1	µg kg-1	#	µg kg-1	µg kg-1	µg kg-1	#	µg kg-1	µg kg-1	µg kg-1	#	µg kg-1	µg kg-1	µg kg-1	#	µg kg-1	µg kg-1	µg kg-1
	> LOD[†]	mean	SD	max	> LOD	mean	SD	max	> LOD	mean	SD	max	> LOD	mean	SD	max	> LOD	mean	SD	max	> LOD	mean	SD	max
DON[§]	44	34	31	138	16	25	35	127	36	44	107	718	10	49	125	442	5	18	30	91	111	42	87	718
3-ADON	21	14	22	74	14	16	20	59	34	31	63	431	12	30	22	69	8	45	45	116	89	25	43	431
15-ADON	20	9	16	45	4	7	11	45	30	10	27	194	12	26	18	55	10	7	8	27	76	16	35	218
DON-3G	27	34	68	425	12	21	30	103	31	13	17	63	11	33	37	96	10	28	33	97	91	26	46	425
β-ZEL	12	7	17	86	6	13	25	96	26	17	33	147	2	5	14	47	4	8	17	46	53	14	30	186
α-ZEL	16	6	19	110	3	6	13	60	32	43	92	515	2	3	9	32	5	10	22	68	61	19	57	515
ZEN	23	29	54	230	14	38	52	157	32	76	165	450	7	9	19	51	8	41	55	85	84	47	104	885
ZEN-14G	15	15	43	154	2	18	43	155	25	39	75	369	0	nd[‡]	nd	nd	5	12	26	91	48	23	54	369
ZEN-14S	4	4	24	176	2	4	24	143	17	23	72	417	1	1	3	12	2	4	10	36	24	10	45	45
β-ZEL-14G	10	7	24	153	2	6	26	153	18	11	32	206	1	1	3	10	2	2	4	10	33	9	26	206
α-ZEL-14G	5	3	10	63	1	0.3	2	12	16	11	30	192	0	nd	nd	nd	1	1	3	10	22	5	19	192
HT-2	36	14	17	49	8	14	17	48	40	13	14	42	2	3	10	35	9	20	32	118	95	19	42	418
T-2	33	16	17	45	5	6	10	45	38	8	8	33	3	4	10	26	9	9	10	34	88	13	13	45

† >LOD: amount of samples with a concentration detected above the limit of detection (LOD); LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 $\mu g\ kg^{-1}$), 3-ADON (10, 8, 7 and 5 $\mu g\ kg^{-1}$), 15-ADON (9, 7, 6 and 5 $\mu g\ kg^{-1}$), DON-3G (13, 12, 8 and 7 $\mu g\ kg^{-1}$), β-ZEL (9, 8, 7 and 7 $\mu g\ kg^{-1}$), α-ZEL (9, 9, 5 and 6 $\mu g\ kg^{-1}$), ZEN (8, 9, 7 and 6 $\mu g\ kg^{-1}$), ZEN-14G (10, 10, 7 and 9 $\mu g\ kg^{-1}$), ZEN-14S (12, 11, 12 and 12 $\mu g\ kg^{-1}$), β-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), α-ZEL-14G(12, 11, 10 and 10 $\mu g\ kg^{-1}$), HT-2 (9, 9, 5 and 6 $\mu g\ kg^{-1}$) and T-2 (11, 10, 9 and 9 $\mu g\ kg^{-1}$)

‡ nd: not detected

§ DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G= α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

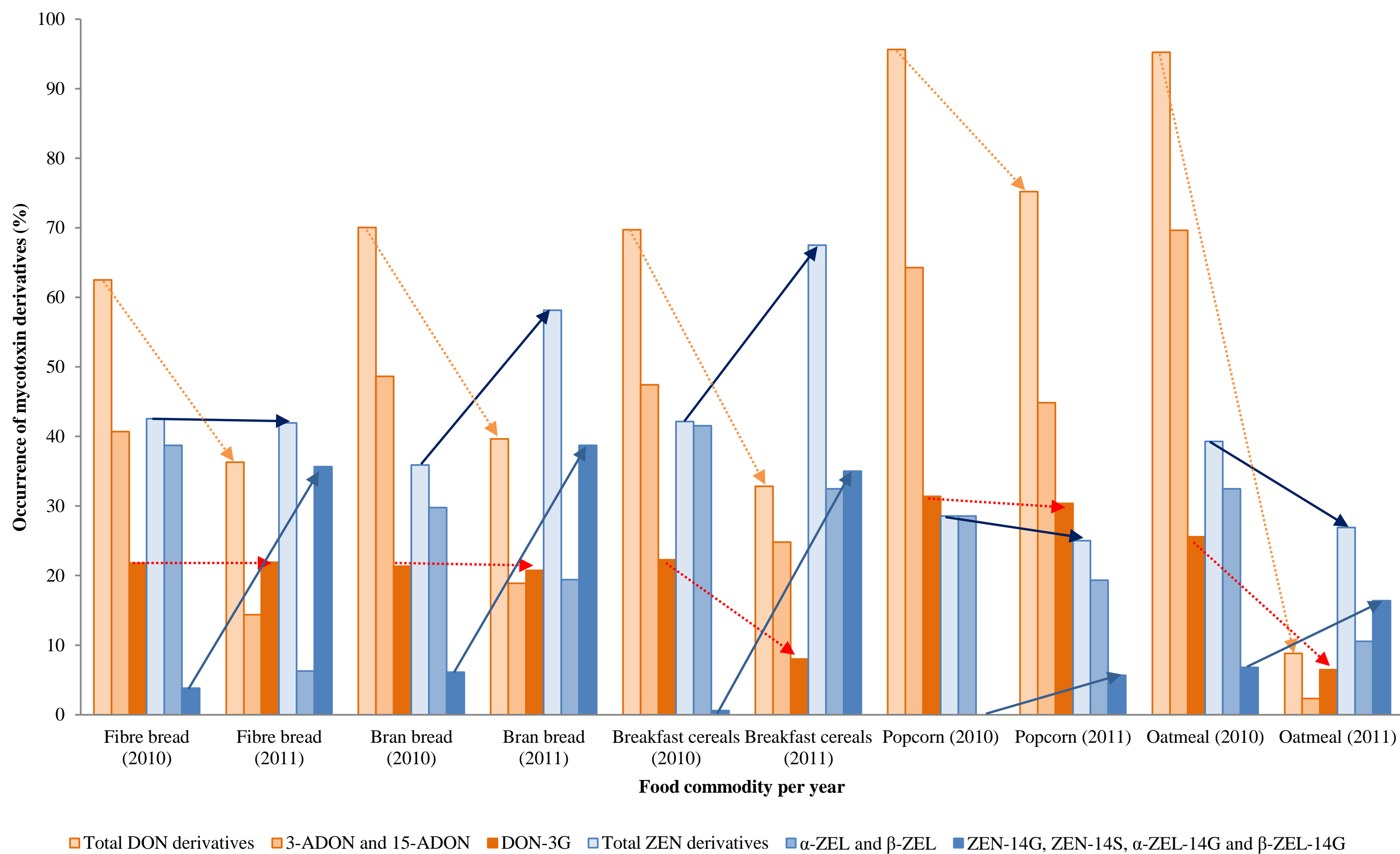


Figure 3.1 Occurrence of mycotoxin derivatives in food products per year (% of analyzed samples with mycotoxin derivative concentrations above the limit of detection). Total deoxynivalenol (DON) derivatives: 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and deoxynivalenol-3-glucoside (DON-3G); total zearalenone (ZEN) derivatives: α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN-14G), zearalenone-14-sulfate (ZEN-14S), α-zearalenol-14-glucoside (α-ZEL-14G) and β-zearalenol-14-glucoside (β-ZEL-14G).

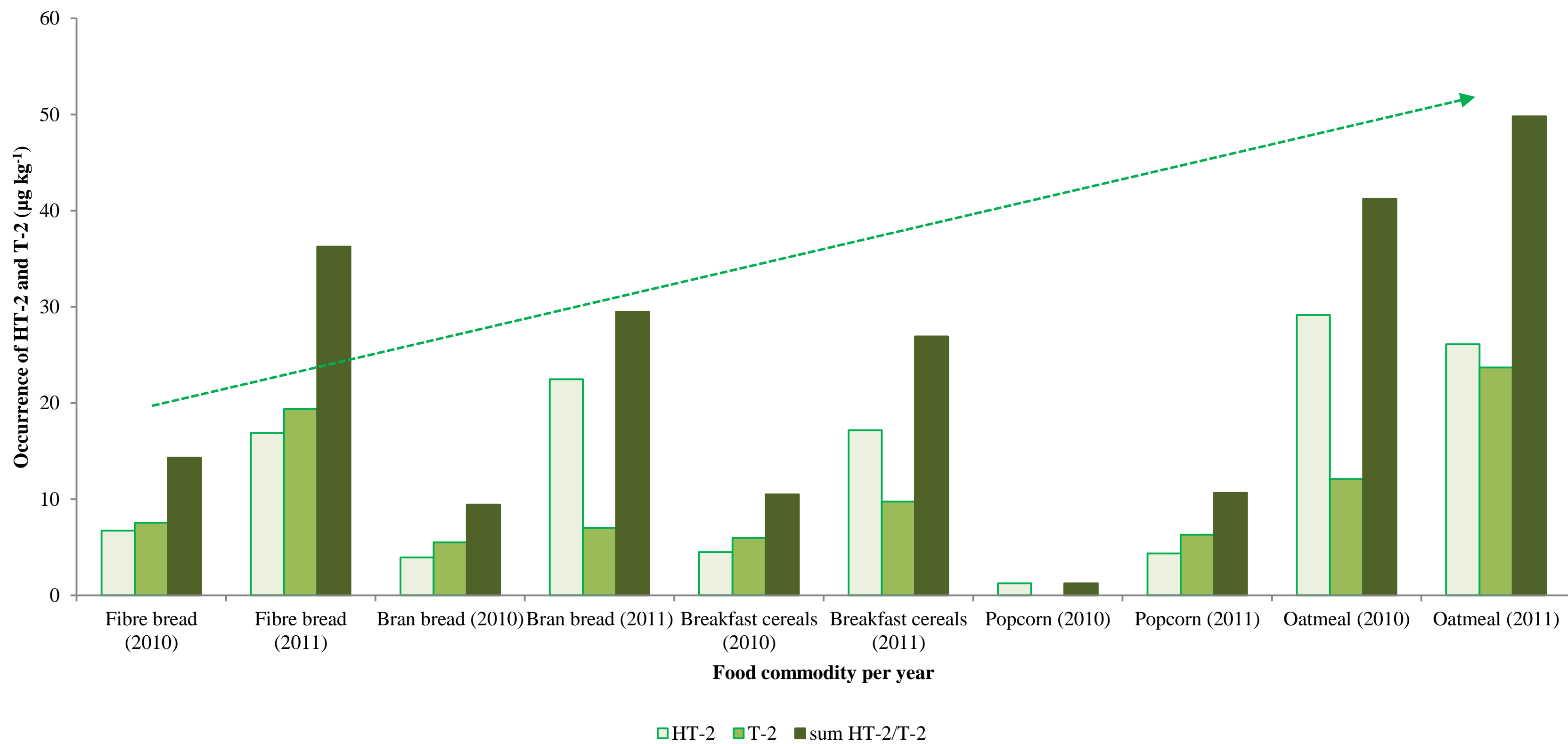


Figure 3.2 Mean concentrations (µg kg^{-1}) of T-2 toxin (T-2) and HT-2 toxin (HT-2) in food commodities per year

The glucosylated form, DON-3G occurred in 53% of the bran-enriched samples with an average concentration of 21 μg kg^{-1} (13 μg kg^{-1} to 103 μg kg^{-1}). As for fibre-enriched bread, no difference on occurrence of DON-3G between the consecutive years was proven (21.4% versus 20.8%) (P=0.98).

HT-2 was present in 58% of the samples with an average of 14 μg kg^{-1} (max 48 μg kg^{-1}), while T-2 in 39% with a mean concentration of 6 μg kg^{-1} (max 48 μg kg^{-1}). Twenty-eight percent was contaminated with both T-2 and HT-2, whereas 31% exceeded the indicative level of the sum of T-2 and HT-2 (25 μg kg^{-1}). As for fibre-enriched bread, there was a discrepancy between the values of 2010 and 2011 (**Table 3.1**). **Figure 3.2** was pointing an upward trend for HT-2 and T-2 to 2011.

Fifty-six % of the analyzed bran-enriched bread samples underscored an average ZEN contamination of 38 μg kg^{-1} (8 μg kg^{-1} to 157 μg kg^{-1}). A major concern was attributed to the 22% of the samples exceeding the maximum permissible limit of 50 μg kg^{-1}. As mentioned before, taking the sum of ZEN-equivalents into account, even 50% of the samples exceeded the EU-maximum limit, if total conversion to the parent ZEN would occur.

In 2010, 20% and 25% of the bran-enriched bread was contaminated with α-ZEL and β-ZEL, respectively, in contrast to the levels of 6% and 9%, respectively observed in 2011. The maximum concentrations for both α-ZEL and β-ZEL were 60 μg kg^{-1} (mean 6 μg kg^{-1}) and 96 μg kg^{-1} (mean 13 μg kg^{-1}), respectively.

Two samples were solely contaminated with the sulfated form, ZEN-14S (12 μg kg^{-1} and 143 μg kg^{-1}). The glucosylated ZEN-forms were also present: 36% (ZEN-14G, max 155 μg kg^{-1}), 3% (α-ZEL-14G, max 12 μg kg^{-1}) and 19% (β-ZEL-14G, max 153 μg kg^{-1}). The total ZEN-derivatives did not differ significantly in 2010 versus 2011 (P=0.69), and the contamination levels of α-ZEL and β-ZEL were relatively lower in 2010.

3.3.1.3 Breakfast cereals

DON, 3-ADON and 15-ADON appeared in 66%, 70% and 65% of the samples, respectively, which is high according to previously reported literature (JECFA, 2010; Vendl *et al.*, 2010). An average DON concentration of 44 μg kg^{-1} was indicated with a maximum of 718 μg kg^{-1}, which exceeded the maximum limit of 500 μg kg^{-1}. For 3-ADON and 15-ADON the mean values were 31 μg kg^{-1} and 10 μg kg^{-1}, with maxima of 431 μg kg^{-1} and 194 μg kg^{-1}, respectively.

Fifty % of the samples were contaminated with DON-3G with an average of 13 µg kg^{-1} (12 µg kg^{-1} to 63 µg kg^{-1}), considerably lower than the acetylated forms. A noteworthy fact was the occurrence of three samples solely contaminated with DON-3G, which might indicate a complete conversion of DON.

The mean incidence of 3-ADON, 15-ADON and DON-3G for 2010 was 70% with a ratio of 3/1 acetylated to glucosylated forms, however for 2011 only 33% with a ratio of 2.1/1. There was a clear significant difference in the occurrence of DON-derivatives between 2010 and 2011 (P=0.03).

T-2 and HT-2-contamination was rather low, with a relative contamination level of 60% and 63% in a range of <10 µg kg^{-1} to 42 µg kg^{-1} and <9 µg kg^{-1} to 33 µg kg^{-1}, respectively. None of the breakfast cereal samples exceeded the indicative level of 75 µg kg^{-1}.

ZEN was present in 52% of the samples, of which 41% exceeded the maximum limit of 50 µg kg^{-1}. The mean concentration was 76 µg kg^{-1} with a maximum of 450 µg kg^{-1}. Regarding α-ZEL and β-ZEL, 50% and 40% of the breakfast cereals were contaminated with an average concentration of 43 µg kg^{-1} and 17 µg kg^{-1} with substantious maxima of 515 µg kg^{-1} and 147 µg kg^{-1}, respectively.

In 2010, masked ZEN forms were nearly absent and occurred in merely 0.58% of the samples. In proportion to the levels in 2011, 44% of the breakfast cereals with relative values of 26%, 29%, 42% and 24% (α-ZEL-14G, β-ZEL-14G, ZEN-14G, ZEN-14S, respectively) were observed. α-ZEL-14G and β-ZEL-14G were both present at a concentration of 11 µg kg^{-1} on average with maxima of 192 µg kg^{-1} and 206 µg kg^{-1}, respectively, while ZEN-14G and ZEN-14S contained average levels of 39 µg kg^{-1} and 23 µg kg^{-1} with maxima of 369 µg kg^{-1} and 417 µg kg^{-1}, respectively.

In **Table 3.1**, the mean and maximum concentrations clearly display the exalted levels in breakfast cereals. Regarding the percentage of ZEN-derivatives in breakfast cereals, there was a clear significant difference between 2010 and 2011 ($P=2\times10^{-3}$).

3.3.1.4 Popcorn

DON is a major contaminant in the other matrices, and also 83% of the popcorn samples were contaminated with an average concentration of 49 µg kg^{-1} (6 µg kg^{-1} to 442 µg kg^{-1}). A remarkable fact was that 3-ADON and 15-ADON occurred in 100% and DON-3G in 92% of the samples, which is substantious in comparison to the parent form. The maximum

concentrations measured were 69 µg kg^{-1}, 55 µg kg^{-1} and 96 µg kg^{-1} for 3-ADON, 15-ADON and DON-3G.

In 2010, 96% of the DON-equivalents were attributed to the derivatives of DON with a ratio of 2/1 acetylated forms to glucosylated forms. In 2011, 75% were 3-ADON, 15-ADON and DON-3G in a ratio of 1.5/1. No statistical difference could be demonstrated among the percentages of acetylated and glucosylated forms in 2010 (P=0.27) and 2011 (P=0.27).

T-2 and HT-2 were present in only 25% of the samples in a range of <5 µg kg^{-1} to 61 µg kg^{-1} (sum of T-2 and HT-2). One sample (61 µg kg^{-1}) indicated an excess of the indicative level for cereal snacks of 25 µg kg^{-1}. There was no clear significant difference (p=0.31) in contamination, however, in 2011 contamination levels were higher than in 2010 (**Figure 3.2**).

The incidence of ZEN was obviously lower (58%) with a mean value of 9 µg kg^{-1} and maximum of 46 µg kg^{-1}. In average, 27% of samples were contaminated with ZEN-derivatives; no significant difference between 2010 and 2011 was found.

One sample contained 93 µg kg^{-1} of ZEN-equivalents, due to the high amount of ZEN and β-ZEL. β-ZEL-14G and ZEN-14S occurred solely in one sample (10 µg kg^{-1} and 12 µg kg^{-1}, respectively). None of the samples contained α-ZEL-14G nor ZEN-14G.

3.3.1.5 Oatmeal

Thirty-nine % of the oatmeal samples were contaminated with DON with average values of 18 µg kg^{-1} and a maximum of 91 µg kg^{-1}. According to a non-parametric Wilcoxon-test (P=0.04), there was a clear statistical difference between 2010 and 2011. In 2010, 95% 3-ADON, 15-ADON and DON-3G was formed, while in 2011, only 9% occurred. The DON contamination was rather low in 2010 and 2011 for oatmeal, but metabolization was substantial in 2010 (100%). The average contamination for 3-ADON, 15-ADON and DON-3G was 45 µg kg^{-1}, 7 µg kg^{-1} and 28 µg kg^{-1}; maximum levels were 116 µg kg^{-1}, 27 µg kg^{-1} and 97 µg kg^{-1}, respectively.

Oats is known to be susceptible for contamination with T-2 and HT-2 due to *Fusarium* (*e.g. F. poae*) contamination (Thrane *et al.*, 2004; Edwards, 2009b; Edwards *et al.*, 2009; van der Fels-Klerx and Stratakou, 2010). In 69% of the samples, specifiable levels of T-2 and HT-2 were detected. Averages of 29 µg kg^{-1} HT-2 and 13 µg kg^{-1} T-2 were found. One sample was contaminated with 118 µg kg^{-1} HT-2 and 34 µg kg^{-1} T-2. According to the indicative level of 200 µg kg^{-1} for the sum of T-2 and HT-2, no samples exceeded the mentioned value. In

Figure 3.2 an upward trend for T-2, and a small downward tendency for HT-2 was observed, however taking the sum of both mycotoxins into account, an inclination was noticed.

Sixty-two % of the analyzed samples were contaminated with ZEN (6 µg kg^{-1} to 85 µg kg^{-1}). The maximum permissible limit has been set at 100 µg kg^{-1}, consequently, all samples met the regulatory level (EC, 2006). In contrast to the DON-equivalents, there was no significant difference in the ZEN contamination levels between 2010 and 2011 (P=0.47). While 50% and 63% of the oatmeal was contaminated with α-ZEL and β-ZEL in 2010, only 9% was in 2011. The maximum concentrations for both α-ZEL and β-ZEL were 68 µg kg^{-1} and 46 µg kg^{-1} with averages of 10 µg kg^{-1} and 8 µg kg^{-1}, respectively.

Two samples were contaminated with ZEN-14S and β-ZEL-14G with a maximum of 36 µg kg^{-1} and 10 µg kg^{-1}, respectively. ZEN-14G appeared with an average of 12 µg kg^{-1} (9 µg kg^{-1} to 91 µg kg^{-1}). α-ZEL-14G was found in one sample in a quantifiable concentration of 10 µg kg^{-1}.

3.3.1.6 Comparison of mycotoxin occurrence in the different food products

A non-parametric Kruskall Wallis test was executed to reveal if there were differences in mycotoxin contamination between the various food products. If significant differences were noticed, a post-hoc test was performed to define the differences. **Table 3.2** indicates the mean concentrations of the mycotoxins and their derivatives (µg kg^{-1}) in the five food products collected in 2010 and 2011. The values followed by a different letter are significantly different at α = 0.05/10, with 10 being the number of pair wise comparisons between the five food products. There were no significant differences between the mycotoxins and derivatives measured in the wheat-based products (fibre-enriched bread and bran-enriched bread). In 2010, all masked mycotoxin concentrations measured in the maize products (popcorn and breakfast cereals) were not significantly different, in contrast to 2011 for some masked forms. In 2011, oatmeal was less heavily contaminated than in 2010.

In view of *Fusarium spp.* being field pathogens, their presence and metabolic activity is subordinate to weather conditions. High humidity and low temperature are conducive for *Fusarium* development (Peraica *et al.*, 1999; Richard *et al.*, 2007; Yazar and Omurtag, 2008). Therefore, weather data from 2010 and 2011 were analyzed in order to disentangle the discrepancies in masked and mycotoxin concentrations between 2010 and 2011. Daily weather data were collected from several field trials throughout Belgium and France.

Table 3.2 Mean concentrations (µg kg^{-1}) of mycotoxins and derivatives in the five food products collected in 2010 and 2011. The values followed by a different letter are significantly different at α = 0.05/10 (with 10 being the number of pair wise comparisons between the 5 food products)

	2010										2011									
Product	**Fibre-enriched bread**		**Bran-enriched bread**		**Breakfast cereals**		**Popcorn**		**Oatmeal**		**Fibre-enriched bread**		**Bran-enriched Bread**		**Breakfast cereals**		**Popcorn**		**Oatmeal**	
DON[§]	15[†]	a	17	a	28	a	3	a	10	a	43	b	31	a	53	a	72	ab	30	ab
3-ADON	23	a	32	a	36	a	32	a	74	b	10	a	3	a	29	a	30	b	nd	ab
15-ADON	5	a	6	a	18	a	10	a	11	a	11	a	8	a	6	a	34	b	2	ab
DON-3G	15	a	17	a	26	a	21	a	41	a	43	a	25	a	7	a	40	ab	7	a
ZEN	17	a	40	a	72	a	15	a	32	a	34	a	37	a	78	a	6	a	57	a
ZEN-14G	1	a	6	a	2	a	nd	a	3	a	21	a	28	ab	59	b	nd	a	25	ab
ZEN-14S	1	a	nd	a	nd	a	nd	a	2	a	5	a	8	a	35	b	2	a	7	a
α-ZEL	12	a	10	a	2	a	1	a	17	a	3	a	2	a	54	b	4	a	nd	a
α-ZEL-14G	0	a	1	a	nd	a	nd	a	1	a	4	a	nd	a	16	a	nd	b	nd	b
β-ZEL	18	a	19	a	16	a	14	a	13	a	2	a	8	ab	17	b	nd	a	nd	a
β-ZEL-14G	0	a	2	a	nd	a	nd	a	1	a	10	a	10	a	17	a	1	a	2	a
Total DON derivatives (%)[‡]	62	ab	70	ab	68	ab	96	bc	95	c	36	a	40	a	33	a	75	a	9	a
3-ADON and 15-ADON (%)	41	a	49	a	45	a	64	a	70	a	14	a	19	a	25	a	45	b	2	a
DON-3G (%)	22	a	21	a	23	a	31	a	26	a	22	a	21	a	8	a	30	a	6	a
Total ZEN derivatives (%)	43	a	36	a	45	a	29	a	39	a	42	a	58	a	68	a	25	a	41	a
ZEN-14G, α-ZEL-14G and β-ZEL-14G (%)	4	a	6	a	1	a	nd	a	7	a	36	a	39	a	35	a	6	a	10	a

[†] Mean = mean concentrations (µg kg^{-1}); nd: not detected; values followed by a different letter are significantly different at α = 0.05/10; a = lowest significance, c = highest significance

[‡] Mean values (%) = [(sum of absolute values of derivatives)/(sum of absolute values of equivalents)]×100.

[§] DON = deoxynivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ZEN = zearalenone; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; α-ZEL-14G= α-zearalenol-14-glucoside; β-ZEL-14G = β-zearalenol-14-glucoside; Total DON derivatives: 3-ADON, 15-ADON and DON-3G; Total ZEN derivatives: α-ZEL, β-ZEL, ZEN-14G, α-ZEL4G, β-ZEL-14G and ZEN-14S.

The observations were obtained by automated weather monitoring equipment of the 'Agricultural Centre Potatoes (Proefcentrum Aardappelteelt, PCA)' near (< 5 km) the field experiments, and these data were supplemented with weather data from Wolfram Mathematica 7.0 (Wolfram Research, Illinois, United States of America) for Belgium and France. The latter is a software package for scientific computing, but includes tools for retrieving historical time series of a large number of weather variables. The quality of the data and the coverage of weather stations are relatively high for Belgium and France. In **Figure 3.3 a** and **3.3 b** the monthly average temperature (°C), the average relative humidity (%) and the average rainfall (mm) were included for the two years, 2010 and 2011 (Landschoot *et al.*, 2012).

The fibre-enriched and bran-enriched bread samples originated from Belgian and French wheat. Different patterns can be observed in **Figure 3.3 a** and **3.3 b**. Concerning the wheat-based samples, the focus was located in May, since this period is the most susceptible for infection. There was up to four times more rainfall in May 2010 than in 2011 (48.97 mm versus 12.25 mm) in Belgium, and a same trend was observed for the monthly rainfall in France (41.40 mm versus 1.00 mm). The temperature was relatively lower in May 2010 in Belgium and France (10.76°C versus 14.44°C; 13.03°C versus 14.01°C). These two parameters were an indication that wheat was growing slowly, and the infection period was more concentrated in June 2010.

Maize-derived products originated from different countries other than Belgium or France. Regarding the maize products (various breakfast cereals and popcorn), the period of bloom would be more situated in July. In terms of average rainfall, more mycotoxins were expected in 2011, however the overall relative humidity was much lower in 2011, which could have weakened the difference (Kenny and Harrison, 1992; Lopez-Bellido *et al.*, 2004; Schaafsma and Hooker, 2007; Klimaat-en-landbouw, 2012).

The authors relate the high incidence of mycotoxins in 2010 and the statistical differences of the wheat-based samples to changes in weather conditions. However, these conclusions have to be taken with caution as parameters such as agronomical factors, cereal milling and other processing steps of raw materials, surely had an influence on the formation of parent and masked mycotoxins in the cereal-based food products (Llorens *et al.*, 2004; Lancova *et al.*, 2008a; Magan and Aldred, 2007; Kostelanska *et al.*, 2011).

Langseth and Rundberget (1999) proved a correlation between the amount of T-2 and HT-2 of 0.73 for Norwegian raw cereal samples, which is expected as these two mycotoxins are

produced by the same species on the same metabolic pathway. In the analyzed cereal-based foods a correlation of 0.40 was found, whereas in samples found to contain T-2 the concentration on average was 59% of that of HT-2 (18% - 100%). Indeed, Edwards (2009) showed that in processed cereals lower correlations were observed due to the lower contents found.

In addition, a statistical analysis was performed to assess whether there was a relationship between the derivatives and the parent mycotoxins. Schneweis *et al.* (2002) described a first estimation of ZEN-14G to the total content of ZEN (r = 0.86). Vendl *et al.* (2010) reported that ZEN-14S occurred in 10% to 18% to the total ZEN-level in cereal-based food.

Figure 3.3 Weather variables for 2010 and 2011 in Belgium (a) and France (b): temperature (°C), relative humidity (%) and rainfall (mm)

Figure 3.3 a: Belgium

Figure 3.3 b: France

Temperature (°C)
25 20 15 10 5 0 -5
Nov Dec Jan Feb Mar Apr May June July
2010 2011

relative humidity (%)
95 90 85 80 75 70 65 60
Nov Dec Jan Feb Mar Apr May June July
2010 2011

rainfall (mm)
120 100 80 60 40 20 0
Nov Dec Jan Feb Mar Apr May June July
2010 2011

DON-3G occurred in cereals in 5 mol% to 46 mol% according to a study of Berthiller *et al.* (2009a). In the present study, the relationship between 3-ADON, 15-ADON and DON-3G, and their parent DON was investigated. The Spearman rank correlation coefficient indicated a significant relationship (ρ=0.49). The same approach was applied for ZEN and its derivatives ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G and β-ZEL-14G which also proved a significant relation (ρ=0.46). This implies, in principle, that the more parent mycotoxins are formed, the higher the percentage of derivatives will be. However, there is a limitation in this respect because probably more conjugation products are available in the plant, which are not included in the analysis and the calculations made.

3.3.1.7 The fate of processing: an additional experiment on wheat processing and DON-3G

Food processing influences the mycotoxin content as already extensively described in *Chapter 1*. Therefore, an additional experiment was executed to consider the fate of baking and the contamination level of DON and DON-3G. Both mechanic and heat treatment (process of milling and baking) was investigated on the occurrence of DON and its masked form, DON-3G.

During the production of bread, two types of wheat were used, non-DON-contaminated and highly DON-contaminated cereals (classified according to ELISA results). Cereals were milled using the Bühler milling machinery (**Figure 3.4**). The mill consists of small cyclones, 3 breaker rollers, 3 smooth rollers (r) and several sieves (s). The breaker roller has a ribbed surface, crushing the raw cereals and removing the seed coat from the endosperm.

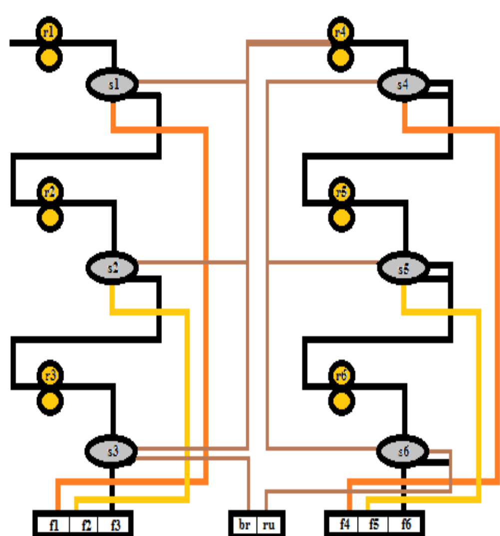


Figure 3.4 Schematic outline of the Bühler milling machinery

After every passage, 3 fractions were sieved: the flour, semolina and the raw material. The latter consisted of the bran and the endosperm, and was transferred to the subsequent roller. After the third passage the remaining fraction was transferred to the bran fraction. The semolina-fraction was transferred to the first smooth roller, milling it to flour. The smaller particles were transferred to the waste, namely runt. The bran was brushed by the bran-finisher because of the remaining endosperm which can be recuperated. After the complete process, 3 fractions were obtained: flour (f) (± 69%, mass percentage, mp), bran (br) (± 17% mp) and runt (ru) (± 7% mp).

Hundred grams of the bran and flour fraction were weighed. According to the Belgian standard baking procedure (30 min, 230 °C), white (only flour) and grey (flour/bran, 85/15) bread were made from both types of wheat. The bread samples were analyzed on the occurrence of DON and DON-3G using the developed LC-MS/MS procedure (*Chapter 2*). In **Figure 3.5** the contamination levels (μg kg^{-1}) for DON and DON-3G of the different fractions are pointed out. **Figure 3.6** shows the discrepancies between the contamination level (μg kg^{-1}) of DON and DON-3G in white and grey bread. The results of the highly contaminated wheat were pointed out.

In conclusion, runt and bran contained high concentrations of DON, probably because of the outer lying position of bran and the seed coat in the kernel. Analyzing the four repetitions (**Figure 3.6**), the DON content remained in white bread, so *in casu* heat treatment did not affect the DON-content. An incline of approximately 300 µg kg^{-1} was observed for grey bread in comparison to the flour/bran (85/15)-mixture.

In general, grey bread contained a higher content of DON and DON-3G than white bread. The mentioned mycotoxins are heat-stable and can not be reduced by heat-treatment. An increase of both forms is even observed. The baking process of grey bread clearly influenced the contamination levels of both DON and DON-3G.

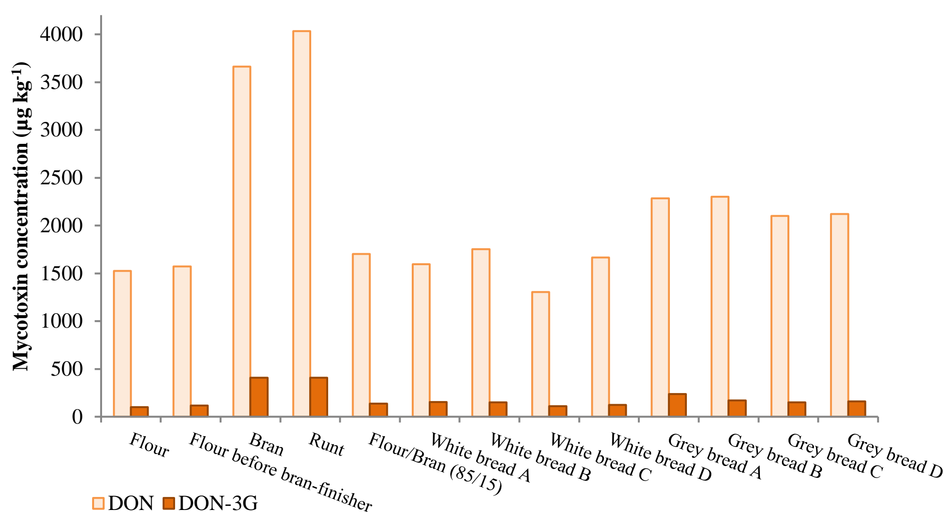


Figure 3.5 Contamination levels (µg kg^{-1}) for DON and DON-3G of the different wheat fractions

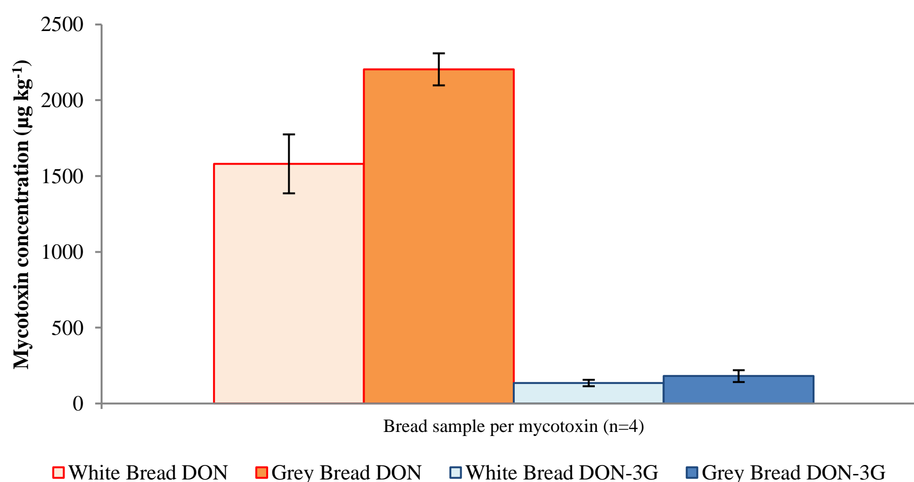


Figure 3.6 Discrepancies between the contamination levels (µg kg^{-1}) of DON and DON-3G in white and grey bread (*n*=4)

3.3.2 Part 2: occurrence data for (masked) mycotoxins in feed from the Belgian market

Sixty-seven compound feeds were analyzed on the occurrence of DON, 3-ADON, 15-ADON, T-2, HT-2, ZEN, α-ZEL and β-ZEL and their respective masked forms. The composition of each feed mixture was known. In addition, feed materials (*n*=19), which were used to prepare the compound feeds, were collected by the manufacturer and analyzed. In **Table 3.3** mean and maximum concentrations of the parent mycotoxins and derivatives are shown for the different compound feeds. **Table 3.4** represents the concentrations measured in the feed materials.

3.3.2.1 Deoxynivalenol and derivatives

The feed materials beet pulp, sunflower seed meal, soy bean, soy peel, oats and barley were hardly contaminated with DON, 3-ADON, 15-ADON and DON-3G (range 6 µg kg^{-1} to 26 µg kg^{-1}) (**Table 3.4**). Feed materials mostly contaminated with DON and derivatives were maize and its by-products, maize germs and maize gluten feed (DON-3G, 542 µg kg^{-1}). Contamination with DON derivatives was rather low in wheat-commodities. A reflection of these results was observed in the feed mixtures, which is subsequently described in detail.

As for poultry feed, all compound feeds contained maize (mean 37%, min 13%, max 100%) and wheat (mean 32%, min 12%, max 45%), and were all contaminated with DON in a range of 32 to 894 µg kg^{-1}. 3-ADON, 15-ADON and DON-3G occurred in 71%, 100% and 64% of the samples, respectively. The total mean of the DON-derivatives was 46%, whereof 34% acetylation and 12% glucosylation.

All piglet feeds were contaminated with DON (**Figure 3.7**), as the main constituent was maize (mean 15%, min 12%, max 45%) with an average of 318 µg kg^{-1} in a range of 6 µg kg^{-1} to 892 µg kg^{-1}. The contamination level of the derivatives was moderate. 15-ADON was present in all of the samples. Considering the sum of the DON-equivalents, 25% of the piglet feed exceeded the recommended limit (900 µg kg^{-1}) ranging from 916 µg kg^{-1} to 1,024 µg kg^{-1}. The DON-derivatives contributed to 26% of the total DON concentrations, and was low in comparison to the other compound feeds.

A similar tendency was observed for sow feed. These matrices contained on average 18% wheat, 10% maize, and 20% barley. All samples were contaminated with DON in an average concentration of 135 µg kg^{-1} in a range of 6 µg kg^{-1} to 245 µg kg^{-1}. 3-ADON, 15-ADON, DON-3G appeared in 33%, 80% and 60% of samples, respectively. The values were relatively moderate ranging from 14 µg kg^{-1} (3-ADON) to 96 µg kg^{-1} (DON-3G). The total (mean) of

the derivatives was 31%, which comprises 19% acetylation and 12% glucosylation. None of the samples exceeded the EU recommended limit of 900 µg kg^{-1}.

Feed for fattening pigs was composed of high amounts of wheat, maize and barley (mean 31%, 23% and 19%). Hence, these feed materials are all susceptible to *Fusarium* infection, therefore almost 75% of the mixtures is considered as a hazardous environment. In consequence, a high DON-contamination was assumed. One hundred % of the samples were infected, the values ranged from 42 µg kg^{-1} to 1,250 µg kg^{-1}. Two samples exceeded the EU recommended limit of 900 µg kg^{-1}. In **Table 3.3** the mean concentrations were described for 3-ADON, 15-ADON and DON-3G. One sample contained 1,304 µg kg^{-1} DON-3G and 1,078 µg kg^{-1} DON, indicating a metabolization rate for DON of 55%. Sixty-two %, 100% and 69% of the samples were contaminated with the three derivatives, respectively, which proves the impact of the commodity maize and its by-products in the feed samples.

The compound feed for horses consisted of large amounts of barley (mean 21%, min 6%, max 39%), while maize (mean 6%) and wheat (mean 21%) occurred in rather low amounts. The occurrence of DON and acetylated derivatives in barley has been described (Edwards, 2009a), consequently a reflection in the compound feed could been noticed. Eighty-six % of the horse feed samples were contaminated with the parent DON while the DON-derivatives, 3-ADON, 15-ADON and DON-3G were present in 21%, 71% and 36%, respectively. The levels of the derivatives were rather low, ranging from 16 µg kg^{-1} (15-ADON) to 85 µg kg^{-1} (DON-3G).

All the analyzed samples of the dairy cattle and young stock were contaminated with DON with an average of 90 µg kg^{-1} in a range of 6 µg kg^{-1} to 151 µg kg^{-1}. No glucosylated forms occurred, however 67% 3-ADON and 100% 15-ADON were present. The total mean of the acetylated forms was 40%, which was relatively high compared with recently published literature (JECFA, 2010).

3.3.2.2 T-2 and HT-2 toxin

Oats were highly contaminated with T-2 and HT-2, as previously reported (**Table 3.4**). Sums of T-2 and HT-2 of 239 µg kg^{-1} were not exceptional. For this reason, horsefeed were mainly included in the study, because of the amount of oats in the feed composition (mean 15% (min. 13% - max. 17%)). Eight of the 14 samples were contaminated in a range of 9 µg kg^{-1} to 142 µg kg^{-1} (sum T-2 and HT-2). Only two samples of sowfeed contained oats and were contaminated with T-2 and HT-2 at 125 µg kg^{-1} and 124 µg kg^{-1}, respectively.

Table 3.3 Mean and maximum concentrations (μg kg^{-1}) of parent mycotoxins and derivatives and mean percentages (%) of (masked) derivatives in the compound feeds

Mycotoxin	Poultry (*n*=14)			Piglets (*n*=8)			Sows (*n*=15)			Fattening pigs (*n*=13)			Horses (*n*=14)			Dairy cattle and young stock (*n*=3)		
	mean	SD	max	mean	SD	max	mean	SD	max	mean	SD	max	mean	SD	max	mean	SD	max
DON[§]	197[†]	239	894	318	284	892	135	66	245	291	399	1,250	200	226	874	90	57	151
3-ADON	47	53	182	25	47	136	11	17	53	29	43	155	13	27	87	44	71	126
15-ADON	50	52	190	42	25	86	20	12	44	38	33	144	44	76	296	22	5	27
DON-3G	30	30	90	43	65	166	29	35	96	121	357	1,304	34	57	179	nd	nd	nd
ZEN	483	542	1,778	459	520	1,548	167	136	433	249	433	1,585	266	337	1,076	129	223	386
ZEN-14G	28	76	282	28	57	164	11	21	64	8	13	36	27	78	296	nd	nd	nd
ZEN-14S	4	8	25	28	41	127	11	12	38	15	24	64	4	13	47	nd	nd	nd
α-ZEL	56	97	316	213	243	609	71	109	339	24	69	250	134	326	1,205	nd	nd	nd
α-ZEL-14G	11	19	75	11	15	75	9	15	96	5	13	418	nd[††]	nd	nd	4	7	97
β-ZEL	23	32	92	89	110	316	4	10	36	13	29	106	63	128	466	2	4	6
β-ZEL-14G	15	23	72	14	26	35	10	25	53	37	115	44	3	7	21	32	56	13
HT-2	13	13	47	24	18	61	20	21	66	12	14	46	25	30	82	14	13	23
T-2	9	16	56	17	18	56	17	19	59	7	16	55	16	27	60	10	10	21

	Poultry	Piglets	Sows	Fattening pigs	Horses	Dairy cattle and young stock
Total DON derivatives (%)[‡]	46	26	31	39	27	40
3-ADON and 15-ADON (%)	34	19	19	25	18	40
DON-3G (%)	12	7	12	14	9	0
Total ZEN derivatives (%)	37	11	24	10	13	22
ZEN-14G, α-ZEL-14G, β-ZEL-14G (%)	21	10	10	10	6	14

[†] Mean = mean concentrations; SD = standard deviation; max = maximum concentrations (all in μg kg^{-1})
[††] nd: not detected.
[‡] Mean values (%) = [(sum of absolute values of derivatives)/(sum of absolute values of equivalents)]×100.
[§] DON = deoxynivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; α-ZEL-14G= α-zearalenol-14-glucoside; β-ZEL-14G = β-zearalenol-14-glucoside; T-2 = T-2 toxin; HT-2 = HT-2 toxin. Total DON derivatives: 3-ADON, 15-ADON and DON-3G; Total ZEN derivatives: α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S.

Table 3.4 Concentrations (μg kg^{-1}) of parent mycotoxins and derivatives in the feed materials.

Commodity	beet pulp	sunflower seed meal	soy bean	soy bean	soy peel	oats	barley	maize germs	maize gluten feed	maize	maize	maize	wheat bran pellets	wheat bran	wheat gluten feed	wheat gluten feed	wheat gluten feed	wheat	wheat
DON	21[†]	nd[‡]	18	26	nd	31	31	335	1,533	586	352	734	29	49	44	217	441	1,381	nd
3-ADON	<LOD[§]	nd	<LOD	nd	16	nd	<LOD	nd	213	70	107	271	nd	nd	nd	<LOD	<LOD	125	nd
15-ADON	nd	23	23	nd	20	nd	nd	43	164	51	65	194	nd	<LOD	nd	<LOD	<LOD	87	<LOD
DON-3G	25	24	25	nd	nd	26	nd	132	542	26	28	63	nd	<LOD	nd	<LOD	<LOD	<LOD	<LOD
ZEN	nd	81	84	nd	175	nd	nd	285	1,829	2,130	90	3,172	278	nd	nd	nd	73	424	15
ZEN-14G	19	nd	398	nd	nd	nd	nd	71	33	nd	nd	199	nd	<LOD	nd	35	95	26	nd
ZEN-14S	nd	nd	167	nd	nd	nd	nd	nd	nd	nd	nd	206	18	21	nd	71	75	144	nd
α-ZEL	nd	nd	nd	<LOD	nd	<LOD	<LOD	nd	nd	16	nd	nd	nd	895	nd	730	830	nd	nd
α-ZEL4G	nd	<LOD	31	nd	nd	nd	29.50	nd	nd	24	51	29	nd	<LOD	nd	<LOD	16	<LOD	<LOD
β-ZEL	<LOD	nd	nd	<LOD	40	nd	nd	nd	nd	<LOD	<LOD	<LOD	nd	nd	25	nd	nd	22	nd
β-ZEL-14G	nd	39	23	nd	nd	74	40	nd	nd	105	<LOD	22	68	<LOD	nd	29	210	30	<LOD
HT-2	nd	nd	nd	nd	nd	196	nd	<LOD	32	22	nd	nd	23	22	31	34	24	<LOD	27
T-2	nd	<LOD	nd	nd	20	42	nd	<LOD	15	nd	nd	<LOD	20	20	<LOD	<LOD	<LOD	nd	<LOD

[†] Mean = mean concentrations (all in μg kg^{-1}); [‡]nd: not detected; [§]<LOD: not quantifiable

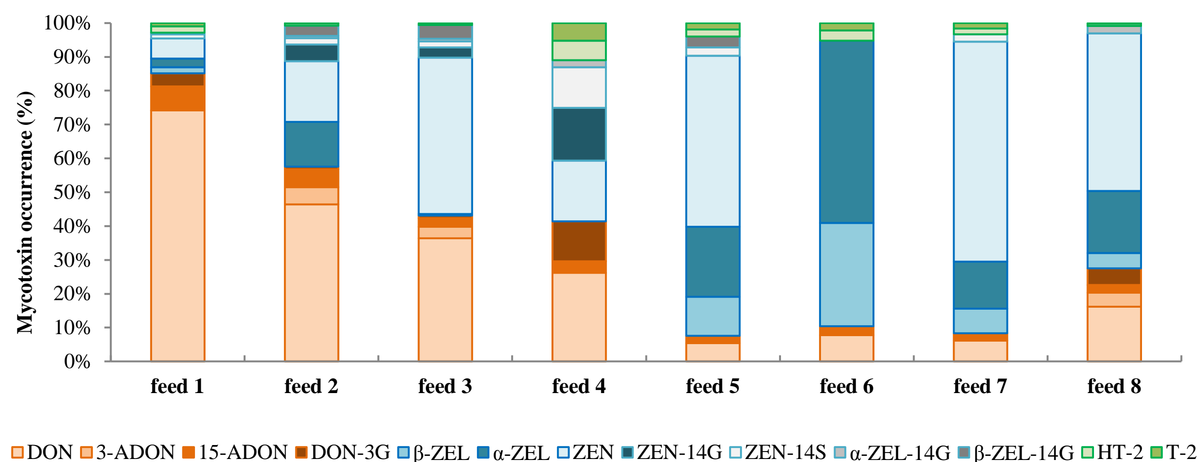


Figure 3.7 Occurrence of parent mycotoxins and derivatives in piglet feed.

