DISPOSITION OF 3- AND 15-ACETYL-DEOXYNIVALENOL AND DEOXYNIVALENOL-3-β-D-GLUCOSIDE IN BROILER CHICKENS AND PIGS

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“The great aim of education is not knowledge but action”

H. Spencer

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LIST OF ABBREVIATIONS

15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivalenol
ADME	absorption, distribution, metabolism, excretion
ADONs	acetylated forms of deoxynivalenol
AF	aflatoxine
ATA	alimentary toxic aleukia
ATP	adenosine triphosphate
AUC	area under the plasma concentration-time curve
BBB	blood brain barrier
BC	before Christ
BioADON	absorbed fraction that enters systemic circulation as ADON
bw	body weight
Caco-2	human colonic adenocarcinoma cell line
CD_{xx}	xx% cytotoxic dose
CEC	Commission of the European Communities
Cl	clearance
C_{max}	maximal plasma concentration
DEQ	deoxynivalenol equivalent (see also DONeq)
DNA	deoxyribonucleic acid
DOM-1	de-epoxy-deoxynivalenol
DON	deoxynivalenol
DON15GlcA	deoxynivalenol-15-β-D-glucuronide
DON3G	deoxynivalenol-3-β-D-glucoside
DON3GlcA	deoxynivalenol-3-β-D-glucuronide
DON3S	deoxynivalenol-3-α-sulfate
DONeq	deoxynivalenol equivalent (see also DEQ)
DON-GlcA	deoxynivalenol-glucuronide
EC	European Commission
EC_{xx}	effective concentration inhibiting cell viability by xx%
EFSA	European Food Safety Authority
ERK	extracellular signal-regulated kinases
ESI	electrospray ionisation
EtOH	ethanol
F	absolute oral bioavailability (%)
FB	fumonisine B
FHB	*Fusarium* head blight
FITC	fluorescein isothiocyanate
FRAC	absorbed fraction of the administered dose
FUS-X	fusarenon-X
GAP	Good Agricultural Practices
GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal
gof	goodness-of-fit coefficient
GSTs	glutathione S-transferases

Hck	hematopoietic cell kinase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HRMS	high resolution mass spectrometry
HT-2	HT-2 toxin
HT2-G	HT-2 toxin-glucoside
i.d.	internal diameter
IARC	International Agency for Research on Cancer
IC_{50}	half maximal inhibitory concentration
IEC	intestinal epithelial cells
IL	interleukin
ILSI	International Life Science Institute
IP	intraperitoneal
IPEC-1	porcine intestinal epithelial cell line
IPEC-J2	porcine intestinal epithelial cell line derived from the jejunum
IV	intravenous
ka	absorption rate constant
ke	elimination rate constant
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD_{xx}	median lethal dose for xx% of subjects
LOD	limit of detection
LOQ	limit of quantitation
MAPK	mitogen-activated protein kinases
MeOH	methanol
MM	molecular mass
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/A	not available
NIV	nivalenol
NK	natural killer
OTA	ochratoxine A
p.a.	post administration
PBS	phosphate buffered saline
P-gp	P-glycoprotein
PI	propidium iodide
PKR	double-stranded RNA-activated protein kinase
PO	*per os*
POMC	proopiomelanocortin
Pres.Hydr.	presystemic hydrolysis
PS	phosphatidylserine
pTDI	provisional tolerable daily intake
Q	intercompartmental flow
r	correlation coefficient
R_A	apparent recovery
R_E	extraction recovery
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviation

SC	subcutaneous
SCF	Scientific Committee for Food
SD	standard deviation
SSE	signal suppression/enhancement
$t_{1/2el}$	plasma elimination half-life
T-2	T-2 toxin
T2-G	T-2 toxin-glucoside
TDI	tolerable daily intake
Th	T helper cell
t_{max}	time to maximum plasma concentration
TNF-α	tumour necrosis factor-α
UGTs	UDP-glucuronosyltransferases
USA	United States of America
USSR	Union of Soviet Socialist Republics
Vc	central volume of distribution
Vd	volume of distribution
Vp	peripheral volume of distribution
ZEN	zearalenone
ZEN14G	zearalenone-14-glucoside
ZEN14S	zearalenone-14-sulfate
ZEN16G	zearalenone-16-glucoside
α-ZEL	α-zearalenol
β-ZEL	β-zearalenol

GENERAL INTRODUCTION

Based on:

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

1. Introduction

1.1. The mycotoxin problem

Mankind has long been aware of the hazards related to fungi and their toxins, as shown by the book of Leviticus, The Bible: “The priest is to order the house to be emptied before he goes in to examine the mould … He is to examine the mould on the walls, and if it has greenish or reddish depressions that appear to be deeper than the surface of the wall, the priest shall go out the doorway of the house and close it up for seven days” (Moses definitely BC). Despite of this millennia-old knowledge, the compounds causing these toxic effects have only recently been discovered and characterised.

The problem remained unidentified up until the year 1960 when more than 100,000 turkeys mysteriously died in England, together with domesticated birds, by an affliction that was called Turkey-X disease. The cause of the disease was traced to a company called ‘Oil Cake Mills’, which produced feed for turkeys. After extensive research, the source of toxicity was identified as a fungus called *Aspergillus flavus*, which had infected the peanut supply of the company. The compound causing Turkey-X was isolated from the fungus and named ‘aflatoxin’, after *A. flavus* and toxin (Cole 1986; Spensley 1963).

The toxic agents produced by fungi, such as aflatoxins, are secondary metabolites, *i.e.* organic compounds which have no direct role in major metabolic pathways of the fungus. Fungi can produce an immense variety of secondary metabolites. From an evolutionary point of view their advantage is to discourage predators to consume the fungi or to suppress competition. Some of these fungi are known to have a great importance and applicability (antibiotics, cheese production, etc...), but others, like the producers of aflatoxins, are known to exhibit major toxic effects in humans as well as in animals. The poisonous compounds they produce are called mycotoxins (from Greek μύκης (mykes, mukos) ‘fungus’ and Latin (toxicum) ‘poison’) (Keller et al. 2005).