All other samples showed lower T-2 and HT-2 concentrations ranging from 8 µg kg^{-1} to 55 µg kg^{-1} for T-2, and 7 µg kg^{-1} to 61 µg kg^{-1} for HT-2. According to 2013/165/EU, compound feed are regulated with an indicative level for the sum of T-2 and HT-2 of 250 µg kg^{-1}. None of the analyzed samples exceeded the reported value. Regarding the dilution factor, other matrices than oats contributed to the contamination level of T-2 and HT-2. Wheat gluten feed (horses, 7%; sows 11%), maize (horses, 6%; sows 10%) and wheat (horses 21%; sows 24%) showed to be susceptible to contamination with T-2 and HT-2.

3.3.2.3 Zearalenone and derivatives

Beet pulp and sunflower seed meal were hardly contaminated with ZEN and its derivatives. One soy bean sample contained approximately 398 µg kg^{-1} ZEN-14G and 167 µg kg^{-1} ZEN-14S. Oats and barley were hardly contaminated, except for β-ZEL-14G (74 µg kg^{-1} and 40 µg kg^{-1}). The feed materials that were highly contaminated with ZEN were maize and its by-products maize-germs and maize gluten feed. One sample of maize even exceeded the guidance value of 3,000 µg kg^{-1}. Indeed, the glucosylated and sulfated forms occurred in large amounts: 199 µg kg^{-1} (ZEN-14G) and 206 µg kg^{-1} (ZEN-14S). Even, wheat and its by-products contained α-ZEL (wheat gluten feed and wheat bran) and ZEN (wheat) in a range up to 210 µg kg^{-1} (β-ZEL-14G) to 895 µg kg^{-1} (α-ZEL).

For poultry, all compound feeds contained maize, wheat and wheat gluten feed (mean 3.27%, min 2.94%, max 3.88%). ZEN was present with an average of 483 µg kg^{-1} in a range up to 1,778 µg kg^{-1}. In general, concerning the mycotoxin derivatives the contamination level was acceptable considering the amounts found in the feed materials.

The compound feeds of piglets contained meanly 9% wheat (min. 5%- max. 12%), maize (mean, 20%; min. 15%- max. 25%) and 9% wheat gluten feed (min. 8%-max. 10%). The occurrence level of each ZEN-equivalent is shown in **Figure 3.7**. The contamination level was very high considering the guidance value of 100 µg kg^{-1}. Six of the 8 analyzed samples exceeded the limit of ZEN in a range of 135 µg kg^{-1} to 1,548 µg kg^{-1}, which is of concern. Also, the amounts of α-ZEL and β-ZEL were concerningly high, ranging from 100 µg kg^{-1} to 609 µg kg^{-1}. One sample (feed 6) was only contaminated with α-ZEL and β-ZEL. The amount of sulfated and glucosylated forms was rather low. Assuming that the EU-recommended limit for piglet feed for the sum of the ZEN-equivalents would be equal to the parent ZEN (100 µg kg^{-1}), all of the observed samples would exceed the guidance value ranging from 145 µg kg^{-1} to 2,380 µg kg^{-1}. The high contamination levels were probably attributed to maize and wheat gluten feed.

A similar tendency was observed for sows and fattening pigs. Feed for sows and fattening pigs contained 18% and 31% wheat, 10% and 23% maize, and 11% and 7% wheat gluten feed, respectively. Twenty-six % of the sow feed-samples and 15% of the fattening pig feed samples exceeded the EU recommended limit (250 µg kg^{-1}). Taking the ZEN-equivalents into account, 47% and 39% of the samples would exceed the limit, ranging from 252 µg kg^{-1} to 2,002 µg kg^{-1}.

Horse feed consisted of large amounts of oats and barley (>50%). Maize (mean 6%) and wheat (mean 21%) were present in rather low amounts. A mean concentration of 266 µg kg^{-1} ZEN was observed with levels up to 1,076 µg kg^{-1}. The percentage of ZEN-derivatives was rather low with a mean of 13%, although α-ZEL-14G was not observed.

These results are pointed out in **Table 3.3**. Oats and barley were hardly contaminated. Sixty-six % of the dairy cattle and young stock samples were not contaminated with ZEN-equivalents: ZEN occurred with an average of 129 µg kg^{-1} with a maximum of 386 µg kg^{-1}. ZEN-14G, ZEN-14S and α-ZEL were not present in the analyzed samples.

3.3.3 Part 3: occurrence data for (masked) mycotoxins in maize under natural infection conditions

3.3.3.1 Incidence of (masked) mycotoxins in the maize varieties

Different mycotoxins (DON, ZEN, sum of T-2 and HT-2, DON-derivatives and ZEN-derivatives) were studied in the maize field trial. In casu, DON-derivatives included the fungal and plant conjugates of DON and implied the sum of DON-3G, 3-ADON and 15-ADON, while ZEN-derivatives referred to the sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G.

DON was found in a range of <LOD to 5,657 μg kg^{-1} (mean, 845 μg kg^{-1} ± 1,262 μg kg^{-1}), whereas its masked forms were present up to 2,099 μg kg^{-1}. An amount of 18% exceeded the maximum permissible limit of DON (1,750 μg kg^{-1}), and 21% if the derivatives were taken into account.

T-2 and HT-2 were found in relatively small amounts (mean, 5 μg kg^{-1} ± 31 μg kg^{-1}). An indicative level of 200 μg kg^{-1} for the sum of T-2 and HT-2 is attributed to unprocessed maize, and based on the analyzed results none of the samples exceeded this value.

The most prevalent mycotoxin was ZEN (mean, 2,175 μg kg^{-1} ± 2,906 μg kg^{-1}, max. 15,677 μg kg^{-1}) with its masked forms ranging from <LOD to 9,788 μg kg^{-1} (mean 732 μg kg^{-1} ± 1,977 μg kg^{-1}) (**Table 3.5**). Based on these results, it was proven that 32% of maize samples exceeded the maximum permissible limit of 350 μg kg^{-1}, and taking the derivatives into account a disturbing value of 73% was observed.

Table 3.5 Incidence (minimum, mean, median and maximum, μg kg^{-1}) of the different mycotoxins in the maize field trial in Bottelare

Mycotoxin	Minimum	Mean	Median	Maximum
DON	<LOD†	845	275	5,657
DON-derivatives‡	<LOD	202	90	2,099
ZEN	<LOD	2,175	925	15,677
ZEN-derivatives§	<LOD	732	41	9,788
T-2 and HT-2	<LOD	5	0	103

† LOD: limit of detection; ‡ DON-derivatives: sum of 3-ADON, 15-ADON and DON-3G;
§ ZEN-derivatives: sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G

Concerning the relative importance of the various mycotoxins, ZEN contents were significantly higher than other toxins, whereas the amount of type A trichothecenes T-2 and HT-2 was significantly lower. It is assumed that this is due to the low production of T-2 and HT-2 by *F. poae* or *F. langsethiae*, which was also found by Vogelgsang *et al. (Vogelgsang et al.*, 2008) and Vanheule (Vanheule, 2012).

The higher contents observed for ZEN and DON indicated that *F. graminearum* is probably the most occurring species in the studied maize field. A species and chemotype analysis should be carried out to confirm this hypothesis. Previous studies revealed a wide range of *Fusarium spp*. present in maize fields in Belgium. Furthermore, most of them have already been detected on plants from the R1-anthesis stage (Scauflaire *et al.*, 2011).

As previously described, environmental conditions have a significant impact on the incidence of ear rot in maize and consequently production of mycotoxins. Red ear rot has been associated with high humidity and low temperature (Ellend *et al.*, 1997; Peraica *et al.*, 1999; Richard *et al.*, 2007; Schaafsma and Hooker, 2007; Yazar and Omurtag, 2008; Xu *et al.*, 2008; Skrbic *et al.*, 2011). **Figure 3.8** describes the climatological parameters of the field trial in Bottelare including rainfall (mm), temperature (°C) and relative humidity (%) during the period of bloom. In conclusion, high rainfall (high humidity) and relative lower temperatures proved the mycotoxin accumulation within the field.

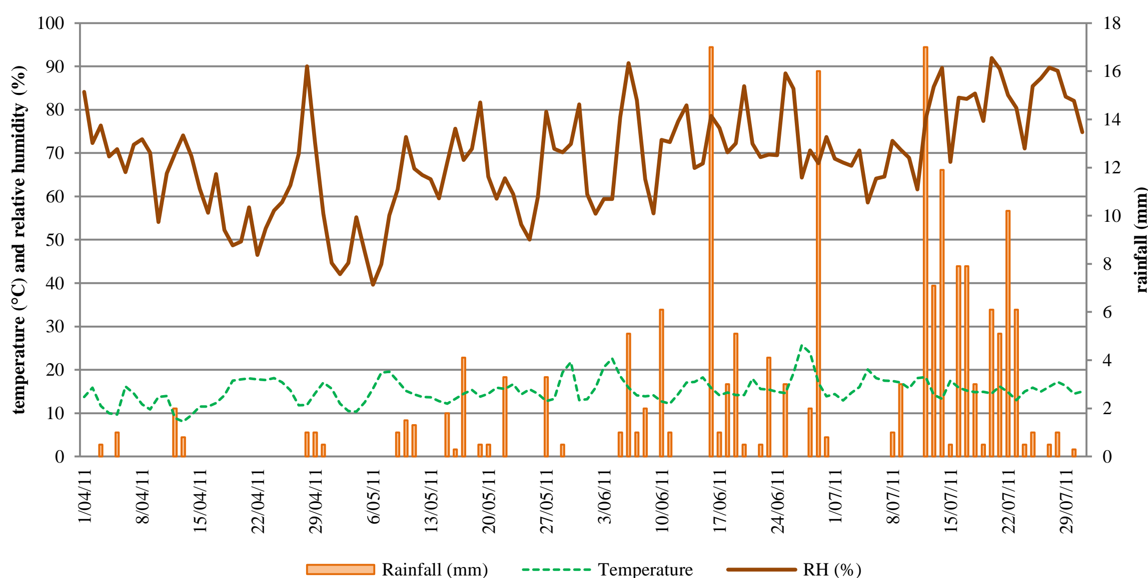


Figure 3.8 Climatological parameters of the field trial in Bottelare including rainfall (mm), temperature (°C) and relative humidity (%)

Box plots in **Figure 3.9** represent the distribution of the measured mycotoxin contents (µg kg^{-1}). A one-sided pair wise Wilcoxon signed rank test evinced that the ZEN content per sample was significantly higher than the other toxins at α=0.05/10 (with 10 the number of pair wise comparisons between the five measured toxins). The T-2 and HT-2 contents were significantly lower than the other mycotoxins (P<0.005). There was no significant difference between masked DON and ZEN concentrations (P=0.51).

Additionally, a correlation analysis between the different mycotoxins was performed using the non-parametric Spearman rank correlation coefficients (**Figure 3.10**). All pair-wise correlations between toxins were positive whereby correlations higher than 0.165 were significant at α = 0.05. Furthermore, the highest correlations were observed between the toxins and their corresponding derivatives (DON and DON-derivatives, ρ = 0.79; ZEN and ZEN-derivatives, ρ = 0.47). The lowest correlation was detected between DON and the sum of HT-2/T-2 (ρ = 0.042).

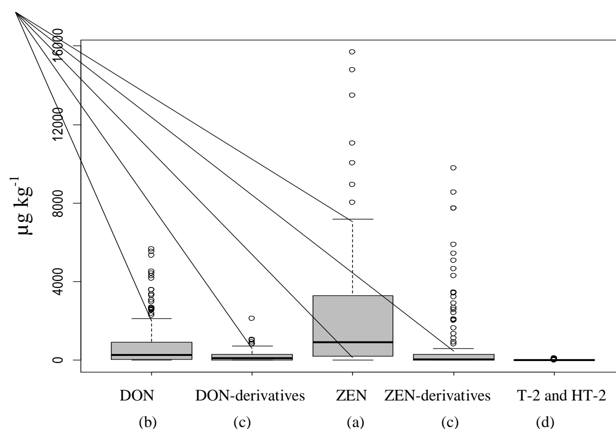
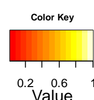


Figure 3.9 Box plot of the measured mycotoxin contents (µg kg^{-1}) in the different maize varieties in the field trial. Significant differences according to the Wilcoxon signed rank test at α=0.05/10 are indicated with a different letter. The line in the box indicates the median value of the data. The upper edge of the box indicates the 75th percentile of the data, and the lower hinge indicates the 25th percentile. The whiskers extend to a maximum of 1.5 times the interquartile range. The points outside the end of the whiskers describe outliers. DON-derivatives include the sum of DON-3G, 3-ADON and 15-ADON, while ZEN-derivatives comprise the sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G.



DON	ZEN	DON-derivatives	Sum of T-2 and HT-2	ZEN-derivatives	
1	0.447	0.786	0.042	0.141	**DON**
0.447	1	0.358	0.39	0.467	**ZEN**
0.786	0.358	1	0.023	0.105	**DON-derivatives**
0.042	0.39	0.023	1	0.406	**Sum of T-2 and HT-2**
0.141	0.467	0.105	0.406	1	**ZEN-derivatives**

Figure 3.10 Graphical representation of the Spearman rank correlation coefficients between the different toxins and their masked forms. Correlations higher than 0.165 were significant at α = 0.05. DON-derivatives include the sum of DON-3G, 3-ADON and 15-ADON, while ZEN-derivatives comprise the sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G.

The correlation analysis revealed that all relations between the mycotoxins were positive, and most of them were statistically significant. This finding indicated that a higher contamination

of a particular toxin is coupled with an elevated load of the other mycotoxins. This confirms that mycotoxins rarely occur as single contaminants. Food and feed co-contaminated with multiple mycotoxins can potentially trigger synergistic interactions as the toxic effect of a single mycotoxin may be amplified due to the presence of other contaminants (Sergent *et al.*, 2008; Grenier and Oswald, 2011). Therefore, the maximum limits laid down by the European Commission may be too high and must be reconsidered *e.g.* by including a sum of mycotoxins (parent mycotoxin and its masked forms). Also, more research on the additional and synergistic effects of mycotoxin mixtures is needed.

Furthermore, the results found in the paragraph related to food and feed (paragraph 3.3.1 and 3.3.2, respectively) were confirmed: a significant correlation between the parent toxins and their masked forms. Most routine mycotoxin analytical methods lack the ability of detecting masked forms, therefore it is expected that highly contaminated samples are in fact even more contaminated due to the presence of the masked forms.

In the correlation studies, DON and the sum of T-2 and HT-2 showed the lowest correlation coefficients. Not surprisingly, as these mycotoxins cannot be formed by the same *Fusarium* species. Also, the high correlation between the parent toxins and their masked forms highlights the importance of masked mycotoxins in unprocessed maize.

3.3.3.2 Influence of the maize variety on mycotoxin accumulation

Since the studied maize varieties exhibit different levels of resistance towards *Fusarium* and toxin accumulation, a study was performed investigating whether significant differences in mycotoxin accumulation between the commercial maize varieties have been noticed. According to the Kruskall-Wallis test there was no significant difference between the toxin accumulation in the 36 different maize varieties (at significance level $\alpha = 0.05$). The *P*-values were respectively 0.07, 0.78, 0.76, 0.08 and 0.47 for DON, ZEN, the sum of T-2 and HT-2, DON-derivatives and ZEN-derivatives. For DON-accumulation the *P*-value was only slightly higher than 0.05, thus for this toxin the maize variety significantly influenced the DON accumulation at $\alpha = 0.1$. So, only for DON the maize variety significantly influenced the mycotoxin accumulation, which can partially be explained by the fact that breeding programs mainly focus on developing hybrids with a low DON-accumulation. The fact that the maize variety significantly influenced the DON-accumulation is probably due to a DON-type II-resistance, more specifically the resistance against spreading within the ear following initial infection of the separate ears (Schroeder and Christensen, 1963).

Furthermore, a similar analysis was performed to investigate if there were any significant differences among the four replications, and shedding light at the relevance of the position in the field. For DON and its conjugated forms 3-ADON, 15-ADON and DON-3G there were no significant differences between the four replications (*P*-value = 0.60 and 0.12), whereas for the other toxins, ZEN, ZEN-derivatives, T-2 and HT-2, the *P*-values were < 0.001. A post-hoc test revealed, as can be noticed in the heat maps, that the mycotoxin content measured in replications three and four (especially at the right side of the field) was significantly higher than in the other two replications (**Figure 3.11, 3.12** and **3.13**). To gain insight on the overall mycotoxin contamination levels within the field, a subdivision in 6 subplots was executed (K1-K6) based on these findings. Performing a Kruskall Wallis test, a *P*-value of 0.004 revealed that mycotoxin concentrations were significantly different as to the total mycotoxin content (DON, DON-derivatives, HT-2 and T-2, ZEN and ZEN-derivatives) among the several subsections.

To reveal differences among the subplots, a post-hoc Kruskall Wallis test was executed which revealed that K6 (right side of the field) was affected most, although not significantly more than K4 an K5. In addition, cluster K1 was significantly less infected than K5, K4 and K6. **Figure 3.14** describes the several subplots of the maize field trial and presents a dendrogram revealing the significance of the mycotoxin contamination levels among the subplots. The more correlation among subplots, the more close the K-parameters in the dendrogram.

Actually, the analysis of clusters is used to describe primary inoculum patterns as well as the spread of the pathogen of interest. It was observed that *Fusarium* diseases are distributed in field in clusters. These subdivisions are arrangements of fungal entities relative to each other and to the architecture of the host crop (Gilligan, 1982). These hotspots are mostly referred to as the primary inoculum of the fungus and indicate the heterogeneity of environmental conditions (Waggoner and Aylor, 2000; Oerke *et al.*, 2010). The occurrence of clusters of mycotoxins on the field was observed. The position significantly influenced the incidence of the different toxins on the field. This can be due to an inhomogeneous distribution of crop residues, surrounding verges or spores (Oerke *et al.*, 2010). Also, these statements underscore the importance of taking a homogeneous sample to obtain an unbiased view on the mycotoxin content of a certain field.

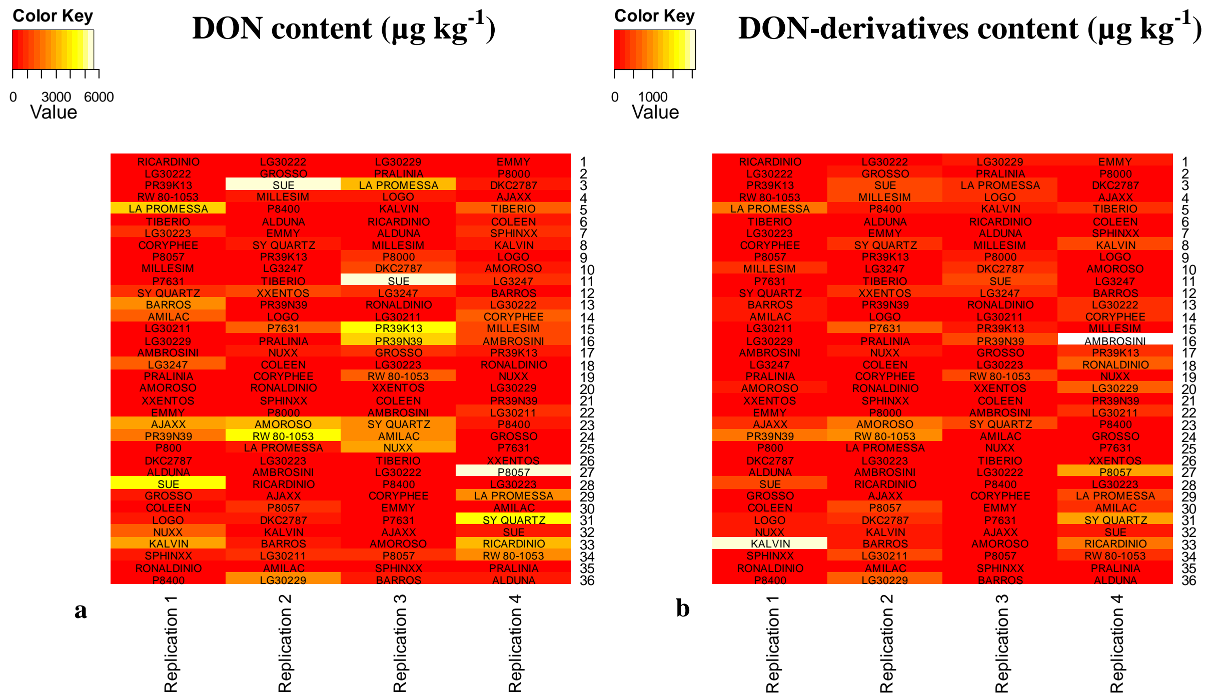


Figure 3.11 Heat map of the deoxynivalenol (DON) content (a) and its derivatives (b) measured in the different varieties and replications in the maize field trial. The contents for DON ranged from <LOD till 5,657 μg kg^{-1}and for the derivatives from <LOD till 2,099 μg kg^{-1}.

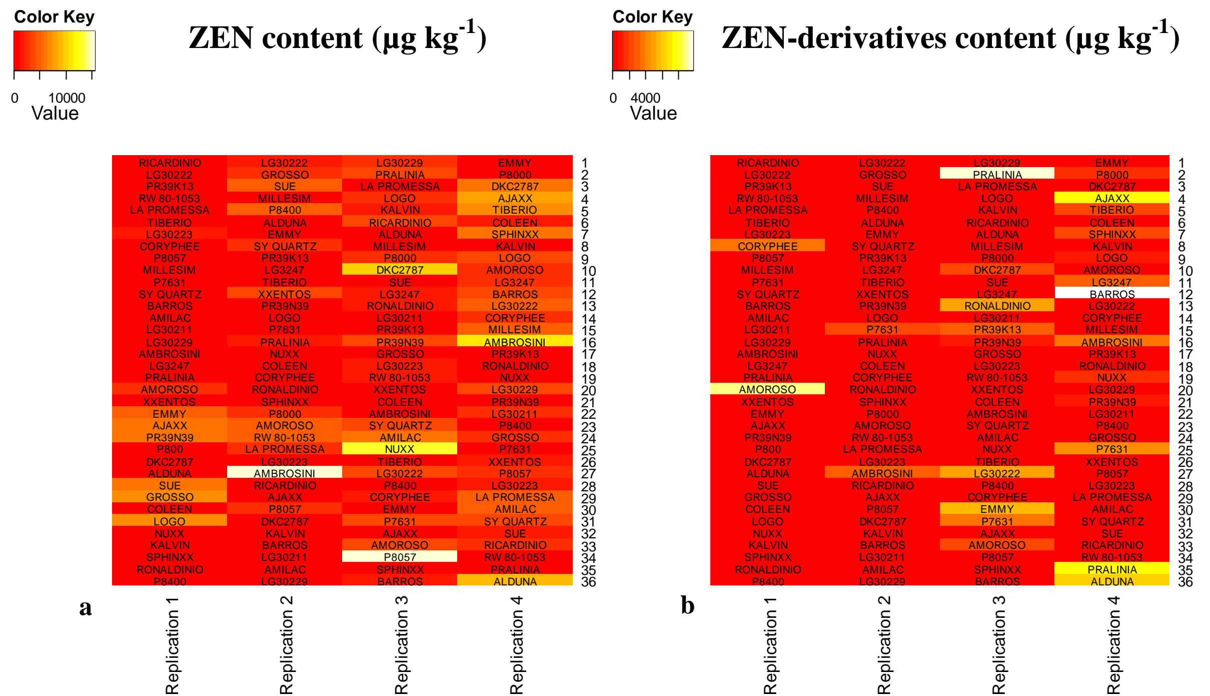


Figure 3.12 Heat map of the zearalenone (ZEN) content (a) and its derivatives (b) measured in the different varieties and replications in the maize field trial. The contents for ZEN ranged from <LOD till 15,677 μg kg^{-1} and for the derivatives from <LOD till 9,788 μg kg^{-1}.

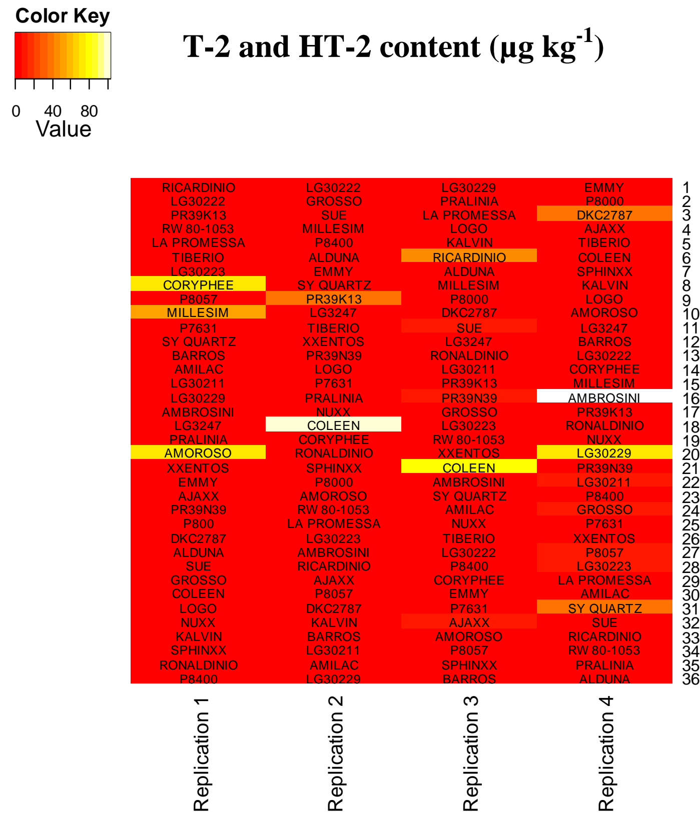
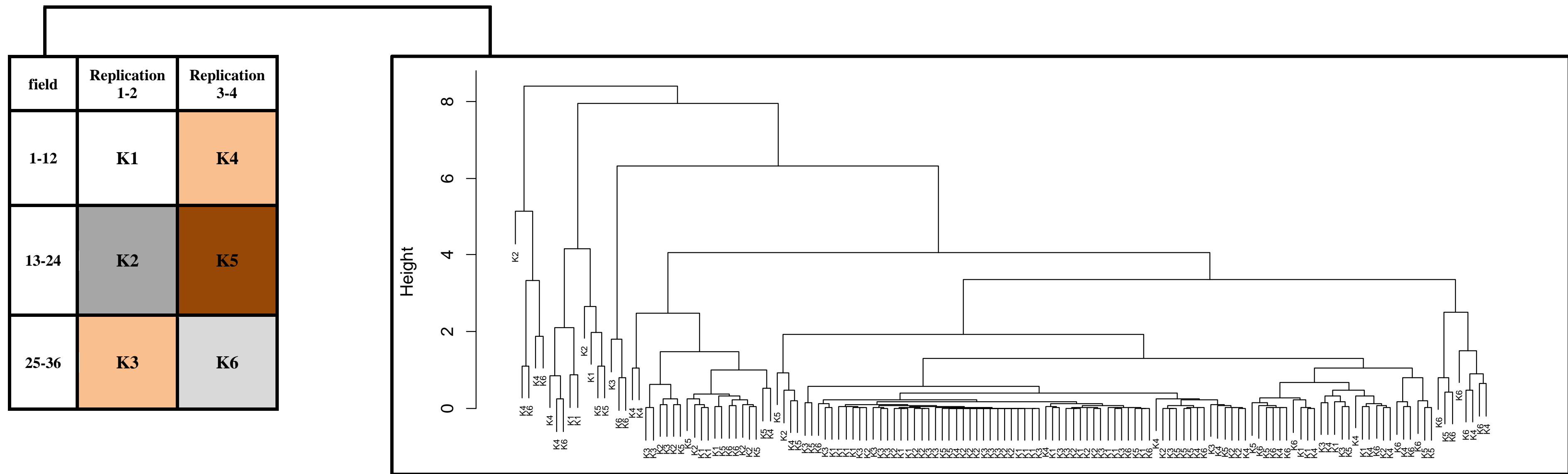


Figure 3.13 Heat map of the HT-2-toxin/T-2-toxin (HT-2/T-2) content measured in the different varieties and replications in the maize field trial. The contents for T-2/HT-2 ranged from <LOD till 103 µg kg^{-1}.



field	Replication 1-2	Replication 3-4
1-12	K1	K4
13-24	K2	K5
25-36	K3	K6

Figure 3.14 Subdivision of the maize field trial into 6 clusters (K1-K6); K1, K2 and K3 represent replication 1 and 2; K4, K5 and K6 represent replication 3 and 4. A dendrogram expresses the significant differences and correlations of the mycotoxin contamination level of the different clusters. According to a post-hoc Kruskall Wallis test large distances reveal statistical differences, while small alterations comprise non-statistical differences. Different colors indicate significant differences among the plots.

3.4 CONCLUSION

The results of the three surveys of highly consumed food products, animal feed and maize clearly show that masked mycotoxins should no longer be neglected.

The JECFA report 2010 (JECFA, 2010) accounted that the acetylated levels were typically less than 10% of those reported for DON. According to the values reported in **Figure 3.1** and **Table 3.3**, the obtained results were not corresponding with the statements made by JEFCA. Also, Placinta *et al.* (1999) and Monbaliu *et al.* (2010) described co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed. *Chapter 3* underscored the co-occurrence of DON, ZEN, T-2 and HT-2, however extended these findings with the co-occurrence of their respective masked forms.

Definitely, there was a correlation between the compound feeds and the feed materials concerning contamination with parent mycotoxins and derivatives. Beet pulp, sunflower seed meal, soy bean and soy peel were hardly contaminated. The feed materials which were mostly infected with DON, 3-ADON, 15-ADON and DON-3G were maize and its by-products (maize germs and maize gluten feed), and wheat. The feed materials which were highly contaminated with DON and ZEN were maize and its by-products, maize-germs and maize gluten feed. Also, the glucosylated and sulfated forms occurred in substantial amounts. As well, wheat and its associated by-products were contaminated with α-ZEL (wheat gluten feed and wheat bran) and ZEN (wheat). In conclusion, the amount of maize, wheat, oats, barley and their by-products definitely played a role in the contamination level of the compound feeds.

To date, no studies have investigated the occurrence of several mycotoxins and their masked forms on different maize varieties. The results showed that maize varieties were co-contaminated with different mycotoxins. Significant positive correlations between the occurrence of the parent and the masked mycotoxins were detected. Additionally, the amount of mycotoxins found was not only dependent on the maize variety, also clusters with higher mycotoxin levels within the field were observed. These results demonstrate that constant monitoring of the entire spectrum of *Fusarium* mycotoxins in maize is necessary to guarantee food and feed safety. Furthermore, it was shown that also masked mycotoxins, which are not measured by the commonly used commercial screening methods, highly contribute to the contamination.

Based on the results, a review of the legislation and the guidance values should be considered, especially for ZEN. Edwards (2011) already mentioned the risk of ZEN in European wheat.

The results in *Chapter 3* confirmed the statements made. The choice of feed depends on the feedstuffs provided, the cost and the suitability with the nutritional needs of the animal. However, with the contamination level of some matrices in mind, other feed materials can be proposed.

As a general conclusion, it is an added value to determine multiple mycotoxins and their masked forms. According to the obtained results, co-occurrence of parent and masked mycotoxins has to be taken into account in future toxicity studies, investigating the impact on animal health and by consequence the extrapolation to the human health.

CHAPTER 4

DIETARY EXPOSURE TO MYCOTOXINS AND THEIR MASKED FORMS THROUGH CEREAL-BASED FOODS AND FEED IN BELGIUM

Redrafted after:

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Diana Di Mavungu, J., Audenaert, K., Maene, P., Kolsteren, P., De Meulenaer, B. and De Saeger, S.

Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium.

Toxicology Letters 218 (2013), 281-292

CHAPTER 4 DIETARY EXPOSURE TO MYCOTOXINS AND THEIR MASKED FORMS THROUGH CEREAL-BASED FOODS AND FEED IN BELGIUM

SUMMARY

In *Chapter 3* contamination data of mycotoxins and their masked forms in food and feed were highlighted. *However, can a risk for the Belgian population be defined according to the obtained contamination levels?* To answer this research question, the results of a dietary exposure assessment to mycotoxins and their masked forms through cereal-based foods and feed in Belgium, are presented in *Chapter 4*.

A quantitative dietary exposure assessment of mycotoxins and their masked forms in cereal-based food was conducted on a national representative sample of the Belgian population using the contamination data of cereal-based foods. Cereal-based food products (*n*=175) were analyzed for the occurrence of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, zearalenone, α-zearalenol, β-zearalenol, T-2 toxin, HT-2 toxin, and their respective masked forms, including, deoxynivalenol-3-glucoside, zearalenone-14-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside and zearalenone-14-sulfate. The habitual intake of these food groups was estimated from a national representative food intake survey. According to a probabilistic exposure analysis, the mean (and 95th percentile (P95)) mycotoxin intake for the sum of the DON-equivalents, ZEN-equivalents, and the sum of HT-2-and T-2 for all cereal-based foods was 0.1162 (0.4047, P95), 0.0447 (0.1568, P95) and 0.0258 (0.0924, P95) µg kg^{-1} body weight day^{-1}, respectively. These values were below the tolerable daily intake (TDI) levels for DON, ZEN and the sum of T-2 and HT-2 (1.0, 0.25 and 0.1 µg kg^{-1} body weight day^{-1}, respectively). However, there was a subpopulation exceeding these safety values. The absolute level exceeding the TDI for all cereal-based foods was calculated, and recorded 0.85%, 2.75% and 4.11% of the Belgian adult population, respectively. Regarding feed, samples (*n*=86) were analyzed for the occurrence of all previously described mycotoxins. A deterministic analysis was implemented, as a probabilistic exposure assessment was not executable due to the lack of detailed consumption data of species. Values were concluded according to the no observed effect level (NOEL) or the lowest observed adverse effect level (LOAEL). No serious concerns were observed, however, the intake of pig feed exceeded the NOEL or the mean contamination level for DON (28.8972 µg kg^{-1} body weight day^{-1}), ZEN (41.7585 µg kg^{-1} body weight day^{-1}) and the sum of T-2 and HT-2 (3.4293 µg kg^{-1} body weight day^{-1}).

Key words: masked mycotoxins, dietary exposure assessment, Monte Carlo simulation

4.1 INTRODUCTION

Chapter 3 clearly displayed the relevance of occurrence of both mycotoxins and their masked forms. However, contamination levels in terms of means and maxima do not hold adequate information concerning risks for the population. Therefore, a detailed exposure assessment was needed quantifying the risk for each individual exposed to certain cereal-based foods from Belgian supermarkets and feed.

As mentioned earlier, a potential risk for consumers is the possible hydrolysis of masked mycotoxins into their toxic parent forms during mammalian digestion (Grabley *et al.*, 1992; Berthiller *et al.*, 2011). The evaluation of masked mycotoxins is however not (yet) available due to the lack of occurrence, bioavailability and toxicological data, notwithstanding the Joint European Commission FAO/WHO Expert Committee (JECFA) considered DON-3G and the acetylated forms 3-ADON and 15-ADON to be an additional contributing factor of the total dietary exposure to DON (JECFA, 2010). Masked forms of ZEN were not considered. Poppenberger *et al.* (2003) already proved that DON-3G dramatically reduces the ability to inhibit protein synthesis of wheat ribosomes *in vitro*. Recently, Berthiller *et al.* (2011) and Dall'Erta *et al.* (2013) showed the toxicological relevance of masked mycotoxins by demonstrating hydrolysis occurring *in vitro* (Berthiller *et al.,* 2011; Dall'Erta *et al.*, 2013). However, Nagl *et al.* (2012) stated that DON-3G was only partially bioavailable in rats. The majority of the administered DON-3G was cleft during digestion and subsequently excreted via faeces. Thus, DON-3G present in food and feed seems to have a significantly lower toxic equivalence compared to DON. However, due to differences regarding anatomy and gut microbiota, bioavailability and metabolization may be species dependent and should be experimentally determined.

Concerning masked ZEN-forms Ayed *et al.* (2011) argued that ZEN and α-ZEL exhibited the same range of cytotoxicity and genotoxicity. Recently, a study was executed on the amount of these forms in rats (Versilovskis *et al.*, 2012). After administration of ZEN-14G, ZEN was found in the stomach suggesting that hydrolysis was possible as proven for the acetylated DON-forms. Small amounts of ZEN-14G were detectable in the small and large intestines suggesting that they were not fully hydrolyzed. But, the large incidence of α-ZEL (Videmann *et al.*, 2012) and a sharp decrease of ZEN-14G in the smal intestine proved hydrolysis (Versilovskis *et al.*, 2012). As a consequence, the total human exposure and risk assessment to mycotoxins might be underestimated.

To be sure of the statements made for the masked forms of DON and ZEN, a full metabolism study should be carried out, preferably by incorporation of mycotoxins in the feed.

The European Union (EU) has set maximum levels for certain mycotoxins as a risk management strategy, and to achieve a high level of public health protection (EFSA, 2013). The EFSA has adopted opinions laying down a tolerable daily intake (TDI) for several toxins. It has established a TDI for DON of 1.00 µg kg^{-1} body weight (bw) day^{-1}, a provisional TDI of 0.25 µg kg^{-1} bw day^{-1} for ZEN, and recently the European Food Safety Authority (EFSA) has established a combined TDI of 0.10 µg kg^{-1} bw day^{-1} for the sum of T-2 and HT-2 (EFSA, 2011a; EFSA, 2011c).

To date however, no risk assessments were performed for masked mycotoxins. Therefore, the objective of *Chapter 4* was to determine the estimate exposure through cereal-based foods and feed for mycotoxins and their masked forms in food (4.3.1) and feed (4.3.2).

4.2 MATERIALS AND METHODS

4.2.1 Reagents and chemicals

All reagents and chemicals used were described in detail in paragraph 2.2.1.

4.2.2 Collection of samples for (masked) mycotoxin analysis in food and feed

In the framework of a regular Belgian sampling program, a total of 175 cereal-based food products were collected between April 2010 and October 2011, including fibre-enriched bread (n=52), bran-enriched bread (n=36), breakfast cereals (n=62), popcorn (n=12) and oatmeal (n=13). Representative feed samples (n=86) were obtained from several Belgian manufacturers of the feed industry. The collection of these samples is detailed in paragraph 3.2.2.

4.2.3 Determination of (masked) mycotoxin concentrations in food and feed

Popcorn, oatmeal, breakfast cereals, bread and feed were pulverized with the Ika Werke® (Staufen, Germany) or the Moulinette 320-grinder (Moulinex, Barcelona, Spain). A cleaning and decontamination routine of the equipment was performed using water and disinfectol® after each milling practice. The sample preparation for food and feed was executed according to the procedure described in paragraph 2.2.

4.2.4 Consumption data

The food consumption data were obtained from the Belgian National Food Consumption Survey of 2004. The survey is a representative sample of food consumption data of 3,083 participants residing in Belgium at the age of 15 years or older (De Vriese *et al.*, 2005). The consumption data of foodstuffs analyzed for their mycotoxin content were extracted from the survey using the food category and food name data (IBM SPSS 19). The usual intake of each of the food groups was estimated from the 2 day 24h recalls by correcting for the intra person variability (German Institute of Human Nutrition Postdam-Rehbrücke (DIfe), 2011; Harttig *et al.*, 2011; Haubrock *et al.*, 2011). All subjects were considered habitual consumers of the cereal products. The usual food intake was determined from the total data set including the 'zero intakes' (when a certain food type is not consumed by an interviewed individual) with the Multiple Source Method program (German Institute of Human Nutrition Postdam-Rehbrücke (DIfe), 2011). The usual food intake was expressed as µg kg^{-1} body weight (bw) day^{-1} using the self-reported bw-data collected during the survey.

Detailed feed consumption data were unavailable as a determination of individual feed intakes of different species was difficult to achieve. Therefore, a literature research was executed to obtain mean intake levels for each species (Ewing, 1997; Leeson and Summers, 2005).

4.2.5 Deterministic exposure assessment

A deterministic analysis was performed, in a first attempt to assess the dietary exposure of the parent and masked mycotoxins. Calculations were executed using Microsoft Excel 2007 (Redmond, WA, USA). Three different scenarios were included for the mycotoxin dietary exposure assessment in relation to the data treatment of the non-detects (< limit of detection (LOD)): lower, medium and upper bound. Non-detects were considered as zero, 1/2 LOD and LOD for lower, medium and upper bound, respectively.

The estimated intakes of the parent and masked mycotoxins were determined by multiplying the mean mycotoxin concentrations by the mean, maximum or 95th percentiles (P95) of the consumption data. To minimize the risk to the consumer, the mean mycotoxin concentration was considered as fixed, while the consumption data were variable (mean, maximum, P95) (Vromman *et al.*, 2010). The three scenarios (lower, medium and upper) were determined with regard to the data treatment. Deterministic estimations of exposures assume that all individuals consume the cereal-based foods and feed in the same period of time and at a same level. In addition, the parent mycotoxins and their masked forms are considered to be continuously present at an average level. As this kind of analysis might cause an overestimation, an additional probabilistic analysis was performed in order to allow a more detailed exposure assessment.

4.2.6 Probabilistic exposure assessment

Calculations were executed using the @Risk® software for Microsoft Excel version 5.5 (Palisade Corporation, USA). Best fit distributions were formed to the lower, medium and upper bound scenario of all mycotoxin concentrations in the six different food categories and to the respective consumption data. The type of distribution selected as *best fit* for the upper bound was applied to the other two scenarios (lower-medium). Best fit was based on chi-square statistics. Also, the probability/probability plots (P/P) and the quantile/quantile plots (Q/Q), resulting from the cumulative distributions, were retained as a parameter when the cumulative distributions corresponded to the theoretical cumulative distributions. First order Monte Carlo simulations were performed considering 10,000 iterations. The estimated intake of the parent and masked mycotoxins (mean, standard deviation, maximum and percentiles)

was determined separately per food category. The total intake (all cereal-based foods) was calculated by considering all concentration data of the food categories and the consumption data according to the estimates from the 2 day 24h recalls by correcting for the intra-person variability. Hence, the sum of the means of the subcategories intake is not necessarily equal to the mean intake of the overall category.

4.3 RESULTS AND DISCUSSION

In *Chapter 3* a detailed summary on the contamination data of the food and feed was described concerning DON, 3-ADON, 15-ADON, DON-3G, T-2, HT-2, ZEN, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G, ZEN-14G and ZEN-14S. The next paragraph is divided into two sub-sections: the dietary exposure assessment in food (4.3.1) and in feed (4.3.2).

4.3.1 Dietary exposure assessment in food

4.3.1.1 Determination of (masked) mycotoxins in foodstuffs

A total of 175 cereal-based samples were analyzed for the occurrence of DON, 3-ADON, 15-ADON, DON-3G, T-2, HT-2, ZEN, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G, ZEN-14G and ZEN-14S. Five food categories were considered: fibre-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal. The difference between fibre- and bran-enriched bread was made according to the labeling of the product. The definition was based on the percentage of fibre/bran per weight depending on the processing of the grains. **Table 4.1** represents the mean concentration, standard deviation and maximum concentration (μg kg^{-1}) of the mycotoxins and their masked forms found in each food category. The analyzed foods were contaminated with an average of 2 to 6 different mycotoxins (median = 4) including 1 to 3 masked forms (median = 1). These masked forms comprised DON-3G, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G. The percentage of mycotoxins occurring in the samples was calculated by the amount of samples (> LOD) divided by the total amount of samples analyzed.

4.3.1.2 Consumption data

The Belgian National Food Consumption Survey of 2004 provided data of a sample of 3,083 persons about their eating habits as to cereal-based foods. Approximately 30% consumed the foodstuffs investigated on the interview days. Oatmeal and popcorn were consumed by few persons (**Table 4.1**). For the exposure assessment, the foods were grouped into five categories: fibre-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal. Also, a sixth category “all cereal based foods”, was included and contained the total of the five categories.

4.3.1.3 Deterministic exposure assessment

In **Table 4.2** the estimated intakes (μg kg^{-1} bw day^{-1}) by the Belgian adult population for the different food categories are presented for the lower and upper bound, respectively.

The total mean intake of the consumption of all cereal-based food for the mycotoxins DON, 3-ADON, 15-ADON, DON-3G is 0.040, 0.024, 0.016, 0.025 µg kg^{-1} bw day^{-1}, respectively (upper bound scenario). Insofar as no TDI's for 3-ADON, 15-ADON and DON-3G are described, the TDI of the parent DON was used. These values are approximately 25 to 63 times lower than the TDI set for DON (1.00 µg kg^{-1} bw day^{-1}). However, extrapolating this value to DON-derivatives is notably a worst-case scenario, assuming that these toxins are equally toxic as their parent or that total hydrolysis occurs. Therefore, the sum of the DON-equivalents was incorporated, which is the sum of DON, 3-ADON, 15-ADON and DON-3G, and compared to the DON-TDI. A mean intake for fibre-enriched bread, bran-enriched bread, breakfast cereals, popcorn, oatmeal and all cereal based foods of 0.125, 0.077, 0.050, 0.019, 0.106 and 0.104 µg kg^{-1} bw day^{-1} was obtained, which is 8 to 53 times lower than the DON TDI-value.

High consumers of fibre-enriched bread exceeded the TDI of 0.10 µg kg^{-1} bw day^{-1} for the sum of HT-2 and T-2 (P95, 0.128 µg kg^{-1} bw day^{-1}; max., 0.227 µg kg^{-1} bw day^{-1}). Also, consumers, who daily consume the maximum of all cereal-based foods, were exposed to a potential risk to HT-2 and T-2 (max intake, 0.146 µg kg^{-1} bw day^{-1}).

Of all the investigated mycotoxins the highest exposure was attributed to the myco-estrogens. High consumers of breakfast cereals and all cereal-based foods nearly exceeded the TDI of 0.25 µg kg^{-1} bw day^{-1} for ZEN (0.201 µg kg^{-1} bw day^{-1} and 0.219 µg kg^{-1} bw day^{-1} intake, respectively). The sum of the ZEN-equivalents was designated as the sum of ZEN, α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G, and β-ZEL-14G. Fibre-enriched bread, bran-enriched bread and breakfast cereals were responsible for a high intake of these toxins with a maximal exceeding factor of 1.8, 1.1 and 2.3 times the ZEN TDI. The maximum consumption of all cereal-based foods included a 0.594 µg kg^{-1} bw day^{-1} intake, which is of high concern. Popcorn and oatmeal were consumed without exceeding TDI, possibly due to lower consumption.

An additional calculation was investigated concerning the sum of the mean (and P95) intakes of the five different cereal-based food matrices. The mean DON-exposure for consuming bread, breakfast cereals, popcorn and oatmeal was 0.146 (0.319, P95) µg kg^{-1} bw day^{-1}, which does not imply any risk. For the sum of T-2 and HT-2 an excess of the TDI was observed for the mean and P95 intake, 0.101 and 0.220 µg kg^{-1} bw day^{-1}, respectively. As to ZEN, consumers were not exposed to any risk for the mean intake (0.119 µg kg^{-1} bw day^{-1}, nevertheless the TDI was achieved for P95 (0.250 µg kg^{-1} bw day^{-1}).

Table 4.1 Concentrations of mycotoxins and their masked forms (mean, standard deviation and maximum) ($\mu g\ kg^{-1}$) in the different food categories analyzed

	Fibre-enriched bread				Bran-enriched bread				Breakfast cereals				Popcorn				Oatmeal				All cereal-based foods			
Consumption data (*n*)	**397**				**243**				**237**				**22**				**20**				**919**			
Occurrence data (*n*)	**52**				**36**				**62**				**12**				**13**				**175**			
Mycotoxin	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	#	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$	$\mu g\ kg^{-1}$
	>LOD†	mean	SD	max	>LOD	mean	SD	max	>LOD	mean	SD	max	>LOD	mean	SD	max	>LOD	mean	SD	max	>LOD	mean	SD	max
DON§	44	34	31	138	16	25	35	127	36	44	107	718	10	49	125	442	5	18	30	91	111	42	87	718
3-ADON	21	14	22	74	14	16	20	59	34	31	63	431	12	30	22	69	8	45	45	116	89	25	43	431
15-ADON	20	9	16	45	4	7	11	45	30	10	27	194	12	26	18	55	10	7	8	27	76	16	35	218
DON-3G	27	34	68	425	12	21	30	103	31	13	17	63	11	33	37	96	10	28	33	97	91	26	46	425
β-ZEL	12	7	17	86	6	13	25	96	26	17	33	147	2	5	14	47	4	8	17	46	53	14	30	186
α-ZEL	16	6	19	110	3	6	13	60	32	43	92	515	2	3	9	32	5	10	22	68	61	19	57	515
ZEN	23	29	54	230	14	38	52	157	32	76	165	450	7	9	19	51	8	41	55	85	84	47	104	885
ZEN-14G	15	15	43	154	2	18	43	155	25	39	75	369	0	nd‡	nd	nd	5	12	26	91	48	23	54	369
ZEN-14S	4	4	24	176	2	4	24	143	17	23	72	417	1	1	3	12	2	4	10	36	24	10	45	45
β-ZEL-14G	10	7	24	153	2	6	26	153	18	11	32	206	1	1	3	10	2	2	4	10	33	9	26	206
α-ZEL-14G	5	3	10	63	1	0.3	2	12	16	11	30	192	0	nd	nd	nd	1	1	3	10	22	5	19	192
HT-2	36	14	17	49	8	14	17	48	40	13	14	42	2	3	10	35	9	20	32	118	95	19	42	418
T-2	33	16	17	45	5	6	10	45	38	8	8	33	3	4	10	26	9	9	10	34	88	13	13	45

† >LOD: amount of samples with a concentration detected above the limit of detection (LOD) (%); LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 $\mu g\ kg^{-1}$), 3-ADON (10, 8, 7 and 5 $\mu g\ kg^{-1}$), 15-ADON (9, 7, 6 and 5 $\mu g\ kg^{-1}$), DON-3G (13, 12, 8 and 7 $\mu g\ kg^{-1}$), β-ZEL (9, 8, 7 and 7 $\mu g\ kg^{-1}$), α-ZEL (9, 9, 5 and 6 $\mu g\ kg^{-1}$), ZEN (8, 9, 7 and 6 $\mu g\ kg^{-1}$), ZEN-14G (10, 10, 7 and 9 $\mu g\ kg^{-1}$), ZEN-14S (12, 11, 12 and 12 $\mu g\ kg^{-1}$), β-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), α-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), HT-2 (9, 9, 5 and 6 $\mu g\ kg^{-1}$) and T-2 (11, 10, 9 and 9 $\mu g\ kg^{-1}$)

‡ nd: not detected

§ DON = deoxynivalenol, 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

Table 4.2 Deterministic analysis of mycotoxins and their masked forms estimated intake (mean, P95-percentile and maximum) by Belgian adult population from the different food categories (μg kg^{-1} bw day^{-1})

LOWER BOUND[§] **(μg kg^{-1} bw day^{-1})**		**DON**[†]	**3-ADON**	**15-ADON**	**DON-3G**	**sum DON-eq**[††]	**HT-2**	**T-2**	**sum HT-2/T-2**	**β-ZEL**	**α-ZEL**	**ZEN**	**ZEN-14G**	**ZEN-14S**	**β-ZEL-14G**	**α-ZEL-14G**	**sum ZEN-eq**[‡]
Fibre-enriched bread	**mean**	0.031	0.015	0.009	0.040	0.095	0.015	0.014	0.029	0.008	0.007	0.036	0.027	0.007	0.008	0.002	0.095
	P95	0.070	0.034	0.021	0.092	0.217	0.035	0.032	0.067	0.018	0.016	0.083	0.062	0.016	0.018	0.005	0.218
	max	0.124	0.060	0.036	0.163	0.383	0.062	0.056	**0.118**	0.032	0.027	0.146	0.110	0.028	0.032	0.008	**0.385**
Bran-enriched bread	**mean**	0.027	0.014	0.004	0.015	0.060	0.009	0.005	0.014	0.009	0.003	0.002	0.029	0.004	0.002	0.000	0.045
	P95	0.056	0.029	0.009	0.030	0.124	0.018	0.010	0.029	0.019	0.006	0.005	0.060	0.007	0.003	0.000	0.094
	max	0.120	0.061	0.020	0.064	0.266	0.040	0.022	0.061	0.041	0.014	0.010	0.129	0.016	0.007	0.000	0.201
Breakfast cereals	**mean**	0.020	0.016	0.004	0.006	0.046	0.005	0.002	0.007	0.008	0.019	0.036	0.018	0.010	0.004	0.004	0.099
	P95	0.039	0.030	0.009	0.011	0.089	0.009	0.004	0.014	0.015	0.038	0.070	0.034	0.020	0.008	0.008	0.192
	max	0.106	0.083	0.024	0.031	0.245	0.026	0.011	0.037	0.041	0.103	0.192	0.093	0.054	0.022	0.022	**0.527**
Oatmeal	**mean**	0.003	0.008	0.001	0.005	0.017	0.003	0.001	0.004	0.001	0.002	0.007	0.002	0.001	0.000	0.000	0.013
	P95	0.008	0.019	0.002	0.011	0.038	0.008	0.002	0.009	0.003	0.004	0.016	0.004	0.002	0.000	0.000	0.028
	max	0.009	0.022	0.002	0.013	0.045	0.009	0.002	0.011	0.003	0.004	0.019	0.005	0.002	0.000	0.000	0.033
Popcorn	**mean**	0.046	0.021	0.018	0.018	0.104	0.000	0.002	0.002	0.003	0.001	0.002	0.002	0.000	0.000	0.000	0.008
	P95	0.108	0.048	0.041	0.043	0.241	0.000	0.004	0.004	0.008	0.003	0.005	0.004	0.000	0.000	0.000	0.019
	max	0.124	0.056	0.048	0.049	0.276	0.000	0.005	0.005	0.009	0.003	0.006	0.005	0.000	0.000	0.000	0.022
All cereal-based foods	**mean**	0.035	0.023	0.009	0.021	0.089	0.010	0.006	0.017	0.010	0.016	0.037	0.026	0.009	0.005	0.003	0.107
	P95	0.080	0.053	0.021	0.048	0.202	0.023	0.015	0.038	0.023	0.037	0.085	0.058	0.020	0.012	0.008	0.243
	max	0.171	0.114	0.045	0.104	0.434	0.050	0.031	0.081	0.050	0.080	0.181	0.125	0.043	0.025	0.016	**0.521**

[†] DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

[§] Lower and upper bound correspond to data (< LOD) treated as 0 and LOD, respectively. LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 μg kg^{-1}), 3-ADON (10, 8, 7 and 5 μg kg^{-1}), 15-ADON (9, 7, 6 and 5 μg kg^{-1}), DON-3G (13, 12, 8 and 7 μg kg^{-1}), β-ZEL (9, 8, 7 and 7 μg kg^{-1}), α-ZEL (9, 9, 5 and 6 μg kg^{-1}), ZEN (8, 9, 7 and 6 μg kg^{-1}), ZEN-14G (10, 10, 7 and 9 μg kg^{-1}), ZEN-14S (12, 11, 12 and 12 μg kg^{-1}), β-ZEL-14G (12, 11, 10 and 10 μg kg^{-1}), α-ZEL-14G (12, 11, 10 and 10 μg kg^{-1}), HT-2 (9, 9, 5 and 6 μg kg^{-1}) and T-2 (11, 10, 9 and 9 μg kg^{-1}). Colored values indicate that exposure exceeds the TDI.

[††] DONeq: DON, 3-ADON, 15-ADON and DON-3G

[‡] ZENeq: ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G

UPPER BOUND[§] ($\mu g\ kg^{-1}$ bw day^{-1})		DON[†]	3-ADON	15-ADON	DON-3G	sum DON-eq[††]	HT-2	T-2	sum HT-2/T-2	β-ZEL	α-ZEL	ZEN	ZEN-14G	ZEN-14S	β-ZEL-14G	α-ZEL-14G	sum ZEN-eq[‡]
Fibre-enriched bread	**mean**	0.038	0.017	0.024	0.046	0.125	0.036	0.020	0.056	0.010	0.009	0.037	0.029	0.008	0.012	0.004	0.108
	P95	0.087	0.039	0.056	0.105	0.287	0.083	0.045	**0.128**	0.024	0.020	0.085	0.066	0.018	0.027	0.008	0.248
	max	0.153	0.070	0.099	0.185	0.507	**0.147**	0.079	**0.227**	0.042	0.035	0.151	0.117	0.032	0.047	0.015	**0.438**
Bran-enriched bread	**mean**	0.035	0.014	0.010	0.018	0.077	0.012	0.010	0.022	0.016	0.005	0.031	0.005	0.004	0.006	0.001	0.063
	P95	0.072	0.030	0.021	0.037	0.159	0.025	0.021	0.046	0.032	0.010	0.064	0.010	0.008	0.012	0.001	0.129
	max	0.154	0.064	0.044	0.079	0.341	0.054	0.044	0.098	0.069	0.022	0.137	0.021	0.017	0.026	0.003	**0.278**
Breakfast cereals	**mean**	0.022	0.016	0.006	0.007	0.050	0.007	0.006	0.013	0.009	0.020	0.038	0.018	0.011	0.006	0.005	0.108
	P95	0.042	0.030	0.011	0.014	0.097	0.014	0.011	0.025	0.017	0.040	0.073	0.035	0.021	0.012	0.011	0.209
	max	0.115	0.083	0.030	0.038	0.267	0.038	0.031	0.069	0.048	0.109	0.201	0.096	0.058	0.033	0.029	**0.573**
Oatmeal	**mean**	0.003	0.008	0.002	0.005	0.019	0.004	0.003	0.007	0.002	0.002	0.008	0.003	0.001	0.001	0.000	0.015
	P95	0.007	0.019	0.004	0.012	0.042	0.009	0.006	0.014	0.004	0.005	0.017	0.006	0.002	0.001	0.001	0.034
	max	0.009	0.022	0.005	0.014	0.050	0.010	0.007	0.017	0.004	0.006	0.020	0.007	0.002	0.001	0.001	0.040
Popcorn	**mean**	0.048	0.021	0.019	0.018	0.106	0.001	0.002	0.003	0.005	0.002	0.005	0.002	0.001	0.001	0.000	0.015
	P95	0.111	0.048	0.045	0.043	0.247	0.002	0.006	0.007	0.011	0.006	0.011	0.004	0.002	0.002	0.000	0.035
	max	0.127	0.056	0.051	0.049	0.283	0.002	0.006	0.008	0.013	0.007	0.012	0.005	0.002	0.002	0.000	0.040
All cereal-based foods	**mean**	0.040	0.024	0.016	0.025	0.104	0.018	0.012	0.030	0.013	0.018	0.045	0.022	0.010	0.009	0.005	0.122
	P95	0.091	0.055	0.035	0.056	0.237	0.041	0.028	0.068	0.030	0.042	0.102	0.049	0.022	0.020	0.011	**0.277**
	max	0.194	0.118	0.076	0.120	0.508	0.087	0.059	**0.146**	0.065	0.090	0.219	0.105	0.048	0.042	0.024	**0.594**

[†] DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

[§] Lower and upper bound correspond to data (< LOD) treated as 0 and LOD, respectively. LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 $\mu g\ kg^{-1}$), 3-ADON (10, 8, 7 and 5 $\mu g\ kg^{-1}$), 15-ADON (9, 7, 6 and 5 $\mu g\ kg^{-1}$), DON-3G (13, 12, 8 and 7 $\mu g\ kg^{-1}$), β-ZEL (9, 8, 7 and 7 $\mu g\ kg^{-1}$), α-ZEL (9, 9, 5 and 6 $\mu g\ kg^{-1}$), ZEN (8, 9, 7 and 6 $\mu g\ kg^{-1}$), ZEN-14G (10, 10, 7 and 9 $\mu g\ kg^{-1}$), ZEN-14S (12, 11, 12 and 12 $\mu g\ kg^{-1}$), β-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), α-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), HT-2 (9, 9, 5 and 6 $\mu g\ kg^{-1}$) and T-2 (11, 10, 9 and 9 $\mu g\ kg^{-1}$). Colored values indicate that exposure exceeds the TDI.

[††] DON-eq: DON, 3-ADON, 15-ADON and DON-3G

[‡] ZEN-eq: ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G

4.3.1.4 Probabilistic exposure assessment

In probabilistic analysis, every possible value that each variable can have and the weight of each possible scenario for the probability of its occurrence is taken into consideration (Vose, 1996), therefore allowing a more accurate (masked) mycotoxin intake estimation. Best fit distributions were formed for respectively all mycotoxin concentrations in the six different categories and all consumption data. In case constructing a distribution was impossible due to insufficient numbers of observed data for parent or masked mycotoxins, the uniform distribution analysis method was applied (**Table 4.1 and 4.3**). The more data were available, the more the theoretical cumulative distributions approximated the observed cumulative distributions. Best fit distributions determined for the upper bound scenario of parent and masked mycotoxin concentrations in the food categories and food intakes, further applied to the probabilistic calculations, are listed in **Table 4.3**. An illustration of the different distribution curves, constructed with @RISK® software is pointed out in **Figure 4.1**, **4.2** and **4.3**.

Figure 4.1 represents the distribution curve of breakfast cereals resulting in an inverse Gauss curve. Concentration data of the sum of the DON-equivalents proved a theoretical extreme value distribution as presented in **Figure 4.2**. A Monte Carlo simulation of these two curves implementing 10,000 iterations proved that 90% of the Belgian population reached TDI-values in a range from -0.008 µg kg^{-1} bw day^{-1} to 0.153 µg kg^{-1} bw day^{-1} (**Figure 4.3**).

Table 4.3 Best fit distributions, mean, minimum and maximum determined for the upper bound scenario of mycotoxins and their masked forms in the food categories and food intakes further applied for probabilistic calculations

Mycotoxin[††]	Food Category: Fibre-enriched bread				Food Category: Bran-enriched bread				Food Category: Breakfast cereals			
	Function	**min**	**mean**	**max**	**Function**	**min**	**mean**	**max**	**Function**	**min**	**mean**	**max**
DON	RiskLoglogistic(-16.953,42.612,3.1963)	-16.95	33.38	+∞	RiskLoglogistic(-14.821,40.948,2.3495)	-14.82	41.46	+∞	RiskLogistic(25.378, 32.394)	-∞	25.38	+∞
3-ADON	RiskUniform(-1.3875,74.928)	-1.39	36.77	74.93	RiskNormal(17.079,20.376)	-1.83	29.30	60.43	RiskExpon(31.929, RiskShift(-0.49889))	-0.499	31.43	+∞
15-ADON	RiskNormal(20.651,50.46)	-∞	20.65	+∞	RiskBetaGeneral(0.13844,0.36641,0,44.64)	0.00	12.24	44.64	RiskNormal(11.698,26.047)	-∞	11.70	+∞
DON-3G	RiskNormal(38.085,72.668)	-∞	38.09	+∞	RiskNormal(21.254,28.106)	-3.11	49.73	102.56	RiskNormal(14.73,17.35)	-∞	14.73	+∞
sum DON-eq[‡]	RiskLoglogistic(-19.915,92.999,2.2041)	-19.92	114.05	+∞	RiskGamma(2.7917,38.672,RiskShift(-16.287))	-16.29	91.67	+∞	RiskExtvalue(56.212,69.488)	-∞	96.32	+∞
HT-2	RiskExpon(30.301,RiskShift(-0.55092))	-0.551	29.75	+∞	RiskBetaGeneral(0.1326,0.33371,0,47.83)	0.00	13.60	47.83	RiskUniform(-0.66349,42.463)	-0.663	20.90	42.46
T-2	RiskLoglogistic(-16.953,42.612,3.1963)	-∞	15.41	+∞	RiskTriang(0,0,51.265)	0.00	17.09	51.27	RiskLogistic(12.3318,6.4341)	-∞	12.33	+∞
sum HT-2/T-2	RiskBetaGeneral(0.33017,6.2809,0,930.26)	0.00	46.46	930.26	RiskTriang(0,0,95.275)	0.00	31.76	95.28	RiskTriang(0,0,72.837)	0	24.28	72.84
β-ZEL	RiskNormal(8.5633,17.052)	-1.60	43.20	88.00	RiskTriang(0,0,196.08)	0.00	65.36	196.08	RiskTriang(0,0,150.19)	0	50.06	150.19
α-ZEL	RiskNormal(7.2815,18.54)	0.00	37.48	112.45	RiskTriang(0,0,62.361)	0.00	20.79	62.36	RiskNormal(41.839,88.612)	-∞	41.84	+∞
ZEN	RiskTriang(0,0,176.93)	0.00	58.98	176.93	RiskNormal(36.706,53.266)	-∞	36.71	+∞	RiskGamma(0.78681,189.96)	0	149.46	+∞
ZEN-14G	RiskTriang(0,0,173.33)	0.00	57.78	173.33	RiskUniform(-1.6219,53.522)	-1.62	25.95	53.52	RiskNormal(36.705,73.016)	-∞	36.71	+∞
ZEN-14S	RiskNormal(6.6638,30.425)	-3.26	87.95	179.16	RiskUniform(-4.4566,147.07)	-4.46	71.31	147.47	RiskExpon(22.288, RiskShift(-0.34825))	-0.348	21.94	+∞
β-ZEL-14G	RiskUniform(-2.8328,155.8)	-2.83	76.48	155.80	RiskTriang(0,0,196.08)	0.00	17.94	53.83	RiskTriang(0,0,209.43)	0	69.81	209.43
α-ZEL-14G	RiskUniform(-1.1754,64.645)	-1.18	31.73	64.65	nd[†]	nd	nd	nd	RiskTriang (0,0,0.19564)	0	65.21	195.64
sum ZEN-eq[§]	RiskExpon(90.412,RiskShift(-1.6439))	-1.64	88.77	+∞	RiskExpon(74.639,RiskShift(-2.2618))	-2.26	72.58	+∞	RiskExpon(220.1,RiskShift(-3.4391))	-3.44	216.66	+∞
Food intake	RiskPearson5(4.5468,0.0048739, RiskShift(-0.00016448))	-0.000164	0.00121	+∞	RiskGamma(1.7098,0.00033989, RiskShift(0.00025779))	0.000258	0.000839	+∞	RiskInvgauss($5.8945*10^{-4}$, 0.002771, RiskShift(-0.0000998969))	$-9.99*10^{-5}$	0.000490	+∞

[†] nd: no distribution can be made
[‡] DON-eq: DON, 3-ADON, 15-ADON and DON-3G
[§] ZEN-eq: ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G
[††] DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

Mycotoxin	Food Category: Popcorn				Food Category: Oatmeal				Food Category: All cereal-based foods			
	Function	min	mean	max	Function	min	mean	max	Function	min	mean	max
DON	RiskExpon(91.559,RiskShift(-6.104))	-6.10	85.46	+∞	RiskExpon(17.764,RiskShift(-1.2689))	-1.27	16.50	+∞	RiskExpon(41.726,RiskShift(-0.23053))	-0.231	41.50	+∞
3-ADON	RiskExtvalue(28.286,19.731)	-∞	39.68	+∞	RiskExtvalue(21.768,33.235)	0.00	40.18	115.50	RiskLogistic(19.563,17.273)	-∞	19.56	+∞
15-ADON	RiskExpon(26.93,RiskShift(8.2047))	8.20	35.13	+∞	RiskExtvalue(5.8245,6.3466)	0.00	9.48	26.90	RiskExpon(16.253,RiskShift(-0.089797)	-0.0898	16.16	+∞
DON-3G	RiskExpon(35.414,RiskShift(-2.3609))	-2.36	33.05	+∞	RiskInvgauss(30.164,11.211,RiskShift(-2.9572))	-2.96	27.21	+∞	RiskExpon(25.695,RiskShift(-0.14196))	-0.142	25.55	+∞
sum DON-eq	RiskLoglogistic(15.275,108.51,1.8762)	15.28	197.95	+∞	RiskUniform(-14.831,207.63)	-14.83	96.40	207.63	RiskLoglogistic(-18.127,92.66,2.0616)	-18.13	123.23	+∞
HT-2	RiskExpon(1.3333,RiskShift(-0.088889))	-0.0889	1.24	+∞	RiskExpon(19.814,RiskShift(-1.4153))	-1.42	18.40	+∞	RiskExpon(18.703,RiskShift(-0.10333))	-0.103	18.60	+∞
T-2	RiskUniform(-1.8643,27.964)	-1.86	13.05	27.96	RiskExpon(13.107,RiskShift(-0.93622)	-2.60	16.90	36.40	RiskTriang(0,0,49.256)	0.00	16.42	49.26
sum HT-2/T-2	RiskNormal(5.8933,10.806)	-∞	5.89	+∞	RiskExpon(32.921,RiskShift(-2.3515))	-3.35	30.57	+∞	RiskLogistic(26.868,16.405)	-∞	26.87	+∞
β-ZEL	RiskNormal(9.0533,16.384)	-∞	9.05	+∞	RiskNormal(8.4357,16.309)	-∞	8.44	+∞	RiskNormal(13.911,29.613)	-∞	13.91	+∞
α-ZEL	RiskTriang(0,0,33.864)	0.00	11.29	33.86	RiskExtvalue(3.4327,9.9077)	0.00	17.22	67.60	RiskNormal(19.319,56.593)	-∞	19.32	+∞
ZEN	RiskNormal(8.76,12.398)	-∞	8.76	+∞	RiskLogistic(29.491,26.259)	0.00	53.90	190.20	RiskExpon(47.038,RiskShift(-0.25988))	-0.260	46.78	+∞
ZEN-14G	nd†	nd	nd	nd	RiskNormal(12.672,24.858)	-∞	12.67	+∞	RiskExtvalue(5.6293,20.963)	-∞	17.73	+∞
ZEN-14S	nd	nd	nd	nd	RiskTriang(0,0,39.088)	0.00	13.03	39.09	RiskNormal(10.308,45.107)	-∞	10.31	+∞
β-ZEL-14G	nd	nd	nd	nd	RiskUniform(-1.5385,21.538)	-1.54	10.00	21.54	RiskTriang(0,0,207.22)	0.00	69.07	207.22
α-ZEL-14G	nd	nd	nd	nd	nd	nd	nd	nd	RiskTriang(0,0,193.54)	0.00	64.51	193.54
sum ZEN-eq	RiskLogistic(25.73,18.343)	-∞	25.73	+∞	RiskExtvalue(42.761,56.954)	-∞	75.64	+∞	RiskExpon(127.34,RiskShift(-0.70351))	-0.704	126.64	+∞
Food intake	RiskExtvalue(0.00033024,0.00032185)	-∞	0.000516	+∞	RiskTriang(0.00000000558878, 0.00000000558878,0.00059316)	$5.589*10^{-9}$	0.000198	0.000593	RiskInvgauss(0.00087228,0.00171559, RiskShift(0.0000838565),	$8.386*10^{-5}$	0.000956	+∞

† nd: no distribution can be made

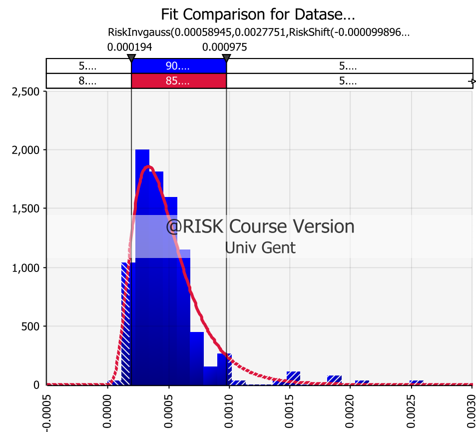


Figure 4.1 Distribution curve: consumption in breakfast cereals

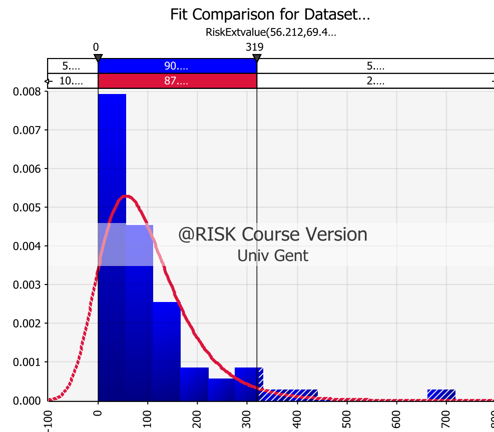


Figure 4.2 Distribution curve: concentration sum DON-eq in breakfast cereals

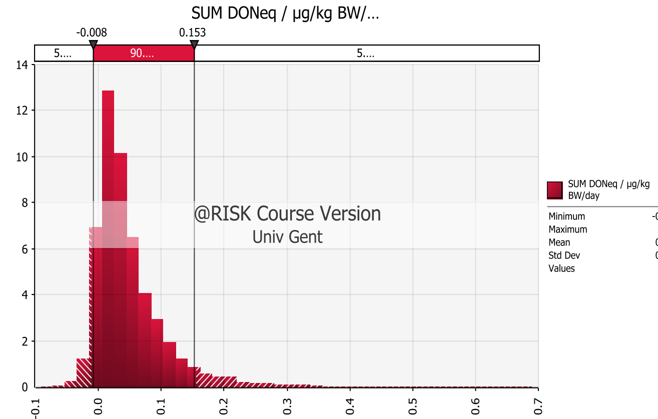


Figure 4.3 Cumulative distribution curve: sum DON-eq in breakfast cereals

Table 4.4 (a, b, c, d and e) represents the probabilistic estimates of the parent and masked mycotoxin intake (mean, standard deviation, maximum and percentiles) (µg kg^{-1} bw day^{-1}) from the different food categories by the Belgian population for the upper bound (worst-case scenario).

Table 4.4 Probabilistic analysis of mycotoxins and their masked forms estimated intake (mean, standard deviation, maximum and percentiles) (µg kg^{-1} bw day^{-1}) by Belgian adult population from the different food categories. Upper bound corresponds to data (< LOD) treated as LOD.

Table 4.4 a

DON	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn	All cereal-based foods
mean	0.0403	0.0311	0.0126	0.0032	0.0446	0.0396
stdev	0.0587	0.0411	0.0340	0.0048	0.0729	0.0550
max	2.1268	0.4197	0.3180	0.0804	1.1904	1.2583
P50	0.0248	0.0186	0.0094	0.0015	0.0199	0.0219
P75	0.0494	0.0396	0.0264	0.0042	0.0559	0.0488
P90	0.0888	0.0755	0.0498	0.0086	0.1185	0.0942
P95	0.1273	0.1102	0.0688	0.0127	0.1763	0.1371
P99.5	0.3419	0.2425	0.1442	0.0270	0.4320	0.3221
TDI†	1.0	1.0	1.0	1.0	1.0	1.0
% TDI excess‡	0.02	0.00	0.00	0.00	0.03	0.01
Population excess§	2,168	0	0	0	3,252	1,084

† TDI = tolerable daily intake
‡ % TDI excess = percent of population which exceeded the tolerable daily intake
§ Population excess = number of people exceeding the TDI. Colored values indicate that exposure exceeds the TDI.

Table 4.4 b

SUM DON-eq††	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn	All cereal-based foods
mean	0.1385	0.0768	0.0469	0.0190	0.1004	0.1162
stdev	0.4379	0.0739	0.0556	0.0229	0.2143	0.2228
max	37.6842	0.9007	0.5803	0.2148	7.3518	7.3421
P50	0.0715	0.0565	0.0322	0.0114	0.0515	0.0573
P75	0.1550	0.1004	0.0661	0.0268	0.1107	0.1247
P90	0.2986	0.1626	0.1122	0.0494	0.2218	0.2536
P95	0.4499	0.2145	0.1498	0.0665	0.3426	0.4047
P99.5	1.5577	0.4252	0.3114	0.1225	1.1041	1.3310
TDI†	1.00	1.00	1.00	1.00	1.00	1.00
% TDI excess‡	1.15	0.00	0.00	0.00	0.68	0.85
Population excess§	124,659	0	0	0	73,711	92,139

† TDI = tolerable daily intake
‡ % TDI excess = percent of population which exceeded the tolerable daily intake
§ Population excess = number of people exceeding the TDI
†† sum of DON, 3-ADON, 15-ADON and DON-3G. Colored values indicate that exposure exceeds the TDI.

Table 4.4 c

SUM T-2 and HT-2	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn	All cereal-based foods
mean	0.0577	0.0266	0.0119	0.0060	0.0032	0.0258
stdev	0.1236	0.0253	0.0116	0.0092	0.0076	0.0383
max	2.8701	0.2726	0.0955	0.1276	0.0761	0.3651
P50	0.0140	0.0193	0.0084	0.0027	0.0016	0.0188
P75	0.0603	0.0369	0.0165	0.0076	0.0059	0.0386
P90	0.1604	0.0593	0.0269	0.0160	0.0122	0.0676
P95	0.2522	0.0765	0.0350	0.0233	0.0172	0.0924
P99.5	0.7052	0.1335	0.0630	0.0541	0.0334	0.1950
TDI†	0.10	0.10	0.10	0.10	0.10	0.10
% TDI excess‡	16.60	1.89	0.00	0.02	0.00	4.11
Population excess§	1,799,424	204,874	0	2,168	0	445,520

† TDI = tolerable daily intake
‡ % TDI excess = percent of population which exceeded the tolerable daily intake
§ Population excess = number of people exceeding the TDI. Colored values indicate that exposure exceeds the TDI.

Table 4.4 d

ZEN	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn	All cereal-based foods
mean	0.0713	0.0304	0.0729	0.0058	0.0045	0.1199
stdev	0.0790	0.0524	0.1038	0.0122	0.0089	0.1585
max	**1.1428**	**0.5032**	**1.8826**	0.1195	0.0747	**2.1112**
P50	0.0480	0.0250	0.0379	0.0032	0.0025	0.0682
P75	0.0934	0.0540	0.0915	0.0102	0.0077	0.1503
P90	0.1614	0.0920	0.1787	0.0204	0.0149	**0.2861**
P95	0.2146	0.1217	0.2576	0.0281	0.0210	**0.4094**
P99.5	**0.4491**	0.2348	**0.6130**	0.0564	0.0418	**0.9662**
TDI[†]	0.25	0.25	0.25	0.25	0.25	0.25
% TDI excess[‡]	5.81	0.95	8.20	0.00	0.00	1.73
Population excess[§]	629,798	102,979	888,872	0	0	187,530

[†] TDI = tolerable daily intake
[‡] % TDI excess = percent of population which exceeded the tolerable daily intake
[§] Population excess = number of people exceeding the TDI. Colored values indicate that exposure exceeds the TDI.

Table 4.4 e

SUM ZEN-eq[††]	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn	All cereal-based foods
mean	0.1074	0.0607	0.1058	0.0150	0.0136	0.0447
stdev	0.1571	0.0771	0.1354	0.0204	0.0247	0.0595
max	**2.8203**	**1.1829**	**1.8673**	0.1781	0.2442	**0.7236**
P50	0.0591	0.0355	0.0604	0.0081	0.0081	0.0248
P75	0.1331	0.0786	0.1370	0.0216	0.0224	0.0556
P90	**0.2570**	0.1451	**0.2546**	0.0405	0.0416	0.1073
P95	**0.3568**	0.2082	**0.3609**	0.0554	0.0583	0.1568
P99.5	**0.9180**	**0.4696**	**0.7892**	0.1081	0.1178	**0.3603**
TDI[†]	0.25	0.25	0.25	0.25	0.25	0.25
% TDI excess[‡]	14.80	5.27	14.91	0.00	0.02	2.75
Population excess[§]	1,614,867	572,106	1,616,131	0	2,168	297,667

[†] TDI = tolerable daily intake
[‡] % TDI excess = percent of population which exceeded the tolerable daily intake
[§] Population excess = Number of people exceeding the TDI
[††] sum of ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G and β-ZEL-14G. Colored values indicate that exposure exceeds the TDI.

Table 4.4 a shows for DON that 0.02%, 0.03% and 0.01% of the Belgian population exceeded the TDI of 1.00 µg kg^{-1} bw day^{-1} for fibre-enriched bread, popcorn and all cereal-based foods. The share of DON intake relative to its equivalents was determined by dividing the excess DON by the excess of the DON-equivalents. The share of DON relative to the estimated intake of the DON-equivalents for fibre-enriched bread, popcorn and all cereal-based foods, was 2%, 4% and 1%, respectively (**Table 4.4 b**). These results suggest that the intake consisted mainly of the DON-derivatives, 3-ADON, 15-ADON and DON-3G. The sum of the DON-equivalents underscores that high consumption of fibre-enriched bread, popcorn and all cereal-based foods enhances the exposure. As the percentage of population, which exceeded the TDI, is difficult to interpret, the risk was expressed on the absolute Belgian population (10,839,905, (Statbel, 2012)). 124,659; 73,711 and 92,139 persons were daily exposed to threshold-exceeding DON-equivalents for fibre-enriched bread, popcorn and all cereal-based foods, respectively. However, based on the lack of toxicity data for the DON-derivatives no conclusions regarding the possible risk can be drawn.

The mean (P99.5) intakes for breakfast cereals, oatmeal and popcorn for the sum of T-2 and HT-2 were 0.0119 (0.0630), 0.0060 (0.0541) and 0.0032 (0.0334) µg kg^{-1} bw day^{-1}, respectively. These values were below the provisional TDI of 0.10 µg kg^{-1} bw day^{-1} **(Table 4.4 c)**. Nevertheless, fibre-enriched bread, bran-enriched bread and all cereal-based foods had T-2 and HT-2 intakes (P 99.5) of 0.7052, 0.1335 and 0.1950 µg kg^{-1} bw day^{-1}, respectively, potentially indicating that the concentrations found in the analyzed foods, when consumed on a daily scale, cause a high exposure to the Belgian population (1,799,424; 204,874; 445,520 persons exceeding the TDI, respectively).

Data in **Table 4.4 d** represents a high ZEN-exposure with P99.5 of 0.4491, 0.2348, 0.6130, and 0.9662 µg kg^{-1} bw day^{-1} for high consumers of fibre-enriched bread, bran-enriched bread, breakfast cereals and all cereal-based foods, respectively. These values correspond to an excess of 629,798, 102,979, 888,872 and 187,530, respectively of the Belgian population, which is of high concern. The attribution of ZEN to the ZEN-equivalents was determined by dividing the excess of ZEN by the overplus of its equivalents **(Table 4.4 e).** The share of ZEN for fibre-enriched bread, breakfast cereals and all cereal-based foods was 39%, 55% and 63%, respectively, which was high, in contrast to the attribution of DON to its DON-equivalents. Concerning the sum of the ZEN-equivalents, only oatmeal can be consumed without any risk due to low consumption (mean, 0.0150 µg kg^{-1} bw day^{-1}; P 99.5, 0.1081 µg kg^{-1} bw day^{-1}). P99.5 intakes of 0.9180, 0.4696, 0.7892, 0.1178 (max 0.2442), and 0.3603 µg kg^{-1} bw day^{-1} were observed for the other five foodstuff groups, which correspond to a population exposure ranging from 2,168 to 1,616,131 consumers. The latter high value is especially disturbing since these adults consume breakfast cereals on a daily basis and thus are exposed to a corresponding amount of ZEN and equivalents.

In **Figure 4.4, 4.5 and 4.6** relative contributions of the sum of the DON-equivalents, T-2 and HT-2, and the ZEN-equivalents are indicated among the six different food categories. The values (%) show the percentages of the examined population which exceed the TDI. It is clearly proven that the consumption of bread, highly-enriched with fibres, as well as the consumption of all cereal-based food causes a risk impact as their shares in the graphs are most prevalent.

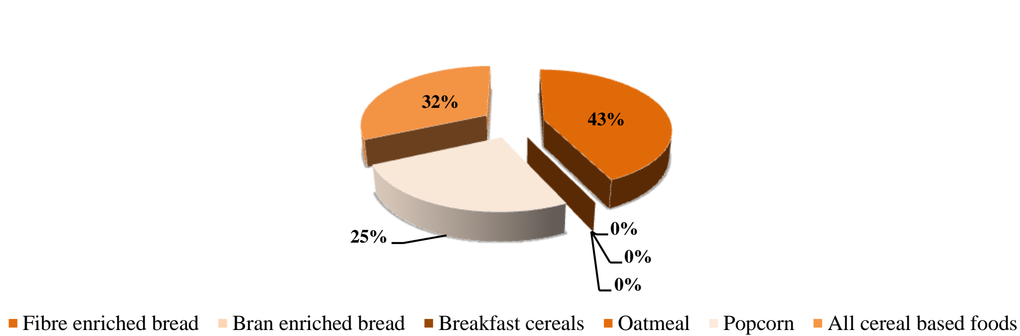


Figure 4.4 Relative contributions of the sum of the DON-equivalents among the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices.

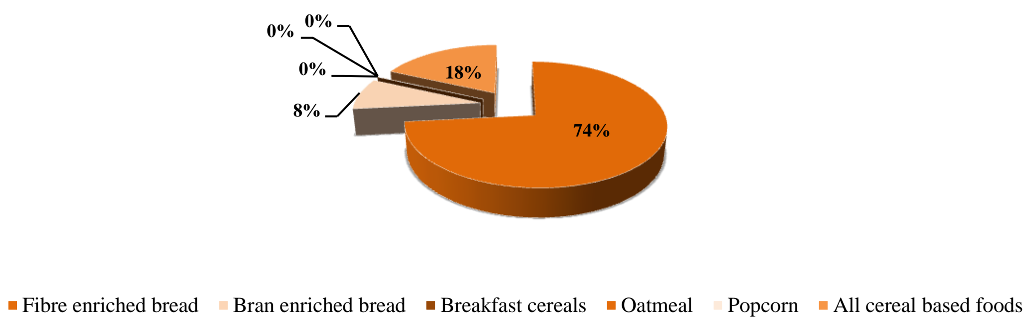


Figure 4.5 Relative contributions of T-2 and HT-2 for the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices.

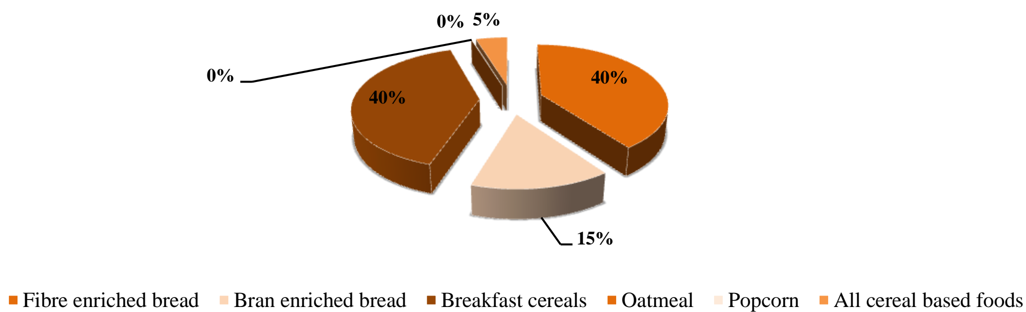


Figure 4.6 Relative contributions of the ZEN-equivalents for the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices.

4.3.1.5 Uncertainty related to exposure assessments

Uncertainties associated with exposure assessments need to be considered with a view to a correct interpretation. A possible overestimation of the subgroup 'all cereal-based foods' is acknowledged inasmuch high consumers of bread might not be high consumers of breakfast cereals within the same time frame. Current software however, does not allow for incorporation of these relationships in the exposure assessment estimations.

Also, the hypothesis that masked mycotoxins are totally converted to their parents during ingestion is assumed here.

The most recent food consumption data of the Belgian population were applied in this study. Although the data originate from 2004, our findings are of public health importance to date in Belgium. Eating habits might change over time, however, changes in the consumption quantities of cereal-based food are limited (Kearney, 2010).

Furthermore, this study applied a dietary assessment characterized by inherent under or over-reporting of the consumption data. Despite this, the 24 hour recall is the recommended method to estimate food intake in large food consumption surveys in Europe (de Boer *et al.*, 2011).

Mycotoxin (DON, ZEN, sum of T-2 and HT-2) dietary estimations have been performed by several EU-countries (Scoop 2003, 2003; JECFA, 2010; Reddy *et al.*, 2010; Bail *et al.*, 2005; Binder *et al.*, 2007; Harcz *et al.*, 2007; Maragos, 2011). From 2002 to 2005, Harcz *et al.* investigated the exposure for DON and ZEN in Belgium, where similar results were obtained (Harcz *et al.*, 2007).

According to SCOOP (2003) the DON-levels for adults were below the TDI, which tends to be confirmed in the observed results (**Table 4.4**). Mean estimates of chronic dietary exposure to the sum of T-2 and HT-2 based on the available occurrence data varied within a range of 0.0034 µg kg^{-1} bw day^{-1} to 0.018 µg kg^{-1} bw day^{-1}, with P95 ranging from 0.0072 µg kg^{-1} bw day^{-1} to 0.039 µg kg^{-1} bw day^{-1} (EFSA, 2011a). In addition, our study proved the relevance of T-2 and HT-2 in cereal-based foods. The EFSA report concluded that in the adult population the mean dietary exposure to ZEN across survey studies ranged from 0.0024 µg kg^{-1} bw day^{-1} to 0.0290 µg kg^{-1} bw day^{-1}, whereas the P95 ranged from 0.0047 µg kg^{-1} bw day^{-1} to 0.0540 µg kg^{-1} bw day^{-1} (EFSA, 2011c). These results were not in accordance with the high values obtained in this study (mean 0.1199, P95 0.4094 µg kg^{-1} bw day^{-1}). The data obtained were transferred to the national risk management authority for further monitoring of the data. The

largest contribution to human health risk can be attributed to breakfast cereals, which was also observed in the EFSA-report.

To date however, no risk assessments were performed for masked mycotoxins. The multiple exposure assessments in other countries indicated that small children were exposed to high levels of the mycotoxins investigated in contrast to the adult population (Serrano *et al.*, 2012; Gauchi *et al.*, 2002; Cano-Sancho *et al.*, 2011). The average intakes for regions such as the Mediterranean area estimate that the exposure is generally below the levels deemed as intolerable. However, certain regions and developing countries will encounter problems (*e.g.* climate, high humidity) for certain mycotoxins, particularly in subpopulations where contamination of cereal-based foods is substantious (Kearney, 2010).

The discrepancies between studies so far could be explained by the use of various methodologies, quantification strategies and food consumption surveys.

4.3.2 Dietary exposure assessment in feed

4.3.2.1 Determination of (masked) mycotoxins in feed

A total of 86 compound feeds were analyzed for the occurrence of DON, 3-ADON, 15-ADON, DON-3G, T-2, HT-2, ZEN, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G, ZEN-14G and ZEN-14S. Six feed categories were considered according to their importance on the Belgian market: feed for poultry, pigs, fattening pigs, sows, horses, and dairy cattle and young stock.

Table 4.5 shows the incidence, mean concentration, and standard deviation (μg kg^{-1}) of the mycotoxins and their masked forms observed in each feed category. The percentage of mycotoxins occurring in the samples was calculated by the amount of samples (> LOD) divided by the total amount of samples analyzed. The compound feeds were contaminated with an average of 5 to 11 different mycotoxins (median = 8) including nil to 4 masked forms (median = 2). These masked forms comprised DON-3G, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G.

Table 4.5 Incidence and mean concentrations (μg kg^{-1}) of parent mycotoxins and derivatives in the compound feeds.

Mycotoxin[§]	Poultry (*n*=14)			Piglets (*n*=8)			Sows (*n*=15)			Fattening pigs (*n*=13)			Horses (*n*=14)			Dairy cattle and young stock (*n*=3)		
	>LOD[†]	mean	SD	>LOD	mean	SD	>LOD	mean	SD	>LOD	mean	SD	>LOD	mean	SD	>LOD	mean	SD
DON	100	197	239	100	318	284	100	135	66	100	291	399	100	200	226	100	90	57
3-ADON	64	47	53	38	25	47	33	11	17	54	29	43	54	13	27	33	44	71
15-ADON	100	50	52	100	42	25	80	20	12	100	38	33	100	44	76	100	22	5
DON-3G	50	30	30	38	43	65	60	29	35	38	121	357	38	34	57	nd	nd	nd
ZEN	79	483	542	88	459	520	87	167	136	69	249	433	69	266	337	33	129	223
ZEN-14G	43	28	76	25	28	57	20	11	21	31	8	13	31	27	78	nd	nd	nd
ZEN-14S	43	4	8	38	28	41	27	11	12	31	15	24	31	4	13	nd	nd	nd
α-ZEL	36	56	97	75	213	243	40	71	109	23	24	69	23	134	326	nd	nd	nd
α-ZEL-14G	43	11	19	25	11	15	20	9	15	15	5	13	nd[‡]	nd	nd	33	4	7
β-ZEL	43	23	32	63	89	110	13	4	10	31	13	29	31	63	128	nd	nd	nd
β-ZEL-14G	43	15	23	38	14	26	20	10	25	8	37	115	8	3	7	nd	nd	nd
HT-2	57	13	13	75	24	18	60	20	21	38	12	14	38	25	30	66	14	13
T-2	21	9	16	50	17	18	47	17	19	15	7	16	15	16	27	33	10	10

[†] >LOD: amount of samples with a concentration detected above the limit of detection (LOD) (%); LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 μg kg^{-1}), 3-ADON (10, 8, 7 and 5 μg kg^{-1}), 15-ADON (9, 7, 6 and 5 μg kg^{-1}), DON-3G (13, 12, 8 and 7 μg kg^{-1}), β-ZEL (9, 8, 7 and 7 μg kg^{-1}), α-ZEL (9, 9, 5 and 6 μg kg^{-1}), ZEN (8, 9, 7 and 6 μg kg^{-1}), ZEN-14G (10, 10, 7 and 9 μg kg^{-1}), ZEN-14S (12, 11, 12 and 12 μg kg^{-1}), β-ZEL-14G (12, 11, 10 and 10 μg kg^{-1}), α-ZEL-14G (12, 11, 10 and 10 μg kg^{-1}), HT-2 (9, 9, 5 and 6 μg kg^{-1}) and T-2 (11, 10, 9 and 9 μg kg^{-1})

[‡] nd: not detected

[§] DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

4.3.2.2. Consumption data

Detailed feed consumption data were unavailable as the gathering of data on individual feed intakes of various species is difficult to achieve. Therefore, only mean intake levels for each species were taken into account (Ewing, 1997; Leeson and Summers, 2005). The mean intake determined for poultry, pigs, fattening pigs, sows, horses, and dairy cattle and young stock were 0.06, 0.091, 0.03, 0.03, 0.01 and 0.017 kg kg^{-1} bw day^{-1}, respectively.