The unmistakable toxicity of these mycotoxins can be demonstrated by their use in biological warfare throughout history. The most notorious example was during the Vietnam War with the release of concentrated T-2 toxin (T-2) trichothecene mycotoxin over remote jungle areas in Laos. This caused over 6,300 deaths between 1975 and 1981. These biological

attacks were named ‘Yellow Rain’ because of the eyewitnesses descriptions of a yellow oily liquid being released from low flying aircrafts (Ashton et al. 1983; Spyker and Spyker 1983).

In retrospect, mycotoxins have been proven or are suspected to be responsible for several diseases. The very important role of *Fusarium spp.* as mycotoxin producers appears to have remained largely unknown until the 1970s. Research has now unequivocally established the role of *Fusarium spp.* as the cause of alimentary toxic aleukia (ATA), previously known as human mycotoxicosis epidemic, an affliction which caused the death of an estimated 100,000 people between 1942 and 1948 in the Union of Soviet Socialist Republics (USSR). Next to ATA, Akakabi poisoning in Japan and mouldy corn disease in the United States of America (USA) are presumably caused by intake of trichothecenes of *Fusarium* species as well (Beardall and Miller 1994; Pitt 2000; Ueno et al. 1973; Yagen and Joffe 1976).

In spite of the increasing awareness of mycotoxigenic fungi and the mycotoxins they produce, this problem is very much a contemporary issue. In the 1940s up to 1960s a Green Revolution attempted to tackle famine and starvation by means of the development of high-yielding varieties of cereal grains, expansion of irrigation infrastructure, modernisation of management techniques and distribution of hybridised seeds, synthetic fertilizers, and pesticides to farmers. Following this Green Revolution, much of the world's wheat crops are genetically very similar. This has led to concerns about the susceptibility of the food supply to pathogens that cannot be controlled by agrochemicals, as well as the permanent loss of many valuable genetic traits bred into traditional varieties over thousands of years. This knowledge together with the long storage times associated with an industrialised food industry, thereby possibly increasing further fungal spread and contamination, creates a potential impact of pathogens on agricultural production and food safety worldwide (Conway and Barbier 1988; Shiva 1991; Yapa 1993).

The total number of mycotoxins is unknown, but it is estimated that several thousands of fungal metabolites exist. Mycotoxins that frequently occur and have pronounced toxic effects are aflatoxins, trichothecenes, fumonisins, zearalenone (ZEN), ochratoxins and ergot alkaloids (Richard, 2003), see **Figure 1**.

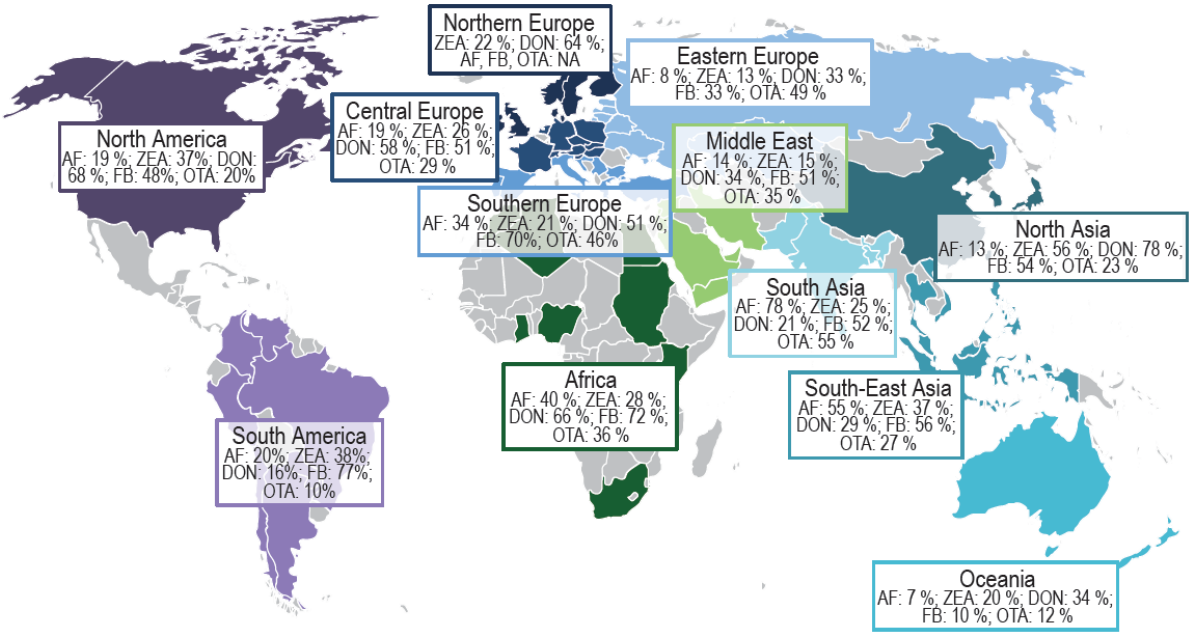


Figure 1. *Global mycotoxin prevalence in surveyed regions. Finished feed and maize accounted for 27% of the samples each. The pool of samples further comprised wheat and wheat bran (9%), barley (8%), silage (8%), soybean meal (4%), distillers dried grain with solubles (DDGS; 2%), corn gluten meal (1%), rice and rice bran (1%), straw (1%) and other feed ingredients (e.g. cotton seed, sorghum, cassava, peanut, copra, etc.; 12%). Number of samples analysed for aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB), ochratoxin A (OTA), respectively: North America: 812; 832; 844; 820; 265; South America: 1,521; 784; 768; 1544; 360; Northern Europe (ZEA; DON): 596; 789; others not analysed (NA); Central Europe: 241; 3,632; 5,521; 206; 235; Southern Europe: 299; 381; 463; 233; 242; Africa: 302; 227; 286; 271; 47; Eastern Europe: 59; 106; 111; 70; 86; Middle East: 167; 172; 170; 156; 69; South Asia: 495; 489; 478; 486; 433; South-East Asia: 2,383; 2,350; 2,237; 2,357; 1,623; Oceania: 859; 873; 873; 842; 681; North Asia: 4,723; 4,799; 4,855; 4,365; 3,352. Adopted from (Schatzmayr and Streit 2013).*