4.3.2.3 Deterministic exposure assessment

A deterministic exposure assessment was executed according to the obtained concentration and consumption data. Exposure was determined using the mean consumption per species and the concentration data (mean, P95 and max). The intake was compared to the no observed effect levels (NOELs), the lowest observed adverse effect levels (LOAELs) or the mean exposure levels based on literature review and EFSA reports. Insofar as no NOELs, LOAELS or mean contamination levels were described for masked mycotoxins, the levels described were fit with those of the parent mycotoxin. In **Table 4.6** the estimated intakes (µg kg^{-1} bw day^{-1}) (mean, P95 and max) for the several compound feeds per species are presented. These data are discussed in three subparagraphs: DON and DON-derivatives, ZEN and ZEN-derivatives and the sum of HT-2 and T-2.

DON and DON-derivatives

A reduced weight gain of the liver was observed in poultry at an exposure level of 4,500 µg kg^{-1} bw day^{-1}, and an increase of abnormalities in youngsters was noticed from 1,250 µg kg^{-1} bw day^{-1}. No value exceeded the described NOAELs.

An NOEL for pigs was described between 18 and 27 µg kg^{-1} bw day^{-1}. Mean exposure for DON was designated as 28.8972 µg kg^{-1} bw day^{-1}, while these for DON-equivalents increased up to 39.1108 µg kg^{-1} bw day^{-1}, which is 1.5 times higher than the NOEL described for DON. As already mentioned in the food section, extrapolating this value to DON- equivalents is notably a worst-case scenario, assuming either that these toxins are equally toxic as their parent or that total hydrolysis occurs.

For fattening pigs and sows no toxicity values were determined.

Horses exhibit no significant issues with DON-feeding at ppm level.

Preliminary values for dairy cattle and young stock were described ranging from 10.7 µg kg^{-1} bw day^{-1} to 14.0 µg kg^{-1} bw day^{-1}. On the basis of the obtained results no excess could be concluded.

ZEN and ZEN-derivatives

For poultry an NOEL of 10,000 µg kg^{-1} bw day^{-1} was set, and according to the described assessment, the intakes did not exceed this value.

In pigs, a formation of tertiary follicles was observed (Bauer *et al.*, 1987) at an exposure of 50 µg kg^{-1} bw day^{-1}, while hyperestrogenism was proven from 200 µg kg^{-1} bw day^{-1} (Obremski

et al., 2003). An NOEL of 10 µg kg^{-1} bw day^{-1} was set, and from **Table 4.6** it can be noticed that the ZEN-intake exceeds this level, even without taking into account α-ZEL en β-ZEL, which are highly abundant derivatives, and play a crucial factor in the overall toxicity of ZEN. So, taking the ZEN-equivalents into account, a general excess was observed.

Fattening pigs should observe an NOEL of 90 µg kg^{-1} bw day^{-1}, but no mycotoxin intakes exceeded this limit.

In current literature, no toxicity data are available for ZEN and ZEN-derivatives concerning feed intake for horses, dairy cattle and young stock.

sum of T-2 and HT-2

Mucosal damage in the oral cavity was observed at an exposure from 40 µg kg^{-1} bw day^{-1}, while a reduction of the egg production and infertility was obtained from 120 µg kg^{-1} bw day^{-1}. The exposure assessment did not meet these levels.

Pigs, however, are the most susceptible species to T-2. Inconvenient immunological and hematological effects were observed from 29 µg kg^{-1} bw day^{-1}. EFSA described mean exposure levels between 0.28 en 0.87 µg kg^{-1} bw day^{-1}. **Table 4.6** indicates that all mean exposure levels exceeded the mentioned values, which is of high concern, however, adverse clinical outcomes (29 µg kg^{-1} bw day^{-1}) will not manifest.

Mean exposure levels between 0.39 en 0.76 µg kg^{-1} bw day^{-1} were described for sows and fattening pigs; also in these species values pointed to an excess of these mean levels.

No toxicity data were described for horses, however EFSA described mean contamination levels between 1.1 and 1.2 µg kg^{-1} bw day^{-1}. Values in **Table 4.6** prove that at P95 and at maximum level the sum of HT-2 and T-2 has been exceeded.

For dairy cattle and young stock intakes ranged from 0.75 to 1.70 µg kg^{-1} bw day^{-1}; none of the calculated assessments exceeded these levels.

Table 4.6 Deterministic analysis of mycotoxins and their masked forms estimated intake (mean, P95-percentile and maximum) for the different compound feeds ($\mu g\ kg^{-1}$ bw day^{-1})

LOWER BOUND† **($\mu g\ kg^{-1}$ bw day^{-1})**		**DON**††	**3-ADON**	**15-ADON**	**DON-3G**	**sum DON-eq**‡	**HT-2**	**T-2**	**sum HT-2/T-2**	**β-ZEL**	**α-ZEL**	**ZEN**	**ZEN-14G**	**ZEN-14S**	**β-ZEL-14G**	**α-ZEL-14G**	**sum ZEN-eq**§
Poultry	**mean**	11.8080	2.7686	3.0234	1.6923	19.2922	0.6891	0.4380	1.1271	1.3416	3.3645	29.0013	1.6304	0.1084	0.4151	0.7958	36.6571
	P95	37.5071	7.8333	9.6370	5.0352	52.2374	2.1459	2.1153	4.2612	5.2707	13.0704	92.9669	8.1905	0.5313	2.4895	3.5533	97.8704
	max	53.6328	10.9038	11.3994	5.3940	79.3170	2.8440	3.3360	6.1800	5.5320	18.9360	106.6914	16.9140	1.5180	4.2938	4.4760	106.6914
Pigs	**mean**	**28.8972**	2.2681	3.8030	3.8240	**38.7923**	**2.0598**	**1.3696**	**3.4293**	8.0468	**19.3787**	**41.7585**	2.2879	2.0873	0.9883	1.0998	**75.6473**
	P95	**69.9372**	9.2725	7.4572	13.7578	**89.7164**	**4.5809**	**4.0154**	**8.5963**	**23.4607**	**53.8388**	**118.3905**	**10.8805**	8.4248	2.9831	5.1162	**177.3144**
	max	**81.1602**	12.3578	7.7878	15.0605	**93.1412**	**5.5237**	**5.0505**	**10.5742**	**28.7924**	**55.4281**	**140.8225**	**14.9149**	**11.5206**	3.1395	6.7886	**216.5982**
Fattening pigs	**mean**	8.7335	0.8596	1.1396	3.5738	14.3065	0.3097	0.1770	**0.4867**	0.3831	0.7323	7.4812	0.2308	0.4183	0.1023	1.0816	10.4297
	P95	34.4098	2.8799	2.4560	17.1954	57.7538	**1.0134**	**1.0494**	**2.0628**	1.6519	3.9758	29.1080	1.0670	1.8941	0.5320	5.9304	34.7398
	max	37.4964	4.6572	4.3131	39.1230	72.5160	**1.3860**	**1.6560**	**3.0420**	3.1740	7.4970	47.5380	1.0872	1.9215	1.3299	12.5310	60.0690
Sows	**mean**	4.0361	0.3093	0.6139	0.8572	5.8166	**0.5341**	**0.4336**	**0.9677**	0.1045	1.9974	4.6656	0.2723	0.2247	0.2103	0.2848	7.7596
	P95	6.9613	1.2398	1.0824	2.7016	8.9164	**1.9701**	**1.7475**	**3.7176**	0.6591	8.1267	12.0888	1.7207	0.9255	1.0274	1.4138	19.5393
	max	7.3590	1.5783	1.3119	2.8872	9.9534	**1.9890**	**1.7580**	**3.7470**	1.0944	10.1700	12.9750	1.9278	1.1529	1.5975	2.8800	20.5710
Horses	**mean**	8.7335	0.8596	1.1396	3.5738	14.3065	0.3097	0.1770	0.4867	0.3831	0.7323	7.4812	0.2308	0.4183	0.1023	1.0816	10.4297
	P95	34.4098	2.8799	2.4560	17.1954	57.7538	1.0134	1.0494	**2.0628**	1.6519	3.9758	29.1080	1.0670	1.8941	0.5320	5.9304	34.7398
	max	37.4964	4.6572	4.3131	39.1230	72.5160	**1.3860**	**1.6560**	**3.0420**	3.1740	7.4970	47.5380	1.0872	1.9215	1.3299	12.5310	60.0690
Dairy cattle and young stock	**mean**	1.5310	0.7112	0.3800	0.0000	2.6222	0.2451	0.1167	0.3618	0.0000	0.0000	2.1896	0.0000	0.0000	0.0000	0.5480	2.7376
	P95	2.4456	1.9202	0.4586	0.0000	4.6726	0.3822	0.3152	0.6974	0.0000	0.0000	5.9119	0.0000	0.0000	0.0000	1.4795	7.3914
	max	2.5600	2.1335	0.4668	0.0000	4.9825	0.3859	0.3502	0.7361	0.0000	0.0000	6.5688	0.0000	0.0000	0.0000	1.6439	8.2127

UPPER BOUND† **($\mu g\ kg^{-1}$ bw day^{-1})**		**DON**††	**3-ADON**	**15-ADON**	**DON-3G**	**sum DON-eq**‡	**HT-2**	**T-2**	**sum HT-2/T-2**	**β-ZEL**	**α-ZEL**	**ZEN**	**ZEN-14G**	**ZEN-14S**	**β-ZEL-14G**	**α-ZEL-14G**	**sum ZEN-eq**§
Poultry	**mean**	11.8080	2.8286	3.0234	1.8123	19.4722	0.9206	0.8237	1.7443	1.4187	3.4416	29.0013	1.7332	0.4299	0.8266	1.1044	37.9557
	P95	37.5071	7.8333	9.6370	5.0352	52.2374	2.1459	2.1153	4.2612	5.2707	13.0704	92.9669	8.1905	1.5537	2.4895	3.5533	97.8704
	max	53.6328	10.9038	11.3994	5.3940	79.3170	2.8440	3.3360	6.1800	5.5320	18.9360	106.6914	16.9140	1.6200	4.2938	4.4760	106.6914
Pigs	**mean**	**28.8972**	2.2681	3.8030	4.1425	**39.1108**	**2.4693**	**1.9838**	**4.4531**	8.2516	**19.3787**	**41.7585**	2.5609	2.9063	1.2613	1.9188	**78.0361**
	P95	**69.9372**	9.2725	7.4572	13.7578	**89.7164**	**4.5809**	**4.0154**	**8.5963**	**23.4607**	**53.8388**	**118.3905**	**10.8805**	8.4248	2.9831	5.1770	**177.3144**
	max	**81.1602**	12.3578	7.7878	15.0605	**93.1412**	**5.5237**	**5.0505**	**10.5742**	**28.7924**	**55.4281**	**140.8225**	**14.9149**	**11.5206**	3.1395	6.7886	**216.5982**
Fattening pigs	**mean**	8.7335	0.9011	1.1396	3.7400	14.5142	0.4343	0.3847	0.8190	0.4939	0.7323	7.4812	0.2862	0.4737	0.2131	1.2478	10.9281
	P95	34.4098	2.8799	2.4560	17.1954	57.7538	1.0134	1.0494	2.0628	1.7016	3.9758	29.1080	1.0670	1.8941	0.9640	5.9304	34.7398
	max	37.4964	4.6572	4.3131	39.1230	72.5160	1.3860	1.6560	3.0420	3.1740	7.4970	47.5380	1.0872	1.9215	1.3299	12.5310	60.0690
Sows	**mean**	4.0361	0.4173	0.6139	0.8572	5.9246	0.6061	0.5416	1.1477	0.1405	1.9974	4.6656	0.3075	0.4647	0.3063	0.3328	8.2148
	P95	6.9613	1.2398	1.0824	2.7016	8.9164	1.9701	1.7475	3.7176	0.7063	8.1267	12.0888	1.7207	0.9255	1.0274	1.4138	19.5393
	max	7.3590	1.5783	1.3119	2.8872	9.9534	1.9890	1.7580	3.7470	1.0944	10.1700	12.9750	1.9278	1.1529	1.5975	2.8800	20.5710
Horses	**mean**	8.7335	0.9011	1.1396	3.7400	14.5142	0.4343	0.3847	0.8190	0.4939	0.7323	7.4812	0.2862	0.4737	0.2131	1.2478	10.9281
	P95	34.4098	2.8799	2.4560	17.1954	57.7538	1.0134	1.0494	**2.0628**	1.7016	3.9758	29.1080	1.0670	1.8941	0.9640	5.9304	34.7398
	max	37.4964	4.6572	4.3131	39.1230	72.5160	**1.3860**	**1.6560**	**3.0420**	3.1740	7.4970	47.5380	1.0872	1.9215	1.3299	12.5310	60.0690
Dairy cattle and young stock	**mean**	1.5310	0.8132	0.3800	0.0000	2.7242	0.2451	0.2187	0.4638	0.1360	0.0000	2.1896	0.0000	0.0000	0.1360	0.5480	3.0096
	P95	2.4456	1.9508	0.4586	0.0000	4.6726	0.3822	0.3458	0.7280	0.3672	0.0000	5.9119	0.0000	0.0000	0.3672	1.4795	7.7994
	max	2.5600	2.1335	0.4668	0.0000	4.9825	0.3859	0.3502	0.7361	0.4080	0.0000	6.5688	0.0000	0.0000	0.4080	1.6439	8.6207

† Lower and upper bound correspond to data (< LOD) treated as 0 and LOD, respectively. LODs for bread, breakfast cereals, popcorn and oatmeal, respectively: DON (10, 9, 6 and 5 $\mu g\ kg^{-1}$), 3-ADON (10, 8, 7 and 5 $\mu g\ kg^{-1}$), 15-ADON (9, 7, 6 and 5 $\mu g\ kg^{-1}$), DON-3G (13, 12, 8 and 7 $\mu g\ kg^{-1}$), β-ZEL (9, 8, 7 and 7 $\mu g\ kg^{-1}$), α-ZEL (9, 9, 5 and 6 $\mu g\ kg^{-1}$), ZEN (8, 9, 7 and 6 $\mu g\ kg^{-1}$), ZEN-14G (10, 10, 7 and 9 $\mu g\ kg^{-1}$), ZEN-14S (12, 11, 12 and 12 $\mu g\ kg^{-1}$), β-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), α-ZEL-14G (12, 11, 10 and 10 $\mu g\ kg^{-1}$), HT-2 (9, 9, 5 and 6 $\mu g\ kg^{-1}$) and T-2 (11, 10, 9 and 9 $\mu g\ kg^{-1}$); ‡ DONeq: DON, 3-ADON, 15-ADON and DON-3G; § ZENeq: ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G. Colored values indicate that exposure exceeds the NOEL/LOEL or NOAEL.

‡ DON-eq: DON, 3-ADON, 15-ADON and DON-3G

§ ZEN-eq: ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G

†† DON = deoxynivalenol, 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; β-ZEL = β-zearalenol; α-ZEL = α-zearalenol; ZEN = zearalenone; ZEN-14G = zearalenone-14-glucoside; ZEN-14S = zearalenone-14-sulfate; β-ZEL-14G = β-zearalenol-14-glucoside; α-ZEL-14G = α-zearalenol-14-glucoside; HT-2 = HT-2 toxin; T-2 = T-2 toxin

4.4 CONCLUSION

To our knowledge, this is the first study assessing the exposure of the Belgian population to DON, 3-ADON, 15-ADON, ZEN, α-ZEL, β-ZEL, T-2, HT-2 and their masked forms. The population is expected to be exposed to moderate levels of several mycotoxins. Although the majority of the Belgian population does not exceed the TDI of 1.0, 0.2 and 0.1 µg kg^{-1} bw day^{-1} (DON, ZEN and the sum of T-2 and HT-2, respectively), there is a large subpopulation, exceeding those safety values. A reduction of (masked) mycotoxin concentrations in cereal-derived foodstuffs is a prerequisite to reach the safety levels. In view of the ZEN exposure in cereal-based food reported in our study, a call for an overhaul of legislation focusing on ZEN is necessary. Edwards (Edwards, 2011) reported already the risk of ZEN in European wheat; our study indubitably confirms his statements.

According to the obtained data for feed exposure, the highest concern is attributed to the consumption of pig compound feeds. For all parent and masked mycotoxins analyzed, safety levels underscored an excess. More samples need to be analyzed to confirm these statements of concern.

The focus of official instances is on levels of the individual mycotoxins that exceed the maximum limits. From a scientific point of view, however, also the mycotoxin derivatives and masked forms should be taken into consideration. Nevertheless, the evaluation of masked mycotoxins should be treated with caution due to the lack of bioavailability and toxicological data. Based on the performed risk assessment a systematic monitoring of mycotoxins and their masked forms in food and feed remains necessary to gain more contamination data and specific toxicokinetic data for the masked forms.

CHAPTER 5

MYCOTOXIN GLUCOSYLATION IN COMMERCIAL WHEAT VARIETIES: IMPACT ON RESISTANCE TO *FUSARIUM GRAMINEARUM* UNDER LABORATORY AND FIELD CONDITIONS

Redrafted after:

Audenaert, K.[†], De Boevre, M.[†], Vanheule, A.[†], Callewaert, J., Bekaert, B., Höfte, M. De Saeger, S., and Haesaert, G.

Mycotoxin glucosylation in commercial wheat varieties: impact on resistance to *Fusarium graminearum* under laboratory and field conditions.

Food Control (in press, 2013; http://dx.doi.org/10.1016/j.foodcont.2013.06.019)

CHAPTER 5 MYCOTOXIN GLUCOSYLATION IN COMMERCIAL WHEAT VARIETIES: IMPACT ON RESISTANCE TO *FUSARIUM GRAMINEARUM* UNDER LABORATORY AND FIELD CONDITIONS

SUMMARY

In *Chapter 3* the contamination levels of masked forms were highlighted. *However, what is the glucosylation capacity and can this capacity quantitatively be determined?* In *Chapter 5* a combined answer to this question and to the above mentioned impact hypothesis is formulated based on *in vitro* and *in vivo* experiments in wheat.

Fusarium head blight (FHB) is a devastating disease of small cereal crops and is primarily caused by *Fusarium graminearum*. FHB typically is associated with a contamination of the infected grains with mycotoxins, which can act as virulence factors for the advancing fungus. Some wheat plants have evolved a typical resistance designated as *Fhb1* resistance, which is associated with a glucosyltransferase function, capable of detoxifying the deposited deoxynivalenol, a mycotoxin abundantly produced by *F. graminearum*. However, the resulting masked mycotoxin deoxynivalenol-3-glucoside may be metabolized in the digestive tract in animals, releasing the native toxin anew. *Chapter 5* aims to assess the glucosylation capacity in the Belgian commercial wheat-pool and to determine the importance of DON glucosylation in the complex background of natural field circumstances. Clear indications were observed that several Belgian commercial wheat cultivars, to some extent, do possess a glucosyltransferase function capable of detoxifying significant amounts of DON. However, the level of glucosylation in the field did not correlate well with disease severity. Results presented in *Chapter 5* reflect the conclusion of a first large-scale assessment of this glucosylation capacity in Belgian commercial wheat cultivars.

Key words: *Fusarium graminearum*, *Fhb1*, resistance, deoxynivalenol-3-glucoside, masked mycotoxins

5.1 INTRODUCTION

The toxigenic fungal pathogen *Fusarium graminearum* is the primary causal agent of *Fusarium* head blight (FHB) on wheat. FHB is caused by a complex of up to 17 different *Fusarium* species, each producing a highly specific blend of mycotoxins (*e.g.* trichothecenes such as deoxynivalenol (DON) and its acetylated forms, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON)). The national economy is affected by the high costs of quality control, regulatory monitoring programs, research and development, and most importantly the cost of human illnesses caused by these toxins. As a result of mycotoxin contamination, a worldwide 10% to 40 % reduction of crop yields can be observed (Charmley *et al.,* 1995).

The role and importance of these mycotoxins in the overall fungal metabolism have not yet been fully elucidated, but an increasing amount of evidence supports a recently proposed central oxidative stress theory for mycotoxin biosynthesis (Reverberi *et al.*, 2010). Indeed, recent studies have proven the fungus' response to oxidative stress to be dependent on whether a DON-chemotype is present (Ponts *et al.,* 2009), and have shown increased toxin production to be a response to oxidative stress (Audenaert *et al.*, 2010). DON acts as a virulence factor and is imperative in the spreading of *F. graminearum* after initial infection of the wheat plant (Maier *et al.*, 2006). The fungus interferes with the plant's defense system, and it has been shown that DON hijacks the pathways leading to the typical oxidative burst and programmed cell death.

Phytotoxicity of DON and other trichothecenes has been shown and therefore it makes sense that wheat has evolved a broad array of detoxification processes among which conjugation to probably less toxic compounds is the most important. The detoxification process and the concomitant formation of masked mycotoxins is an emerging health issue as these conjugated forms remain latently present in the plant tissue, ready to be released upon exposure to enzymes in the digestive system or upon food processing (Berthiller *et al.*, 2012; Dall'Erta *et al.*, 2013).

The ratio DON-3G/DON varied in relation to years and genotypes, but reached levels up to 29% (Berthiller *et al.*, 2009b) and even to 70% (De Boevre *et al.*, 2012a). A recent survey of unprocessed Belgian wheat showed levels of DON-3G up to 800 µg kg^{-1} (Vanheule *et al.*, 2013), while Berthiller *et al.* (2009b) found values up to 1,070 µg kg^{-1}. As described in *Chapter 1*, after processing, levels of contamination can differ. However, an increasing

number of the consumers prefer cereal products, not or less processed (organic foods), and, hence, it is advisable to further assess the exposure to these contaminants by focusing more extensively on cereals and cereal-based foods.

In search of an explanation for this active conversion of toxins to glucosylated derivatives, Miller *et al.* (1988) were among the first to speculate that formation of a less toxic DON conjugate might be responsible for partial FHB resistance of wheat. Pursuing a genetic approach, Lemmens *et al.* (2005) demonstrated that the ability of wheat lines to convert DON to DON-3G was linked to a quantitative trait locus (QTL), namely Qfhs.ndsu-3B which had previously been reported to be associated with FHB resistance against spreading of *Fusarium* infection (*Fhb1* resistance). They hypothesized that this QTL encodes for a DON-glucosyltransferase, or regulates the expression hereof. In contrast to the overwhelming amount of information on DON-glucosylation, information on other mycotoxins in this regard is limited. Lemmens *et al.* (2005) stated the possibility that the glucosyltransferase function is only effective against *Fusarium* strains that produce DON or structurally highly similar trichothecenes (*e.g.* nivalenol (NIV)). This would fundamentally change the current perception of resistance against FHB in wheat plants, which has always been considered to be species-independent (Parry *et al.*, 1995).

Lemmens *et al.* (2008) investigated the situation for NIV, and were not able to conclusively prove a link between *Fhb1*-mediated glucosylation and plant resistance against *F. graminearum* infection. In any case, it is tempting to speculate on the importance of DON glucosylation in the field, where plants are confronted with highly diverse populations of different *Fusarium* species with divergent chemotypes. Interestingly, the toxins for which *Fhb1*-mediated glucosylation has been investigated, NIV and DON, are both type B trichothecenes while several fairly prevalent *Fusarium* species such as *F. poae* also produce type A trichothecenes (*e.g.* neosolaniol (NEO) and diacetoxyscirpenol (DAS)). Reports on the phytotoxicity and availability for glucosylation of these toxins are scarce to non-existent.

In the present study, an artificial ear infection trial with *F. graminearum* and several commercial cultivars was set up. Levels of DON, its acetylated forms and DON-3G were monitored *in planta*, as well as the localization of these compounds within the ear. Over two consecutive years, the importance of DON-glucosylation as a component of total FHB resistance in the complex background of a field trial was evaluated for a large number of cultivars.

5.2 MATERIALS AND METHODS

5.2.1 Plant genotypes, cultivars and fungal isolates

The international reference isolate *F. graminearum* 8/1 (Jansen *et al.*, 2005) was used in all artificial inoculations in this study. The isolate was grown on potato dextrose agar (PDA, Oxoid; 39 g L^{-1}) for 7 days at 20 °C and kept at 4 °C until use. Conidia were obtained by incubating a mycelium plug on a PDA plate for 7 days under a light regime of UV/darkness (12h (365 nm 10W) $12h^{-1}$). Conidia were harvested by adding distilled water amended with 0.01% Tween 80 (Merck, Germany) to the fully grown PDA plates and by rubbing the sporulated mycelium with a spatula. Conidia were counted with a Bürker counting chamber and diluted to a final concentration of 10^6 conidia mL^{-1}.

Commercial wheat varieties used in the *in vivo* and field experiments are presented in **Table 5.1**. Levels of resistance were determined as outlined in Landschoot *et al.* (2012).

Table 5.1 Summary of the commercial wheat cultivars used in this study. Experiment 1 is the *in vivo* artificial infection of ears while experiment 2 is the 2-year field trial.

COMMERCIAL CULTIVARS					
Cultivar	**Distributor (country)**	**Resistance (gene)**	**Chromosomal location**	**Experiment**	**Reference**
EXPERT	Syngenta Seeds (Belgium)	Unknown	Unknown	2	LCG Flanders 2011
HOMEROS	Limagrain (Belgium)	Fairly sensitive	Unknown	1,2	LCG Flanders 2011
OZON	Unknown	Fairly sensitive	Unknown	2	LCG Flanders 2011
RAZZANO	RAGT (France)	Fairly sensitive	Unknown	2	LCG Flanders 2011
SAHARA	Aveve (Belgium)	Resistant	Unknown	1,2	LCG Flanders 2011
SCOR	Unisigma (Belgium)	Fairly sensitive	Unknown	2	LCG Flanders 2011
TABASCO	Von Borries (Germany)	Fairly resistant	Unknown	2	LCG Flanders 2011
VISCOUNT	CPBT Wyford (Belgium)	Unknown	Unknown	2	LCG Flanders 2011
LEXUS	Limagrain (Belgium)	Sensitive	Unknown	1	LCG Flanders 2011
LIMES	Unknown	Fairly resistant	Unknown	1	LCG Flanders 2011
CONTENDER	Limagrain (Belgium)	Fairly resistant	Unknown	1	LCG Flanders 2011

Table 5.2 shows which cultivars were used in each of the experiments in this study. Cultivars were chosen for the artificial inoculation with attention to an even distribution between the resistance categories.

Table 5.2 Representation of the commercial and reference wheat cultivars used in each of the separate experiments

***In vivo* infection of ears**	**Field trial**
HOMEROS	HOMEROS
SAHARA	SAHARA
LIMES	EXPERT
CONTENDER	OZON
LEXUS	RAZZANO
	SCOR
	TABASCO
	VISCOUNT

5.2.2 *In vivo* ear infection of commercial wheat varieties under laboratory conditions

Five commercial wheat cultivars specified in **Table 5.2** were used. *F. graminearum* macroconidia were obtained, and harvested as previously described. A conidial suspension of 10^6 conidia mL^{-1} was prepared. Ten ears of the wheat plants at flowering stage (Zadok's stage 60) (Zadoks *et al.*, 1974) were infected with 2 droplets of 20 µL of conidial suspension. The wheat plants were placed in a growth chamber at 22 °C under a relative humidity of 100% for 2 days to guarantee the conidial germination and penetration. After 2 days, the plants were incubated for 12 days in a growth chamber at 22 °C under a light regime of 16h light/8h dark. Fourteen days after inoculation, the infection was assessed based on the surface of the ear covered with *Fusarium* symptoms and the disease index (DI) was subsequently calculated as outlined by Landschoot *et al.* (2012). The experiment was repeated twice. After the completion of the symptom's assessment, ears were detached and dissected into two parts, namely grains and chaff. These were analyzed by the HPLC-MS/MS method as described in *Chapter 2*. The ratio of DON, 3-ADON, 15-ADON and DON-3G in both the grains and chaff was determined.

5.2.3 Field experiments to assess FHB resistance and DON-3G presence in commercial wheat varieties

In order to extrapolate the laboratory results to the field, a large-scale field experiment was conceived and executed over two consecutive years. Eight commercial wheat cultivars as stated in **Table 5.1** were incorporated. In these field trials, no artificial inoculation was performed. The experiment was carried out in Bottelare, Belgium (sandy loam ground) in growing seasons 2010-2011 and 2011-2012 with sowing on November 3rd 2010 and October 21st 2011, respectively. The experimental design consisted of a completely randomized block design with four replications. The elementary plot size was 15 m^2. Normal crop husbandry measures were taken including three nitrogen fertilizations according to the N-index established by the Pedological Services Belgium, and pre- and post-emergence herbicide applications. Two fungicide treatments were executed at growth stage GS39 (the last but one leaf has fully emerged) and GS55-GS59 (ear has been formed) (Zadoks *et al.*, 1974). Assessment of FHB resistance was performed in June 2011 and June 2012. In each elementary plot, 100 ears originating from distinct plants were randomly sampled at GS 69 and scored using an ordinal scoring system based on the surface of the ear covered with *Fusarium spp.* symptoms. The DI was again calculated according to Landschoot *et al.* (2012).

Fields were harvested on August 1st 2011 and August 11th 2012, with grains from every elementary plot being kept separate. Analysis for DON and DON-3G was performed on representative samples originating from every elementary plot as described further.

5.2.4 Determination of the FHB population composition in the field

In order to obtain a representative image of the FHB population at each location, two symptomatic ears from each variety and each parallel were harvested during the disease assessment period in June. The FHB population composition on these samples was carried out as described in Audenaert *et al.* (2009).

5.2.5 LC-MS/MS methodology for the quantification of DON, 3-ADON, 15-ADON and DON-3G

An LC-MS/MS method was optimized for the determination and quantification of the parent DON, its acetylated derivatives 3-ADON and 15-ADON, and its glucosylated form DON-3G (2.2). The grain kernels, orginating from both the *in vivo* infection experiment and the field trials, were ground applying the M20-grinder (Ika Werke, Staufen, Germany), while the chaff from the *in vivo* infection experiment was treated with liquid nitrogen to pulverize these into dusty particles using the Moulinette 320-grinder (Moulinex, Barcelona, Spain). The extraction was performed in duplicate for each treatment. The extraction procedure and the actual analysis are detailed in paragraph 2.2.

DON-3G/TOTAL DON ratios represent the relative amount of DON that was glucosylated to DON-3G (*i.e.* glucosylation capacity).

5.2.6 Statistical analysis

Data processing and calculations were performed using IBM SPSS 19 (Armonk, NY, USA). The correlation made between the disease index and the DON-3G/TOTAL DON ratio in the artificial inoculation experiment, was investigated using the coefficient of determination (R^2), which indicates the degree of approximation of the real data points to the regression line in a power-curve. The correlation between the DI and the DON deposition in the same experiment was made using the Pearson correlation coefficient (r).

5.3 RESULTS

5.3.1 Presence and localization of DON and DON-3G after artificial *in vivo* infection of wheat ears

Figure 5.1 shows the levels of DON and DON-3G that were found in the grains. **Table 5.3** shows the results for the artificial ear inoculation. Clearly, artificial infection under optimal laboratory conditions leads to elevated DON levels. It was possible to attribute an important role to the level of DON production by *F. graminearum* in the consequent disease severity (**Figure 5.2 a**, Pearson correlation coefficient, r = 0.831). DON deposition in the commercial wheat cultivars was met with rather efficient glucosylation. **Figure 5.1** states that the wheat grains were able to glucosylate between 8% and 30% of the DON which is deposited by the advancing *F. graminearum* strain.

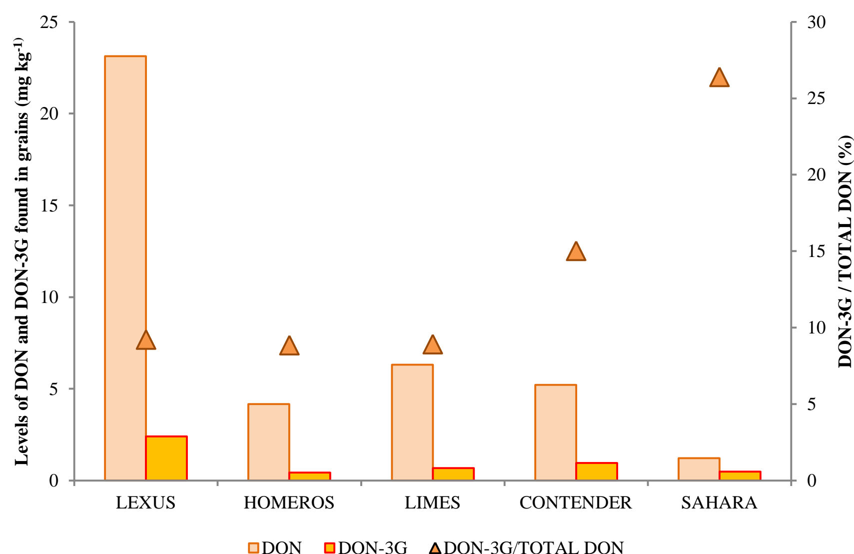


Figure 5.1 *In vivo* DON and DON-3G concentrations (bars) in the grains of five commercial wheat cultivars, after artificial inoculation of the ears with *F. graminearum*. Concentrations of DON and DON-3G were determined after LC-MS/MS analysis and are presented as the means of 3 pooled batches, originating from 10 infected ears per cultivar. Discrete data points represent the glucosylation capacity, calculated as the relative amount of DON that was glucosylated to DON-3G.

Interestingly, the correlations between the DON content and DI, and DON to DON-3G glucosylation and DI, are only valid for the grains of the inoculated ears, and not the chaff. This clearly indicates that while the fungus ultimately targets the entire wheat ear, the plant's physiological defense centers at the developing grain.

Table 5.3 Quantitative results of the LC-MS/MS analysis of the grains of artificially inoculated commercial wheat cultivars

WHEAT GRAINS	LEXUS	HOMEROS	LIMES	CONTENDER	SAHARA
DON (µg kg^{-1})	23,132	4,174	6,317	5,217	1,219
3-ADON (µg kg^{-1})	139	191	313	117	66
15-ADON (µg kg^{-1})	361	183	285	110	70
DON-3G (µg kg^{-1})	2,401	442	678	963	486
TOTAL DON (µg kg^{-1})	26,034	4,989	7,593	6,407	1,840
DON-3G/TOTAL DON (%)	9	9	9	15	26
3-ADON/TOTAL DON (%)	1	4	4	2	4
15-ADON/TOTAL DON (%)	1	4	4	2	4
ADON/TOTAL DON (%)	2	7	8	4	7

WHEAT CHAFF	LEXUS	HOMEROS	LIMES	CONTENDER	SAHARA
DON (µg kg^{-1})	3,112	7,992	6,957	8,515	2,966
3-ADON (µg kg^{-1})	866	759	865	1,432	308
15-ADON (µg kg^{-1})	809	675	777	1,242	296
DON-3G (µg kg^{-1})	905	811	1,070	2,338	1,278
TOTAL DON (µg kg^{-1})	5,693	10,238	9,669	13,527	4,848
DON-3G/TOTAL DON (%)	16	8	11	17	26
3-ADON/TOTAL DON (%)	15	7	9	11	6
15-ADON/TOTAL DON (%)	14	7	8	9	6
ADON/TOTAL DON (%)	29	14	17	20	12

Importantly, the share of DON that has been glucosylated to DON-3G in the grains of the wheat plant is highly correlated with the disease index (**Figure 5.2 b**). **Figure 5.1** and **Table 5.3** also clearly illustrate that cultivars with higher resistance elicit least DON from the fungus.

For all cultivars except Lexus, slightly more DON was deposited in the chaff than in the grains. In Lexus, the most susceptible variety, drastically more DON was deposited in the grain up to an alarmingly high value of 23,000 µg kg^{-1} (**Table 5.3**). The deposition of ADON was however more unambiguous. A consistently higher amount, and simultaneously a higher share of the total DON load, of both 3-ADON and 15-ADON was detected in the chaff. Glucosylation rates were similar in the grains and the chaff for most cultivars.

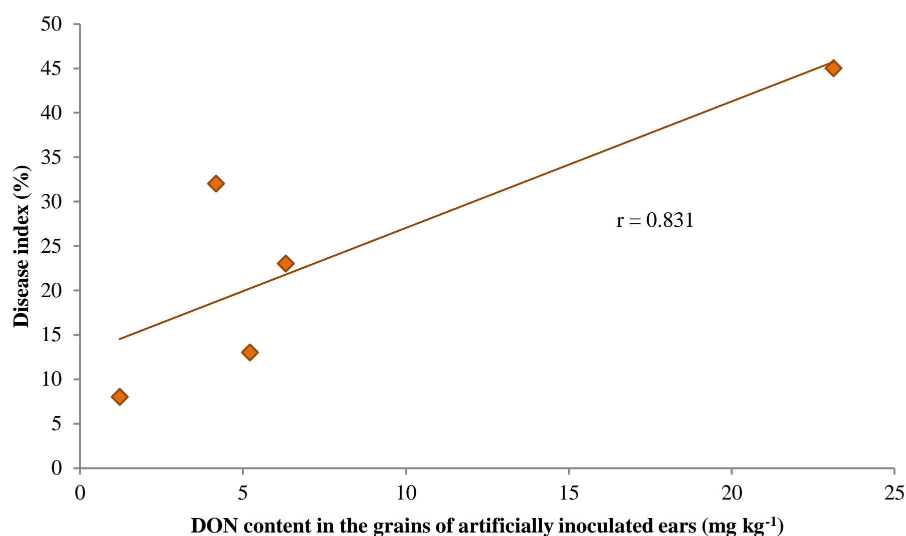


Figure 5.2 a Relation between DON levels in wheat grains of commercial cultivars and disease index, under *in vivo* laboratory conditions (Pearson correlation coefficient, r = 0.831). The DON content was determined by LC-MS/MS analysis of the grains after artificial inoculation with *F. graminearum*.

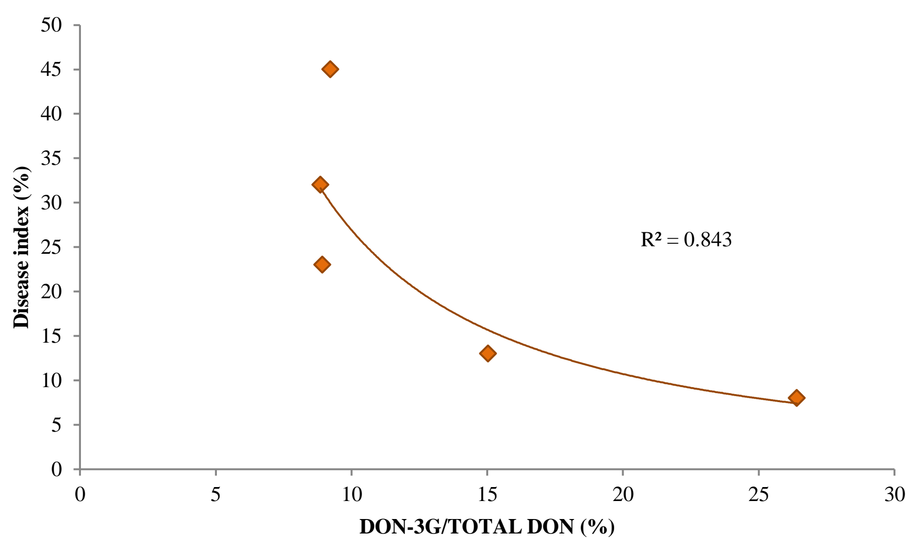


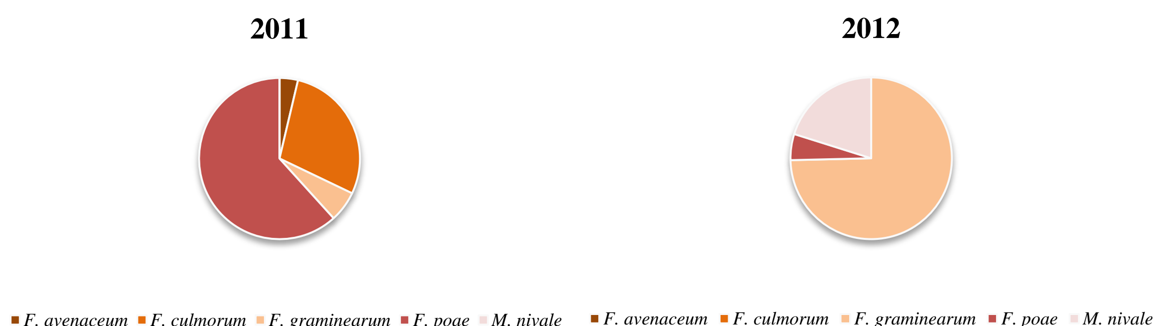
Figure 5.2 b Relation between DON-3G / TOTAL DON ratio and disease index. DON and DON-3G were determined by LC-MS/MS analysis of the complete grains of five commercial wheat varieties, after artificial *in vivo* inoculation with *F. graminearum*.

5.3.2 Field experiments with commercial wheat varieties in the 2010-2011 growing season

A field trial was set up in Bottelare (Belgium) to assess the importance of glucosylation under natural circumstances. Sampling of the *Fusarium* population showed that only 5% of the symptomatic ears contained *F. graminearum* in 2011, while this was the case for all ears in 2012 (**Figure 5.3**). **Figure 5.4** shows the relevant results of the field trial. Clearly, DON levels were considerably lower in natural circumstances when compared to the levels after *in vivo* artificial infection of wheat ears. However, in 2012, remarkably higher depositions of DON could be noted for all cultivars tested when compared to 2011.

Despite rather large standard deviations, it can also be concluded from **Figure 5.4** that several commercial wheat varieties manage to glucosylate a relatively large amount of DON to DON-3G in the field. The range of percentages seems to be relatively well in accordance with the glucosylation capacities that were found in the *in vivo* ear infection experiments, as in 2011 from 18% to 47% could be glucosylated, with Sahara being the exception. In 2012, glucosylation capacities from 8% to 25% were observed.

Figure 5.3 Incidence of 5 *Fusarium* species in Bottelare (Belgium), presented as relative contribution to the other species



It was not possible for either of the two years to correlate the DON content in the grains with disease severity in the field trial. The glucosylation capacity could also not be correlated with the DI as observed in **Figure 5.5**, while once again this was established in the previous experiments under controlled conditions.

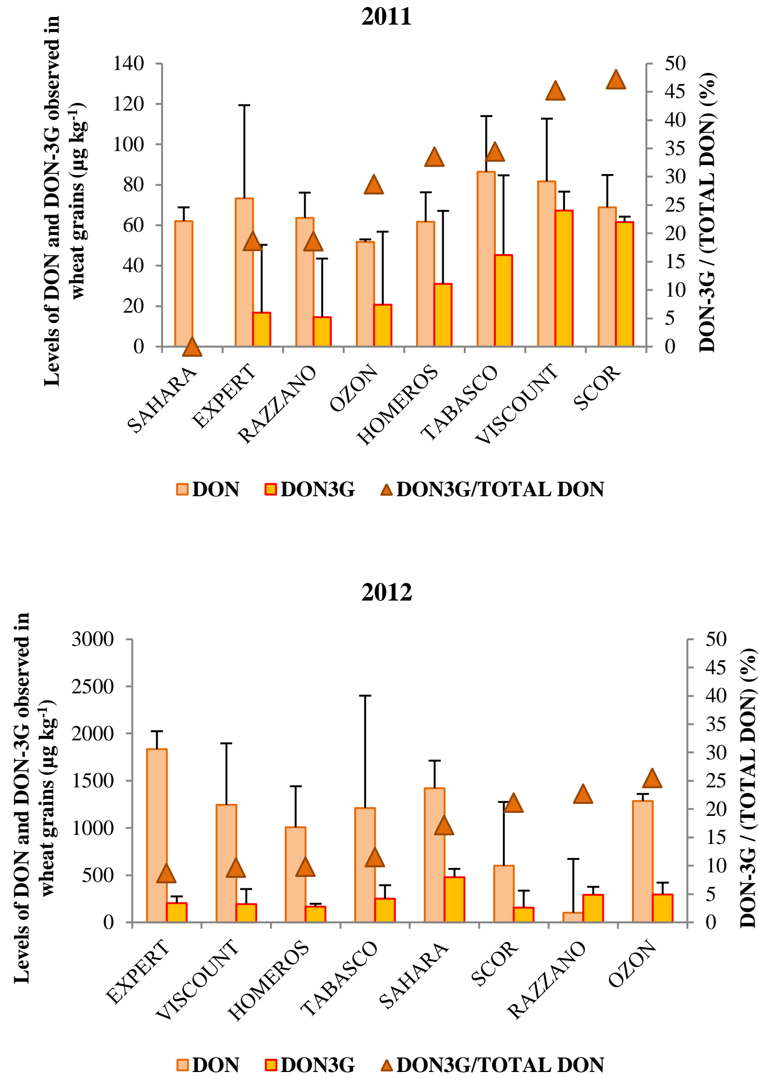


Figure 5.4 ***In planta*** **DON and DON-3G concentrations (bars) in 8 commercial wheat cultivars in the field, under natural conditions in 2011 and 2012. Concentrations were determined after LC-MS/MS analysis of the grains. Each concentration was calculated as the mean of four replications, with standard errors presented. Discrete data points represent glucosylation capacity, calculated as the relative amount of DON that was glucosylated to DON-3G.**

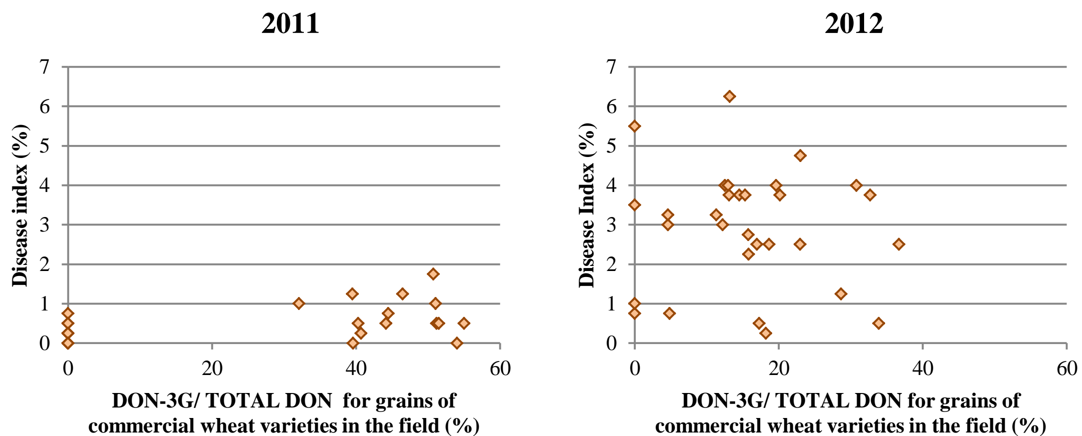


Figure 5.5 Relation between DON-3G/TOTAL DON ratio, and disease index for 8 commercial wheat cultivars in the field. Field trials were executed under normal infection. Four replications per cultivar were used, and all 32 data points are plotted on the graph. DON and DON-3G levels were determined after LC-MS/MS analysis of the grains.

5.4 DISCUSSION AND CONCLUSION

According to Lemmens *et al.* (2008) *Fhb1* is one of the most prevalent resistance sources in resistance breeding against *Fusarium*, and has always been associated with highly efficient resistance against disease spreading in the wheat ears, the so-called type II resistance. This gene has also been shown to be highly important for the detoxification of DON (Lemmens *et al.*, 2005), consequently, it is possible that breeding practices over the last decades have inadvertently incorporated a capacity for glucosylation in modern wheat varieties. However, commercial wheat cultivars have never been screened for this characteristic on a large scale, nor is its importance in disease containment known in the Belgian setting. For the purpose of this study, glucosylation is regarded as the share of DON-3G in the sum of DON-equivalents (*in casu* DON and DON-3G), while other possible metabolites of DON such as DON-glutathione were ignored (Gardiner *et al.*, 2010).

Assuming that glucosylation is a key component and characteristic of *Fhb1* resistance (Lemmens *et al.*, 2005), this study offers the first proof that several Belgian commercial wheat cultivars do possess this resistance to some extent (**Figures 5.1** and **5.4**). This approach leans heavily on the presumption of glucosylation as a symptom of *Fhb1* resistance. The *Fhb1* QTL is the only gene to which DON glucosylation and FHB resistance have been linked, however, the strong link between the two, found in the *in vivo* ear infection trial, would therefore seem to indicate an *Fhb1* presence in the commercial cultivars.

The correlation visible in **Figure 5.2 b** is reminiscent of the relation described by Lemmens *et al*. (2005). These authors compared near-isogenic lines either containing or lacking the *Fhb1* QTL. They found that the lines containing the QTL were associated with high glucosylation and low DI, as with cultivars Sahara and Contender in our study, while the lines lacking the QTL resulted in low glucosylation and high DI, as with cultivars Homeros, Limes and Lexus. A genetic investigation into *Fhb1* presence in the commercial cultivars used here, would be able to determine whether the two similar relations discussed here are in fact based on the same physiological principle.

Analysis of the *in vivo* ear infection data suggests that Sahara glucosylates DON most efficiently. In this context, it needs to be stated that Sahara also elicits least DON production from the infecting *F. graminearum* strain. Therefore, perhaps Sahara is not necessarily most efficient at glucosylation; it might be in fact the cultivar which is simply best equipped to interrupt *F. graminearum* before DON production. The strong correlation between DON

glucosylation and DI, however, does support the hypothesis that efficiency of DON glucosylation plays an important role in commercial cultivars indeed.

The consistently higher values of ADON in the chaff when compared to the grains in the artificial inoculation trials (**Table 5.3**) illustrates nicely the usefulness of sampling 'grain dust' (of which the chaff is a component) for mycotoxin contamination. Indeed, several authors have reported on this technique (Krysinska-Traczyk *et al.*, 2007; Sanders *et al.*, 2013). The astoundingly high value for DON in the grains of the very susceptible cultivar Lexus and not the chaff, however, does question the robustness of this approach.

The low amount of DON that was detected in Sahara in the *in vivo* ear infection could not be reproduced in the field trials. In fact, the second to most amount of DON was found in Sahara in 2012. Interpreting the glucosylation capacities of this highly resistant cultivar is troublesome as well, with no DON-3G being found in 2011, and most of all cultivars in 2012. Interestingly, De Boevre *et al.* (2013b) found that in the setting of a maize field trial (3.3.3), there was a significant spatial distribution of DON and DON-3G levels, assigning a large importance to inoculum-hotspots in the field.

Therefore, care should always be taken when interpreting results from field trials under natural circumstances. Indeed, several authors have reported on the importance of inoculum hotspots and local topography in the field, and the accompanying variability in the spatial distribution of DON and, consequently, DON-3G (Xu *et al.*, 2008).

During the field trial, only the harvested grains were tested for DON and DON-3G content. A remarkable difference between the two growing seasons could be observed. In 2012, tremendously higher DON levels than in 2011 were found. In fact, in all cultivars except Scor, there was at least one replication where the maximum allowed DON concentration of 1,250 μg kg^{-1} (1881/2006/EC, 2006) was exceeded. The maximum DON level of 2,866 μg kg^{-1} and the maximum DON-3G level of 477 μg kg^{-1} were still well below the maximum levels found by Berthiller *et al.* (2009b) in Austrian, Germanian and Slovakian wheat samples.

Interestingly, many authors have reported a strong correlation between the incidence of *F. graminearum* and DON (Waalwijk *et al.*, 2004). While a quantitative approach, where concentrations of fungal DNA are measured *in planta* and correlated with DON levels, is preferable for set-ups like these, it is of importance to remark on the ubiquitous presence of *F. graminearum* in the population, and the highly elevated DON levels in 2012, when compared to 2011.

The increase in DON over the 2 years was met with a somewhat less than equal increase in DON-3G, resulting in overall lower glucosylation capacities for the second year. It is noteworthy that these percentages better approximate the range of values found in the *in vivo* ear trials trials, while it should however also be kept in mind that only two cultivars were used in both set-ups. The high glucosylation capacities that were observed in 2011 are likely to be exceptional, merely possible by merit of the extremely low DON production in that year. The shift to a more *F. graminearum*-dominated population in 2012, associated with higher DON production, exposed the more realistic values of DON-3G glucosylation that were also observed in the *in vivo* ear infection trial.

It is noteworthy that similar glucosylation capacities for the *in vivo* ear infection trial and the second year of the field trials were observed, considering there is a remarkable difference in DON levels between the two. For Homeros the glucosylation capacity was 10% in both the *in vivo*- and the field trial (2012). It could be inferred that the up-regulation of the UDP-glucosyltransferases responsible for the detoxification of DON to DON-3G is directly proportional to and triggered by the concentration of deposited DON. Interestingly, in an *in vitro* test developed by our research group to quickly assess the glucosylation capacity of wheat cultivars using their detached leaves, DON-3G could only be detected at high DON levels, potentially confirming DON's role as a proportional trigger (*Chapter 6*).

DON levels in the field were logically lower than those encountered during the experiments in the laboratory. As is evident from **Figure 5.5**, the high correlation between DI and glucosylation that was observed under laboratory conditions, did not hold up in the field for either of the two evaluated years. In fact, it was shown by Landschoot *et al*. (2012) that in Belgian winter wheat, over a period of 10 years, it was not possible to determine a clear correlation between disease severity and DON incidence. These authors argued that DON content is a function of many more factors than just visible disease severity.

Therefore, the lack of a correlation between DI and DON-3G is a logical consequence. Still, it is interesting to note that when both years are compared as a whole, rather than comparing all cultivars within each year, the higher DON content is associated with a dramatically higher DI in 2012.

Finally, it must be remembered that up to 17 species of *Fusarium* can infect wheat, each producing their own specific blend of toxins. Efficacy of *Fhb1* against toxins other than DON and the closely related NIV has not yet been described, while potential type A trichothecene

producers such as *F. poae* are in some years quite important within the *Fusarium* population (**Figure 5.3**).

However, it is clear that in *F. graminearum*-dominated populations, large amounts of DON can be glucosylated to DON-3G in commercial wheat cultivars, which in a controlled environment was confirmed to have a profound effect on *Fusarium* disease limitation.

CHAPTER 6

DETACHED LEAF ASSAY FOR MASKED MYCOTOXIN BIOSYNTHESIS AND SUBSEQUENT ANALYSIS OF (UNKNOWN) CONJUGATES BY (UN)TARGETED LC-MS/MS

Redrafted after:

De Boevre, M., Vanheule, A., Audenaert, K., Bekaert, B., Diana Di Mavungu, J., Werbrouck, S., Haesaert, G. and De Saeger, S.

Detached leaf assay for masked mycotoxin biosynthesis and subsequent analysis of (unknown) conjugates by (un)targeted LC-MS/MS.

Journal of Agricultural and Food Chemistry (submitted, 2013)

CHAPTER 6 DETACHED LEAF ASSAY FOR MASKED MYCOTOXIN BIOSYNTHESIS AND SUBSEQUENT ANALYSIS OF (UNKNOWN) CONJUGATES BY (UN)TARGETED LC-MS/MS

SUMMARY

The analytical scientist is faced with an ever-expanding spectrum of mycotoxins and derivatives. In *Chapter 1* the *need for identification of other masked forms via untargeted screening of samples* was highlighted. To answer this emerging demand, *Chapter 6* details the development of an *in vitro* model plant system using detached leaves. This *in vitro* detached leaf assay provided biosynthesis of other masked mycotoxins, and was firstly applied to deoxynivalenol and zearalenone with satisfying results.

The biosynthesis of deoxynivalenol-3-glucoside, zearalenone-14-glucoside, α-zearalenol-14-glucoside, β-zearalenol-14-glucoside and zearalenone-14-sulfate was confirmed using their respective reference standards. Mono- and tri-glucoside derivatives of T-2 toxin and HT-2 toxin, T-2-3-glucoside, T-2-3-tri-glucoside and HT-2-3-glucoside were identified and characterized using Orbitrap™ high-resolution mass spectrometry. This is the first report on the biosynthesis of a tri-glucoside of T-2 toxin. The technology allowed obtaining molecular structure details by measuring exact masses of main characteristic fragments. Using the application of collision-induced dissociation, fragmentation patterns proved glucosylation at C_3 position.

The discovery of new masked forms implies the importance of the development of analytical methods for their detection, the constitution of toxicity studies, and proving the relevance of presence in the food and feed chain.

Key words: untargeted analysis, high resolution mass spectrometry, Orbitrap™, masked mycotoxins, detached leaf

6.1 INTRODUCTION

In recent years, scientific awareness on masked mycotoxins in food, feed and unprocessed samples has increased. Focus has traditionally been directed towards the glucosylated conjugate of the trichothecene mycotoxin deoxynivalenol (DON), namely deoxynivalenol-3-glucoside (DON-3G). DON is abundantly produced by *Fusarium graminearum* as a pathogenicity factor produced during infection of wheat or other hosts. Plants have evolved a battery of sophisticated mechanisms to circumvent the phytotoxic effects of DON. These mechanisms comprise of chemical modification such as glucosylation followed by compartmentation of the DON-3G mycotoxin in the vacuoles (Berthiller *et al.*, 2009b; De Boevre *et al.*, 2012a). Also, the unavailability of reference standards for masked conjugated mycotoxins and the possible occurrence of unknown masked forms currently constitute two major complications in routine analytical methods. The detection of these unknowns and inclusion in surveys are of crucial importance. However, due to the complexity of various chemical structures of mycotoxins, this matter remains a challenge (Berthiller *et al.*, 2012).

To date, the occurrence of DON-3G has been extensively described (3.3.1, 3.3.2 and 3.3.3). The identification of ZEN-glucoside conjugates in naturally contaminated wheat (Berthiller *et al.*, 2006) revealed that the conjugation mechanism is not limited to trichothecenes. Recently, some research groups detected more unknown conjugates. Nakagawa *et al.* (2011) proved the presence of glucosides of fusarenon-X (FUS-X) and nivalenol (NIV) in wheat grain, and the same authors observed a mono-glucoside derivative of 15-monoacetoxyscirpenol (MAS) (Nakagawa *et al.*, 2013). However, the main focus has been on T-2 and HT-2 mono-glucosides (T-2-3G and HT-2-3G), and their presence has already been proven in corn powder, wheat, oats, and barley, as well as cultures of *F. sporotrichioides* (Busman *et al.*, 2011; Lattanzio *et al.*, 2012b; Nakagawa *et al.*, 2012; Veprikova *et al.*, 2012). Nakagawa *et al.* (2013) revealed the existence of di-glucosylated derivatives of T-2 and HT-2 in corn powder. These masked mycotoxins were identified as T-2-di-glucoside (T-2-GG) and HT-2-di-glucoside (HT-2-GG). However, the absolute structure of T-2-GG was not clarified, and when considering the structure of T-2 two glucose molecules were suggested to be conjugated at the C_3-OH position in tandem. The structure specification seems to be more complicated in the case of HT-2-GG, since HT-2 has two possible glucosylation positions (C_3 and C_4) (**Figure 6.1**) (Nakagawa *et al.*, 2013).

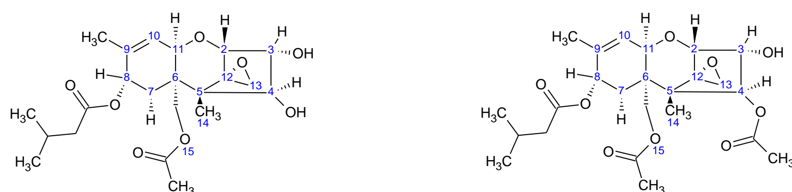


Figure 6.1 Structures of HT-2-toxin (left) and T-2-toxin (right)

Several strategies for masked mycotoxins synthesis have been described. Chemical synthesis was delineated for conjugates of ZEN using the Koenigs-Knorr reaction (Zill *et al.*, 1990; Grabley *et al.*, 1992). Also, DON-3G and deoxynivalenol-15-glucoside (DON-15G) were synthesized according to a modified protocol in a two-step reaction from 1-β-bromo-1-deoxy-2,3,4,6-tetra-*O*-acetyl-α-glucose and the acetylated forms of DON (Savard, 1991). The execution of chemical synthesis offers a high yield, which however, can be laborious and expensive. Another approach was a fermentation procedure for the production of zearalenone-14-glucoside (ZEN-14G), using an engineered *Saccharomyces cerevisiae* strain, expressing the *Arabidopsis thaliana* UDP-glucosyltransferase UGT73C6 (Berthiller *et al.*, 2009c). In addition, the same results were observed for *Rhizopus spp.* (Kamimura, 1986). Recently, yeasts of the *Trichomonascus clade* were used for the production of T-2 glucosides (Mc Cormick *et al.*, 2012).

Furthermore, the feeding of pure toxin to model systems is promising as well. For example, Mc Cormick *et al.* (2012) selected species of the *Trichomonascus clade* yeast as potential candidates for trichothecene modification because these were earlier found to metabolize compounds such as adenine, xanthine, glycine, uric acid, putrescine, and branched-chain aliphatic compounds (Middelhoven and Kurtzman, 2003). These researchers found that three *Blastobotrys* species converted T-2 into T-2-3G (Mc Cormick *et al.*, 2012).

In recent years, new methods for production of glucosylated mycotoxins have been developed in close association with molecular plant pathology studies. The glucosylation of DON to DON-3G constitutes a major resistance trait for the wheat plant (Lemmens *et al.*, 2005), a great deal of research has been dedicated to elucidating which genes are responsible for this transformation. These genes are of considerable importance to breeders, but their potential as sources for DON-3G and other glucosides for the analytical community should not be underestimated. Poppenberger *et al.* (2003) described the *Arabidopsis* gene AtUGT73C5, which effectively provides resistance against DON in yeast, but has undesired phenotypic side effects in mature plants under over-expression. Schweiger *et al.* (2010) identified a barley glucosyltransferase (HvUGT13248) conferring resistance against DON, which was then

cloned into *Arabidopsis* and was shown to be capable of glucosylating significant amounts of DON in this model system (Shin *et al.*, 2012).

Another natural approach is the treatment of cereals with mycotoxins or *Fusarium* strains. Treatment of cereals with DON or T-2, or alternatively spray-inoculation with either *F. graminearum* or *F. sporotrichioides* at anthesis was described by Buerstmayr *et al.* (2004) (Berthiller *et al.*, 2005; Busman *et al.*, 2011). However, the glucoside extraction procedure from naturally or artificially contaminated grain samples is quite troublesome.

Mc Cormick *et al.* (2011) proposed possible trichothecene biosynthetic pathways illustrating the divergence into type A-B and type D (**Figure 6.2**). Type A and B trichothecenes are synthesized on the basis of isotrichotriol, where several subgroups are formed according to three different pathways. The apparent similarity in structures between these groups of mycotoxins suggests that both types are able to be converted into analogous conjugates (**Figure 6.2**, blue and green box). To date, most widely investigated masked forms of trichothecenes are their glucoside derivatives. Berthiller *et al*. (2006) characterized DON-3G in wheat, and in accordance to this conjugate, theoretical reaction mechanisms for conjugates of T-2 and HT-2 were proposed. Due to the presence of OH-functions, many conjugation products can be formed. An *in vivo* trial in rats described unknown T-2 metabolites via hydrolysis at C_8 and C_{15} (Wu *et al.*, 2010). Also, acetylation on C_3 seems to be plausible, even conjugation with glucose. The existing acetyl functions on T-2 and HT-2 were reported as targets of biotransformation. Recently, evidence has been reported that glutathione adducts constitute yet another possible detoxification mechanism in plants (Gardiner *et al.*, 2010).

Current multi-mycotoxin methods are based on the separation and detection either by analysis of preselected compounds with triple quadrupole MS or by the untargeted detection in full scan mode with high resolution mass spectrometry (HRMS). With the latter method, the preselection of targeted compounds is no prerequisite and compound detection depends on the predefined full scan range. Furthermore, the exploitation of full scan acquisition data to obtain additional information on the complex fungal metabolome has been investigated (Cirlini *et al.*, 2012).

In *Chapter 6* an *in vitro* model system was developed to detect and characterize new conjugates of *Fusarium* mycotoxins in a fast set-up. In this model system, pure toxin was inserted to an *in vitro* set-up of detached leaves of wheat and seedlings of maize. Toxin choice fell to DON, ZEN and T-2 for which legislation is in place. The analysis of possible conjugates after an incubation step, using state-of-the-art Orbitrap^{TM} technology is discussed.

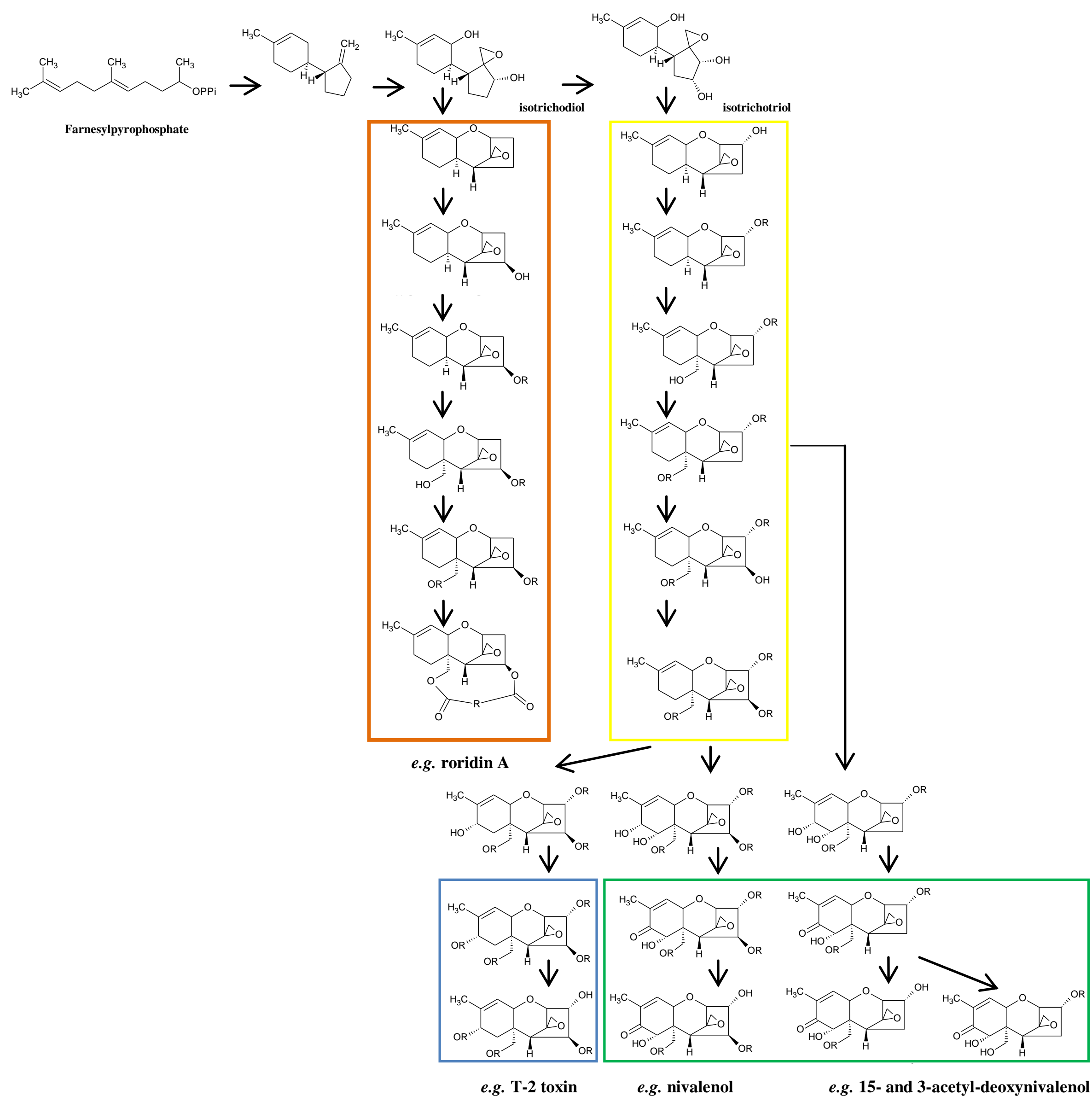


Figure 6.2 Proposed trichothecene biosynthetic pathways illustrating the divergence into the D-type (from isotrichodiol) (orange box) and the A-B-type (from isotrichotriol) (yellow box) trichothecenes. The blue box indicates type A trichothecenes, while the green box indicate type B trichothecenes (Mc Cormick *et al*., 2011).

6.2 MATERIALS AND METHODS

6.2.1 Reagents and chemicals

Methanol (LC-MS grade) was purchased from BioSolve BV (Valkenswaard, The Netherlands), while acetonitrile (Analar Normapur), n-hexane (Hipersolv Chromanorm) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany). Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Potassium hydroxide (KOH) and sucrose were obtained by Sigma Aldrich NV/SA (Bornem, Belgium). Dimethylsulfoxide (DMSO) was supplied from Labconsult (Brussels, Belgium); agar from Duchefa Biochemie (Haarlem, The Netherlands) and Tween 20 was obtained from Sigma Aldrich (Diegem, Belgium). Sodium hypochlorite (NaOCl) was purchased from the local supermarket.

Individual mycotoxin solid standards (25 mg) of DON, ZEN and T-2 (molecular weight 296, 318 and 466 g $mole^{-1}$, respectively) were obtained from Fermentek (Jerusalem, Israel). The mycotoxin solid standards were dissolved in DMSO/0.01N KOH (10/90, v/v), and storable for a minimum of 1 year at -18 °C (Spanjer *et al.*, 2008). Solid standards (1 mg) of deepoxydeoxynivalenol (DOM) and zearalanone (ZAN) were purchased from Sigma Aldrich NV/SA (Bornem, Belgium) and diluted with methanol to 1 mg mL^{-1}.

DON-3G (50.2 ng μL^{-1} in acetonitrile) was the only masked mycotoxin commercially available, and was purchased by Biopure Referenzsubstanzen GmbH (Tulln, Austria). Reference standards of the ZEN conjugates, namely ZEN-14G, α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G) and zearalenone-14-sulfate (ZEN-14S) were kindly provided by Dr. Franz Berthiller (Christian Doppler Laboratory for Mycotoxin Metabolism, Vienna, Austria).

6.2.2 Detached leaf assay

In order to determine the capacity for trichothecene detoxification through glucosylation, an *in vitro* detached leaf assay system was developed. This procedure stems from the notion that one-week old detached leaves in a fully controlled environment are able to metabolize mycotoxins, as adult plants can.

Successful germination of the maize seeds (*Zea mays* “Dominator”, *Advanta*) was acquired through the incubation on an artificial germination medium. Before incubation, seeds were wrapped in filter paper, sterilized during 15 minutes in 0.75% NaOCl and 25 µL L^{-1} Tween

20, and rinsed three times with sterile deionized water. The germination medium was based on 20 mL solid Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} fyto agar. The pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving (121 °C, 15 min). Each seed was germinated in a glass tube (24 mm x 140 mm) during 5 days at 26 ± 2 °C under a 16h photoperiod provided by cool white fluorescent lights (40 µE m^{-2} s^{-1}). When seedlings had a length of approximately 9 cm, a 3 cm stem part was cut and antiseptically transferred, per 4 parts, in a 15 mL falcon tube filled with 10 mL of liquid Murashige and Skoog medium, supplemented with 30 g L^{-1} sucrose in order to maintain a liquid medium (Murashige, 1962) (**Figure 6.3**).

Figure 6.3 Seedlings of *Zea Mays Advanta*

In **Table 6.1** the composition of the Murashige and Skoog medium is pointed out.

Table 6.1 Composition of the Murashige and Skoog medium

MACRO NUTRIENTS	MICRO NUTRIENTS
Ammonia nitrate (NH_4NO_3): 1,650 mg L^{-1}	*i*-Inositol: 100 mg L^{-1}
Boric acid (H_3BO_3): 6.2 mg L^{-1}	Niacin: 0.5 mg L^{-1}
Calcium chloride ($CaCl_2$): 440 mg L^{-1}	Pyridoxine HCl: 0.5 mg L^{-1}
Cobalt chloride ($CoCl_2$): 0.025 mg L^{-1}	Thiamin HCl: 0.1 mg L^{-1}
Magnesium sulfate ($MgSO_4$): 370 mg L^{-1}	IAA protein 1: 30 mg L^{-1}
Copper sulfate ($CuSO_4$): 0.025 mg L^{-1}	Kinetin: 0.04–10 mg L^{-1}
Potassium phosphate (KH_2PO_4): 170 mg L^{-1}	Glycine (recrystallized): 2.0 g L^{-1}
Iron sulfate ($FeSO_4$): 27.8 mg L^{-1}	Edamin S: 1.0 g L^{-1}
Potassium nitrate (KNO_3): 1,900 mg L^{-1}	Sucrose: 20 g L^{-1}
Manganese sulfate ($MnSO_4$): 22.3 mg L^{-1}	Agar†: 10 g L^{-1}
Potassium iodide (KI): 0.83 mg L^{-1}	
Sodium molybdate (Na_2MoO_4): 25 mg L^{-1}	
Zinc sulfate ($ZnSO_4$): 8.6 mg L^{-1}	
$Na_2EDTA.2H_2O$: 37.2 mg L^{-1}	

† solid medium: 10 g L^{-1}; liquid medium: 1 g L^{-1}

At this point, the parent mycotoxins DON, ZEN or T-2 (> 99% purity), diluted in DMSO/0.01N KOH (10/90, v/v) to concentrations of 1 mg L^{-1} and 10 mg L^{-1} were added to the medium and the Falcon tube was placed on a vertically shaker (Innova 4000, Artisan Scientific Corporation, Illinois, USA), at 28 °C under a 16h photoperiod provided by cool

white fluorescent lights (20 µE m^{-2} s^{-1}). After 3 days, detached leaves and the remaining medium were processed.

Detached leaf assays with wheat were set up in a similar manner as with maize. The Chinese cultivar Sumai3 bearing amongst others the QTL Qfhs.ndsu-3BS was utilized, and incubation with parent mycotoxins lasted for 10 days. Mycotoxins DON, ZEN and T-2 were investigated.

6.2.3 Sample preparation

Sample preparation was based on the method described in paragraph 2.2.3, however minor adjustments were introduced. In detail, both stem parts and medium were homogenized with an Ultraturrax® mixer coupled to a disposing tool (Ika® Werke T8.01 S8N-5G, Staufen, Germany) until a green viscous mass was obtained. An extraction with 20 mL acetonitrile/water (84/16, v/v), combined with a hexane defatting (10 mL) was performed using the Agitator decanter overheadshaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 60 minutes for the whole procedure. Defatting was executed mainly to get rid of the chlorophyll.

Subsequently, stem parts and the medium viscous mass were centrifuged at 3,000 g for 15 minutes, afterwards, the supernatant (hexane layer) was removed, while the aqueous layer was filtered through an Ederol-filter paper (12.5 cm, quality 15, VWR, Belgium). The filtrates were evaporated to dryness under a gentle N_2-stream at 40 °C using an evaporator module (Grant Instruments Ltd, Cambridge, United Kingdom), and were redissolved in 100 µL injection solvent, consisting of methanol/water (50/50, v/v) and 10 mM ammonium acetate, adjusted to pH 4 with glacial acetic acid. Finally, the redissolved sample was vigorously vortexed for 3 minutes, collected in an Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) and centrifuged for 10 minutes at 10,000 g. Samples were stored in vials in the dark at 4 °C until analysis. Matrix blanks, to which no parent mycotoxin was added were prepared accordingly.

6.2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions

In paragraph 2.2.2 the method used for the analysis of conjugates of both DON and ZEN has been detailed. LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA). MassLynx™ version 4.1. and QuanLynx® version 4.1. software (Micromass, Manchester, UK) were used for data acquisition and processing.

6.2.5 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) conditions

Concerning unknown T-2 conjugates, LC-HRMS was applied using the LC-Orbitrap^{TM} MS Exactive (ThermoFisher Scientific, San José, USA) according to the method described in 2.2.2. Chromatographic separation was performed applying a ZORBAX Eclipse XDB C18-column (3.5 µm, 100 mm x 4.6 mm) (Agilent Technologies, Diegem, Belgium). The column was kept at room temperature (21.5 °C; range [21 °C-22.4 °C]), while the autosampler was set at 10 °C. A mobile phase consisting 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 to pH 4 with glacial acetic acid was used at a flow rate of 0.2 mL min^{-1}. The gradient elution program started at 50% mobile phase A for 2 min with a linear increase to 100% mobile phase B in 8 min. An isocratic gradient of 100% mobile phase B was initiated at 10 min for 5 min. Before analysis, the mobile phases were filtered through a 0.22 µm PVDF-filter. The duration of each HPLC run was 20 minutes, including reequilibration. The HPLC system was composed of an Accela pump and a Thermo PAL auto injector (ThermoFisher Scientific, San José, USA). The MS parameters include the following settings: ESI source block temperature 150 °C; desolvation temperature 300 °C and capillary voltage 4 kV. For data acquisition Xcalibur® software (Thermo Scientific, San José, USA) was applied.

6.3 RESULTS AND DISCUSSION

The newly-developed method is based on the assumption that detached leaves are able to metabolize mycotoxins. Before testing the method on T-2, the assumption was validated on DON and ZEN. Calibration standards for both DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S were on hand, therefore LC-MS/MS could be used as a confirmatory method with detached leaves inserted with parent mycotoxins DON and ZEN. HRMS was applied then further on for the detection of unknown conjugates of T-2. The application of the maize or wheat detached leaf assay and subsequent LC-MS/MS methodologies per mycotoxin is detailed in the following sections.

6.3.1 Biosynthesis of DON-3G

DON-3G was determined according to the LC-MS/MS method described in 2.2.2, using DOM as the internal standard (100 μg kg^{-1}). The Chinese cultivar Sumai3 was utilized for the glucosylation tests of DON with wheat. This cultivar has previously been described to have a significant resistance against DON and to contain a QTL associated with DON glucosylation, designated as the *Fhb1* QTL (Lemmens *et al.*, 2005). Interestingly, no DON-3G could be detected in the liquid medium or plant extract after application of 1,000 μg kg^{-1} DON, while detection was only possible after feeding up to 10,000 μg kg^{-1} DON.

This role for DON as a proportional trigger for glucosylation is well in accordance with Audenaert *et al.* (2013). These authors investigated glucosylation capacities in both an artificial inoculation experiment and a field trial under natural circumstances. While DON levels were obviously very different between the two set-ups, the relative glucosylation capacities (expressed as share of DON-3G to the sum of DON and DON-3G) were very similar. Also, the same authors found that in the grains of naturally contaminated wheat, levels of approximately 1,000 μg kg^{-1} DON were steadily associated with DON-3G levels of 150 μg kg^{-1} to 300 μg kg^{-1}. Detached leaves are clearly less effective at glucosylating DON than wheat ears (*Chapter 5*).

However, addition of 10,000 μg kg^{-1} DON to the detached wheat leaf assay did lead to detectable DON-3G levels. Unfortunately, biological variation over three repetitions was quite large. Several factors may have influenced this variation, such as difference in weight of the leaf fraction between the repetitions, and discrepancies in maturity of the various leaf parts. Detection of 100 μg kg^{-1}, 200 μg kg^{-1} and 1,000 μg kg^{-1} was registered.

6.3.2 Biosynthesis of masked forms of ZEN

Up to now, reference standards for the masked forms of ZEN, namely ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S are not commercially available. However, amounts of the standards were made available for this study, allowing for the possibility to evaluate the detached leaf assay for ZEN. An experimental design was set up using maize cell suspension cultures. The production of undifferentiated, totipotent cells (*i.e.* callus) was however critical and time-consuming, consequently another procedure was developed with the aid of the maize detached leaf assay.

The optimal incubation time of the parent mycotoxin ZEN in order to obtain a maximal production of the masked forms was verified. Conjugates were quantified by LC-MS/MS using ZAN as an internal standard (100 µg kg^{-1}). After 72h an equilibration phase was observed, which probably indicated a saturation of the cytosolic UDP-(glucosyl)-transferases. Consequently, sample treatment of the maize stem parts was performed after 3 days of incubation (*n*=3) (**Figure 6.4**).

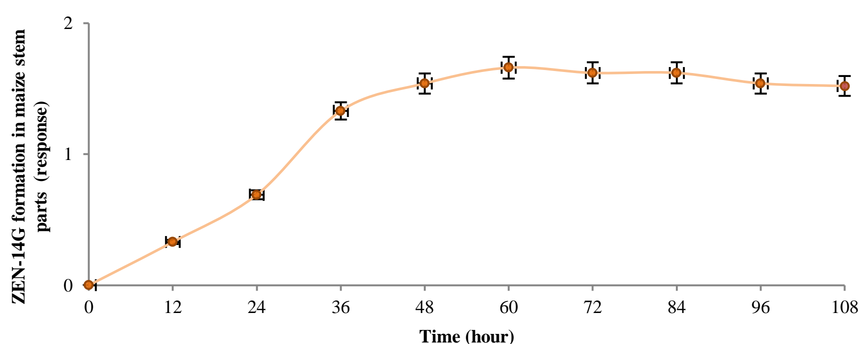


Figure 6.4 Production of ZEN-14G in incubated maize stem parts (*n*=3)

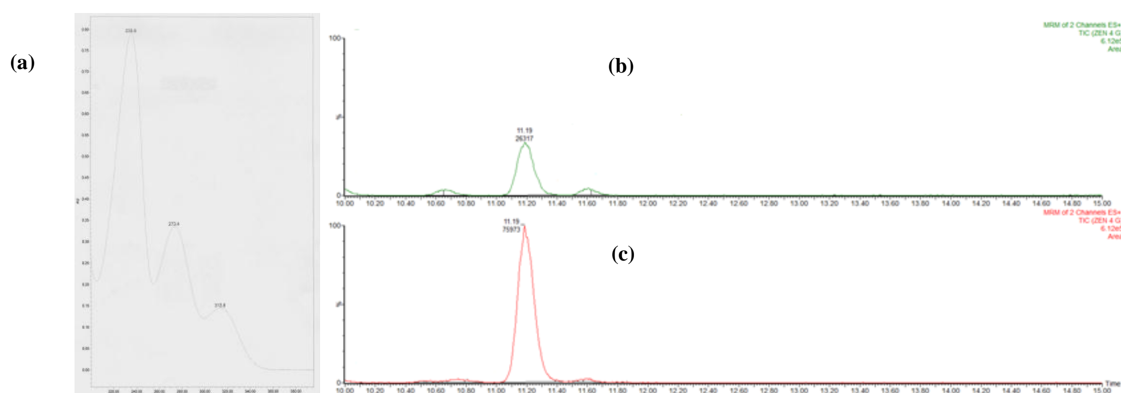


Figure 6.5 PDA spectrum of ZEN-14G at 254 nm (a) and comparison of the chromatogram of ZEN-14G of the obtained reference standard (b) and the detached leaf procedure (c)

Fractions of the masked ZEN conjugates, namely ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S were collected using the Waters Fraction Collector III (Waters, Milford, MA, USA). According to their retention time, the fractions were individually compiled in glass tubes. Firstly, using a photo diode array detector (PDA, Sercolab, Belgium), the masked ZEN forms were identified at 254 nm as shown in **Figure 6.5**. Secondly, identification of the masked forms was accomplished performing a full scan and establishing collision-induced fragmentation patterns. Full scans and fragmentation patterns were compared with those obtained from the masked ZEN-reference standards for ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S. According to the developed LC-MS/MS method, the retention times and total ion chromatograms (TIC) of the obtained masked mycotoxins were compared with those of the reference standards (**Figure 6.5**).

An illustration of a full scan of the ZEN-14S fraction after single fractionation (**Figure 6.6 a**) showed many interferences, in proportion to the target compound, therefore, two additional fractionations were executed (**Figure 6.6 b**). Also, for this reason, nuclear magnetic resonance spectra were obtained for ZEN-14S to confirm the compound's identity and to assure its purity.

α-ZEL-14G and β-ZEL-14G were fragmented into the same fragment ions, however with different abundance. Therefore, co-eluting mycotoxins could be differentiated by applying the ion ratios. For these mycotoxins the ion ratios (*m/z* 483) / (*m/z* 321) and (*m/z* 483) / (*m/z* 283) were determined, resulting in mean values of 0.156 (confidence interval (CI): [0.143, 0.395]) and 0.954 (CI: [0.856, 1.325]), respectively. According to a t-test ($p > 0.05$), means of the compounds described above were evaluated and pointed no significant difference. By applying different quantifier ions in the SRM transitions and bringing into consideration the difference in the ratio of the two product ions, no determination problems occurred.

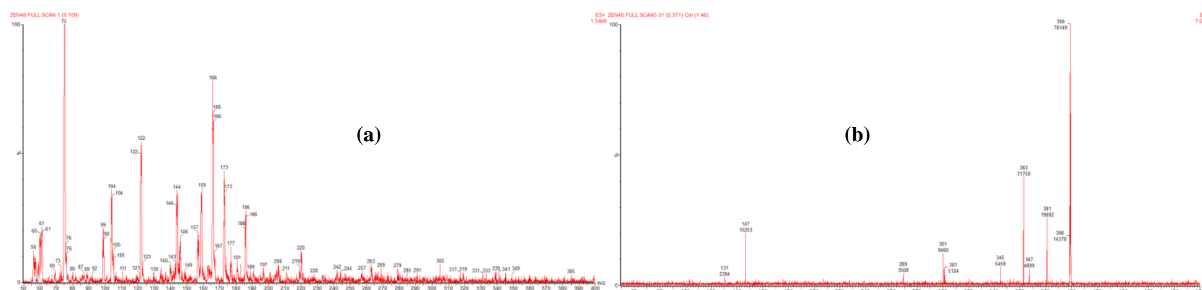


Figure 6.6 Spectra for ZEN-14S after single fractionation (a) and multiple fractionation (b)

Based on the results, a yield of 30% of masked ZEN metabolites could be obtained.

6.3.3 Biosynthesis of masked forms of T-2

The results of DON and ZEN clearly indicated that the model system using detached leaves of wheat and maize was able to produce known conjugates such as DON-3G, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S. It was the author's goal to subject the highly toxic trichothecene T-2 to this validated system.

Prior to the analysis of T-2 spiked detached leaves, the HRMS equipment was calibrated for optimal detection of T-2 conjugates, using two working solutions containing T-2 (10 ng μL^{-1} and 100 ng μL^{-1}). During calibration of the equipment, radio frequency and voltage parameters were adjusted to gain a maximum mass transfer of the C-trap to the Orbitrap™. For an optimal detection and stable electrospray, the T-2 standard was tuned. A spectrum was obtained showing a peak at mass to charge ratio (*m/z*) of 484.25. Possible according molecular masses for 484.25 were pointed out (**Table 6.2**). The exact mass of $C_{24}H_{38}O_9N$ was set taking the $[M+NH_4]^+$ adduct into account. Ideal parameters for T-2 were necessary to be found as conjugates of T-2 were searched. Other parameters like capillary temperature (275 °C), capillary voltage (67.5 V), tube lens voltage (120 V), skimmer voltage (18 V) and heater temperature (200 °C) were optimized. Indeed, after setting these parameters an increase of 62% of the signal was observed.

Table 6.2 Elemental composition search on mass 484.25 (*m/z* 479.25 - 489.25). The bold value indicates the chosen composition of $C_{24}H_{38}O_9N$, namely T-2-toxin

Theoretical mass (*m/z*)	Delta (ppm)	composition
484.25	0.16	$C_{22}H_{36}O_8N_4$
484.25	-1.17	$C_{23}H_{38}O_9N$
484.25	**-1.18**	$\mathbf{C_{24}H_{38}O_9N}$
484.25	1.50	$C_{21}H_{40}O_{12}$
484.25	1.51	$C_{20}H_{34}O_7N_7$
484.26	-2.52	$C_{25}H_{34}O_5N_5$
484.25	2.84	$C_{19}H_{38}O_{11}N_3$
484.25	2.85	$C_{18}H_{32}O_6N_{10}$

The electrospray positive (ESI^+) operation mode was appointed. Scanning in the mass range of *m/z* 80 to 1,000 at a resolving power of 100,000 full width at half maximum (FWHM) was accomplished. A full scan with collision induced dissociation (CID) was performed in different chromatographic runs. The mass spectrometer was capable of generating fragmentation information in a non-selective manner using a collision cell without precursor ion selection. The Orbitrap™ technology provided that ions passed through the C-trap into a multipole collision cell were fragmented. Thereafter, ions were transferred into the C-trap from where they were injected into the Orbitrap™ for detection.

Additional post-data acquisition mathematical tools were applied to perform molecular identification. According to all possible molecular weights (MW) of conjugation products including positive adducts (H^+, NH_4^+, Na^+ and K^+) of both T-2 and HT-2, a database was established to extrapolate data obtained from the inoculated stem parts (100 µg kg^{-1} and 1,000 µg kg^{-1}).

Full scans from *m/z* 80 to *m/z* 1,000 were recorded. The obtained chromatograms were investigated and peak values were determined. Interference peaks were recorded at *m/z* 157 and *m/z* 179. Authors attribute these values to DMSO used as a solvent for T-2. To obtain higher efficiency, the full scan was recorded from *m/z* 200 to *m/z* 1,000. Besides peaks relevant to T-2 and HT-2, three peaks were attributable to the mono-glucoside derivatives of T-2 and HT-2, and the triglucoside of T-2. The measured MW 604.29593 was attributed to the molecular formula of $C_{28}H_{46}O_{13}N$, corresponding to $[\text{HT-2-glucoside} + NH_4]^+$ with a mass accuracy of -0.52002 (sensitivity of 5.77 x E^7). The mass value of 646.30665 suggested to be $[\text{T-2-glucoside} + NH_4]^+$ with a molecular formula of $C_{30}H_{48}O_{14}N$ with a mass accuracy of -0.50991 (sensitivity of 2.82 x E^7). The earlier elution of T-2-GGG (8.99 min) compared with those of T-2-G (9.87 min) and T-2 (10.85 min) suggested that T-2-GGG is more hydrophilic than T-2-G and T-2. The mass value of T-2-GGG (MW 971.41321, $C_{42}H_{68}O_{24}N$) was detected at a reasonable deviation (-1.06 ppm).

Exact mass values of the ions found are pointed out in **Table 6.3**. In accordance with the European Commission guideline, mass deviation <5 ppm from the theoretical value was used as the criterion for compound identification (ISO/IEC 17025, 2011).

Table 6.3 Elemental composition of masses observed for HT-2-3-glucoside, T-2-3-glucoside and T-2-triglucoside

mycotoxin	theoretical mass	delta (ppm)	composition	exact mass	Retention time (min)
T-2 toxin	484.3	-1.18	$C_{24}H_{38}O_9N$	484.25310	10.85
HT-2-3-glucoside	604.3	-0.52	$C_{28}H_{46}O_{13}N$	604.29593	9.00
T-2-3-glucoside	646.3	-0.51	$C_{30}H_{48}O_{14}N$	646.30665	9.87
T-2-triglucoside	971.4	-1.06	$C_{42}H_{68}O_{24}N$	971.41321	8.99

Previous reports described the presence of T-2-GG and HT-2-GG, however ions with MW 808 and 766 could not be extracted. Full scans were scrutinized with the calculated masses of T-2-G and HT-2-G, and clear peaks at 9.87 min and 9.00 min were detected (**Figure 6.7**). The analysis was repeated twice with the recording of full scans from *m/z* 500 to *m/z* 700 and *m/z* 800 to *m/z* 1,000.

Assuming that similar fragmentation occurs for T-2, T-2-G and T-2-GGG, a full scan chromatogram for all observed forms was acquired using fragmentation by the collision cell

(collision energy, 10 eV). The hypothesis of the structures was confirmed by the presence of main fragment characteristics, as detected in the spectrum of the precursor ion. Neutral losses in trichothecenes (*Chapter 1*) are mostly observed. The loss of isovaleric acid, acetic acid and formaldehyde was proven by measuring mass values of 544.2394, 514.2053, 484.2531 (T-2-G); 502.2292, 442.2445 and 412.1974 (HT-2-G), respectively. For T-2-GGG similar fragments were formed with the inclusion of the neutral loss of the glucose fragment, 646.3067. In previous studies, it was assumed that glucosylation of HT-2 mainly occurs at C_3 rather than at C_4, because T-2 consists of only one OH-function at C_3 and was therefore concurrently glucosylated.

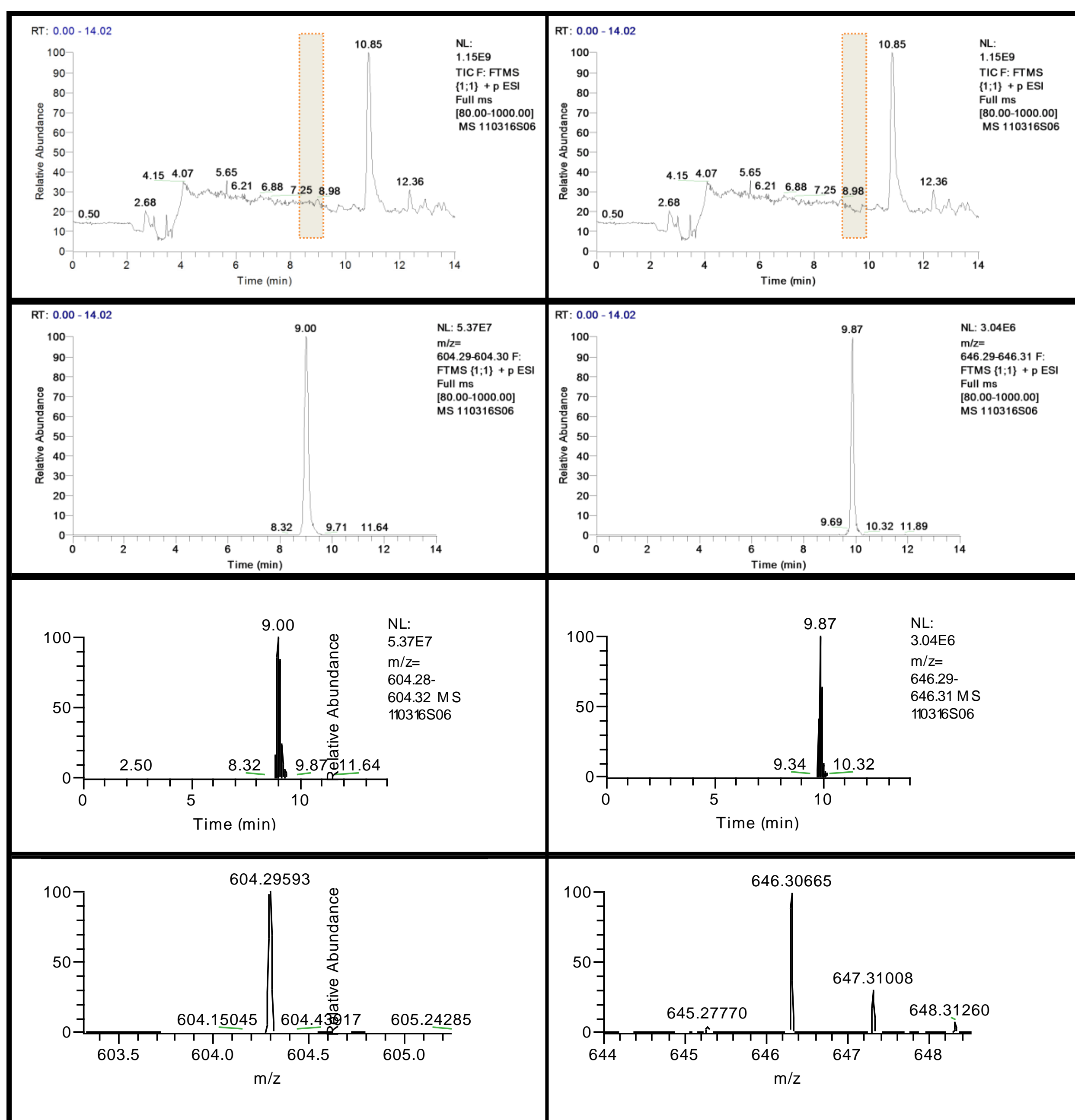


Figure 6.7 Extracted chromatograms of HT-2-3-glucoside (a) and T-2-3-glucoside (b)

A full scan spectrum of the ammonium adduct of T-2-GGG is shown in **Figure 6.8**. Since a mass spectrometer separates and detects ions of slightly different masses, it easily distinguishes different isotopes of a given element. This was also pointed out in the spectra of [T2-GGG + NH_4]$^+$ (MW 971.40) (**Figure 6.8**).