Figure 2. *From left to right wheat increasingly affected by* Fusarium *Head Blight (FHB).*

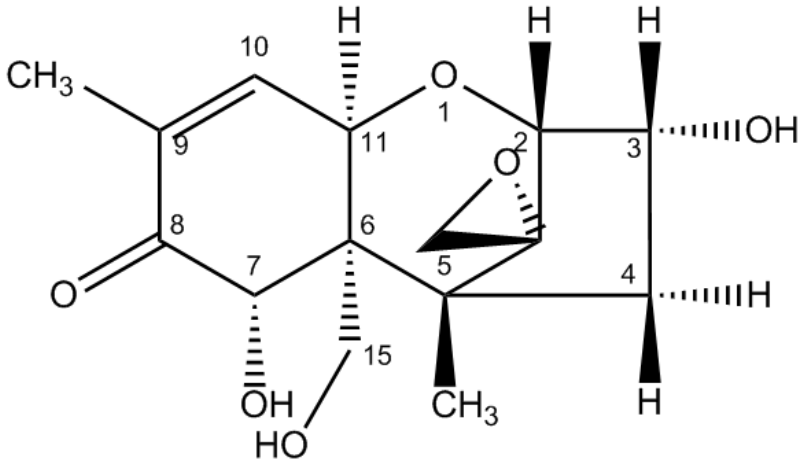


Figure 3. *Chemical structure of deoxynivalenol (DON), a trichothecene mycotoxin mainly produced by* Fusarium *fungi.*

In Northern and Central Europe, as well as in North America, North Asia and Oceania, deoxynivalenol (DON) is the most prevalent mycotoxin with pronounced toxic effects. Consequently, the focus of this thesis will be on the trichothecene DON, produced mainly by *Fusarium* fungi, responsible for *Fusarium* Head Blight (FHB) in wheat, as depicted in **Figure 2**. The chemical structure of DON is presented in **Figure 3** (Richard 2003; Richard 2007).

1.2. Mycotoxin management

Because of the detrimental effects caused by mycotoxins, a number of preventive strategies have been developed to reduce the growth of mycotoxigenic fungi and mycotoxin production (pre- and post-harvest strategies), to detoxify contaminated feed and to lower the systemic availability once mycotoxins are ingested by the animal (mycotoxin detoxifying agents).

1.2.1. Pre- and post-harvest strategies

The **pre-harvest methods** correspond largely to the implementation of good agricultural practice (GAP). Drought, insect infestation, primary inoculum and delayed harvesting are important external factors that contribute to fungal contamination. Some of these factors are environmental and difficult to control. However, GAP measures such as cultivar/variety choice, crop rotation, crop residue management, fungicide usage, minimizing animal and mechanical damage, irrigation, and harvest and storage conditions, are preventive actions

that are regarded as primary mechanisms to reduce *Fusarium* mycotoxins in cereals and cereal products (Bullerman et al. 1984; Edwards 2004; Prandini et al. 2009).

Management at the level of the plant or crop consists of developing mycotoxigenic fungi resistant cultivars, one of the major goals of numerous breeding programs across the world. Regarding FHB-resistant varieties, reviews are published by Kazan et al. 2012; Kolb et al. 2001; Rudd et al. 2001; Snijders 2004.

Crop rotation focuses on breaking the chain of infectious material, for example by wheat/non-cereal crop rotations. Including maize in the rotation should be avoided, as it is very susceptible to *Fusarium* spp. infestations. Crop residue management consists of destruction, removal or burial of the infected crop, thereby reducing the *Fusarium* inoculum for the following crop. The benefits of such crop practices have been demonstrated by several research groups (Dill-Macky and Jones 2000; Obst et al. 1997; Schaafsma et al. 2001).

In the field, there is conflicting evidence as to the ability of fungicides to reduce FHB and to reduce contamination by *Fusarium* mycotoxins. The triazole fungicides, e.g. metconazole and tebuconazole, are active against *Fusarium* species. Correctly timed and dosed these fungicides result in a reduction of FHB and DON contamination (Edwards et al. 2001; Jennings et al. 2000). However, a number of fungicides at sub-lethal concentrations stimulate mycotoxin production *in vitro* (D'Mello et al. 1998; Matthies et al. 1999). Field trials have proven that application of other fungicides (e.g. azoxystrobin) can result in a reduction in FHB, but in an increase in DON contamination (Simpson et al. 2001). Promising results have been published in the field of biological control of *Fusarium* spread. The application of non-mycotoxigenic fungi or other microorganisms allows active competition with fungal pathogens for space and nutrients from the host plant (Diamond and Cooke 2003; Schisler et al. 2002a; Schisler et al. 2002b).

Compromised kernel integrity is another predisposing factor to fungal invasion. Appropriate use of insecticides combined with adequate pest control should minimize the damage resulting from insects, rodents and birds. Furthermore, these animals may act as a vector for fungal spores. *Fusarium* species have been readily isolated from a wide range of insects (Miller et al. 1998). Mechanical damage during harvest, handling and processing should be avoided as well (Dill-Macky and Jones 2000; Fandohan et al. 2006; Fiscus et al. 1971).

Reducing plant stress by irrigation is also valuable to prevent fungal infestation. All plants in the field need an adequate water supply, as there are indications that drought stress may compromise host plant defences against pathogens, or possibly lead to increased insect herbivory (Kabak et al. 2006; Miller 2001). However, excess irrigation, such as mist irrigation, during flowering (anthesis) can also make conditions favorable for *Fusarium* infection (Dill-Macky and Jones 2000).

The **post-harvest methods** consist firstly of bulk drying where necessary and separation of diseased from unaffected material (Aldred and Magan 2004; Jard et al. 2011). At present, the use of chemical preservatives in wheat-based food production only becomes important in the later processing stages. However, tests on the use of preservatives in wheat have shown a 90% reduction in DON and nivalenol (NIV) accumulation (Aldred et al. 2008; Cairns and Magan 2003; Hope et al. 2003). The cornerstone of post-harvest mycotoxin management consists of a well-controlled storage, with special regards to aspects such as moisture, temperature, insect and pest control. Although chemical methods are not allowed in the European Union (European Commission 2001), several chemical detoxification methods have also been described. In all cases, they aim to destroy or inactivate mycotoxins by generating non-toxic products but still warrant the nutritional value and technological properties of the food and/or feed. The wide variety of chemical decontamination processes includes radiation, oxidation, reduction, ammonisation, alkalisation, acidification and deamination (Jard et al. 2011; Kabak et al. 2006).

For more information on post-harvest control strategies, following reviews were published: Aldred and Magan 2004; Magan and Aldred 2007; Magan et al. 2010; Mylona et al. 2012.

1.2.2. Mycotoxin detoxifying agents

Despite these strenuous prevention measures, contamination of various agricultural commodities with mycotoxins can never be fully eliminated. Consequently mycotoxin contaminated feed can cause disorders in animals as well as lead to economic losses due to subclinical toxicity.

One strategy for further reducing the exposure to mycotoxins is to decrease their bioavailability by including so called mycotoxin-adsorbing agents or mycotoxin binders in the

compound feed, leading to a reduction of mycotoxin uptake. Mycotoxin-adsorbing agents are large molecular weight compounds that should be able to bind mycotoxins if contaminated feed is consumed, without dissociating in the gastrointestinal (GI) tract of the animal. In this way the toxin-adsorbing agent complex passes through the GI tract and is eliminated via the faeces. Mycotoxin-adsorbing agents can be silica-based inorganic compounds or carbon-based organic polymers (EFSA 2009; Kolosova and Stroka 2011).

Another strategy is the degradation of mycotoxins into non-toxic metabolites by using mycotoxin-biotransforming agents such as bacteria, yeasts, fungi or enzymes (McCormick 2013). Based on a literature review by the European Food Safety Authority (EFSA), the inventory of mycotoxin-adsorbing and mycotoxin-biotransforming agents is summarised in **Table 1** (EFSA 2009). For more information on mycotoxin detoxifying agents, several reviews were published: EFSA 2009; Kolosova and Stroka 2011; McCormick 2013; Devreese et al. 2013.

Table 1. *Overview of different classes and subclasses of mycotoxin detoxifiers (adapted from EFSA 2009 and Devreese et al. 2013).*

Mycotoxin binders	*Inorganic*	*Aluminosilicates*	*Phylosilicates*	*Bentonites* *Montmorillonites* *Hydrated sodium calcium aluminosilicate* *Smectites* *Kaolinites* *Illites*
			Tectosilicates	*Zeolites*
		Activated charcoal		
		Polymers	*Dietary fibre* *Polyvinylpyrrolidone* *Cholestyramine*	
	Organic	*Saccharomyces cerevisiae*	*Live yeast*	
			Yeast cell wall components	*Glucomannans*
		Lactic acid bacteria	*Lactococcus* *Lactobacillus* *Leuconostoc* *Pediococcus*	
Mycotoxin modifiers	*Bacteria*	*Eubacterium BBSH 797* *Nocardia asteroides* *Corynebacterium rubrum* *Mycobacterium fluoranthenivorans* *Rhodococcus erythropolis* *Flavobacterium aurantiacum* *Pseudomonas fluorescens* …		
	Yeasts	*Trichosporon mycotoxinivorans* *Phaffia rhodozyma* *Xanthophyllomyces dendrorhous* *Saccharomyces cerevisiae* …		
	Fungi	*Aspergillus sp.*	*A. flavus* *A. niger*	
		Rhizopus sp.	*R. stolonifer* *R. oryzae* *R. microsporus*	
		Penicillium raistrickii *Exophiala spinifera* *Rhinocladiella atrovirens* …		
	Enzymes	*Epoxidase* *Lactonohydrolase or Lactonase* *Carboxypeptidase A* *α-Chymotrypsin* *Carboxylesterase*		

2. Deoxynivalenol

DON is chemically described as 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-one ($C_{15}H_{20}O_6$, MW: 296.32 g/mol, CAS 51481-10-8) and is stable at high temperatures (stable at 120 °C, moderately stable at 180 °C). It is soluble in water and in some polar solvents (e.g. aqueous methanol, acetonitrile, and ethyl acetate) (EFSA 2004).

DON belongs to the trichothecenes, a group of structurally related compounds with a common tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system. The approximately 170 identified trichothecenes can be classified into four types (A-D) according to their functional hydroxyl and acetoxy side groups (Grove 1988, 1993), as depicted in **Figure 4**. Type A trichothecenes are mainly represented by HT-2 toxin (HT-2) and T-2. Type B is most frequently represented by DON. Types C (crotocin) and D (verrucarin, roridin A, satratoxin H) include some trichothecenes of lesser importance in a food-related context (Barthel et al. 2012; Ueno 1984).