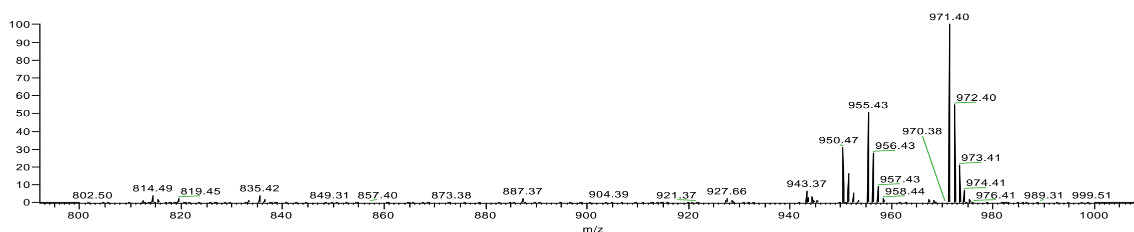


Figure 6.8 Full scan spectrum of T-2-triglucoside

No NMR measurements of the T-2 and HT-2 conjugates were recorded as the available quantity of these forms were too scarce.

The analysis of detached leaves spiked with T-2 allowed for the first time detection of a triglucosylated form of T-2. Further analysis of cereal-based products needs to executed to reveal the true relevance of these conjugated mycotoxins. Previous findings (Lattanzio *et al.*, 2012b; Veprikova *et al.*, 2012; Nakagawa *et al.*, 2013) proved the widespread presence of trichothecene glucosides in cereal grains. The results of this study underscore the importance of the conjugation mechanism *in planta*. Furthermore, the relevance of this newly detected compound, and untargeted screening using model systems, was proven by its detection in a highly contaminated oat field sample (data not shown).

The same detached leaf set-up was applied for both NIV and FUS-X. Glucosides of these mycotoxins were already reported in wheat samples, unfortunately these forms could not yet be confirmed through the detached leaf assay (Nakagawa *et al.*, 2012).

Quantification of the T-2 masked forms was not possible due to the lack of reference standards, however, based on the observed peak areas the formation was estimated to be less than 10% of the parent toxin. According to these estimations, glucosylation rates in the detached leaf assay showed similar results of previous reports of other trichothecene glucosides in wheat, while it must be kept in mind that the detached leaves are only moved to glucosylation at a higher dosage of toxin addition than wheat ears.

6.4 CONCLUSION

Conjugates of DON and ZEN were confirmed to be present in the newly-developed detached leaf assay, designating the system as a reliable tool to synthesize glucosylated conjugates. The existence of mono-glucosylated T-2 and HT-2 and a triglucoside of T-2 was endorsed by means of LC-Orbitrap™ MS.

The newly described triglucoside of T-2 could also be found in this set-up, nicely illustrating the usefulness of the developed assay. The insertion of other parent mycotoxins to the set-up will in the future allow for identification of even more conjugates. This method provides financial and time-efficient advantage, as the untargeted screening can be limited to a few iterations of the developed *in vitro* system. The information on new conjugates gleaned from this HRMS analysis, can then be used in toxicological studies and targeted routine screening of field samples.

CHAPTER 7

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

GENERAL CONCLUSIONS

The research presented focused on the chemical risks related to food and feed containing masked *Fusarium* mycotoxins. The final goal was to gain insight in the overall risks of masked mycotoxins in Belgium.

First of all, there was a need to develop a sensitive and reliable method for the quantification of the mycotoxins of interest. These included deoxynivalenol (DON), zearalenone (ZEN), T-2-toxin (T-2), HT-2-toxin (HT-2), and fungal and plant conjugates thereof, comprising 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), deoxynivalenol-3-glucoside (DON-3G), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN-14G), α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G) and zearalenone-14-sulfate (ZEN-14S) in cereals and cereal-based food and feed.

A liquid chromatography-tandem mass spectrometry method in electrospray positive mode (LC-ESI^+-MS/MS) was successfully developed and validated for the simultaneous determination of 13 mycotoxins, including their masked conjugates, for several matrices such as maize, wheat, oats, breakfast cereals and bread. A clean-up procedure was not included as low recoveries were obtained for both parent and masked forms. An extraction with acetonitrile/water/acetic acid (79/20/1, v/v/v) was performed with an additional hexane defatting step resulting in relatively cleaner extracts, which allowed increasing the sensitivity. All validation parameters in terms of linearity, limit of detection, limit of quantification, precision, apparent recovery and expanded measurement uncertainty were in accordance with Regulation 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC, which establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. The developed LC-MS/MS method was the foundation of the whole research project.

Analysis of highly-consumed food products, animal feed and maize in Belgium clearly revealed the presence of various masked forms. Other international research groups stated similar results, which proved that masked mycotoxins could no longer be neglected. In some samples masked forms even occurred at higher levels than their parent forms.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010) specified that 3-ADON and 15-ADON were typically less than 10% of those reported for DON. According to the values reported in *Chapter 3* the obtained results were not in correspondence with the

statements made by JEFCA. Also, the presented results demonstrated the co-occurrence of DON, ZEN, T-2 and HT-2 with their respective masked forms.

Moreover, a correlation between compound feeds and feed materials was proven concerning contamination with parent mycotoxins and derivatives. Beet pulp, sunflower seed meal, soy bean and soy peel were hardly contaminated. Feed materials mostly infected with DON, 3-ADON, 15-ADON and DON-3G were maize and its by-products (maize germs and maize gluten feed), and wheat. Feed materials which were highly contaminated with ZEN were maize and its by-products, maize germs and maize gluten feed. Also, the glucosylated and sulfated forms occurred in substantial amounts. Wheat and its associated by-products were contaminated with α-ZEL (wheat gluten feed and wheat bran) and ZEN (wheat) as well. In conclusion, the individual mycotoxin load present in maize, wheat, oats, barley and their by-products definitely play a role in the contamination level of the compound feeds.

To date, no studies have investigated the natural occurrence of different mycotoxins and their masked forms in a range of different maize varieties. The results in *Chapter 3* revealed that maize varieties were co-contaminated with different mycotoxins. Significant positive correlations between the occurrence of the parent and the masked forms were detected. Additionally, the amount of mycotoxins found was not only dependent on the maize variety, also clusters with higher mycotoxin levels within the field were observed. These results demonstrated that constant monitoring of the entire spectrum of *Fusarium* mycotoxins in maize is necessary to guarantee food and feed safety. Furthermore, it was shown that also masked mycotoxins, which are not measured by the commonly used commercial screening methods, highly contribute to the overall contamination. Therefore, being able to determine multiple mycotoxins and their masked forms just using one single method constitutes an added value.

Scientific insights in toxicological, analytical, and food chemistry fields have contributed greatly to the current knowledge of chemical hazards in food. The potential adverse health effects that mycotoxins may cause are not only related to their toxicological efects but also to the ingested amount, and this can be evaluated by means of a risk assessment. To our knowledge the results presented in *Chapter 4* are the first enabling the assessment of the exposure of the Belgian population to DON, 3-ADON, 15-ADON, ZEN, α-ZEL, β-ZEL, T-2, HT-2 and their masked forms. In general, the population is expected to be exposed to just moderate levels of mycotoxins, whatever their type. Although the majority of the Belgian population does not exceed the tolerable daily intake (TDI) of 1.0, 0.25 and 0.1 μg kg^{-1} bw

day^{-1} DON, ZEN and the sum of T-2 and HT-2, respectively, there is a large subpopulation, exceeding those safety values. A reduction of (masked) mycotoxin concentrations in cereal-derived foodstuffs is a prerequisite to keep within their safety boundaries. However, complete eradication of mycotoxins is not feasible. In view of ZEN exposure in cereal-based food reported in our study, a call for an overhaul of legislation focusing on ZEN is necessary. Edwards (2011) reported already the risk of ZEN in European wheat; the reported results indubitably confirm his statements.

Concerning feed, the highest concern is attributed to the consumption of pig compound feeds. For all parent mycotoxins analyzed, an excess was noted. In addition, an excess for masked conjugates was noticed at the same time. However, more samples need to be analyzed to confirm these concerning statements. Based on the results of the Belgian risk assessment a systematic monitoring of mycotoxins and their masked forms in food and feed remains necessary to gain more concentration data and also specific toxicokinetic data for the masked forms are needed. According to the obtained results, co-occurrence of parent and masked mycotoxins has to be taken into account in future toxicity studies investigating the impact on animal health and its concomitant extrapolation to human health.

In a next chapter, a field trial was set up to establish the glucosylation capacity in the Belgian wheat pool. Assuming that glucosylation is a key component and characteristic of *Fhb1* resistance, the results in *Chapter 5* offer the first proof that several Belgian commercial wheat cultivars do possess this resistance to some extent. The correlation of glucosylation and DON incidence is reminiscent of the relation described by Lemmens *et al*. (2005). Analyzing the *in vivo* grain infection data, it would seem that Sahara glucosylates DON most efficiently. Perhaps Sahara is not necessarily most efficient at glucosylation, it might be in fact the cultivar which is simply best equipped to interrupt *F. graminearum* before DON production.

The strong correlation between DON glucosylation and disease index (DI) *in vitro*, however, does support the hypothesis that indeed efficiency of DON glucosylation plays an important role in commercial cultivars.

During the field trial in 2012, tremendously higher DON levels than in 2011 were found. In fact, in all cultivars except Scor, there was at least one replication where the maximum allowed DON concentration of 1,250 µg kg^{-1} was exceeded (1881/2006/EC, 2006). The increase in DON over the 2 years was met with a somewhat less than equal increase in DON-3G, resulting in overall lower glucosylation capacities for the second year. It is noteworthy that these percentages better approximate the values found in the *in vivo* trials. The high

glucosylation capacities that were observed in 2011 are likely to be exceptional, merely possible by merit of the extremely low DON production in that year.

A high correlation between disease index (DI) and glucosylation capacity was observed under laboratory conditions, but did not hold up in the field for either of the two evaluated years. In fact, it was shown by Landschoot *et al*. (2012) that in Belgian winter wheat, over a period of 10 years, it was not possible to determine a clear correlation between disease severity and DON incidence. The lack of a correlation between DI and DON-3G is a logical consequence as many other factors have an influence on the contamination.

It is clear that in *F. graminearum*-dominated populations, large amounts of DON can be glucosylated to DON-3G in commercial wheat cultivars, which, in a controlled environment, was confirmed to have a profound effect on *Fusarium* disease limitation.

The analytical scientist is faced with an ever-expanding spectrum of mycotoxins and derivatives. The need of identification of new masked forms via untargeted screening of samples is an emerging research topic. To answer this demand, the occurrence of conjugates of T-2 and HT-2 was described. The presence of glucoside derivatives of T-2 and HT-2 in a newly-developed detached leaf assay was proven. Mono- and tri-glucoside derivatives of T-2 and HT-2, T-2-3-glucoside, T-2-3-triglucoside and HT-2-3-glucoside were identified and characterized, using Orbitrap™ HRMS. The technology allowed obtaining molecular structure details by measuring exact masses of main characteristic fragments. Using the application of collision induced dissociation, fragmentation patterns proved glucosylation at C_3 position for both T-2 and HT-2. Occurrence of new masked forms implies the importance of the development of analytical methods for their detection, the initiation of toxicity studies, and of proving the relevance of their presence in the food and feed chain. In addition, a detached leaf assay for the production of masked glucosides has been reported for the first time. Potential large-scale availability of masked forms will lead to the opportunity to perform toxicological trials.

FUTURE PERSPECTIVES

From the available literature and the information obtained during this PhD-dissertation from 2009 until 2013, it has become clear that masked mycotoxins could represent a potential additional risk for both animals and humans. However, despite the enormous progress many research challenges remain since essential information is still lacking and more studies are urgently needed to unravel the true masked mycotoxin problemacy.

To begin with, sampling is still a major issue in the global mycotoxin analysis due to the sometimes very heterogeneous distribution of secondary metabolites in agricultural commodities and products intended for human and animal consumption. The selection of an appropriate sample is essential for the production of analytical data. As already described in *Chapter 1*, many uncertainties are related with a mycotoxin test procedure. Consequently it is difficult to determine with 100% certainty the true contamination of a bulk lot. The associated variance is the sum of sampling, sample preparation and analytical discrepancies. To date, methods have been developed to predict the total number of lots accepted and rejected, the amount of mycotoxin in these lots and the costs associated with mycotoxin inspection programs for several commodities (Whitaker *et al.*, 1993). However, these methods have only been applied by the USDA in peanut aflatoxin-testing programs. A future perspective is the expansion of these methods to other relevant mycotoxins and their masked forms.

Up to now, not many clean-up procedures (or devices) are available for the purification or enrichment of mycotoxin conjugates for their application in multi-mycotoxin analysis. Probably immuno-affinity columns, already on the market for a number of mycotoxins, might show some potential for the purification of masked mycotoxins. When wanting to get a clear picture of the masked mycotoxin level, the precise impact of cross reactivity remains an unsolved problem. The development of immunochemical approaches on field for the determination of a range of mycotoxins is in its infancy. The knowledge on the cross reactivity in current immunochemical methods remains limited, which hampers correct data interpretation. Optimization and validation of rapid test systems will contribute to effective screening of masked mycotoxins.

Still, LC-MS/MS is the method of choice in multi-masked mycotoxin analysis. It can be expected that further improvements of LC-MS/MS instrumentation and its availability at lower price will further contribute to LC-MS/MS becoming the major tool for the analysis of multi-contaminants in an amalgam of matrices. MS-based screening has played a pivotal role in the discovery of novel mycotoxin conjugates in the past and it is believed that this will also

continue in the future. The power of HRMS facilitates the discovery of new masked forms. Judging from the expanding available literature, it is obvious that more masked forms will be identified. The relevance of these new conjugates needs to be proven by the elaboration of occurrence surveys in cereal-based matrices.

Also, the use of HRMS offers new opportunities in the field of mycotoxin research through screening of a wider group of metabolites. An emerging research topic in mycotoxin analysis is *metabolomics*. The extensive identification of conjugates or metabolites can reveal new detoxification routes of the fungus and unravel new metabolization patterns of mycotoxins in biological fluids.

The constant monitoring of masked mycotoxins in food and feed remains important to map the masked mycotoxin contamination pattern. Equally important is to acquire knowledge about the stability and formation of masked forms during processing and their influence on some technological processes. To date, inconclusive results about technological parameters on the masked form levels are pointed out.

The topic of conjugated mycotoxins is also of great interest to the community of plant breeders, who are aiming for *Fusarium*-resistant varieties of certain crops. In *Chapter 5* high levels of DON-3G were found in wheat, and previous reports correlated these findings with the overall *Fusarium* resistance. It is expected that the sowing of such *Fusarium*-resistant (*e.g.* wheat or maize) lines will increase in the near future, which will directly result in a higher occurrence of masked mycotoxins in cereals.

Nevertheless, most concerns are related to the potential health effects of conjugated forms of mycotoxins. Either direct or indirect toxicity via hydrolysis to their parent forms can be attributed to masked mycotoxins. Only few reports indicated the possible toxic relevance of conjugated forms. In the near future full scale metabolism studies could reveal relevant toxicity data by incorporating masked mycotoxins in the feed.

However, a major challenge for this specific future work and for the progress in the area of immunochemical methods is to provide large amounts of masked mycotoxin reference standards. In *Chapter 6* a promising technique using detached leaves of maize and wheat has been described. This method, however, should be ameliorated to obtain larger quantities in terms of mg of masked mycotoxins. Straightforward chemical synthesis techniques for glucuronides were described (Mikula *et al.*, 2012). Other approaches using extensive enzymatic reconversion of parent mycotoxins or the application of fungal strains should be

considered in future developments. Hopefully, commercial suppliers might pick up this topic in the near future, which will lead to the availability of conjugates of most common mycotoxins.

Risk assessment is undoubtedly an obligatory tool to quantify the true exposure of mycotoxins and their conjugates through food and feed. The results obtained in *Chapter 4* have been a first step to determine the risk related to the dietary intake of mycotoxins. However, many additional parameters which contribute to the true risk of exposure are lacking. The presence of airborne mycotoxins is not taken into account, also other highly consumed matrices (*e.g.* beer, wine, grapes) should be considered. An interesting, more holistic approach is the risk analysis of a full week's diet of individuals. The summary of the total dietary exposures will provide a clearer view on the overall mycotoxin contamination levels. Nevertheless, current risk analyses do not take into account possible mycotoxin metabolization products or biomarkers. Reports have not yet delivered satisfactory information because only a few biomarkers have been validated for some mycotoxins whereas more than 30 (masked) mycotoxins are reported to contaminate food and feed. Firstly, future research needs to focus on untargeted screening to discover new valuable biomarkers. Secondly, exposure assessment studies need to improve by analyzing the impact of mycotoxins on human and animal health. This is best achieved by using biomarkers of exposure in several biological matrices like blood, urine, feces, liver, kidney and breast milk.

Concerning biomarkers, the potential correlation of human diseases with the occurrence of relevant mycotoxin biomarkers should be investigated to get a more integrated view on (masked) mycotoxin exposure and associated adverse health effects.

Legislation clearly intends to improve food and feed safety. National and international authorities ensure the implementation of regulations or recommendations of mycotoxins in food and feed. Recently, indicative values for the sum of T-2 and HT-2 were published after full consideration of occurrence and toxicological data. For masked mycotoxins these guidance values or maximum levels are lacking. However, according to the extensive research it can be expected that legislation for the sum of a group of (masked) mycotoxins will be introduced. Official instances are focused on the levels of the individual mycotoxins that exceed the maximum limits. From a scientific point of view however, the mycotoxin derivatives should also be taken into consideration.

The European aflatoxin crisis in feed (spring 2013) was an eye-opener for national instances and the public community. Researchers hope that in future authorities will consider the true issue of all mycotoxins and not restrict themselves to a few parent mycotoxins only.

In the course of the research, it became obvious that mycotoxins are clearly not as well known to the consumer as other agents *e.g.* pesticides, dioxins or antibiotic residues. Only agricultural representatives are involved in this problematic matter. It can be relevant to inform the consumer population of the described topic.

To end, a total eradication of mycotoxins is not feasible. Nonetheless, the expanding knowledge on these xenobiotics helps industry to tackle this issue *from farm to fork*. The implementation of mycotoxin binders or detoxifiers in compound feed is an expanding commercial industry. However, no single application has been developed equally effective against a wide variety of mycotoxins. Future studies can focus on this specific matter to tackle this issue. In general, the primary line of defense for mycotoxins is Good Agricultural Practices (GAP) followed by the implementation of Good Manufacturing Practices (GMP) during handling, storage, processing and distribution of cereals. All these parameters can be included in a complementary management system or Hazard Analysis Critical Control Point (HACCP) manual for the control of mycotoxins. The correct implementation of these parameters and the proper monitoring of legislation, will ease the issue of mycotoxins.

Terminating these future perspectives, the author concludes that masked-mycotoxin research will be a never ending story.

SUMMARY

The manuscript entitled '*Chemical risks related to food and feed containing masked Fusarium mycotoxins*' contributes both to the understanding and awareness of the problematic nature, and to the risk assessment of masked mycotoxins in cereals, cereal-based foods and feedstuffs.

A general introduction has been presented in ***Chapter 1*** on the basis of an extensive literature review. An overview of the chemical and toxic properties of the studied fusariotoxins, trichothecenes and myco-estrogens is described. These mycotoxins are included in the study because of their known occurrence in cereal crops and their proved negative economic impact. The research focuses, however, on masked mycotoxins. The second part of *Chapter 1* describes the formation, prevention and toxicological profile of these conjugates, and presents an extensive discussion of the applied analytical techniques. In *Chapter 4* a risk assessment study on masked mycotoxins is expounded, for this reason, the adjoining final section of *Chapter 1* is devoted to risk analysis specified to (masked) mycotoxins.

Both food and feed crops can be affected through an amalgam of mycotoxin-producing fungi. Multiple occurrence of mycotoxins produced by one fungal species is plausible, however, the plant or fungus is capable of converting these xenobiotics into masked mycotoxins. Generating data on these conjugated forms is crucial and for this reason the development, validation and application of a multi-mycotoxin LC-MS/MS method is a prerequisite.

In ***Chapter 2*** an LC-MS/MS method was developed and validated for the simultaneous determination of deoxynivalenol (DON), zearalenone (ZEN), T-2-toxin (T-2), HT-2-toxin (HT-2), and both their fungal and plant conjugates, including 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), deoxynivalenol-3-glucoside (DON-3G), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN-14G), α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G) and zearalenone-14-sulfate (ZEN-14S) in maize, wheat, oats, breakfast cereals and bread, using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer.

Extraction was performed with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. After filtration, the extract was evaporated and the residue was redissolved in mobile phase for injection. A sample clean-up procedure was not included, because of the low recoveries of parent and masked mycotoxins and their differences in chemical structure and subsequent polarity. The method was validated for several parameters such as linearity, apparent recovery, limit of detection, limit of quantification, precision, expanded measurement uncertainty and specificity. The values for the limit of detection

varied from 5 µg kg^{-1} to 13 µg kg^{-1}, those for the limit of quantification from 10 µg kg^{-1} to 26 µg kg^{-1}. The performance characteristics of the LC-MS/MS method were well in agreement with the criteria mentioned in the Commission Regulation (EC) No 2002/657. The developed LC-MS/MS method was the basis for the execution of research described in *Chapter 3, 4, 5* and *6*.

The general question comes to the mind whether these masked forms occur naturally in cereals, cereal-based food and feedstuffs. ***Chapter 3*** comprises the elaboration of three large surveys in cereal-based foods (*part 1*), feed (*part 2*) and maize (*part 3*).

In *part 1* and *2*, 175 cereal-based foodstuffs, 67 compound feeds and 19 raw materials were analyzed as to the natural occurrence of fusariotoxins and their masked forms. Fibre-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal were collected in Belgian supermarkets from April 2010 to October 2011. All food samples analyzed were contaminated with an average of 2 to 6 mycotoxins, including 1 to 3 masked forms. Feed materials that were used in the analyzed compound feeds were collected by the manufacturer. The feed materials were beet pulp, sunflower seed meal, soy bean, soy peel, oats, barley, maize germs, maize gluten feed, maize, wheat gluten feed, wheat bran pellets, wheat bran and wheat. Beet pulp, sunflower seed meal, soy bean and soy peel were hardly contaminated. The feed materials that were mostly infected with DON, ZEN and derivatives were maize and its by-products. Also, the glucosylated and sulfated forms occurred in substantial amounts. Wheat and its by-products were contaminated as well with α-ZEL (wheat gluten feed and wheat bran) and ZEN (wheat). The contamination pattern and level of feed materials were reflected in the corresponding compound feeds.

Regarding maize (*part 3*), a total of 288 maize samples were analyzed for the same mycotoxin batch. To get a better insight, the occurrence of various *Fusarium* (masked) mycotoxins in different maize varieties grown under natural infection conditions in Flanders, Belgium was assessed. The outcome showed that maize varieties were contaminated with a mixture of both parent and masked mycotoxins. Moreover, a positive correlation between the parent and the masked forms was established. This indicates that a higher contamination by a particular mycotoxin is coupled with an elevated load of the other. These results highlight the relevance of screening for multiple mycotoxins, both parent and masked, in order to guarantee food and feed safety. Further, a field analysis was executed to elucidate the distribution of the various mycotoxins in the field. Except for DON, the maize variety did not significantly influence the mycotoxin accumulation. Subdivisions with higher mycotoxin levels in the field were

observed. The characterization of these clusters is of great importance as sampling schemes for field kernels can be constructed.

In *Chapter 3* contamination data of mycotoxins and their masked forms in food and feed were highlighted. Notwithstanding the moderate amounts, the question was raised if the Belgian population was exposed to a true risk posed by (masked) mycotoxins? In ***Chapter 4*** the results of a quantitative dietary exposure assessment to mycotoxins and their masked forms through cereal-based foods and feed in Belgium, were presented.

In the first part a quantitative dietary exposure assessment of mycotoxins and their masked forms in cereal-based food was conducted on a nationally representative sample of the Belgian population using the contamination data of cereal-based foods. The habitual intake of these food groups was estimated from the Belgian National Food Consumption Survey of 2004. According to a probabilistic exposure analysis, the mean (and P95) mycotoxin intake for the sum of the DON-equivalents, ZEN-equivalents, and the sum of HT-2-and T-2 for all cereal-based foods was 0.1162 (0.4047, P95), 0.0447 (0.1568, P95) and 0.0258 (0.0924, P95) µg kg^{-1} body weight (bw) day^{-1}, respectively. These values were below the tolerable daily intake (TDI) levels for DON, ZEN and the sum of T-2 and HT-2 (1.0, 0.25 and 0.1 µg kg^{-1} bw day^{-1}, respectively). The level of the Belgian population exceeding the TDI for all cereal-based foods was calculated, and recorded 0.85%, 2.75% and 4.11%, respectively.

The second part implemented a quantitative deterministic analysis for feed, as a probabilistic exposure assessment was not executable due to the lack of detailed consumption data for animal species. Values were concluded according to the no observed effect level (NOEL) or the lowest observed adverse effect level (LOAEL). No serious concerns were observed, however the intake of pig feed exceeded the NOEL or the mean contamination level for DON (28.8972 µg kg^{-1} bw day^{-1}), ZEN (41.7585 µg kg^{-1} bw day^{-1}) and the sum of T-2 and HT-2 (3.4293 µg kg^{-1} bw day^{-1}).

In ***Chapter 5*** an answer to the glucosylation capacity of certain wheat cultivars is formulated according to *in vitro* and *in vivo* experiments. Some wheat plants have evolved a typical resistance designated *Fhb1* resistance, which is associated with a glucosyltransferase function, capable of detoxifying the deposited DON. *Chapter 5* aims to assess the glucosylation capacity in the Belgian commercial wheat pool and to determine the importance of DON glucosylation in the complex background of natural field circumstances. Clear indications were found that several Belgian commercial wheat cultivars, to some extent, do possess a glucosyltransferase function capable of detoxifying significant amounts of DON. However,

the level of glucosylation in the field did not correlate well with disease severity. Clearly, the obtained results provide the first large-scale assessment of glucosylation capacity in Belgian commercial wheat cultivars.

In *Chapter 1* the relevance of identification of other masked forms via untargeted screening of matrices was highlighted. To answer this demand, ***Chapter 6*** describes the occurrence of conjugates of T-2 and HT-2. The presence of glucoside derivatives of T-2 and HT-2 in a newly-developed detached leaf assay was proven. Mono- and tri-glucoside derivatives of T-2 and HT-2, T-2-3-glucoside, T-2-3-tri-glucoside and HT-2-3-glucoside were identified and characterized using Orbitrap^{TM} high-resolution mass spectrometry. The technology allowed obtaining molecular structure details by measuring exact masses of main characteristic fragments. Using the application of collision-induced dissociation, fragmentation patterns proved glucosylation at C_3 position for both T-2 and HT-2. Occurrence of new masked forms implies the importance of the development of analytical methods for their detection, the constitution of toxicity studies, and the relevance of their riskful presence in the food and feed chain. In addition, a detached leaf assay for the production of masked glucosides has been reported for the first time. Potential large-scale availability of masked forms will lead to the opportunity to perform *e.g. in vivo* toxicity trials.

The knowledge gathered from the different chapters prompts the formulation of general conclusions and future perspectives regarding masked mycotoxins in ***Chapter 7***.

SAMENVATTING

Het doctoraatsonderzoek getiteld '*Chemical risks related to food and feed containing masked Fusarium mycotoxins*' levert een bijdrage tot het begrijpen van de problematiek en tot de risico-inschatting van gemaskeerde mycotoxinen aanwezig in granen, graangebaseerde voeding en voeder.

Vooreerst wordt in ***Hoofdstuk 1*** uitgaande van een uitgebreid literatuuronderzoek, een algemene inleiding weergegeven. Een overzicht betreffende de chemische en toxische eigenschappen van de bestudeerde fusariotoxinen, trichothecenen en myco-oestrogenen wordt beschreven. Deze mycotoxinen worden in het onderzoek opgenomen daar hun voorkomen in graangewassen en het economisch belang reeds uitvoerig bewezen werd. Het doctoraatsonderzoek focust zich echter op gemaskeerde mycotoxinen. Een tweede luik van *hoofdstuk 1* beschrijft daarom de vorming, het voorkomen en het toxicologische profiel van deze conjugaten, alsook een uitvoerige beschouwing betreffende de bestaande analytische technieken. *Hoofdstuk 4* van het doctoraatsproefschrift geeft een risico-evaluatie van de onderzochte mycotoxinen weer. Om deze reden wordt het laatste luik van *hoofdstuk 1* gewijd aan risicoanalyse met een specificatie naar (gemaskeerde) mycotoxinen.

Zowel voedings- als voedergewassen kunnen worden aangetast door een amalgaam van mycotoxinen producerende schimmels. Het meervoudig voorkomen van mycotoxinen geproduceerd door één schimmelspecies is mogelijk. Echter daarnaast is de plant of schimmel in staat om deze xenobiotische vormen om te zetten naar gemaskeerde mycotoxinen. Het genereren van data betreffende deze vormen is van cruciaal belang en om deze reden is de ontwikkeling, validatie en toepassing van een multi-mycotoxine LC-MS/MS methode een vereiste.

In ***Hoofdstuk 2*** wordt een LC-MS/MS methode ontwikkeld en gevalideerd voor de simultane bepaling van deoxynivalenol (DON), zearalenone (ZEN), HT-2 toxine (HT-2), T-2-toxine (T-2) en hun conjugaten 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), deoxynivalenol-3-glucoside (DON-3G), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-14-glucoside (ZEN-14G), α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G) en zearalenone-14-sulfaat (ZEN-14S) in tarwe, maïs, haver, ontbijtgranen en brood met behulp van een Waters Acquity UPLC systeem gekoppeld aan een Quattro Premier XE massa spectrometer.

Door de divergentie in chemische structuur en het daarmee gepaard gaande polariteitsverschil was het een uitdaging om een geschikte opzuiveringsmethode te ontwikkelen. Een extractie met acetonitrile/water/azijnzuur (79/20/1, v/v/v) gevolgd door hexaanontvetting en filtratie

werd aanzien als de meest optimale methode om de verschillende mycotoxinen te detecteren. Hierna werd het extract drooggedampt en het residu heropgelost in mobiele fase voor injectie. Het invoegen van een opzuiveringsmethodiek zoals *solid phase extraction* werd niet toegepast daar een lage terugvinding werd bekomen voor zowel precursor als gemaskeerde mycotoxinen.

Inzake validatie werd voldaan aan de prestatiecriteria beschreven in Verordening Nr. 2002/657/EC. De validatieparameters omvatten lineariteit, relatieve terugvinding, detectielimiet, kwantificatielimiet, precisie, specificiteit en uitgebreide meetonzekerheid in tarwe, maïs, haver, ontbijtgranen en brood. De waarden voor de detectielimiet variëren van 5 $\mu g\ kg^{-1}$ tot 13 $\mu g\ kg^{-1}$, terwijl deze voor de kwantificatielimiet van 10 $\mu g\ kg^{-1}$ tot 26 $\mu g\ kg^{-1}$. De LC-MS/MS methode werd ontwikkeld als basis voor de uitvoering van hoofdstuk 3, 4, 5 en 6.

De algemene vraag rees of deze gemaskeerde vormen natuurlijk voorkomen in granen, graangebaseerde voeding en voeder. ***Hoofdstuk 3*** omvat de beschrijving van 3 grootschalige studies uitgewerkt in graangebaseerde voeding (*deel 1*), voeder (*deel 2*) en maïs (*deel 3*).

In *deel 1* en *2* werden 175 graangebaseerde voedingsstalen, 67 mengvoeders en 19 grondstoffen geanalyseerd op het natuurlijk voorkomen van de reeds vermelde mycotoxinen en gemaskeerde vormen. Vezel- en zemelrijk brood, ontbijtgranen, popcorn en havermout werden gecollecteerd in Belgische supermarkten van april 2010 tot oktober 2011. Alle voedingsstalen bleken gecontamineerd met een gemiddelde van 2 tot 6 mycotoxinen, met inbegrip van 1 tot 3 gemaskeerde vormen. De grondstoffen gebruikt voor het aanmaken van de mengvoeders waren bietenpulp, zonnebloempitmeel, sojabonen, sojaschillen, haver, gerst, maïskiemen, maïsglutenvoeder, maïs, tarweglutenvoeder, tarwezemelenpellets, tarwezemelen en tarwe. Bietenpulp, zonnebloempitmeel, sojaschillen en sojabonen bleken nauwelijks gecontamineerd. De hoogste infectiegraad werd aangetoond in maïs en maïsbijproducten met mycotoxinen zoals DON, ZEN en derivaten. Alsook geglucosyleerde en gesulfateerde vormen waren aanwezig in substantiële hoeveelheden. Tarwe en bijproducten bleken gecontamineerd met α-ZEL (tarweglutenfeed en tarwezemelen) en ZEN (tarwe). Het contaminatiepatroon en -gehalte van de grondstoffen werd duidelijk gereflecteerd in de corresponderende mengvoeders.

Betreffende de maïssurvey (*deel 3*) werden er in totaal 288 maïsstalen geanalyseerd op het natuurlijk voorkomen van de vernoemde mycotoxinen. Om een beter inzicht te krijgen in het voorkomen van gemaskeerde mycotoxinen in maïs, werd een veldproef opgesteld waarin

verschillende maïsvariëteiten geteeld werden onder natuurlijke infectiecondities in Vlaanderen, België. Resultaten toonden aan dat maïsvariëteiten gecontamineerd bleken met zowel precursor als gemaskeerde mycotoxinen. Alsook werd een positieve correlatie gevonden tussen de precursoren en hun gemaskeerde vormen. Dit laatste indiceert dat een hogere contaminatie van een bepaald mycotoxine is gekoppeld aan een verhoogd gehalte van een gemaskeerde vorm. De relevantie met betrekking tot het screenen van multipele mycotoxinen werd wederom aangehaald. Daarenboven werd een analyse uitgevoerd om de distributie van de mycotoxinen in het veld op te helderen. De mycotoxinen-accumulatie werd niet significant beïnvloed door de maïsvariëteiten, behalve voor DON. Om dit vernoemde resultaat te kaderen werden subpopulaties uitgewerkt waarbij hogere contaminatiegehalten in het veld werden geobserveerd. De karakterisering van deze clusters is van groot belang opdat bemonsteringsschema's voor veldstalen zo kunnen worden opgesteld.

Het voorkomen van gemaskeerde vormen wordt geconfirmeerd in hoofdstuk 3, echter de onderzoeksvraag rees of de geobserveerde data een risico inhielden voor zowel mens als dier. In ***Hoofdstuk 4*** worden de resultaten voorgesteld van een risicobeoordeling van mycotoxinen en gemaskeerde vormen in graangebaseerde voeding en voeder in België.

In het eerste luik werd een kwantitatieve deterministische en probabilistische risico-evaluatie uitgewerkt betreffende graangebaseerde voedingsmiddelen op een nationaal representatief staal van de Belgische bevolking. De gebruikelijke inname van de verschillende geanalyseerde matrices werd geschat uitgaande van de Belgische Nationale Voedselconsumptiepeiling (2004). Uitgaande van een probabilistische risico-evaluatie met behulp van Monte Carlo simulaties, werd de gemiddelde (en P95) inname voor de som van de DON-equivalenten, ZEN-equivalenten, en de som van HT-2 en T-2 voor alle voedingsmiddelen geschat op 0,1162 (0,4047, P95); 0,0447 (0,1568, P95) en 0,0258 (0,0924, P95) µg kg^{-1} lichaamsgewicht (lg) dag^{-1}. De opgetekende waarden lagen onder de tolereerbare dagelijkse inname (TDI) voor de precursoren DON, ZEN en de som van T-2 en HT-2 (1,0; 0,25 en 0,1 µg kg^{-1} lg dag^{-1}). Er werd eveneens een extrapolatie uitgevoerd naar het aantal geëxposeerde personen in de Belgische bevolking waarbij de blootstelling hoger was dan de TDI. De waarden bedroegen 0,85%, 2,75% en 4,11% van de Belgische populatie voor respectievelijk DON-equivalenten, ZEN-equivalenten, en de som van HT-2 en T-2.

Anderzijds werd een kwantitatieve deterministische risico-evaluatie uitgevoerd op de geanalyseerde voederstalen. De implementatie van een deterministische risicobepaling werd geopperd daar een probabilistische risicobepaling niet aan de orde was door het ontbreken van

gedetailleerde consumptiedata van de onderzochte species. De bekomen blootstelling werd vergeleken met de *no observed effect level* (NOEL) of de *lowest observed adverse effect level* (LOAEL). Er werden geen bedreigingen vastgesteld, echter de blootstelling van biggen aan het geanalyseerde voeder overschreed de NOEL of het gemiddelde contaminatiegehalte voor DON (28,8972 µg kg^{-1} lg dag^{-1}), ZEN (41,7585 µg kg^{-1} lg dag^{-1}) en de som van T-2 en HT-2 (3,4293 µg kg^{-1} lg dag^{-1}).

Een volgende onderzoeksvraag betrof de algemene glucosylatie-capaciteit van bepaalde tarwevariëteiten. In ***Hoofdstuk 5*** wordt een antwoord geformuleerd aan de hand van *in vitro* en *in vivo* experimenten in tarwe. Bepaalde tarwesoorten hebben een typische resistentie ontwikkeld (*Fhb1* resistentie), geassocieerd met een glucosyltransferase functie die in staat wordt geacht om DON te detoxifiëren tot gemaskeerde geglucosyleerde vormen. De glucosylatiecapaciteit van Belgische lokale tarwerassen werd ingeschat en de relevantie van DON glucosylatie in de complexe achtergrond van veldomstandigheden werd bepaald. Er werden duidelijke aanwijzingen gevonden dat verschillende Belgische commerciële tarwecultivars een glucosyltransferase functie bezitten met een mogelijkheid om significante gehalten van DON te conjugeren. Echter, onder veldcondities correleerde het gehalte van glucosylatie niet voldoende met de ziekte-index. Doch, relevante resultaten werden bekomen en een grootschalige evaluatie van de glucosylatiecapaciteit in Belgische tarwecultivars werd voor het eerst beschreven.

In hoofdstuk 1 werd het belang van de identificatie van andere gemaskeerde vormen via het untargeted screenen van matrices aangehaald. Om op deze nood te antwoorden werd in ***Hoofdstuk 6*** de aanwezigheid van glucosides van T-2 en HT-2 bewezen met behulp van een nieuw ontwikkelde *detached leaf* methodiek. Mono- en tri-glucoside derivaten van T-2 en HT-2, het T-2-3-glucoside, T-2-3-tri-glucoside en HT-2-3-glucoside werden geïdentificeerd en gekarakteriseerd gebruik makende van Orbitrap^{TM} hoge resolutie massaspectrometrie. Deze technologie maakt het mogelijk om details betreffende moleculaire structuren te genereren. Met behulp van collisie geïnduceerde dissociatie, werd fragmentatie bewezen op de C_3 positie van zowel T-2 als HT-2. Het voorkomen van nieuwe gemaskeerde vormen impliceert de ontwikkeling van analytische methodes voor hun detectie, de uitwerking van toxiciteitstudies en het bewijzen van de relevantie van hun aanwezigheid in zowel de voedings- als de voederketen. Gebruik makende van de *detached leaf* methodiek bestaat de mogelijkheid om deze uit te breiden tot een potentieel grote schaal productie van gemaskeerde

vormen. De beschikbaarheid van deze gemaskeerde vormen kan leiden tot het uitvoeren van bijvoorbeeld *in vivo* toxiciteits-trials.

Tot slot worden in ***Hoofdstuk 7*** de algemene conclusies en toekomstperspectieven betreffende de problematiek rond gemaskeerde mycotoxinen beschreven op basis van de vermelde resultaten in de verschillende hoofdstukken.

BIBLIOGRAPHY

A

Abid-Essefi, S., Ouanes, Z., Hassen, W., Baudrimont, I., Creppy, E., & Bacha, H. (2004). Cytotoxicity, inhibition of DNA and protein syntheses and oxidative damage in cultured cells exposed to zearalenone. *Toxicology in Vitro, 18,* 467-474.

Abrunhosa, L., Paterson, R., & Venancio, A. (2010). Biodegradation of ochratoxin A for food and feed decontamination. *Toxins, 2,* 1078-1099.

Anastassiades, A. (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International, 86,* 412-431.

Apfelthaler, E., Bicker, W., Lammerhofer, M., Sulyok, M., Krska, R., Lindner, W. et al. (2008). Retention pattern profiling of fungal metabolites on mixed-mode reversed-phase/weak anion exchange stationary phases in comparison to reversed-phase and weak anion exchange separation materials by liquid chromatography-electrospray ionisation-tandem mass spectrometry. *Journal of Chromatography A, 1191,* 171-181.

Arino, A. & Bullerman, L. (1994). Fungal colonization of corn grown in Nebraska in relation to year, genotype and growing conditions. *Journal of Food Protection, 57,* 1084-1087.

Audenaert, K., Broeck, R., Bekaert, B., Witte, F., Heremans, B., Messens, K. et al. (2009). *Fusarium* head blight (FHB) in Flanders: population diversity, inter-species associations and DON contamination in commercial winter wheat varieties. *European Journal of Plant Pathology, 125,* 445-458.

Audenaert, K., Callewaert, E., De Saeger, S., Höfte, M., & Haesaert, G. (2010). Hydrogen peroxide induced by application of triazole fungicides triggers deoxynivalenol (DON) production by *Fusarium graminearum*. *BMC Microbiology, 10,* 1-14.

Audenaert, K., De Boevre, M, Vanheule, A, Callewaert, J, Bekaert, B, De Saeger, S. & Haesaert, G. (2013). Mycotoxin glucosylation in commercial wheat varieties: impact on resistance to *Fusarium graminearum* under laboratory and field conditions. *Food Control (submitted).*

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

B

Baars, B. (1996). *Chromatografie in de praktijk. vol 3*. Ed. Ten Hagen en Stam (The Netherlands)

Bail, M. L., Verger, P., Doré, T., Fourbet, J. F., Champeil, A., Ioos, R. et al. (2005). Simulation of consumer exposure to deoxynivalenol according to wheat crop management and grain segregation: Case studies and methodological considerations. *Regulatory Toxicology and Pharmacology, 42,* 253-259.

Bartholomew, D., Van Dyk, D., Lau, S., O'Keefe, D., Rea, P., & Viitanen, P. (2002). Alternate energy-dependent pathways for the vacuolar uptake of glucose and gluthatione conjugates. *Plant Physiology, 130,* 1562-1572.

Bartók, T., Tolgyesi, L., Szekeres, A., Varga, M., Bartha, R., Szecsi, A. et al. (2010). Detection and characterization of twenty-eight isomers of fumonisin B1 (FB1) mycotoxin in a solid rice culture infected with Fusarium verticillioides by reversed-phase high-performance liquid chromatography/electrospray ionization time-of-flight and ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry, 24,* 35-42.

Bauer, J., Wertmer, R., & Gedek, B. (1980). Zur Kontamination von Futtermitteln mit toxinbildenen Fusarienstämmen und deren Toxinen. *Wiener tieräztliche Montsschrift, 67,* 282-288.

Bauer, J., Gareis, M., Detzler, W., Gedek, B., Heinritzi, K., & Kabilka, G. (1987). Detoxification of mycotoxins in feedstuffs. *Tierärtztliche Umschau, 42,* 70-80.

Bauwin, G. & Ryan, H. (1982). Sampling inspection and grading of grain. In C.M.Christensen (Ed.), Storage of cereal grains and their production, *vol 5*, Minneapolis, AACC, 115.

Beloglazova, N., De Boevre, M., Goryacheva, I. Yu., Werbrouck, S., Guo, Y., & De Saeger, S. (2013). Immunochemical approach for the determination of zearalenone-4-glucoside determination. *Talanta (in press).*

Bennet, J. & Klich, M. (2003). Mycotoxins. *Clinical Microbiological Reviews, 26,* 497-514.

Berger, U., Oehme, M., & Kuhn, F. (1999). Quantitative Determination and Structure Elucidation of Type A- and B-Trichothecenes by HPLC/Ion Trap Multiple Mass Spectrometry. *Journal of Agricultural and Food Chemistry, 47,* 4240-4245.

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

Berthiller, F., Schuhmacher, R., Poppenberger, B., Lucyshyn, D., Lemmens, M., Adam, G. et al. (2006). Determination of masked mycotoxins using HPLC-tandem mass spectrometry. *Mycotoxins and Phycotoxins, Proceedings,* 125-132.

Berthiller, F., Sulyok, M., Krska, R., & Schuhmacher, R. (2007). Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *International Journal of Food Microbiology, 119,* 33-37.

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

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

Berthiller, F., Hametner, C., Krenn, P., Schweiger, W., Ludwig, R., Adam, G. et al. (2009c). Preparation and characterization of the conjugated *Fusarium* mycotoxins zearalenone-4O-b-D-glucopyranoside, alpha-zearalenol-4O-b-D-glucopyranoside and beta-zearalenol-4O-b-D-glucopyranoside by MS/MS and two-dimensional NMR. *Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment, 26,* 207-213.

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

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. D., Haesaert, G., Karlovsky, P. et al. (2012). Masked mycotoxins: a review. *Molecular Nutrition & Food Research, 57,* 165-186.

Binder, E. M. (2007). Managing the risk of mycotoxins in modern feed production. *Animal Feed Science and Technology, 133,* 149-166.

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

Brasel, T., Martin, M., Carriker, C., Wilson, S., & Straus, D. (2005). Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins in the indoor environment . *Applied and Environmental Microbiology, 71,* 7376-7388.

Brazier, M., Cole, D., & Edwards, R. (2002). O-Glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (Alopecurus myosuroides). *Phytochemistry, 59,* 149-156.

Brazier, M., Cole, D. J., & Edwards, R. (2003). Partial purification and characterisation of a 2,4,5-trichlorophenol detoxifying O-glucosyltransferase from wheat. *Phytochemistry, 64,* 419-424.

Buerstmayr, H., Legzdina, L., Steiner, B., & Lemmens, M. (2004). Variation for resistance to *Fusarium* head blight in spring barley. *Euphytica, 137,* 279-290.

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

C

Campbell, K. (1996). *The rethorical act*, *vol 2*.Wadsworth Pub, Belmont, California.

Cano-Sancho, G., Gauchi, J. P., Sanchis, V., Marin, S., & Ramos, A. J. (2011). Quantitative dietary exposure assessment of the Catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives & Contaminants: Part A, 28,* 1098-1109.

CAST (2003). *Council for Agricultural Science and Technology*. http://www.cast.eu/.

Chakrabarti, D. K. & Ghosal, S. (1986). Occurrence of free and conjugated 12,13-epoxytrichothecenes and zearalenone in banana fruits infected with *Fusarium moniliforme*. *Applied and Environmental Microbiology, 51,* 217-219.

Champeil, A., Dore, T., & Fourbet, J. (2004). *Fusarium* head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. *Plant Science, 166,* 1389-1415.

Charmley, L. L., Trenholm, H. L., Prelusky, D. B., & Rosenberg, A. (1995). Economic losses and decontamination. *Natural Toxins, 3,* 199-203.

Chowdhury & Smith, T. K. (2005). Effects of feeding grains naturally contaminated with Fusarium mycotoxins on hepatic fractional protein synthesis rates of laying hens and the efficacy of a polymeric glucomannan mycotoxin adsorbent. *Poultry Science, 84,* 1671-1674.

COCERAL (2009). *Coceral mycotoxins survey: synthesis report 2009*. http://www.coceral.com/data/1354551464final_EXTERNAL_Report_Mycotoxins_2009_.pdf

Cirlini, M., Dall'Asta, C., & Galaverna, G. (2012). Hyphenated chromatographic techniques for structural characterization and determination of masked mycotoxins. *Journal of Chromatography A, 1255,* 145-152.

Cochran, W. & Cox, G. (1957). Experimental design. *2nd ed.*, Wiley and sons, New York.

Codex, (2013). ftp:ftp.fao.org/codex/meetings/CCCF/CCCF5/cf05_11e.PDF. (2013).

Coleman, J., Blake-Kalff, M., & Davies, E. (1997). Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends in Plant Science, 2,* 144-151.

Conkova, E., Laciakova, A., Kovac, G., & Seidel, H. (2003). Fusarial toxins and their role in animal diseases. *Veterinary Journal, 165,* 214-220.

Cousin, M., Riley, R., & Pestka, J. (2005). *Foodborne mycotoxins: chemistry, biology, ecology and toxicology*.

Covello, V. (1992). *Risk communication: an emerging area of health communication research*. Communication yearbook, 359–73. S. Deetz, Newbury Park and London: Sage Publications.

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

D

Dai, J., Park, G., Wright, M., Adams, M., Akman, S., & Manderville, R. (2002). Detection and characterization of a glutathione conjugate of ochratoxin A. *Chemical Research in Toxicology, 15,* 1581-1588.

Dall'Asta, C., Galaverna, G., Aureli, G., Dossena, A., & Marchelli, R. (2008). A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in maize and maize-based products. *World Mycotoxin Journal, 1,* 237-246.

Dall'Asta, C., Mangia, M., Berthiller, F., Molinelli, A., Sulyok, M., Schuhmacher, R. et al. (2009a). Difficulties in fumonisin determination: the issue of hidden fumonisins. *Analytical and Bioanalytical Chemistry, 395,* 1335-1345.

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

Dall'Asta, C., Scarlato, A., Galaverna, G., Brighenti, F., & Pellegrini, N. (2012). Dietary exposure to fumonisins and evaluation of nutrient intake in a group of adult celiac patients on a gluten-free diet. *Molecular Nutrition & Food Research, 56,* 632-640.

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

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., & Dall'Asta, C. (2013). Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chemical Research in Toxicology, 26,* 305-312.

Dänicke, S., Valenta, H., & Döll, S. (2004). On the toxicokinetics and the metabolism of deoxynivalenol (DON) in the pig. *Archives of Animal Nutrition, 58,* 169-180.

de Boer, E. J., Slimani, N., van 't Veer, P., Boeing, H., Feinberg, M., Leclercq, C. et al. (2011). The European Food Consumption Validation Project: conclusions and recommendations. *European Journal of Clinical Nutrition, 65,* S102-S107.

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

De Boevre, M., Diana Di Mavungu, J., Maene, P., Audenaert, K., Deforce, D., Haesaert, G. et al. (2012b). Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food. *Food Additives and Contaminants, 29,* 819-835.

De Boevre, M., Jackxens, L., Lachat, C., Maene, P., Eeckhout, M., Audenaert, K. et al. (2013a). Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium. *Toxicology Letters, 218,* 281-292.

De Boevre, M., Landschoot, S., Audenaert, K., Maene, P., Diana di Mavungu, J., Eeckhout, M., Haesaert, G. & De Saeger, S. (2013b). Within-field variability of *Fusarium* mycotoxins and their masked forms in maize crops in Belgium. *World Mycotoxin Journal (submitted).*

De Nijs, M., Van den Top, H. J., Portier, L., Oegema, G., Kramer, E., van Egmond, H. P. et al. (2012). Digestibility and absorption of deoxynivalenol-3-glucoside in *in vitro* models. *World Mycotoxin Journal, 5,* 319-324.

De Smet, D., Dubruel, P., Van Peteghem, C., & De Saeger, S. (2011). Development of a molecularly-imprinted polymer for patulin in apple juice. *World Mycotoxin Journal, 4,* 375-383.

De Vriese, S., De Backer, G., De Henauw, S., Huybrechts, I., Kornitzer, K., Leveque, A. et al. (2005). The Belgian food consumption survey: aims, design and methods. *Archives of Public Health, 63,* 1-16.

Delmulle, B. S., De Saeger, S. M. D. G., Sibanda, L., Barna-Vetro, I., & Van Peteghem, C. H. (2005). Development of an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. *Journal of Agricultural and Food Chemistry, 53,* 3364-3368.

Desjardins, A., Hohn, T., & McCormick, S. (1993). Trichothecene biosynthesis in *Fusarium* species - chemistry, genetics, and significance. *Microbiological Reviews, 57,* 595-604.

Desjardins, A., Proctor, R., Bai, G., McCormick, S., Shaner, G., Buechley, G. et al. (1996). Reduced virulence of trichothecene-nonproducing mutants of Gibberella zeae in wheat field tests . *Moleculat Plant-Microbe interactions, 9,* 775-781.

Desmarchelier, A. & Seefelder, W. (2011). Survey of deoxynivalenol and deoxynivalenol-3-glucoside in cereal-based products by liquid chromatography electrospray ionization tandem mass spectrometry. *World Mycotoxin Journal, 4,* 29-35.

Diaz, G. J., Cortès, A., & Roldín, L. (2005). Evaluation of the efficacy of four feed additives against the adverse effects of T-2 Toxin in growing broiler chickens. *The Journal of Applied Poultry Research, 14,* 226-231.

Dickens, J., Whitaker, T., Monroe, R., & Weaver, J. (1979). Accuracy of subsampling mill for granular material. *Journal of American Oil Chemistry, 56,* 842.

Döll, S., Gericke, S., Dänicke, S., Raila, J., Ueberschär, K. H., Valenta, H. et al. (2005). The efficacy of a modified aluminosilicate as a detoxifying agent in *Fusarium* toxin contaminated maize containing diets for piglets. *Journal of Animal Physiology and Animal Nutrition, 89,* 342-358.

Dorokhin, D., Haasnoot, W., Franssen, M., Zuilhof, H., & Nielen, M. (2011). Imaging surface plasmon resonance for multiplex microassay sensing of mycotoxins. *Analytical and Bioanalytical Chemistry, 400,* 3005-3011.

E

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

Edwards, S., Barrier-Guillot, B., Clasen, P., Hietaniemi, V., & Pettersson, H. (2009). Emerging issues of HT-2 and T-2 toxins in European cereal production. *World Mycotoxin Journal, 2,* 173-179.

Edwards, S. (2009). *Fusarium* mycotoxin content of UK organic and conventional barley. *Food Additives and Contaminants, 26,* 1185-1190.

Edwards, S. (2011). Zearalenone risk in European wheat. *World Mycotoxin Journal, 4,* 433-438.

Edwards (2012). T-2 toxins. In *Fusarium* Toxin Forum, Brussels.

EFSA (2004). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to deoxynivalenol (DON) as undesirable substance in animal feed (Question N° EFSA-Q-2003-036). *EFSA Journal, 73,* 1-42.

EFSA (2009). *Scientific report submitted to EFSA: Review of mycotoxin-detoxifying agents used as feed additives: mode of action, efficacy and feed/food safety* (Rep. No 2009/01).

EFSA Panel on Contaminants in the Food Chain (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 2011, 9,* 187.

EFSA Panel on Contaminants in the food chain (2011b). Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal, 9,* 2407.

EFSA Panel on Contaminants in the food chain (2011c). Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA Journal, 9,* 2197.

EFSA (2012). Risk assessment of contaminants in food and feed. *EFSA Journal, 10,* s1004.

EFSA (2013). EFSA website. http://www.efsa.europa.eu/

Egmond van, H. & Jonker, M. (2004). Current regulations governing mycotoxin limits in food. In N.Magan & M. Olsen (Eds.), *Mycotoxins in food. Detection and control* (1 ed., pp. 49-68). England: Woodhead Publishing Limited.

El-Sharkawy, S. & Abul-Hajj, Y. (1987). Microbial transformation of zearalenone: formation of zearalenone-4-O-b-glucoside. *Journal of Natural Products, 50,* 520-521.

Ellend, N., Binder, J., Krska, R., & Horvath, E. (1997). Contamination of Austrian corn with *Fusarium* toxins in autumn 1996. *Cereal Research Communications, 25,* 359-360.

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

EPA (2013). Environmental Protection Agency. http://www.epa.gov/

Ewing, J. (1997). *The feeds directory*, *vol 1*, Wageningen academic publishers, Packinton, England.

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

F

Falavigna, C., Cirlini, M., Galaverna, G., & Dall'Asta, C. (2012). Masked fumonisins in processed food: co-occurrence of hidden and bound forms and their stability under digestive conditions. *World Mycotoxin Journal, 5,* 325-334.

FAO (1994). *The state of food and agriculture*. (27 ed.).

FAO (1995). *Food,Nutrition and Agriculture review 15: food safety and Trade*.

FAO (2001). *Food insecurity: the state of food insecurity in the world*.

FAO/WHO Food standards Program (2001). *Proposed draft revised sampling plan for total aflatoxin in peanuts intended for further processing* (Rep. No. Codex Alimentarius Commission).

Filho, E., Xie, W., Mirocha, C., & Hogge, L. (1997). Fragmentation of some zearalenones by fast-atom bombardment mass spectrometry. *Rapid Communications in Mass Spectrometry, 11,* 1515-1520.

Fischhoff, B. (1970). Risk perception and communication unplugged: twenty years of process. *Risk Analysis, 15,* 137-145.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *British Journal of Experimental Pathology, 10,* 226-236.

Fliege, R. & Metzler, M. (1999). The mycotoxin patulin induces intra- and intermolecular protein crosslinks in vitro involving cysteine, lysine, and histidine side chains, and amino groups. *Chemico-Biological Interactions, 123,* 85-103.

Forgacs, J. (1962). Mycotoxicoses: the neglected diseases. *Feedstuffs, 18,* 124-134.

Foster, P., Slater, T., & Patterson, D. (1975). A possible enzymic assay for trichtothecene mycotoxins in animal feedstuffs. *Biochemical Social Transformation, 3,* 875-878.

Frear, D. & Swanson, H. (1978). Behaviour and fate of ^{14}C maleic hydrazide in tobacco plants. *Journal of Agricultural and Food Chemistry, 26,* 660.

G

Galtier, P. (1998). Biological fate of mycotoxins in animals. *Révue de médicine vétérinaire, 149,* 549-554.

Galvano, F., Pietri, A., Bertuzzi, T., Piva, A, Chies, L., & Galvano, M. (2013). Activated carbons: *In vitro* affinity for ochratoxin A and deoxynivalenol and relation of adsorption ability to physicochemical parameters . *Journal of Food Protection, 61,* 469-474.

Gardiner, S., Boddu, J., Berthiller, F., Hametner, C. et al. (2010). Transcriptome analysis of the barley deoxynivalenol interaction: evidence for a role of glutathione in deoxynivalenol detoxification. *Molecular Plant and Microbe Interactions*, *23 (7)*, 962-976.

Gareis, M., Bauer, J., Thiem, J., Plank, G., Grabley, S., & Gedek, B. (1990). Cleavage of zearalenone-glycoside, a masked mycotoxin, during digestion in swine. *Journal of Veterinary Medicine Series B-Zentralblatt fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health, 37,* 236-240.

Gauchi, J. & Leblanc, J. (2002). Quantitative assessment of exposure to the mycotoxin Ochratoxin A in Food. *Risk Analysis, 22,* 219-234.

German Institute of Human Nutrition Postdam-Rehbrücke (DIfe) (2011). *MSM: Multiple Source Method for estimating usual dietary intake from short-term measurement data <https://nugo.dife.de/msm/>*.

Ghosal, S., Chakrabarti, D., Srivastava, A., & Srivastava, R. (1982). Toxic 12,13-epoxytrichothecenes from anise fruits infected with *Trichothecium roseum*. *Journal of Agricultural and Food Chemistry, 30,* 106-109.

Gilligan, C. (1982). Statistical analysis of the spatial pattern of *Botrytis fabae* on *Vicia faba*: a methodological study. *Transactions of the British mycological society, 79,* 193-200.

Gorst-Allman, C., Van Rooyen, P., Wnuk, S., Golinski, P., & Chelkowski, J. (1986). Structural elucidation of an antibiotic from the fungus *Fusarium avenaceum* Fries Sacc - an ammended structure for lateropyrone. *South African Journal of Chemistry, 39,* 116-117.

Goryacheva, I. Yu. & De Saeger, S. (2012). Immunochemical detection of masked mycotoxins: A short review. *World Mycotoxin Journal, 5,* 281-287.

Goswami, R. & Kistler, H. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology, 5,* 515-525.

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

Grenier, B. & Oswald, I. P. (2011). Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. *World Mycotoxin Journal, 4,* 285-313.

Grovey, J. F. (2007). The trichothecenes and their biosynthesis. In W.Herz, H. Falk, & G. W. Kirby (Eds.), *Progress in the Chemistry of Organic Natural Products* (88 ed., pp. 63-130). Springer Vienna.

H

Hanelt, M., Gareis, M., & Kollarczik, B. (1994). Cytotoxicity of mycotoxins evaluated by the MTT cell culture assay. *Mycopathologia, 128,* 167-174.

Harcz, P., De Temmerman, L., De Voghel, S., Waegeneers, N., Wilmart, O., Vromman, V. et al. (2007). Contaminants in organically and conventionally produced winter wheat (Triticum aestivum) in Belgium. *Food Additives and Contaminants, 24,* 713-720.

Harttig, U., Haubrock, J., Knuppel, S., & Boeing, H. (2011). The MSM program: web-based statistics package for estimating usual dietary intake using the Multiple Source Method. *European Journal of Clinical Nutrition, 65,* S87-S91.

Harvey, J., Han, J., & Reiser (1978). Decomposition of oxamyl in soil and water . *Journal of Agricultural and Food Chemistry*.

Haubrock, J., Nathlings, U., Volatier, J. L., Dekkers, A., Ocke, M., Harttig, U. et al. (2011). Estimating usual food intake distributions by using the multiple source method in the EPIC-Potsdam calibration study. *The Journal of Nutrition, 141,* 914-920.

Hayes, J., Judah, D., Mc Lellan, L., & Neal, G. (1991). Contribution of the glutathion S transferases to the mechanisms of resistance to aflatoxin B1. *Pharmacology Therapy, 50,* 443-472.

He, J. W., Zhou, T., Young, J. C., Boland, G. J., & Scott, P. A. (2010). Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: a review. *Trends in Food Science & Technology, 21,* 67-76.

He, X. Q. & Pan, R. N. (1992). Bacteriophage lytic patterns for identification of *salmonellae, shigellae*, *Escherichia coli*, *Citrobacter freundii*, and *Enterobacter cloacae*. *Journal of Clinical Microbiology, 30,* 590-594.

Henrici, A. (1930). *Molds, yeast, and actinomycetes: a handbook for students of bacteriology*. (296 pp) John Wiley & Sons, Incorporated.

Heyndrickx, E., Sioen, I., Diana Di Mavungu, J., Callebaut, A., De Henauw, S. & De Saeger, S. (2013). *Personal communication*. Assessment of mycotoxin exposure in the Belgian population using biomarkers. Presented at *35th Mycotoxin Workshop, 2013* (Ghent, Belgium)

Holt, D., Lay, V., Clarke, E., & Dinsmore, A. (1995). Characterization of the safener-induced glutathione S-transferase isoform II from maize. *Planta, 196,* 295-302.

Howard, P., Churchwell, M., Couch, L., Marques, M., & Doerge, D. (1998). Formation of N-(Carboxymethyl)fumonisin B1, following the reaction of fumonisin B1 with reducing sugars. *Journal of Agricultural and Food Chemistry, 46,* 3546-3557.

Hui, Y. (1999). *Encyclopedia of Food Science and Technology, vol 3.* Wiley, USA.

I

IARC, World Health organization: international agency for research on cancer (2002). *IARC monographs on the evaluation of carcinogenic risks to humans* (Rep. No. 82).

ISO/IEC 17025 (2011). Document No. SANCO/12495/2011, supersedes Document No. SANCO/10684/2009. *Method validation and quality control procedures for pesticide residues analysis in food and feed* (http://ec.europa.eu/food/plant/protection/pesticides/docs/qualcontrol_en.pdf)

IUPAC (1995). Note for IUPAC Recommendations. *International journal of mass spectrometry and ion processes, 142,* 209-240.

J

Jansen, C., von Wettstein, D., Schafer, W., Kogel, K., Felk, A., & Maier, F. (2005). Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences of the United States of America, 102,* 16892-16897.

Jarvis, B., Zhou, Y., Jiang, J., Wang, S., Sorenson, W., Hintikka, E.. et al. (1996). Toxigenic molds in water-damaged buildings: dechlorogriseofulvins from *Memnoniella echinata*. *Journal of Natural Products, 59,* 553-554.

JECFA (2010). WHO Technical Report Series 959: JECFA 2010: Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives: evaluation of certain contaminants in food, 2010. *WHO Technical Report Series 959*.

Jimenez, A. & Vazquez, D. (1975). Quantitative binding of antibiotics to ribosomes from a yeast mutant altered on the peptidyl-transferase center. *European Journal of Biochemistry, 54,* 483-492.

K

Kabak, B., Dobson, A., & Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical reviews in food and nutrition, 46,* 593-619.

Kadota, T., Kimura, M., Hirano, S., Tajima, O., Nakajima, T., Kamata, Y. et al. (2011). Development of a simultaneous liquid chromatography/tandem mass spectrometric method for the determination of type B trichothecenes, their derivatives, and precursors in wheat. *Rapid Communications in Mass Spectrometry, 25,* 3481-3490.

Kamimura, H. (1986). Conversion of zearalenone to zearalenone glycoside by *Rhizopus* sp. *Applied and Environmental Microbiology, 52,* 515-519.

Karlovsky, P. (2011). Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Applied Microbiology and Biotechnology, 91,* 491-504.

Kazan, K., Gardiner, D. & Manners, J. (2012). On the trail of a cereal killer: recent advances in *Fusarium graminearum* pathogenomics and host resistance. *Molecular Plant Pathology, 13 (4)*, 399-413.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the Royal Society, 365,* 2793-2807.

Kelner, A. (1982). Differential sensitivity of estrogen target issues: the role of the receptor. *Endocrinology, 111,* 1986-1985.

Kenny, G. & Harrison, P. (1992). Thermal and moisture limits of grain maize in Europe: model testing and sensitivity to climate change. *Climate Research, 2,* 113-129.

Khoschsorur, G. & Erwa, W. (2004). Liquid chromatographic method for simultaneous determination of mycophenolic acid and its phenol- and acylglucuronide metabolites in plasma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 799,* 355-360.

Kim, E., Scott, P., & Lau, B. (2003). Hidden fumonisin in corn flakes. *Food Additives and Contaminants, 20,* 161-169.

Kimura, M., Tokai, T., Takahashi-Ando, N., Ohsato, S., & Fujimura, M. (2007). Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Bioscience Biotechnology and Biochemistry, 71,* 2105-2123.

Klimaat-en-landbouw (2012) http://www.klimaatenlandbouw.wur.nl/ NL/Informatie+en+data/-Wintertarwe/ Effecten+van+extremen/.(18/04/2012).

Kluger, B., Bueschl, C., Lemmens, M., Berthiller, F., Häubl, G., Jaunecker, G., Adam, G., Krska, R. & Schuhmacher, R. (2013). *Analytical and Bioanalytical Chemistry, 405*, 5031-5036.

Koppen, R., Koch, M., Siegel, D., Merkel, S., Maul, R., & Nehls, I. (2010). Determination of mycotoxins in foods: current state of analytical methods and limitations. *Applied Microbiology and Biotechnology, 86,* 1595-1612.

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, J. 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,* 3187-3194.

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

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

Kroes, R. & Kozianowski, G. (2002). Threshold of toxicological concern (TTC) in food safety assessment. *Toxicology Letters, 127,* 43-46.

Krska, R., Baumgartner, S., & Josephs, R. (2001). The state-of-the-art in the analysis of type-A and -B trichothecene mycotoxins in cereals. *Fresenius J Anal Chem, 371,* 285-299.

Krska, R., Schubert-Ullrich, P., Molinelli, A., Sulyok, M., MacDonald, S., & Crews, C. (2008). Mycotoxin analysis: an update. *Food Additives & Contaminants: Part A, 25,* 152-163.

Krysinska-Traczyk, E., Perkowsky, J., Dutkiewicz, J. (2007). Levels of fungi and mycotoxins in the samples of grain and grain dust collected from five various cereal crops in eastern Poland. *Annals of Agricultural and Environmental Medicine*, *14*, 159-167.

L

Lamoureux, G. L. & Rusness, D. G. (1986). Xenobiotic conjugation in higher-plants. *ACS Symposium Series, 299,* 62-105.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H. et al. (2008a). Fate of trichothecene mycotoxins during the processing: Milling and baking. *Food Additives and Contaminants, 25,* 650-659.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P. et al. (2008b). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food Additives and Contaminants, 25,* 732-744.

Landschoot, S., Waegeman, W., Audenaert, K., Vandepitte, J., Baetens, J., Haesaert, G. et al. (2012). An empirical analysis of explanatory variables affecting *Fusarium* infection and deoxynivalenol production in wheat (2010). *Journal of Plant Pathology*.

Landschoot, S. (2012). *Prediction of Fusarium head blight and deoxynivalenol in winter wheat with regression-based learning algorithms.* Dissertation.

Langseth, W. & Rundberget, T. (1999). The occurrence of HT-2 toxin and other trichothecens in Norwegian cereals. *Mycopathologia, 147,* 157-165.

Lattanzio, V. M. T., Gatta, S. D., Suman, M., & Visconti, A. (2011). Development and in-house validation of a robust and sensitive solid-phase extraction liquid chromatography/tandem mass spectrometry method for the quantitative determination of aflatoxins B1, B2, G1, G2, ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-

2 toxins in cereal-based foods. *Rapid Communications in Mass Spectrometry, 25,* 1869-1880.

Lattanzio, V. M. T., Nivarlet, N., Lippolis, V., Gatta, S. D., Huet, A. C., Delahaut, P. et al. (2012a). Multiplex dipstick immunoassay for semi-quantitative determination of Fusarium mycotoxins in cereals. *Analytica Chimica Acta, 718,* 99-108.

Lattanzio, V. M. T., Visconti, A., Haidukowski, M., & Pascale, M. (2012b). 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.

Leeson, S. & Summers, J. (2013). *Commercial poultry nutrition*. (3 ed.).

Lemmens, M., Scholz, U., Berthiller, F., Dall'Asta, C., Koutnik, A., Schuhmacher, R. 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 and Microbe Interactions, 18,* 1318-1324.

Lemmens, M., Koutnik, A., Steiner, B., Buerstmayr, H., Berthiller, F., Schuhmacher, R. et al. (2008). Investigations on the ability of FHB1 to protect wheat against nivalenol and deoxynivalenol. *Cereal Research Communications, 36,* 429-435.

Lemmens, M. (2011). *Fusarium* head blight in wheat: breeding for resistance and the consequences for the (masked) mycotoxin content in the grain. De Saeger S - 4th International Symposium: Mycotoxins: Challenges and Perspectives , 73-83.

Leonard, K. & Bushnell, W. (2003). *Fusarium head blight of wheat and barley*. WR, APS Press, St-Paul, Minnesota, USA.

Li, F., Wang, W., Ma, J., Yu, C., Lin, X., & Yan, W. (2012). Natural occurrence of masked deoxynivalenol in Chinese wheat and wheat-based products during 2008-2011. *World Mycotoxin Journal, 5,* 221-230.

Lim, E. K. & Bowles, D. J. (2004). A class of plant glycosyltransferases involved in cellular homeostasis. *EMBO Journal, 23,* 2915-2922.

Lin, L., Zhang, J., Wang, P., Wang, Y., & Chen, J. (1998). Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A, 815,* 3-20.

Liu, Y., Walker, F., Hoeglinger, B., & Buchenauer, H. (2005). Solvolysis procedures for the determination of bound residues of the mycotoxin deoxynivalenol in *Fusarium* species infected grain of two winter wheat cultivars preinfected with barley yellow dwarf virus. *Journal of Agricultural and Food Chemistry, 53,* 6864-6869.

Liu, Z., Wei, F., & Feng, Y. Q. (2010). Determination of cytokinins in plant samples by polymer monolith microextraction coupled with hydrophilic interaction chromatography-tandem mass spectrometry. *Analytical Methods, 2,* 1676-1685.

Liu, Q., Xu, L., Ke, Y., Jin, Y., Zhang, F., & Liang, X. (2011). Analysis of cephalosporins by hydrophilic interaction chromatography. *Journal of Pharmaceutical and Biomedical Analysis, 54,* 623-628.

Llorens, A., Mateo, R., Hinojo, M. J., Valle-Algarra, F. M., & Jimenez, M. (2004). Influence of environmental factors on the biosynthesis of type B trichothecenes by isolates of *Fusarium* spp. from Spanish crops. *International Journal of Food Microbiology, 94,* 43-54.

Logrieco, A., Mulé, G., Moretti, A., & Bottalico, A. (2002). Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. *European Journal of Plant Pathology, 108,* 597-609.

Lopez-Bellido, R., Shepherd, C., & Barraclough, P. (2004). Predicting post-anthesis N requirements of bread wheat with a Minolta SPAD meter. *European Journal of Agronomy, 20,* 313-320.

Lyubenova, L., Goetz, C., & Golan-Goldhirsh, A. (2007). Direct effect of Cd on glutathion S transferase and glutathion reductase from *Calystegia sepium*. *International Journal of phytoremediation, 9,* 465-473.

M

Maestroni, B & Cannavan, A. (2011). Sampling strategies to control mycotoxins. In *Determing mycotoxins and mycotoxigenic fungi in food and feed*. De Saeger S., Woodhead Publishing (Ed.) (pp. 3-36).

Magan, N. & Aldred, D. (2007). Post-harvest control strategies: minimizing mycotoxins in the food chain. *International Journal of Food Microbiology, 119,* 131-139.

Maier, F., Miedaner, T., Hadeler, B., Felk, A., Salomon, S., Lemmens, M. et al. (2006). Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. *Molecular Plant Pathology, 7,* 449-461.

Malachova, A., Dzuman, Z., Veprikova, Z., Vaclavikova, M., & Zachariaso (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.

Maragos, C. M. (2011). Zearalenone occurrence and human exposure. *World Mycotoxin Journal, 3,* 369-383.

Marrs, K. (1996). The functions and regulation of glutathione S-transferases in plants. *Annual Review of Plant Physiology and Plant Molecular Biology, 47,* 127-158.

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

McComas, K. (2006). Defining moments in risk communication research: 1995-2005. *Journal of Health Communication, 11,* 75-91.

McCormick, S., Stanley, A., & Stover, N. (2011). Trichothecenes: from simple to complex mycotoxins. *Toxins, 3,* 802-814.

McCormick, S. P., Price, N. P. J., & Kurtzman, C. P. (2012). Glucosylation and other biotransformations of T-2 toxin by yeasts of the *Trichomonascus Clade*. *Applied and Environmental Microbiology, 78,* 8694-8702.

Medeiros Vinci, R. (2011). *A contribution to the risk assessment of genotoxic carcinogens in food - cas studies on benzene and acrylamide*. Dissertation.

Meky, F. A., Turner, P. C., Ashcroft, A. E., Miller, J. D., Qiao, Y. L., Roth, M. J. et al. (2003). Development of a urinary biomarker of human exposure to deoxynivalenol. *Food and Chemical Toxicology, 41,* 265-273.

Middelhoven, W. & Kurtzman, C. (2003). Relation between phylogeny and physiology in some ascomycetous yeasts. *Antonie Van Leeuwenhoek, 83,* 69-74.

Mikula, H., Hametner, C., Berthiller, F., Warth, B., Krska, R., Adam, G. et al. (2012). Fast and reproducible chemical synthesis of zearalenone-14-glucuronide. *World Mycotoxin Journal, 5,* 289-296.

Miller, J., Taylor, A., & Greenhalgh, R. (1983). Production of deoxynivalenol and related compounds in liquid culture by *Fusarium graminearum*. *Canadian Journal of Microbiology, 29,* 1171-1178.

Miller, J. & Greenhalgh, R. (1988). Metabolites of fungal pathogens and plant resistance. In P. Hedin, J. Menn, & R. Hollingworth (Eds.), (379 ed., pp. 117-129).

Miller, S., Reid, L., & Harris, L. (2007). Colonization of maize silks by *Fusarium graminearum*, the causative organism of *gibberella* ear rot. *Canadian Journal of Botany, 85,* 369-376.

Minervini, F., Giannoccaro, A., Cavallini, A., & Visconti, A. (2005). Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. *Toxicology Letters, 159,* 272-283.

Mirocha, C. (1981). Distribution and metabolism of zearalenone in a lactating cow. *Journal of the American oil chemists society, 58,* a597-a598.

Molinelli, A., Grossalber, K., & Krska, R. (2009). A rapid lateral flow test for the determination of total type B fumonisins in maize. *Analytical and Bioanalytical Chemistry, 395,* 1309-1316.

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, 23,* 3-11.

Monbaliu, S., Van Poucke, C., Detavernier, C., Dumoulin, F., Van De Velde, M., Schoeters, E. et al. (2010). Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS Method. *Journal of Agricultural and Food Chemistry, 58,* 66-71.

Monbaliu, S. (2011). *Development of multi-mycotoxin LC-MS/MS methods and application in food and feed analysis.* Dissertation.

Moon, Y. & Pestka, J. (2003). Deoxynivalenol-induced mitogen-activated protein kinase phosphorylation and IL-6 expression in mice suppressed by fish oil. *The Journal of Nutritional Biochemistry, 14,* 717-726.

Mounjouenpou, P., Gueule, D., Fontana-Tachon, A. l., Guyot, B., Tondje, P. R., & Guiraud, J. P. (2008). Filamentous fungi producing ochratoxin a during cocoa processing in Cameroon. *International Journal of Food Microbiology, 121,* 234-241.

Mumma, R. & Hamilton, R. (1979). Xenobiotic metabolism: in vitro methods. *Americal Chemical Society, 97,* 35-76.

Munkvold, G. P. (2003). Epidemiology of *Fusarium*; diseases and their mycotoxins in maize ears. *European Journal of Plant Pathology, 109,* 705-713.

Murashige T, (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant, 15,* 473-497.

N

Nagl, V., Schwartz, H., Krska, R., Moll, W. D., Knasmuller, S., Ritzmann, M. et al. (2012). Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in rats. *Toxicology Letters, 213,* 367-373.

Nakagawa, H., Ohmichi, K., Sakamoto, S., Sago, Y., Kushiro, M., Nagashima, H. et al. (2011). Detection of a new *Fusarium* masked mycotoxin in wheat grain by high-resolution LC-Orbitrap-MS. *Food Additives & Contaminants: Part A, 28,* 1447-1456.

Nakagawa, H., Sakamoto, S., Sago, Y., Kushiro, M., & Nagashima, H. (2012). The use of LC-Orbitrap MS for the detection of *Fusarium* masked mycotoxins: the case of type A trichothecenes. *World Mycotoxin Journal, 5,* 271-280.

Nakagawa, H., Sakamoto, S., Sago, Y., & Nagashima, H. (2013). Detection of type A trichothecene diglucosides produced corn by high resolution liquid chromatography-Orbitrap mass spectrometry. *Toxins, 5,* 590-604.

Njumbe Ediage, E., Diana Di Mavungu, J., Scippo, M. L., Schneider, Y. J., Larondelle, Y., Callebaut, A. et al. (2011). Screening, identification and quantification of glucosinolates in black raddish (*Raphanus sativus L. niger*) based dietary supplements using HPLC-DAD and LC-MS/MS. *Journal of Chromatography A, 1218,* 4395-4405.

Njumbe Ediage, E., Mavungu, J. D., Goryacheva, I. Yu., Peteghem, C., & Saeger, S. (2012). Multiplex flow-through immunoassay formats for screening of mycotoxins in a variety of food matrices. *Analytical and Bioanalytical Chemistry, 403,* 265-278.

O

Obremski, K., Gajecki, M., Zwierzchowski, W., Bakula, T., Apoznaniski, J., & Wojciechoeski, J. (2003). The level of zearalenone and a-zearalenol in the blood of gilts with clinical symptoms of toxicosis, fed diets with a low zearalenone content. *Journal of Animal and Feed Sciences, 12,* 529-538.

Oerke, E. C., Meier, A., Dehne, H. W., Sulyok, M., Krska, R., & Steiner, U. (2010). Spatial variability of *fusarium* head blight pathogens and associated mycotoxins in wheat crops. *Plant Pathology, 59,* 671-682.

Olsen, M., Mirocha, C., Abbas, H., & Johansson, B. (1986). Metabolism of high concentrations of dietary zearalenone by young male turkey poults. *Poultry Science, 65,* 1905-1910.

Ostry, V. (1998). *Filamentous microscopic fungi (moulds), mycotoxins and human health*. (SZU Praha In Czech ed.).

O'Brien, J., Renwick, A., Constable, A., Dybing, E., Muller, D., Schlatter, J. et al. (2006). Approaches to the risk assessment of genotoxic carcinogens in food: A critical appraisal. *Food and Chemical Toxicology, 44,* 1613-1635.

P

Palenchar, M. (2005). A new era of risk communication. *Communication Research Reports, 22 (1),* 59-67.

Palenchar, M. (2008). Risk communication and community right to know: a public relations obligation to inform. *Public Relations Journal, 2,* 1-26.

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.

Papst, C., Zellner, J., Venkataratnam, S., & Eder, J. (2007). *Fusarium* - Problematik bei Körnermais (*Zea Mays L.*). *Gesunde Pflanzen, 59,* 7-16.

Park, J., Scott, P., Lau, B., & Lewis, D. (2004). Analysis of heat-processed corn foods for fumonisins and bound fumonisins. *Food Additives and Contaminants, 21,* 1168-1178.

Parry, D., Jenkinson, P., & Mc Leod, L. (1995). *Fusarium* ear blight (scab) in small grain cereals - a review. *Plant Pathology, 44,* 207-238.

Parsons, M. W. & Munkvold, G. P. (2010). Associations of planting date, drought stress, and insects with *Fusarium* ear rot and fumonisin B1 contamination in California maize. *Food Additives & Contaminants: Part A, 27,* 591-607.

Paustenbach, D. (2000). The practice of exposure assessment: a state of the art review (reprinted from principles and methods of toxicilogy, 4th edition, 2001). *Journal of Toxicology and Environmental Health Part B Critical Reviews, 3,* 179-291.

Pedras, M. S., Irina Zaharia, L., & Ward, D. E. (2002). The destruxins: synthesis, biosynthesis, biotransformation, and biological activity. *Phytochemistry, 59,* 579-596.

Peraica, M., Radic, B., Lucic, A., & Pavlovic, M. (1999). Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization, 77,* 754-766.

Perry, R., Cooks, R., & Noll, R. (2008). Orbitrap mass spectrometry: instrumentation, ion motion and applications. *Mass Spectrometry Reviews, 27,* 661-699.

Pestka, J., Zhou, H.-R., Moon, Y., & Chung, Y. (2004). Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicology Letters, 153,* 61-73.

Pestka, J. & Smolinski, A. (2005). Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Health Part B, 8,* 39-69.

Pflugmacher, S. & Sandermann, J. (1996). Induction of lipid-protein mismatch by xenobiotics: kinetic cooperativity. *Biochimica et Biophysica Acta, 1300,* 219-225.

Pinton, P., Tsybulskyy, D., Lucioli, J., Laffitte, J. l., Callu, P., Lyazhri, F. et al. (2012). Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: differential effects on morphology, barrier function, tight junction proteins, and mitogen-activated protein kinases. *Toxicological Sciences, 130,* 180-190.

Pittet, A. (1998). Natural occurrence of mycotoxins in foods and feeds-an updated review. *Revue de Médecine Vétérinaire, 149,* 479-492.

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

Plasencia, J. & Mirocha, C. (1991). Isolation and characterization of zearalenone sulfate produced by *Fusarium* spp. *Applied and Environmental Microbiology, 57,* 146-150.

Poling, S., Plattner, R., & Weisleder, D. (2002). N-(1-Deoxy-d-fructos-1-yl) fumonisin B1, the initial reaction product of fumonisin B1 and d-glucose. *Journal of Agricultural and Food Chemistry, 50,* 1318-1324.

Pons, S., Pinson-Gadais, L., Atanassova, V., Boutigny, A., Roucolle, J., Carolo, P. et al. (2008). Phenolic acids composition of maize kernels modulates in planta fungal growth and trichothecenes B production by *F. graminearum*? *Cereal Research Communications, 36,* 459-463.

Ponts, N., Couedelo, L., Pinson-Gadais, L., Verdal-Bonnin, M., Barreau, C., & Richard-Forget, F. (2009). *Fusarium* response to oxidative stress by H_2O_2 is trichothecene chemotype-dependent. *Fems Microbiology Letters, 293,* 255-262.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R. et al. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry, 278,* 47905-47914.

Poppenberger, B., Berthiller, F., Bachmann, H., Lucyshyn, D., Peterbauer, C., Mitterbauer, R. et al. (2006). Heterologous expression of *Arabidopsis* UDP-glucosyltransferases in *Saccharomyces cerevisiae* for production of zearalenone-4-O-glucoside. *Applied and Environmental Microbiology, 72,* 4404-4410.

Prelusky, D. B., Veira, D., Trneholm, H., & Foster, B. (1987). Metabolic fate and elimination in milk, urine and bile of deoxynivalenol following administration to lactating sheep. *Journal of Environmental Science and Health part B, 22,* 125-148.

R

Reddy, K., Salleh, B., Saad, B., Abbas, H., Abel, C., & Shier, W. (2010). An overview of mycotoxin contamination in foods and its implications for human health. *Toxin Reviews, 29,* 3-26.

Reid, L., Mather, D., & Hamilton, R. (2006). Distribution of deoxynivalenol in *Fusarium graminearum*-infected maize ears . *Phytopathology, 86,* 110-114.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A., & Fanelli, C. (2010). Natural functions of mycotoxins and control of their biosynthesis in fungi. *Applied Microbiological Biotechnology, 87,* 899-911.

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

Richard, J. (2012). Mycotoxins: an overview. In *Guide to mycotoxins* (4 ed., pp. 1-53).

Rodrigues-Fo, E., Mirocha, C., Xie, W., Krick, T., & Martinelli, J. (2002). Electron ionization mass spectral fragmentation of deoxynivalenol and related tricothecenes. *Rapid Communications in Mass Spectrometry, 16,* 1827-1835.

Romer, J. (1986). Use of small charcoal/alumina cleanup columns in determination of trichothece mycotoxins in foods and feeds. *Journal - Association of Official Analytical Chemists, 69,* 699-703.

Rosenberg, E., Krska, R., Wissiack, R., Kmetov, V., Josephs, R., Razzazi, E. et al. (1998). High-performance liquid chromatography-atmospheric-pressure chemical ionization mass spectrometry as a new tool for the determination of the mycotoxin zearalenone in food and feed. *Journal of Chromatography A, 819,* 277-288.

Rosso, L., Maier, M., & Bertoni, M. (2000). Trichothecenes production by the hypocrealean epibiont of *Baccharis coridifolia*. *Plant Biology, 2,* 684-686.

Ruhland, M., Engelhardt, G., & Wallnofer, P. (1996). Transformation of the mycotoxin ochratoxin A in plants. Time course and rates of degradation and metabolite production in cell-suspension cultures of different crop plants. *Mycopathologia, 134,* 97-102.

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

S

Sabbioni, G., Skipper, P., Buchi, G., & Tannenbaum, S. (1987). Isolation and charakterization of the major serum-albumin adduct formed by aflatoxin-B1 in vivo in rats. *Carcinogenesis, 8,* 819-824.

Saenz de Rodriguez, C., Bougovanni, A., & Conde de Borrego, L. (1985). An epidemic precocious development in Puerto Rican children. *Journal of Pediatrics, 107,* 393-396.

Sanders, M., De Boevre, M., Dumoulin, F., Martens, F., Detavernier, C., Eeckhout, M. et al. (2013). A promising technique in sampling: the sampling of wheat dust and subsequent analysis of deoxynivalenol by LC-MS/MS. *Journal of Agricultural and Food Chemistry, submitted.*

Sasanya, J., Hall, C., & Olf-Hall, C. (2008). Analysis of deoxynivalenol, masked deoxynivalenol, and *Fusarium graminearum* pigment in wheat samples, using liquid chromatography-UV-mass spectrometry. *Journal of Food Protection, 71,* 1205-1213.

Savard, M. E. (1991). Deoxynivalenol fatty-Acid and glucoside conjugates. *Journal of Agricultural and Food Chemistry, 39,* 570-574.

Scauflaire, J., Mahieu, O., Louvieaux, J., Foucart, G., Renard, F., & Munaut, F. (2011). Biodiversity of *Fusarium* species in ears and stalks of maize plants in Belgium. *European Journal of Plant Pathology, 131,* 59-66.

Schaafsma, A. & Hooker, D. (2007). Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. *International Journal of Food Microbiology, 119,* 116-125.

Scherm, B., Balmas, V., Spanu, F., Pani, G., Delogu, G., Pasquali, M. & Mighelli, Q. (2013). *Fusarium culmorum*: causal agent of foot and root rot and head blight on wheat. *Molecular Plant Pathology, 14 (4),* 323-341.

Schiefer, H. & Beasley, V. (1989). Effects of T-2 toxin on the digestive system and energy metabolism. In *Trychothecene Mycotoxicosis: pathophysiologic effects* (2 ed.).

Schmale, D., Shah, D., & Bergstrom, G. (2005). Spatial patterns of viable spore deposition of the corn ear rot pathogen, *Gibberella zeae*, in first-year corn fields. *Canadian Journal of Plant Pathology - revue canadienne de phytopathologie, 27,* 225-233.

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

Schollenberger, M., Müller, H.-M., Ernst, K., Sondermann, S., Liebscher, M., Schlecker, C. et al. (2012). Occurrence and distribution of 13 trichotecene toxins in naturally contaminated maize plants in Germany. *Toxins, 4,* 778-787.

Schröder, P., Scheer, C., Diekmann, F., & Stampfl, A. (2007). How plants cope with foreign compounds. Translocation of xenobiotic glutathione conjugates in roots of barley (*Hordeum vulgare*) (9 pp). *International Environmental Science and Pollution Research, 14,* 114-122.

Schroeder, H. & Christensen, J. (1963). Factors affecting resistance to wheat to scab caused by *Gibberella Zeae*. *Phytopathology, 53,* 831.

Schweiger, W., Boddu, J., Shin, S., Poppenberger, B., Berthiller, F., Lemmens, M. et al. (2010). Validation of a candidate deoxynivalenol-inactivating UDP-glucosyl-transferase from barley by heterologous expression in yeast. *Molecular Plant and Microbe Interactions, 23,* 977-986.

Scoop 2003 (2003). *Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU Member States*. Reports on tasks for Scientific Cooperation: task 3.2.10.

Scott, R. (2003). Liquid chromatography. In *Chrom-Ed Book Series*, *Book 5*, http://libserv.libraryscience.com/book_pdfs.html.

Scott, P. M. (2005). Biomarkers of human exposure to ochratoxin A. *Food Additives and Contaminants, 22,* 99-107.

Seefelder, W., Knecht, A., & Humpf, H. U. (2003). Bound fumonisin B-1: Analysis of fumonisin-B-1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 51,* 5567-5573.

Sergent, T., Ribonnet, L., Kolosova, A., Garsou, S., Schaut, A., De Saeger, S. et al. (2008). Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. *Food and Chemical Toxicology, 46,* 813-841.

Serrano, A., Font, G., Ruiz, M., & Ferrer, E. (2012). Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean are. *Food Chemistry, 135,* 423-429.

Sewald, N., Vongleissenthall, J., Schuster, M., Muller, G., & Aplin, R. (1992). Structure elucidation of a plant metabolite of 4-desoxynivalenol. *Tetrahedron-Asymmetry, 3,* 953-960.

Shapira, R. & Paster, N. (2013). Control of mycotoxins in storage and techniques for their decontamination. In N.Magan & M. Olsen (Eds.), *Mycotoxins in Food. Detection and Control* (1 ed., pp. 190-223). Cambridge, England.

Sheppard, B. (2012). *Understanding risk communication theory: a guide for emergency managers and communicators* (Report to Human factors/Behavioral sciences division, science and technology directorate).

Shifrin, V. & Anderson, P. (1999). Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *The Journal of Biological Chemistry, 274,* 13985-13992.

Shin, S., Torres-Acosta, J., Heinen, S., McCormick, S., Lemmens, M., Paris, M. et al. (2012). Transgenic *Arabidopsis thaliana* expressing a barley UDP-glucosyltransferase exhibit resistance to the mycotoxin deoxynivalenol. *Journal of Experimental Botany, 63,* 4731-4740.

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.

Skrbic, B., Malachova, A., Zivancev, J., Veprikova, Z., & Hasjlova, J. (2011). *Fusarium* mycotoxins in wheat samples harvested in Serbia: A preliminary survey. *Food Control, 22,* 1261-1267.

Sousa, S., Fernandes, P., & Ramos, M. (2006). Protein-ligand docking: Current status and future challenges. *Proteins: Structure, Function, and Bioinformatics, 65,* 15-26.

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

Stanbury, P., Whitaker, A., & Hall, S. (1999). *Principles of fermentation technology*. (2 ed.).

Statbel (2012) http: & statbel.fgov.be/nl/binaries/NL_A5WEBLandbouw2012tcm325-192178.pdf. (3-5-2012). FPS Economy. (http://statbel.fgov.be/nl/statistieken/cijfers/)

Suarez-Quiroz, M., Gonzalez-Rios, O., Barel, M., Guyot, B., Schorr-Galindo, S., & Guiraud, J. P. (2004). Study of ochratoxin A-producing strains in coffee processing. *International Journal of Food Science & Technology, 39,* 501-507.

Subramanian, V. (2002). DNA shuffling to produce nucleic acids for mycotoxin detoxification. *US Patent 6.500.639*.

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry, 20,* 2649-2659.

Suzuki, T. & Casida, J. (1981). Diphenyl ether herbicides remarkably elevate the content in *Spinacia oleracea* of (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxy-3-methoxyphenyl)ethyl]-2-propenamide. *Journal of Agricultural and Food Chemistry, 29,* 1027.

Swamy, H. V., Smith, T. K., Karrow, N. A., & Boermans, H. J. (2004). Effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins on growth and immunological parameters of broiler chickens. *Poultry Science, 83,* 533-543.

T

Tanaka, T., Yoneda, A., Inoue, S., Sugiura, Y., & Ueno, Y. (2000). Simultaneous determination of trichothecene mycotoxins and zearalenone in cereals by gas chromatography-mass spectrometry. *Journal of Chromatography A, 882,* 23-28.

Tangni, E., Motte, J., Callebaut, A., & Pussemier, L. (2010). Cross-reactivity of antibodies in some commercial deoxynivalenol test kits against some fusariotoxins. *Journal of Agricultural and Food Chemistry, 58,* 12625-12633.

Thorton, R. (1959). *Sporidesmium bakeri* and facial eczema of sheep in the field. *Nature, 184,* 1327-1328.

Thrane, U., Adler, A., Clasen, P. E., Galvano, F., Langseth, W., Logrieco, A. et al. (2004). Diversity in metabolite production by *Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*. *International Journal of Food Microbiology, 95,* 257-266.