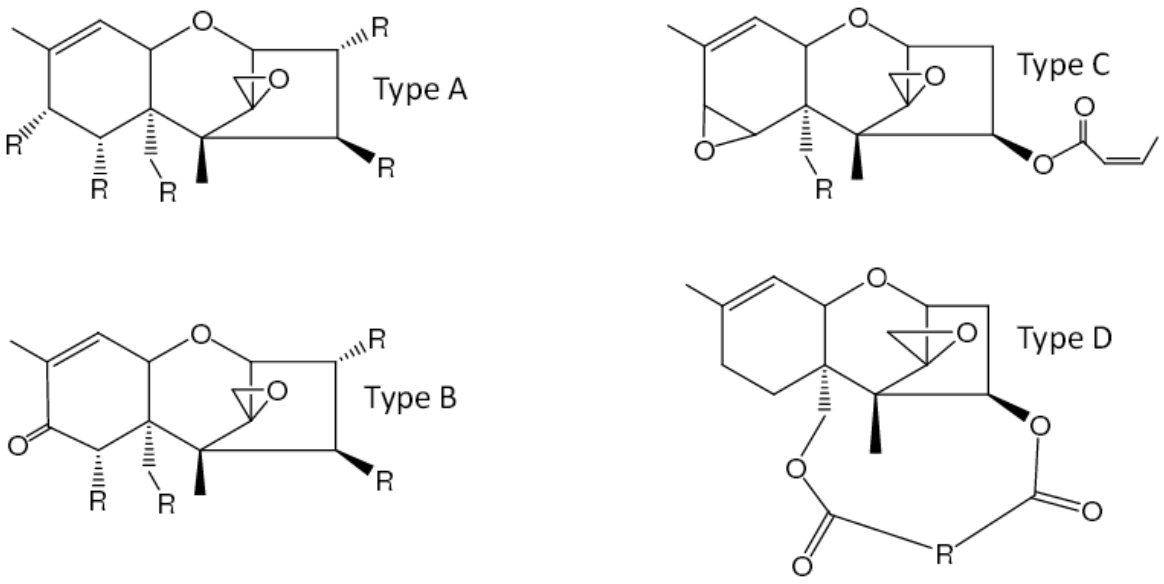


Figure 4. *Classification of trichothecene type structures A-D; R groups may be H, OH, OAcyl, or variations in the macrolide chain (adopted from McCormick et al. 2011).*

2.1. *Fusarium* species

Fusarium spp. belong to the phylum Ascomycota, the largest phylum of the Fungi with over 64,000 species. The defining feature of this fungal group is the 'ascus', a microscopic sexual structure in which non-motile spores, called ascospores, are formed (Walter et al. 2010). A list of the top 10 economically and scientifically important fungal plant pathogens was constructed. The fourth and fifth places comprise two *Fusarium* species, but with contrasting host ranges: with *F. graminearum* causing significant damage mainly to grains and cereals (and few non-cereal species), and *F. oxysporum* having a wide host range, with severe losses in crops as diverse as tomato, cotton and banana (Dean et al. 2012).

The Ascomycota are represented in all land ecosystems worldwide, occurring on all continents including Antarctica. Spores and hyphal fragments are dispersed through the atmosphere and freshwater environments, as well as ocean beaches and tidal zones. The distribution of species is variable; while some are found on all continents, others, as for example the white truffle *Tuber magnatum*, only occur in isolated locations in Italy and Eastern Europe.

F. graminearum, also called *Gibberella zeae* during its sexual reproductive stage, is the most important *Fusarium* species in Europe. *F. graminearum* species complex can be divided into 13 species. These species may be divided into two chemotypes, based on their production of trichothecenes, a NIV- and a DON-chemotype. The latter can be divided into 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) chemotype, producing mainly 3ADON and 15ADON, respectively. The chemotypes have also been called chemotype IA (3ADON), IB (15ADON) and II (NIV). *F. graminearum* can produce, aside from the trichothecenes, another major type of mycotoxin: the estrogenic ZEN (Bily et al. 2004; Geiser et al. 2004; Miller et al. 1991; O'Donnell et al. 2000). Apart from different chemotypes, the mycotoxin production and composition, such as ADON/DON ratio, is also affected by environmental factors, such as temperature, water activity, substrate and competition (Bottalico and Perrone 2002; Logrieco et al. 2002; McMullen et al. 1997; Yli-Mattila 2010).

F. graminearum can infect several plants and agricultural crops such as wheat (*Triticum*), maize (*Zea*), barley (*Hordeum*), rice (*Oryza*) and other small grain cereals (Goswami and Kistler, 2004). Furthermore, *F. graminearum* is the most common causal agent for FHB, a

destructive disease, which affects small grains both in temperate and in semitropical areas. The disease has the capacity to destroy a potentially high-yielding crop within a few weeks of harvest. Nganje et al. (2004) demonstrated the significance of this problem, they estimated the economic losses due to FHB for all crops in the USA from 1998 to 2000 at 2.7 billion US dollars. These small grains (wheat, barley, oats) and maize crops comprise two thirds of cereal supply, which is currently in the order of 350 kg/person/year (Bily et al. 2004; Goswami and Kistler 2004; McMullen et al. 1997; Nganje et al. 2004).

2.2. Occurrence in food and feed

Occurrence surveys of mycotoxins in food and feed matrices have demonstrated that DON is one of the most prevalent food and feed associated mycotoxin, particularly in cereals and cereal-derived products. Due to its widespread occurrence and the potential for economic losses, DON is considered as one of the most important trichothecenes (Streit et al. 2012).

Regarding DON occurrence in individual cereals, wheat has been investigated more widely in comparison with other grains. However, maize shows the highest percentage of positive samples (89%), followed by wheat (61%), rye (50%), barley (47%) and oat (34%). The weighted DON mean ranged from 594 µg/kg for maize to 37 µg/kg for barley, as shown in **Table 2** (Gareis et al. 2003; Marin et al. 2013).

Table 2. *Deoxynivalenol prevalence in cereal samples originating from the European Union (adopted from Gareis et al. 2003).*

Cereal	Samples n	Positive samples n (%)	Mean µg/kg	Maximum µg/kg
Wheat and wheat flour	6,358	3,891 (61%)	205	50,000
Barley	781	367 (47%)	37	619
Oat	595	204 (33%)	95	5,004
Rye and rye flour	271	111 (41%)	42	595
Maize	520	463 (89%)	594	8,850

As DON is mainly present in cereals, pigs and poultry, which have a cereal-based diet, are often exposed to DON. An eight year long (2004-2011) mycotoxin survey in feed and feed raw materials, mainly from Asia (40%) and Europe (38%), showed similar findings to individual cereals. Of all samples (n=15,549), 55% tested positive for DON (n=8,608), with a mean of 535 µg/kg and a maximum of 50.3 mg/kg (Streit et al. 2013a).