Tomaszewski, J., Miturski, R., Semczuk, Koarski, J., & Jakowicki, J. (1998). Tissue zearalenone concentration in normal hyperplasic ans neoplasic human endometrium. *Gynakologe, 69,* 363-366.

Tran, S. & Smith, T. (2011). Determination of optimal conditions for hydrolysis of conjugated deoxynivalenol in corn and wheat with trifluoromethanesulfonic acid. *Animal Feed Science and Technology, 163,* 84-92.

Trucksess, M. (1994). Mycotoxins. *Journal of AOAC International, 77,* 135-142.

Turcotte, J., Hunt, P., & Blaustein, J. (2005). Estrogenic effects of zearalenone on the expression of progestin receptors and sexual behavior in female rats. *Hormones and Behavior, 47,* 178-184.

Turner, N. W., Subrahmanyam, S., & Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta, 632,* 168-180.

Turner, P. C., Burley, V. J., Rothwell, J. A., White, K. L. M., Cade, J. E., & Wild, C. P. (2008). Deoxynivalenol: rationale for development and application of a urinary biomarker. *Food Additives and Contaminants: Part A, 25,* 864-871.

U

Ueno, Y. (1985). The toxicology of mycotoxins. *Critical Reviews Toxicology, 14,* 99-132.

V

van der Fels-Klerx, H. & 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,* 349-367.

Vanheule, A. (2012). *Personal communication*. Presented at *Mytox happening 2012* (Ghent, Belgium).

Vanheule, A., De Boevre, M., Audenaert, K., Bekaert, B., Höfte, M., De Saeger, S. & Haesaert, G. (2013). Occurrence of *Fusarium* species and their associated mycotoxins in unprocessed cereals, food and feed products in Belgium. *Molecular Nutrition & Food Research (submitted).*

Varga, E., Malachova, A., Schwartz, H., Krska, R., & Berthiller, F. (2012). Survey of deoxynivalenol and its conjugates deoxynivalenol-3-glucoside and 3-acetyl-deoxynivalenol in 374 beer samples. *Food Additives and Contaminants: Part A, 30,* 137-146.

Vendl, O., Berthiller, F., Crews, C., & Krska, R. (2009). Simultaneous determination of deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food by LC-MS-MS. *Analytical and Bioanalytical Chemistry, 395,* 1347-1354.

Vendl, O., Crews, C., MacDonald, S., Krska, R., & Berthiller, F. (2010). Occurrence of free and conjugated *Fusarium* mycotoxins in cereal-based food. *Food Additives and Contaminants Part A-Chemistry Analysis Control Exposure & Risk Assessment, 27,* 1148-1152.

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

Versilovskis, A., Huybrecht, B., Tangni, E. K., Pussemier, L., De Saeger, S., & Callebaut, A. (2011). Cross-reactivity of some commercially available deoxynivalenol (DON) and zearalenone (ZEN) immunoaffinity columns to DON- and ZEN-conjugated forms and metabolites. *Food Additives & Contaminants: Part A, 28,* 1687-1693.

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

Videmann, B., Koraichi, F., Mozallon, M., & Lecoeur, S. (2012). Effect of gender, pregnancy and exposure conditions on metabolism and distribution of zearalenone in rats. *World Mycotoxin Journal, 5*.

Vigier, B., Reid, L., Seifert, K., Stewart, D., & Hamilton, R. (1997). Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario . *Canadian Journal of Plant Pathology - revue canadienne de phytopathologie, 19,* 60-65.

Visconti, A. & Pascale, M. (1998). Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A, 815,* 133-140.

Vogelgsang, S., Sulyok, M., Hecker, A., Jenny, E., Krska, R., Schuhmacher, R. et al. (2008). Toxigenicity and pathogenicity of *Fusarium poae* and *Fusarium avenaceum* on wheat. *European Journal of Plant Pathology, 122,* 265-276.

Vose, D. (1996). *Quantitative risk analysis: a guide to Monte Carlo simulation modelling*. West Sussex: J. Wiley and sons.

Vromman, V., Waegeneers, N., Cornelis, C., De Boosere, I., Van Holderbeke, M., Vinkx, C. et al. (2010). Dietary cadmium intake by the Belgian adult population. *Food Additives & Contaminants: Part A, 27,* 1665-1673.

W

Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Costrel-de Corainville, G. et al. (2004). Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology, 110,* 481-494.

Waggoner, P. & Aylor, D. (2000). Epidemiology: a science of patches. *Annual Review of Phytopathology, 38,* 71-94.

Wei, W., Jiao-Jie, M., Chuan-Chuan, Y., Xiao-Hui, L., Hong-Ru, J., Bing, S. et al. (2012). Simultaneous determination of masked deoxynivalenol and some important type B trichothecenes in Chinese corn kernels and corn-based products by ultra-performance liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 60,* 11638-11646.

Whitaker, T., Dickens, J., & Monroe, R. (1974). Variability of aflatoxin test results. *Journal of American Oil Chemistry, 51,* 214-218.

Whitaker, T., Dowell, F., Hagler, W., Giesbrecht, F., & Wu, J. (1993). Variability associated with sampling, sample preparation, and chemically testing farmers stock peanuts for aflatoxin. *Journal of American Oil Chemistry, 77,* 107-116.

Whitaker, T., Truckness, M., Johannson, A., Giesbrecht, F., Hagler, W., & Bowman, D. (1998). Variability associated with testing shelled corn for fumonisin. *Journal of American Oil Chemistry, 81,* 1162-1168.

WHO (2013). World Health Organization website. http://www.who.int/en/

Wiebe, M. (2002). Myco-protein from *Fusarium venenatum*: a well-established product for human consumption. *Appl Microbiol Biotechnol, 58,* 421-427.

Wu, Q., Dohnal, V., Huang, L., Ku-ìa, K., & Yuan, Z. (2010). Metabolic pathways of trichothecenes. *Drug Metabolism Reviews, 42,* 250-267.

X

Xu, X.-M., Parry, D. W., Nicholson, P., Thomsett, M.., Simpson, D., Edwards, S. et al. (2005). Predominance and association of pathogenic fungi causing *Fusarium* ear blight in wheat in four European countries. *European Journal of Plant Pathology, 112,* 143-154.

Xu, X.-M., Parry, D., Nicholson, P., Thomsett, M., Simpson, D., Edwards, S. et al. (2008). Within-field variability of *Fusarium* head blight pathogens and their associated mycotoxins. *European Journal of Plant Pathology, 120,* 21-34.

Y

Yazar, S. & Omurtag, G. Z. (2008). Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences, 9,* 2062-2090.

Yike, I., Distler, A., & Ziady, A. (2006). Mycotoxin adducts on human serum albumin: biomarkers of exposure to *Stachybotrys chartarum*. *Environmental Health perspectives, 114,* 1221-1226.

Young, J. C., Fulcher, R., Hayhoe, J., Scott, P., & Dexter, J. (1984). Effect of milling and baking on deoxynivalenol (vomitoxin) content of eastern canadian wheats. *Journal of Agricultural and Food Chemistry, 32,* 659-664.

Z

Zöllner, P. & Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *Journal of Chromatography A, 1136,* 123-169.

Zachariasova, M., Hajslova, J., Kostelanska, M., Poustka, J., Krplova, A., Cuhra, P. 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., Lacina, O., Malachova, A., Kostelanska, M., Poustka, J., Godula, M. et al. (2010). Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Analytica Chimica Acta, 662,* 51-61.

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.

Zadoks, J., Chang, T., & Konzak, C. (1974). Decimal code for growth stages of cereals. *Weed Research, 14,* 415-421.

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

Zhou, B., Schwarz, P., & He, G.-Q. (2008). Effect of enzyme pretreatments on the determination of deoxynivalenol in barley. *Journal of the American Society of Brewing Chemists, 66,* 103-108.

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

Zill, G., Ziegler, W., Engelhardt, G., & Wallnofer, P. R. (1990). Chemically and biologically synthesized zearalenone-4-Beta-D-glucopyranoside-comparison and convenient determination by gradient HPLC. *Chemosphere, 21,* 435-442.

#

91/414/EEC (1991). Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. *Official Journal European Communities, L230,* 1-32.

96/23/EC (1996). Council Directive 96/23/EC of 29 April on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Official Journal of the European Communities,* L125-10-L125/32.

2002/32/EC (2002). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Official Journal European Communities, L140,* 0010-0022.

2002/657/EC (2002). Commission Decision 2002/657/EC Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal European Communities, L221,* 8-36.

178/2002/EC (2002). Regulation (EC) No 178/2002 of the European Parliament and of the council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal European Communities, L31*, 1-25.

401/2006/EC (2006). Commission Regulation (EC) No 401/2006 of 23 february 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal European Communities, L70,* 12-34.

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

2006/576/EC (2006). Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxine A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). *Official Journal European Communities, L229,* 7-9.

1126/2007/EC (2007). Commission Regulation (EC) No 1126/2007 of 28 september 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Official Journal European Communities, L255,* 14-17.

2013/165/EC (2013). Commission Recommendation of 27/03/2013 on the presence of T-2 and HT-2 in cereals and cereal products. *Official Journal European Communities, L91,* 12-15.

LIST OF ABBREVIATIONS

% TDI excess	percent of population which exceeded the tolerable daily intake
(U)HPLC	(ultra) high performance liquid chromatography
15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
A.	*Aspergillus*
Acc	accuracy or bias
ACN	acetonitrile
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
APCI	atmospheric pressure chemical ionization
ARfD	acute reference dose
ATA	alimentary toxic aleukia
ATP	adenosine-5'-triphosphate
BMDL	benchmark dose lower confidence limit
bw	body weight
CCP	critical control point
CI	confidence interval
CID	collision-induced dissociation
cf.	confer
CO	cut-off level
CR	cross-reactivity
CV	coefficient of variation
DAS	diacetoxyscirpenol
DI	disease index
DMSO	dimethylsulfoxide
DOM-1	deepoxy-deoxynivalenol-1
DON	deoxynivalenol
DON-3G	deoxynivalenol-3-glucoside
DON-15G	deoxynivalenol-15-glucoside
DON-equivalents	DON, 3-ADON, 15-ADON and DON-3G
EC	European Commission
ECD	electron capture detection
EFSA	European Food Safety Authority
e.g.	exempli gratia
ELISA	enzyme linked immunosorbent assays
EPA	Environmental Protection Agency
ESI	electrospray ionization
F.	*Fusarium*
FA	formic acid
FAO	Food and Agricultural Organization
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FDA	Food and Drug Administration
FHB	*Fusarium* head blight
FUS-X	fusarenon-X
FUS-X-G	fusarenon-X-glucoside
FWHM	full width at half maximum
G.	*Gibberella*
GAP	Good Agricultural Practices
GC	gas chromatography
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Points
HBGV	health-based guidance value

HCD	higher energy collision dissociation
HFB_1	hydrolyzed fumonisin B_1
HFB_2	hydrolyzed fumonisin B_2
HFB_3	hydrolyzed fumonisin B_3
HILIC	hydrophilic interaction liquid chromatography
HOAc	acetic acid
HRMS	high resolution mass spectrometry
HT-2	HT-2 toxin
HT-2-3G	HT-2-3-glucoside
HT-2-GG	HT-2-di-glucoside
IAC	immunoaffinity columns
IARC	International Agency for Research on Cancer
i.e.	id est
IS	internal standard
JECFA	Joint Expert Committee on Food Additives
KOH	potassium hydroxide
LC	liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LLE	liquid-liquid extraction
LLS	liquid-liquid separation
lg	lichaamsgewicht
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
m	average of the *n* measured x_i values
M.	*Microdochium*
m/z	mass-to-charge ratio
MAPK	mitogen-activate protein kinase
MAS	monoacetoxyscirpenol
max	maximum
MOE	margin of exposure approach
mp	mass percentage
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multiple tandem mass spectrometric experiments
MW	molecular weight
n	amount of values, amount of samples
NaOCl	sodium hypochlorite
nd	not detected
NEO	neosolaniol
NIV	nivalenol
NIV-G	nivalenol-glucoside
NOAEL	no observed adverse effect level
NOEL	no observed effect level
OC	operation characteristics
OTA	ochratoxin A
P	p-value for Wilcoxon rank sum test, Kruskal Wallis test
P95	95th percentile
P/P	probability/probability plot
p-ADI	provisional acceptable daily intake
PDA	potato dextrose agar
PPP	plant protection products
Q	quantifier ion
q	qualifier ion
Q/Q	quantile/quantile plot

Q1	first quadrupole
Q2	second quadrupole
QTL	quantitative trait locus
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R	Pearson's correlation coefficient
RSD	relative standard deviation
RSD_r	relative standard deviation for intra-day precision
RSD_R	relative standard deviation for inter-day precision
s/n	signal to noise
SAPK/JNK	activated protein kinases/cJun N-terminal kinases
SAX	strong anionic exchange
s_{bias}	mean recovery
SDS	dodecylsulphate
SE	standard error of mean
SLE	solid-liquid extraction
SPE	solid-phase extraction
SPR	surface plasmon resonance
SR	selected reaction monitoring
s_R	standard deviation for intra-laboratory reproducibility
stdev	standard deviation
T-2	T-2 toxin
T-2-3G	T-2-3-glucoside
T-2-GG	T-2-di-glucoside
t-ADI	temporary acceptable daily intake
TCA	tricarballylic acid
TDI	tolerable daily intake, tolereerbare dagelijkse inname
TFMSA	trifluoromethanesulfonic acid
TIC	total ion chromatogram
TLC	thin layer chromatography
TOF	time of flight
Total DON derivatives	3-ADON, 15-ADON and DON-3G
Total ZEN derivatives	α-ZEL, β-ZEL, ZEN-14G, α-ZEL-14G, β-ZEL-14G and ZEN-14S
Tris	tris (hydroxyl methyl) amino methane
TTC	threshold of toxicological concern
U	expanded measurement uncertainty
$U(C_{ref})$	uncertainty associated with the purity of the standard
u_c	combined standard uncertainty
UDP glucosyltransferase	uridine difosfate glucosyltransferase
V	variance
V_t	true value
WHO	World Health Organization
X_i	average of the *n* measured values
x_i	measured value
ZEN	zearalenone
ZEN-14G	zearalenone-14-glucoside
ZEN-14S	zearalenone-14-sulfate
ZEN-equivalents	ZEN, ZEN-14G, ZEN-14S, α-ZEL, β-ZEL, α-ZEL-14G, β-ZEL-14G
α-ZEL	α-zearalenol
α-ZEL-14G	α-zearalenol-14-glucoside
β-ZEL	β-zearalenol
β-ZEL-14G	β-zearalenol-14-glucoside
ρ	Spearman rank correlation coefficient

LIST OF TABLES, FIGURES AND EQUATIONS

TABLES

CHAPTER 1

GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

Table 1.1 Phase I reactions producing metabolites susceptible to glucose conjugation in higher plants (Lamoureux and Rusness, 1986) 15

Table 1.2 Several known mycotoxin conjugates and their origin 21

Table 1.3 Enzymes involved during food processing 28

Table 1.4 Variety of extraction and clean-up used in masked mycotoxin analysis 41

Table 1.5 Overview of LC columns, solvents and MS mode used in masked mycotoxin analysis 41

Table 1.6 Overview of the loss of molecules by CID of trichothecenes (Berger *et al.*, 1999) 43

Table 1.7 Overview of the loss of molecules by CID of myco-estrogens (Filho *et al.*, 1997) 43

Table 1.8 Commercial kits and cross reactivity of ELISA, IACs and sensors applied for masked mycotoxin analysis 51

Table 1.9 Current maximum limits for DON and ZEN in foodstuffs according to No 1881/2006 (EC), guidance values for DON and ZEN in feed according Commission Recommendation No 2006/576 (EC) and indicative values for the sum of T-2 and HT-2 in food and feed according to Commission Recommendation No 2013/165 (EU) 53

Table 1.10 Factors influencing the risk management strategy 59

Table 1.11 Seven stages of risk communication and best practices 64

Table 1.12 Key priorities of a communication strategy according to EFSA 65

Table 1.13 Mycotoxin detoxifying agents according to EFSA (2009) 69

Table 1.14 A hazard analysis critical control point (HACCP) concept with focus on fungal toxins 70

CHAPTER 2

IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE SIMULTANEOUS DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

Table 2.1 Optimized ESI^+ MS/MS-parameters 76

Table 2.2 Chromatographic retention parameters 85

Table 2.3 Recovery rates (%) of the investigated SPE-columns and the dilute-and-shoot method 87

Table 2.4 Validation parameters for the analyzed mycotoxins in wheat 90

Table 2.5 Validation parameters for the analyzed mycotoxins in maize 90

Table 2.6 Validation parameters for the analyzed mycotoxins in oats 91

Table 2.7 Validation parameters for the analyzed mycotoxins in breakfast cereals 91

Table 2.8 Validation parameters for the analyzed mycotoxins in bread 92

CHAPTER 3

NATURAL OCCURRENCE OF MYCOTOXINS AND THEIR MASKED FORMS IN FOOD, FEED AND MAIZE: THE ELABORATION OF 3 SURVEYS

Table 3.1 Concentrations of mycotoxins and their masked forms (mean, standard deviation and maximum) (µg kg^{-1}) in the different food categories analyzed (years 2010-2011) 103

Table 3.2 Mean concentrations (µg kg^{-1}) of mycotoxins and derivatives in the five food products collected in 2010 and 2011. The values followed by a different letter are significantly different at α = 0.05/10 (with 10 being the number of pair wise comparisons between the 5 food products) 110

Table 3.3 Mean and maximum concentrations (µg kg^{-1}) of parent mycotoxins and derivatives and mean percentages (%) of (masked) derivatives in the compound feeds 118

Table 3.4 Concentrations (µg kg^{-1}) of parent mycotoxins and derivatives in the feed materials 119

Table 3.5 Incidence (minimum, mean, median and maximum, µg kg^{-1}) of the different mycotoxins in the maize field trial in Bottelare 122

CHAPTER 4

DIETARY EXPOSURE TO MYCOTOXINS AND THEIR MASKED FORMS THROUGH CEREAL-BASED FOODS AND FEED IN BELGIUM

Table 4.1 Concentrations of mycotoxins and their masked forms (mean, standard deviation and maximum) (µg kg^{-1}) in the different food categories analyzed 143

Table 4.2 Deterministic analysis of mycotoxins and their masked forms estimated intake (mean, P95-percentile and maximum) by Belgian adult population from the different food categories (µg kg^{-1} bw day^{-1}) 144

Table 4.3 Best fit distributions, mean, minimum and maximum determined for the upper bound scenario of mycotoxins and their masked forms in the food categories and food intakes further applied for probabilistic calculations 147

Table 4.4 Probabilistic analysis of mycotoxins and their masked forms estimated intake (mean, standard deviation, maximum and percentiles) (µg kg^{-1} bw day^{-1}) by Belgian adult population from the different food categories. Upper bound corresponds to data (< LOD) treated as LOD. 149

Table 4.5 Incidence and mean concentrations (µg kg^{-1}) of parent mycotoxins and derivatives in the compound feeds 155

Table 4.6 Deterministic analysis of mycotoxins and their masked forms estimated intake (mean, P95-percentile and maximum) for the different compound feeds (µg kg^{-1} bw day^{-1}) 158

CHAPTER 5

MYCOTOXIN GLUCOSYLATION IN COMMERCIAL WHEAT VARIETIES: IMPACT ON RESISTANCE TO *FUSARIUM GRAMINEARUM* UNDER LABORATORY AND FIELD CONDITIONS

Table 5.1 Summary of the commercial wheat cultivars used in this study. Experiment 1 is the *in vivo* artificial infection of ears while experiment 2 is the 2-year field trial. 166

Table 5.2 Representation of the commercial and reference wheat cultivars used in each of the separate experiments 166

Table 5.3 Quantitative results of the LC-MS/MS analysis of the grains of artificially inoculated commercial wheat cultivars 170

CHAPTER 6

DETACHED LEAF ASSAY FOR MASKED MYCOTOXIN BIOSYNTHESIS AND SUBSEQUENT ANALYSIS OF (UNKNOWN) CONJUGATES BY (UN)TARGETED LC-MS/MS

Table 6.1 Composition of the Murashige and Skoog medium 187

Table 6.2 Elemental composition search on mass 484.25 (*m/z* 479.25 - 489.25). The bold value indicates the chosen composition of $C_{24}H_{38}O_9N$, namely T-2-toxin 193

Table 6.3 Elemental composition of masses observed for HT-2-3-glucoside, T-2-3-glucoside and T-2-triglucoside 194

FIGURES

INTRODUCTION & OBJECTIVES

Figure I Schematic outline of the research carried out in this PhD-dissertation XIII

CHAPTER 1

GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

Figure 1.1 Basic structure of trichothecenes and their respective structures 5

Figure 1.2 Cascade of trichothecenes in animals: trichothecenes enter mammalian cells via diffusion and bind to the ribosomes, which activates mitogen-activate protein kinases (MAPKs) by phosphorylation. The activation leads to transcriptional activation, mRNA stability and activation of caspases with subsequent chronic and immunotoxic effects (Pestka *et al.*, 2004) 6

Figure 1.3 Basic structure of zearalenone, α-zearalenol and β-zearalenol 7

Figure 1.4 Cascade of zearalenone in animals: zearalenone interacts with the cytosolic estrogen receptor. The mycotoxin passively crosses the cell membrane and binds to the receptor. The formed complex is transferred to the nucleus where it binds to specific nuclear receptors and generate estrogenic responses via gene activation, resulting in the production of mRNA that codes for proteins, normally expressed by the receptor-estrogen complex-binding (Cousin *et al.*, 2005) 8

Figure 1.5 The life cycle of *Fusarium graminearum* (sexual phase, *Gibberella zeae*), causal agent of *Fusarium* head blight on wheat 10

Figure 1.6 Symptomatic outcome of *Fusarium* head blight, *Fusarium* ear rot and *Fusarium* crown rot 11

Figure 1.7 Structurally elucidated masked mycotoxins 14

Figure 1.8 Metabolization products of zearalenone in *Arabidopsis thaliana* (Berthiller *et al.*, 2006) 18

Figure 1.9 Determination of the uncertainty of a process with parameters as accuracy and precision 31

Figure 1.10 Operation Characteristics (OC) curve for the acceptance probability of mycotoxin sampling in a bulk lot (*e.g.* guidance value ZEN, 100 µg kg^{-1}), defined with possible outcomes when lots are classified as accepted or rejected 33

Figure 1.11 The sequence of ionized ions in the Quattro Premier XE® MS/MS 39

Figure 1.12 Fragmentation patterns of trichothecenes, example of deoxynivalenol (1) (Berger *et al.*, 1999) and T-2-toxin (2) 44

Figure 1.13 Fragmentation patterns of myco-estrogens (Filho *et al.*, 1997) 44

Figure 1.14 Linear regression equation derived using ELISA and LC–MS/MS data for ZEN-14G screening in maize samples (*n=5*) 50

Figure 1.15 Overview of possible sources of chemical hazards in the agricultural food chain 57

Figure 1.16 Schematic framework of the Codex for risk analysis (Codex, 2013) 57

Figure 1.17 Possible mycotoxin reduction key-points *from farm to fork* 66

CHAPTER 2

IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE SIMULTANEOUS DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

Figure 2.1 Cone voltage versus intensity (ESI^+ and ESI^-) of DON-3G in a methanol-based mobile phase 81

Figure 2.2 MS spectra for DON-3G in ACN + 0.1 % formic acid: ESI^- en ESI^+ 82

Figure 2.3 Spectra for product ions of DON-3G: protonated adduct of DON-3G and ammonium adduct of DON-3G in ESI^+-MS/MS scan 82

Figure 2.4 LC-MS/MS chromatograms of the three HILIC-columns and a ZORBAX SB-C18-column 85

Figure 2.5 LC-MS/MS chromatograms of a spiked wheat sample (100 µg kg^{-1}) in their respective retention windows 86

CHAPTER 3

NATURAL OCCURRENCE OF MYCOTOXINS AND THEIR MASKED FORMS IN FOOD, FEED AND MAIZE: THE ELABORATION OF 3 SURVEYS

Figure 3.1 Occurrence of mycotoxin derivatives in food products per year (% of analyzed samples with mycotoxin derivative concentrations above the limit of detection). Total deoxynivalenol (DON) derivatives: 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and deoxynivalenol-3-glucoside (DON-3G); total zearalenone (ZEN) derivatives: α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalenone-4-glucoside (ZEN-14G), zearalenone-4-sulfate (ZEN-14S), α-zearalenol-4-glucoside (α-ZEL-14G) and β-zearalenol-4-glucoside (β-ZEL-14G). 104

Figure 3.2 Mean concentrations (µg kg^{-1}) of T-2 toxin (T-2) and HT-2 toxin (HT-2) in food commodities per year 105

Figure 3.3 Weather variables for 2010 and 2011 in Belgium (a) and France (b): temperature (°C), relative humidity (%) and rainfall (mm) 112

Figure 3.4 Schematic outline of the Bühler milling machinery 114

Figure 3.5 Contamination levels (µg kg^{-1}) for DON and DON-3G of the different wheat fractions 115

Figure 3.6 Discrepancies between the contamination levels (µg kg^{-1}) of DON and DON-3G in white and grey bread (*n*=4) 115

Figure 3.7 Occurrence of parent mycotoxins and derivatives in piglet feed 119

Figure 3.8 Climatological parameters of the field trial in Bottelare including rainfall (mm), temperature (°C) and relative humidity (%). 123

Figure 3.9 Box plot of the measured mycotoxin contents (µg kg^{-1}) in the different maize varieties in the field trial. Significant differences according to the Wilcoxon signed rank test at α=0.05/10 are indicated with a different letter. The line in the box indicates the median value of the data. The upper edge of the box indicates the 75th percentile of the data, and the lower hinge indicates the 25th percentile. The whiskers extend to a maximum of 1.5 times the interquartile range. The points outside the end of the whiskers describe outliers. DON-derivatives include the sum of DON-3G, 3-ADON and 15-ADON, while ZEN-derivatives comprise the sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G. 124

Figure 3.10 Graphical representation of the Spearman rank correlation coefficients between the different toxins and their masked forms. Correlations higher than 0.165 were significant at α = 0.05. DON-derivatives include the sum of DON-3G, 3-ADON and

15-ADON, while ZEN-derivatives comprise the sum of α-ZEL, β-ZEL, ZEN-14G, ZEN-14S, α-ZEL-14G and β-ZEL-14G. 124

Figure 3.11 Heat map of the deoxynivalenol (DON) content (a) and its derivatives (b) measured in the different varieties and replications in the maize field trial. The contents for DON ranged from <LOD till 5,657 µg $kg^{-1}$and for the derivatives from <LOD till 2,099 µg kg^{-1} 127

Figure 3.12 Heat map of the zearalenone (ZEN) content (a) and its derivatives (b) measured in the different varieties and replications in the maize field trial. The contents for ZEN ranged from <LOD till 15,677 µg kg^{-1} and for the derivatives from <LOD till 9,788 µg kg^{-1} 127

Figure 3.13 Heat map of the HT-2-toxin/T-2-toxin (HT-2/T-2) content measured in the different varieties and replications in the maize field trial. The contents for T-2/HT-2 ranged from <LOD till 103 µg kg^{-1} 128

Figure 3.14 Subdivision of the maize field trial into 6 clusters (K1-K6); K1, K2 and K3 represent replication 1 and 2; K4, K5 and K6 represent replication 3 and 4. A dendrogram expresses the significant differences and correlations of the mycotoxin contamination level of the different clusters. According to a post-hoc Kruskal Wallis test large distances reveal statistical differences, while small alterations comprise non-statistical differences. Different colors indicate significant differences among the plots. 129

CHAPTER 4

DIETARY EXPOSURE TO MYCOTOXINS AND THEIR MASKED FORMS THROUGH CEREAL-BASED FOODS AND FEED IN BELGIUM

Figure 4.1 Distribution curve: consumption in breakfast cereals 148

Figure 4.2 Distribution curve: concentration sum DON-equivalents in breakfast cereals 148

Figure 4.3 Cumulative distribution curve: sum DON-equivalents in breakfast cereals 148

Figure 4.4 Relative contributions of the sum of the DON-equivalents among the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices. 152

Figure 4.5 Relative contributions of T-2 and HT-2 among the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices. 152

Figure 4.6 Relative contributions of the ZEN-equivalents among the six different food categories. The values (%) show the percentages of the examined population which exceeds the tolerable daily intake (TDI) in relative terms with respect to the other matrices. 152

CHAPTER 5

MYCOTOXIN GLUCOSYLATION IN COMMERCIAL WHEAT VARIETIES: IMPACT ON RESISTANCE TO *FUSARIUM GRAMINEARUM* UNDER LABORATORY AND FIELD CONDITIONS

Figure 5.1 *In vivo* DON and DON-3G concentrations (bars) in the grains of five commercial wheat cultivars, after artificial inoculation of the ears with *F. graminearum*. Concentrations of DON and DON-3G were determined after LC-MS/MS analysis and are presented as the means of 3 pooled batches, originating from 10 infected ears per cultivar. Discrete data points represent the glucosylation capacity, calculated as the relative amount of DON that was glucosylated to DON-3G. 169

Figure 5.2 a Relation between DON levels in wheat grains of commercial cultivars and disease index, under *in vivo* laboratory conditions (Pearson correlation coefficient, r = 0.831). The DON content was determined by LC-MS/MS analysis of the grains after artificial inoculation with *F. graminearum*. 171

Figure 5.2 b Relation between DON-3G / TOTAL DON ratio and disease index. DON and DON-3G were determined by LC-MS/MS analysis of the complete grains of five commercial wheat varieties, after artificial *in vivo* inoculation with *F. graminearum*. 171

Figure 5.3 Incidence of 5 *Fusarium* species in Bottelare (Belgium), presented as relative contribution to the other species 172

Figure 5.4 *In planta* DON and DON-3G concentrations (bars) in 8 commercial wheat cultivars in the field, under natural conditions in 2011 and 2012. Concentrations were determined after LC-MS/MS analysis of the grains. Each concentration was calculated as the mean of four replications, with standard errors presented. Discrete data points represent glucosylation capacity, calculated as the relative amount of DON that was glucosylated to DON-3G. 173

Figure 5.5 Relation between DON-3G/TOTAL DON ratio, and disease index for 8 commercial wheat cultivars in the field. Field trials were executed under normal infection. Four replications per cultivar were used, and all 32 data points are plotted on the graph. DON and DON-3G levels were determined after LC-MS/MS analysis of the grains. 173

CHAPTER 6

DETACHED LEAF ASSAY FOR MASKED MYCOTOXIN BIOSYNTHESIS AND SUBSEQUENT ANALYSIS OF (UNKNOWN) CONJUGATES BY (UN)TARGETED LC-MS/MS

Figure 6.1 Structures of HT-2-toxin (left) and T-2- toxin (right) 183

Figure 6.2 Proposed trichothecene biosynthetic pathways illustrating the divergence into the D-type (from isotrichodiol) (orange box) and the A-B-type (from isotrichotriol) (yellow box) trichothecenes. The blue box indicates type A trichothecenes, while the green box indicate type B trichothecenes (Mc Cormick *et al*., 2011). 185

Figure 6.3 Seedlings of *Zea Mays Advanta* 187

Figure 6.4 Production of ZEN-14G in incubated maize stem parts (*n*=3) 191

Figure 6.5 PDA spectrum of ZEN-14G at 254 nm (a) and comparison of the chromatogram of ZEN-14G of the obtained reference standard (b) and the detached leaf procedure (c) 191

Figure 6.6 Spectra for ZEN-14S after single fractionation (a) and multiple fractionation (b) 192

Figure 6.7 Extracted chromatograms of HT-2-3-glucoside (a) and T-2-3-glucoside (b) 195

Figure 6.8 Full scan spectrum of T-2-triglucoside 196

EQUATIONS

CHAPTER 1

GENERAL INTRODUCTION: MYCOTOXINS, MASKED MYCOTOXINS AND RISK ANALYSIS

Equation 1.1 Equation for the determination of the accuracy 31
Equation 1.2 Equation for the determination of the variance 31
Equation 1.3 Equation for the determination of the standard deviation 31
Equation 1.4 Equation for the determination of the variation coefficient 31
Equation 1.5 Calculation of exposure assessment 62

CHAPTER 2

IN-HOUSE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODOLOGY FOR THE SIMULTANEOUS DETERMINATION OF MYCOTOXINS AND THEIR MASKED FORMS

Equation 2.1 Formula apparent recovery 79

CURRICULUM VITAE

Marthe De Boevre was born on February 7th, 1986 in Ghent, Belgium. In 2004 she obtained her diploma with distinction at the Saint-Francis institute (Evergem, Belgium), with Latin and Mathematics as major subjects in the 3rd degree. In 2009 she graduated with distinction in Master in Pharmaceutical Sciences at Ghent University (Ghent, Belgium). The master thesis 'Development and validation of an LC-MS/MS procedure for the determination of fluoroquinolones in eggs' was accomplished at the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium) under supervision of Dr. Els Daeseleire and Prof. dr. Sarah De Saeger.

In August 2009 she was admitted to the Laboratory of Food Analysis (Faculty of Pharmaceutical Sciences, Ghent University, Ghent Belgium) to pursue her PhD studies under promotorship of Prof. dr. Sarah De Saeger and Prof. dr. Carlos Van Peteghem.

The research was funded by the Special Research Fund (BOF) of Ghent University (BOF 05V00709). Results of her research were published in a number of peer-reviewed scientific journals, and were presented during several national and international conferences. She is also reviewer of several A1-peer reviewed journals. During the PhD-dissertation she supervised 4 master thesis students.

SCIENTIFIC OUTPUT

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.

De Boevre, M., Diana di Mavungu, J., Maene, P., Audenaert, K., Deforce, D., Haesaert, G., Eeckhout, M., Callebaut A., Berthiller, F., Van Peteghem, C. & De Saeger, S.

Food Additives and Contaminants 29 (5), 819-835 (2012).

Natural occurrence of mycotoxins and their masked forms in food.

De Boevre, M., Diana di Mavungu, J., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.

Invited paper, special issue on masked mycotoxins, World Mycotoxin Journal 5 (3), 207-219 (2012).

Immunochemical approach for zearalenone-4-glucoside determination.

Beloglazova, N., De Boevre, M., Goryacheva, I. Yu., Werbrouck, S., Guo, Y. & De Saeger, S.

Talanta 106, 422-430 (2013).

Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium.

De Boevre, M., Jacxsens, L., Lachat, C., Eeckhout, M., Diana di Mavungu, J., Audenaert, K., Maene, P., Haesaert, G., Kolsteren, P., De Meulenaer, B. & De Saeger, S.

Toxicology Letters 218, 281-292 (2013).

Mycotoxin glucosylation in commercial wheat varieties: impact on resistance to *Fusarium graminearum* under laboratory and field conditions.

Audenaert, K.[†], De Boevre, M[†], Vanheule, A[†], Callewaert, J, Bekaert, B, De Saeger, S. & Haesaert, G.

Food Control (in press), http://dx.doi.org/10.1016/j.foodcont.2013.06.019 (2013).

A promising technique in sampling: the sampling of wheat dust and subsequent analysis of deoxynivalenol by UPLC-MS/MS.

Sanders, M., De Boevre, M., Dumoulin, F., Detavernier, C., Martens, F., Van Poucke, C., Eeckhout, M. & De Saeger, S.

Journal of Agricultural and Food Chemistry (in press), http://dx.doi.org/10.1021/jf401323s (2013).

Within-field variability of *Fusarium* mycotoxins and their masked forms in maize crops in Belgium.

De Boevre, M., Landschoot, S., Audenaert, K., Maene, P., Diana di Mavungu, J., Eeckhout, M., Haesaert, G. & De Saeger, S.

World Mycotoxin Journal (submitted) (2013).

Occurrence of *Fusarium* species and their associated mycotoxins in unprocessed cereals, food and feed products in Belgium.

Vanheule, A.[†], De Boevre, M.[†], Audenaert, K., Bekaert, B., Höfte, M., De Saeger, S. & Haesaert, G.

Molecular Nutrition & Food Research (submitted) (2013).

Detached leaf assay for masked mycotoxin biosynthesis and subsequent analysis of (unknown) conjugates by (un)targeted LC-MS/MS.

De Boevre, M.†, Vanheule, A.†, Audenaert, K., Bekaert, B., Diana Di Mavungu, J., Vanhaecke, L., Werbrouck, S., Haesaert, G. & De Saeger, S.

Journal of Agricultural and Food Chemistry (submitted) (2013)

ACTIVE CONTRIBUTION

Oral presentations

33rd Mycotoxin Workshop, Freising, Germany (29/30/31/05 and 1/6/2011)

De Boevre, M., Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.†

Facts and figures: masked Fusarium mycotoxins in cereals and cereal-derived food.

Invited speaker at Congress of the *Fusarium* group of the Koninklijke Nederlandse Plantenziektenkundige vereniging, Fungal Biodiversity Centre, Utrecht, The Netherlands (26/10/2011)

De Boevre, M.†, Diana di Mavungu, J., Landschoot, S., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.

Analysis and (natural) occurrence of masked Fusarium mycotoxins.

125th AOAC Annual meeting and Exposition, New Orleans, Louisiana, USA (18/21/09/2011)

De Saeger, S.†, Diana Di Mavungu, J., De Boevre, M., Van Peteghem, C., Audenaert, K., Maene, P., Eeckhout, M., Haesaert, G., Versilovskis, A. & Callebaut, A.

Challenges in masked mycotoxin analysis.

126th AOAC Annual meeting and Exposition, Las Vegas, California, USA (19/23/09/2012)

Diana Di Mavungu, J.†, De Boevre, M. & De Saeger, S.

Analytical methodology and survey results for masked mycotoxins in food and feed.

Scientific Committee of the Federal Agency for the Safety of the Food Chain (Belgium) (23/12/2012)

De Boevre, M.† & De Saeger, S.†

Assessment of chemical risks of foods containing masked Fusarium mycotoxins.

35th Mycotoxin Workshop, Ghent, Belgium (22-25/05/2013)

De Boevre, M.†, Jacxsens, L., Lachat, C., Landschoot, S., Audenaert, K., Eeckhout, M., Diana Di Mavungu, J., Maene, P., Haesaert, G. & De Saeger, S.

Masked mycotoxins: facts and figures 2009-2013.

Poster presentations

Jong KVCV-congres, Blankenberge, Belgium (01/02/03/2010)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., Van Peteghem & De Saeger, S.

Investigation of new analytical challenges for mycotoxin determination: masked mycotoxins.

Sixth International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium (1/4/06/2010)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., Van Peteghem & De Saeger, S.

An update: investigation of new analytical challenges for mycotoxin determination: masked mycotoxins.

MS in food and feed, KVCV, Ghent, Belgium (9/6/2010)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., Van Peteghem & De Saeger, S.

Development and validation of an LC-MS/MS-method for the simultaneous determination of deoxynivalenol, zearalenone, T2-toxin and their masked forms in different cereals and cereal based food.

Exchange: "International Symposium: where industry meets academia", Ghent, Belgium (28/10/2010)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G., Van Peteghem & De Saeger, S.

Masked mycotoxins: an emerging issue for food safety.

Fourth International Symposium: "Mycotoxins: challenges and perspectives", Ghent, Belgium (24/05/2011)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.

Development and validation of an LC-MS/MS-method for the simultaneous determination of deoxynivalenol, zearalenone, T2-toxin and their masked forms in different cereals and cereal based food.

Fourth International Symposium: "Mycotoxins: challenges and perspectives", Ghent, Belgium (24/05/2011)

De Boevre, M.[†], Diana di Mavungu, J., Audenaert, K., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.

Masked Fusarium mycotoxins: results of a one-year survey in Flanders.

World Mycotoxin Forum 2012, Rotterdam, The Netherlands (5-8/11/2012)

De Boevre, M.[†], Landschoot, S., Audenaert, K., Diana di Mavungu, J., Eeckhout, M., Maene, P., Haesaert, G. & De Saeger, S.

Masked Fusarium mycotoxins under natural infection conditions.

TUTORSHIP MASTER THESES

2009-2010

Bieke Loosveldt

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Promotor: Prof. Dr. C. Van Peteghem

Ontwikkeling en validatie van een vloeistofchromatografische-massaspectrometrische methode voor de simultane bepaling van vrije en gemaskeerde mycotoxinen in maïs

2010-2011

Hadewich Lempens

Faculty of Applied Bioscience Engineering, University College Ghent, Ghent, Belgium

Promotor: Ir. P. Maene

Onderzoek naar het voorkomen van gemaskeerde mycotoxinen in graan en graangebaseerde voeding met behulp van LC-MS/MS

Stefanie Depoorter

Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

Promotor: Prof. Dr. S. De Saeger

Untargeted analyse van gemaskeerde mycotoxinen met behulp van UHPLC-Orbitrap HRMS

2011-2012

Simona Giampieri

Facoltà di Farmacia, Università degli studi di Camerino, Camerino, Italy

Promoters: Prof. Dr. S. Vittori and Prof. Dr. S. De Saeger

La presenza naturale die micotossine mascherate nel mais e negli alimenti a base di cereali - Natural occurrence of masked mycotoxins in maize and cereal-based food

ACKNOWLEDGEMENTS

Bij het behalen van mijn diploma als apotheker, benadrukte Prof. dr. J.-P. Remon dat doctoreren geen *9 to 5 job* is. Inderdaad, een doctoraat wordt je niet op een schaaltje gepresenteerd en kost je veel zweet en tranen, echter deze zaken vervagen en het is dan ook met een enorme fierheid dat ik u dit doctoraat voorstel.

Gedurende mijn masterthesis in het ILVO te Melle, waren het Els, Luc, Petra en Patricia die me de zin in onderzoek hebben gegeven. Mede door hun enthousiasme, begeleiding en motivatie, werd mij een doctoraat voorgesteld in het Laboratorium voor Bromatologie. Bij deze neem ik de kans om enkele mensen te bedanken die me gedurende 4 jaar met raad en daad hebben bijgestaan.

Professor Van Peteghem, bedankt om mij de kans te geven te starten als doctoraatstudent in uw labo. Uw jarenlange ervaring, opmerkzaamheid en kritische geest hebben mijn onderzoekswerk gedurende de eerste jaren positief beïnvloed. Ik heb kunnen meegenieten van uw laatste jaren als prof, waarbij uw afscheid mij niet onbewogen liet.

Professor De Saeger, Sarah, je hebt van mycotoxinen jouw levenswerk gemaakt waarbij we nu tot de wereldtop behoren in de onderzoekswereld. Je benadrukt steeds het feit dat jouw team je zo ver brengt, maar op elk schip staat een bekwame kapitein die alles in de goede banen leidt. Bedankt voor alle kansen die je me hebt gegeven en de continue appreciatie en positivisme die je me gaf tijdens mijn werk.

Een schip heeft veel matrozen nodig, de een al wat meer dan de ander, maar voor mij zijn er twee *anciens* die extra in de verf dienen gezet te worden. Christ'l en Annie, naast de motor van het schip, zijn jullie twee vrouwen met ballen. Jullie zijn een voorbeeld voor velen!

Christof, ze zeggen wel eens dat iedereen kan gemist worden op de werkvloer, maar ik vrees dat dat bij jou niet het geval is. Jouw ervaring en insteken hebben me in deze 4 jaar geholpen. Bedankt om in de examencommissie te willen zetelen; het is nog maar jouw tweede keer maar uit de grond van mijn hart hoop ik dat er nog meer volgen!

Ellen en Melanie, jullie lijden net als mij aan een eindeloze perfectiedrang: dat kan negatief maar meestal zeer positief uitdraaien! Ik ben er zeker van dat jullie de komende jaren het tot een succes gaan brengen. *Carpe diem* (!) en weet dat jullie op mij kunnen rekenen.

Jeroen, als assistent alleen is het soms niet gemakkelijk: er wordt veel van jou verwacht en alles blijkt evident te zijn. Ik wens je veel succes om het *Alternaria* verhaal tot een mooi einde te brengen.

Gilke, go for it!

PJ, je bent een echte onderzoeker: kritische focus op die ene onderzoeksvraag en weg ben je (naar Zweden)! Veel succes!

Mario en Fré, service work wordt door jullie gedragen! Jullie continue gedrevenheid om de stalen te verwerken en hulp bij acute vragen of problemen, daar kunnen veel personen een voorbeeld aan nemen.

Emmanuel, Natalia, José, Svetlana, Pratheeba, Marianne, Tom, Johan en Kelly, bedankt om op de een of andere manier te hebben bijgedragen tot het welslagen van mijn doctoraat.

Vele ex-collega's, Ilse, Sofie en David verdienen ook een vernoeming in mijn bedankingswoord.

Een bijzondere merci gaat uit naar mijn stagiairs: zonder jullie harde werk en input zou dit doctoraat er heel anders hebben uitgezien! Ik wens jullie al het beste in de toekomst, Bieke, Hadewich, Stefanie en Simona!

MYCOMASK is het eerste onderzoeksproject van de associatieonderzoeksgroep MYTOX dat een doctoraatsproefschrift tot resultaat heeft. Deze interdisciplinaire instelling heeft ervoor gezorgd dat uit alle hoeken hulp en expertise werd geleverd om mijn doctoraat te vervolledigen. Hier ben ik fier op!

Een speciale dank gaat uit naar Prof. dr. Geert Haesaert en Prof. dr. Mia Eeckhout. Jullie input tijdens de vele begeleidingscomités was onontbeerlijk! Bedankt!

Achter elke sterke man/vrouw, staat een ijzersterk team. Dr. Audenaert, Kris, in 4 jaar heb ik nooit iemand zo competent als jou ontmoet: jouw continue inzet om alles tot een hoger niveau te brengen, de manier waarop jij jouw doctorandi begeleidt, het niet beroerd zijn om zelf de handen uit de mouwen te slaan...dit is heel bewonderenswaardig! Peter, jouw gedrevenheid, passie in het onderzoek en recht door zee-mentaliteit maakte het voor mij ontzettend aangenaam om met jou samen te werken. Sofie, door jouw statistisch denkvermogen, jouw accuraatheid en opmerkelijke snelheid maak je het voor iedereen een enorm plezier om met jou samen te werken. Adriaan, ik ben er zeker van: je wordt een expert in jouw vakgebied! Doe zo voort! Boris, alle technische ondersteuning kan jij aan.

Prof. dr. Stefaan Werbrouck, bedankt voor het inoculeren van de maïsplantjes en het intreden tot de MYTOX-groep. Jouw expertise zal nog veel nodig zijn.

Een speciale vermelding voor Liesbeth en Carl van de onderzoeksgroep van Prof. dr. De Meulenaer: *Chapter 4* is mede dankzij jullie ontstaan.

Dr. Callebaut, Fons, bedankt voor de nuttige en kritische insteken in het MYCOMASK-project.

Bedankt aan de volledige lees -en examencommissie die met een kritische blik mijn doctoraat hebben bestudeerd en hopelijk hebben geapprecieerd.

Dr. Dall'Asta, it was an honour to welcome you at Ghent University, and thank you for all comments related to my PhD!

Thank you, Dr. Berthiller, for the reference standards of zearalenone-derivatives; without these I would not have been able to fulfill my research work.

Bedankt aan Mevr. Christine Vinckx en Mevr. Ria Nouwen van de Federale Overheidsdienst voor de Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu voor enerzijds de financiële steun aan het MYCOMASK-project en anderzijds voor de belangrijke contributies gedurende de begeleidingscomités.

Bedankt Marc voor de kritische blik op mijn doctoraat en de vele hulp!

Bedankt aan al mijn fantastische vrienden, familie, mijn schoonouders Gerrit en Brigitte, schoonzusjes Maud en Gaëlle, schoonbroer Loïc en Ainur om steeds voor mij klaar te staan.

Uit het diepste van mijn hart bedank ik mijn ouders en mijn zus Manon voor de onvoorwaardelijke steun.

Thibault, bedankt voor alles. La vie en rose!

Marthe