2.3. Toxicity and toxicodynamics

2.3.1. Cellular effects

At a cellular level, DON primarily affects protein synthesis. Fast dividing cells such as (intestinal) epithelial cells will consequently be more sensitive to this toxic effect of DON. The cellular toxicity has been linked to the presence of the epoxide moiety (Cundliffe et al. 1974; Pestka et al. 2004; Rotter et al. 1996; Ueno et al. 1973). This group allows DON and trichothecenes in general to bind to ribosomes, and causes 'ribotoxic stress response'. This leads to activation of various protein kinases, modulation of gene expression, inhibition of protein synthesis and cellular toxicity. The absence of an epoxide moiety explains the diminished toxicity of de-epoxy-deoxynivalenol (DOM-1), an *in vivo* metabolite of DON (Pestka et al. 2004a; Pestka 2008, 2010b).

At present, the precise nature of the chemical reactions allowing DON to bind to ribosomal ribonucleic acid (rRNA) are unknown. However, DON is known to bind to the A-site of the peptidyl transferase center of the 60S subunit of ribosomes in eukaryotic cells, which normally forms peptide bonds between adjacent amino acids by means of transfer RNA, and thus interferes with protein translation (Pierron et al. 2015). Probably the chemically very reactive epoxide moiety could react with nucleophile functions present on proteins, DNA and RNA nucleotides, i.e. amine moieties, and on the amine, carboxyl, thiol and hydroxyl groups of amino acids, as depicted in **Figure 5** (Maresca 2013).

The present hypothesis on DON toxicity is that DON binds to rRNA through the interaction of its epoxide moiety leading to the rapid activation of the double-stranded RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck), which in turn activate mitogen-activated protein kinases (MAPK). The type of activated MAPK will depend on DON concentrations. For instance, in macrophages, low doses of DON (nM range) activate preferentially extracellular signal-regulated kinases (ERK), causing cell survival, gene expression and inflammation, whereas high doses of DON (µM) activate p38 leading to apoptosis, rRNA cleavage and protein synthesis inhibition (He et al. 2012a, 2012b).

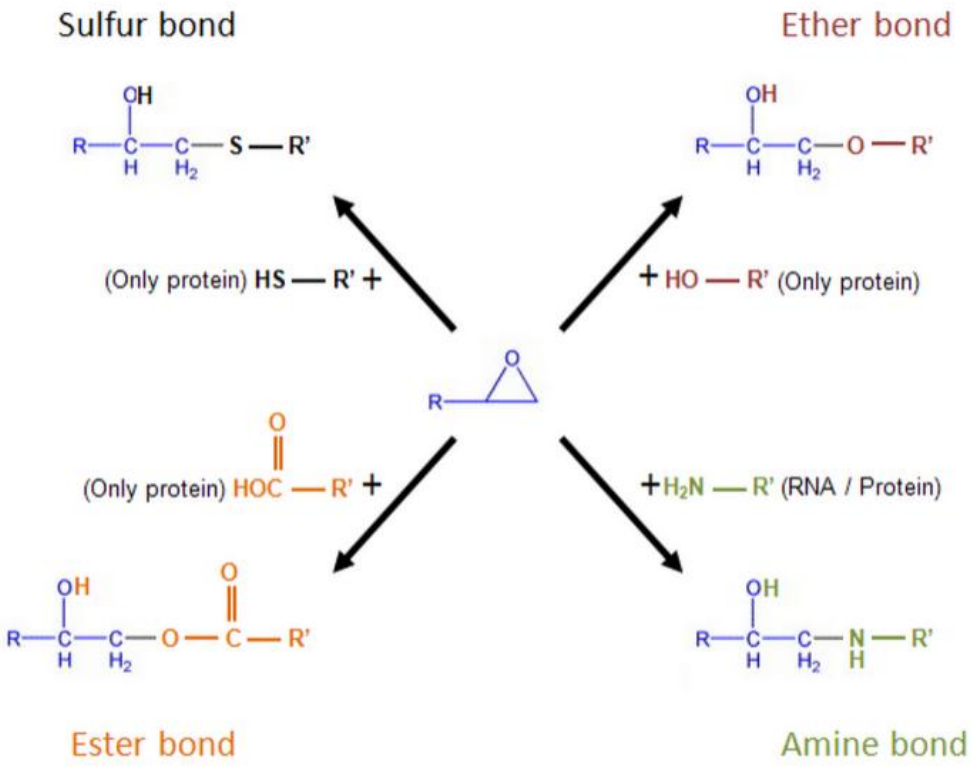


Figure 5. *Chemical reactivity of the epoxide moiety (adopted from Maresca 2013).*

Accordingly, a recent study from Pestka's group demonstrated that DON affects the phosphorylation of 188 proteins, including proteins involved in transcription, epigenetic modulation, cell cycle, RNA processing, translation, ribosome biogenesis, cell differentiation and cytoskeleton organisation (Pan et al. 2013).

2.3.2. Impact on intestinal functions

After ingestion of contaminated food and feed, intestinal epithelial cells (IEC) are the first target of DON. DON affects the proliferation and viability of animal and human IEC. Cell proliferation inhibition in human IEC is observed at low DON doses, with half maximal inhibitory concentration (IC_{50}) in the range of 300 µg/L to 1.5 mg/L. Cytotoxic effects are observed at higher doses (2.5-12 mg/L) (Goossens et al. 2012; Instanes and Hetland 2004; Maresca et al. 2002). Similarly, high doses of DON cause decreased viability and apoptosis in rat and pig IEC. Moreover, toxicity studies on pig IEC show that the status of the cells (undifferentiated versus differentiated) and application site of DON (apical versus basolateral) have a significant effect on the toxicity. Undifferentiated IEC appear to be more

vulnerable and basolaterally applied DON has higher toxicity (Bianco et al. 2012; Diesing et al. 2011a; Diesing et al. 2011b; Vandenbroucke et al. 2011).

Next to inhibition of proliferation and viability of IEC, DON also exerts toxic effects on intestinal functions such as 1) mutagenic effects in enterocytes, 2) modification of the intestinal microbiota, 3) alteration of the mucus composition and production, 4) increase of the transepithelial passage linked to tight junction disruption, 5) modulation of the secretion of proinflammatory cytokines by the intestinal epithelium, 6) increased IgA production, and 7) disruption of the T helper cell (Th) 1/Th 2 immune response balance (Antonissen et al. 2014b; Maresca 2013; Pinton and Oswald 2014).

2.3.3. Immunomodulation

After crossing the intestinal epithelium, the second organ system affected by DON is the immune system. DON may exert immunosuppression or -stimulation depending on the concentration, duration and time of exposure. Both *in vivo* and *in vitro* studies have shown that immune cells, more specifically macrophages, natural killer (NK) cells and lymphocytes, are very sensitive to DON (Escriva et al. 2015; Maresca 2013).

Macrophages are stimulated to secrete inflammatory cytokines interleukin(IL)-1β, IL-2, IL-4, IL-5, IL-6 and tumour necrosis factor-α (TNF-α) and the expression of intracellular proteins involved in the innate immunity in response to low doses of DON (Döll et al. 2009; Pestka et al. 2004; Pestka 2008, 2010a; Sugita-Konishi and Pestka 2001). Simultaneously, low doses of DON also potentiate the stimulatory effects of cytokines/bacterial components on macrophages (Sugiyama et al. 2010). Furthermore, low DON doses also affect the ability to phagocytose and eliminate bacteria, leading to either an increase or a decrease in phagocytosis depending of the type of bacteria (Ayral et al. 1992; Vandenbroucke et al. 2009).

NK cells are an important component of the innate immunity, playing a role in the immune response against tumours and microbial infections (Vivier et al. 2008). Low doses of DON (45-90 µg/L) can inhibit the activity of human NK cells *in vitro,* potentially leading to an increase in the emergence of tumours, in contrast with its International Agency for Research on Cancer (IARC) class 3 classification (Berek et al. 2001).

With regards to lymphocytes, high doses of DON (> 3mg/L) may induce apoptosis of B and T cells. The resulting immunosuppression increases the susceptibility to infections and decreases vaccine efficiency (Antonissen et al. 2014a; Oswald et al. 2005; Pestka et al. 2004; Pestka 2008; Pinton et al. 2008). At lower doses (30-180 µg/L), DON merely suppresses the mitogen-induced proliferation of lymphocytes. Remarkably, at even lower doses (0.3-9 µg/L) DON stimulates the proliferation of lymphocytes (Oswald et al. 2005; Taranu et al. 2010). Furthermore, low doses of DON (µg/L) increase the lymphocytic expression of cytokines such as IL-2, IL-4, IL-6, IL-8 and TNF-α (Meky et al. 2001).

2.3.4. Effects on the neuro-endocrine system

As mentioned before, plasmatic DON is able to cross the blood brain barrier (BBB) (Pestka et al. 2008; Prelusky et al. 1990). When the BBB is crossed, DON can affect the viability and functions of astrocytes and microglial cells *in vitro*. Microglial cells, which are the resident macrophages of the central nervous system, are 100-times more sensitive than astrocytes, with IC_{50} values of 100 µg/L and 10 mg/L, respectively (Razafimanjato et al. 2011). The function of the latter is as biochemical support of endothelial cells but they also ensure provision of nutrients to the nervous tissue and have a role in the repair and scarring process of the brain and spinal cord following traumatic injuries. Astrocytes, when exposed to non-cytotoxic levels of DON, lose their ability to reabsorb the excitatory neurotransmitter glutamate. Inhibition of the glutamate uptake may cause neuronal damage as a consequence of high excitotoxic extracellular glutamate concentrations (Wang and Bordey 2008). Furthermore, the high glutamate concentrations may lead to effects such as an increased brain tumour progression (Varini et al. 2012), pain hypersensitivity (Ren and Dubner 2008) and memory and learning defects (Gibbs et al. 2008).

DON is also referred to as 'vomitoxin', as several *in vivo* studies have demonstrated its emetic (and anorectic) properties in mice and pigs as a consequence of activation of central anorexigenic neurocircuitries, including proopiomelanocortin (POMC) and nesfatin-1 neurons present in areas of the brain responsible for vomiting and feed intake, such as the area postrema, characterized by its extensive vasculature and lack of a normal BBB because its endothelial cells do not contain tight junctions. Consequently, this allows for free

exchange of molecules, such as mycotoxins, between blood and brain tissue (Girardet et al. 2011; Ossenkopp et al. 1994).

In addition to these neural effects, DON also causes endocrine perturbations. It is known to modify the gene expression, viability and synthesis/secretion of steroid hormones by human and animal adrenocortical cells, causing an increase in progesterone and a decrease in testosterone, estradiol and cortisol (Ndossi et al. 2012). In turn, this may potentially lead to reproductive disorders (Kolesarova et al. 2012; Medvedova et al. 2011). Furthermore, DON at nanomolar doses, may lead to growth retardation through inhibition of the growth hormone (Amuzie and Pestka 2010; Voss 2010). Finally, DON increases the secretion of insulin by pancreatic β cells and of hormone peptide YY, both hormones with an anorexic effect (Flannery et al. 2012; Szkudelska et al. 2002). In the pig and poultry industry this may lead to a reduced weight gain and consequently significant economic losses.

2.4. Toxicokinetics

Toxicokinetic studies describe how the animal or human body interacts with a toxin, as a function of dose and time. A number of different models have been developed in order to simplify conceptualisation of the many processes that take place in the interaction between an organism and a xenobiotic. The main processes are commonly referred to as ADME: absorption, distribution, metabolism and excretion. Together with toxicodynamic knowledge, toxicokinetic information is essential to investigate dose-response relations which allow legislative authorities to perform risk assessments and establish guidance values in food and feed.

Most studies have been conducted in laboratory animals and domestic livestock, where interspecies variation in toxicokinetic parameters and susceptibility to trichothecenes has been consistently reported (Sudakin 2003). The susceptibility of animal species to DON can be ranked in the following decreasing order: pigs > mice > rats > poultry ≈ ruminants (Pinton and Oswald 2014). This differential sensitivity may be due to differences in absorption, distribution, metabolism and excretion of DON among animal species. Limited data is available on the toxicokinetics of trichothecenes in humans (Warth et al. 2013). As this thesis will mainly focus on toxicokinetics in broiler chickens and pigs, these species will be discussed in further detail.

2.4.1. Absorption

A key quantitative toxicokinetic parameter to describe absorption is oral bioavailability (F%), a measure for the rate and extent at which an intact mycotoxin reaches the systemic circulation after oral exposure. Absolute oral bioavailability may be calculated based on the ratio between dose-corrected area under the plasma concentration-time curves (AUC) after *per os* (PO) and intravenous (IV) administration. Species-specific differences in oral bioavailability of DON have been demonstrated and can be ranked as follows: pigs (28-139%) > poultry (19-20%) > ruminants (1-10%) (Dänicke and Brezina 2013; Devreese et al. 2015; Osselaere et al. 2013; Prelusky et al. 1986). It needs to be noted that within one species, F may display a large range. Some of the factors influencing this large variation of F within species have been identified. A main contributing factor is acute versus chronic exposure. Chronic feeding of DON to pigs resulted in significantly higher F compared to acute exposure, i.e. single bolus studies (Goyarts and Dänicke 2006), namely 89 versus 54%. This phenomenon can be partly attributed to a compromised function of the intestinal barrier after chronic exposure due to DON mediated influence on tight junctions (Diesing et al. 2011a; Pinton et al. 2010), and by a limited liver clearance capability (Beyer et al. 2010). On the other hand, chronic exposure in broiler chickens tends to decrease oral bioavailability (Osselaere et al. 2012; Yunus et al. 2012). Morphological and functional adaptations, such as decreased villus height, which reduce the absorptive surface of the intestines have been suggested as possible mechanisms for this decrease in F (Devreese et al. 2015; Yunus et al. 2012). The relatively low values for F in chickens can be partially attributed to the short GI transit time in these animals. Short transit time together with a faster absorption are reflected by the time to maximum plasma concentration (t_{max}), in chickens these values vary between 0.4 and 2 h post administration (p.a.), for pigs the t_{max} range is 0.2 – 4.1 h p.a. (Dänicke and Brezina 2013).

2.4.2. Distribution

A quantitative parameter to assess the distribution and tissue penetration of a xenobiotic is the volume of distribution (Vd), a theoretical volume that the total amount of toxin should occupy, to provide a concentration identical to the one in plasma. A large (small) Vd corresponds with a good (limited) tissue distribution. For chickens, a Vd for DON after IV

administration of 4.9±1.2 L/kg has been observed (Osselaere et al. 2013). After a single oral dose of DON to chickens, maximum concentrations were found after 3 h in spleen, liver, heart, kidney, brain and GI tissues. For fat, muscle and oviduct tissues, concentrations were highest at 6 h p.a. Highest concentrations were observed in the liver, kidney and spleen. After 12 days repeated administration of DON, tissue concentrations remained relatively low, indicating a minimal accumulation of DON after chronic exposure (Prelusky et al. 1986). In pigs, Vd values of 1.02-1.62 L/kg (Prelusky and Trenholm 1991) and Vd/F values of 1.19±0.02 L/kg (Devreese et al. 2014) were reported. These relatively high values indicate distribution in total body water and/or in various tissues. Similar to chickens, pigs had the highest DON concentration in kidney tissue, followed by the liver. These high concentrations are probably due to these organs' role in clearing DON from the body (Goyarts and Dänicke 2006; Prelusky et al. 1988). Nonetheless, carry-over of DON from feed into edible tissues of pigs and broiler chickens is regarded as negligible (Dänicke and Brezina 2013).

2.4.3. Elimination

Elimination can be divided into metabolism and excretion. Metabolism describes the biotransformation of DON to certain degradation products by e.g. liver, kidney, intestinal mucosa and GI microbiota. The metabolism of xenobiotics is often divided into two phases. Phase I, modification or transformation, consists mainly of oxidation (e.g. via cytochrome P450), reduction, and hydrolysis reactions. The goal is to convert xenobiotics to more polar (hydrophilic) metabolites. Phase II biotransformation, i.e. conjugation, is comprised mainly of glucuronidation, acetylation, and sulfation reactions in an attempt to convert the modified xenobiotic to more polar (water soluble) metabolites. Excretion is the process of removing a compound and its metabolites from the body.

An *in vivo* metabolite of DON in ruminants is DOM-1 which is facilitated by the rumen microbiota. However, microbial de-epoxidation of DON has also been demonstrated for pigs and poultry. In pigs, de-epoxidation is limited in the proximal part of the GI, increases towards distal parts and reaches nearly 100% in rectal faeces (Dänicke et al. 2004; Eriksen et al. 2002; Kollarczik et al. 1994). In pig blood, the quantity of DOM-1 after DON administration varies between 0-7%. For broiler chickens, no DOM-1 could be detected in blood after single IV and PO administration (Osselaere et al. 2013). In contrast, for turkey

poults chronically administered PO DON, the amount of DOM-1 detected in plasma exceeded that of DON (Devreese et al. 2015). In chicken faeces, recovery as DOM-1 amounted for 7-11% (Dänicke et al. 2013). No DOM-1 was detected in jejunal and ileal content, indicating that the de-epoxidation process is mainly situated at the caecum and colon (Dänicke and Brezina 2013).

The other significant metabolic pathway consists of phase II biotransformation, conjugation with e.g. glucuronic acid. In the case of DON there are three functional groups that can serve as glucuronidation sites, resulting in respectively DON-3-β-D-O-glucuronide (DON3GlcA), DON-7-β-D-O-glucuronide (DON7GlcA) and DON-15-β-D-O-glucuronide (DON15GlcA). *In vitro* incubation tests with liver microsomes demonstrated the species-dependence of the degree and type of hepatic glucuronidation, as shown in **Figure 6**. Pig and chicken liver microsomes both formed DON3GlcA. However, chickens had the lowest glucuronidation capacity of all tested species (human, cow, rat, fish, pig and chicken). Pigs also exhibited a weak glucuronidation activity (Maul et al. 2012). One study demonstrated a complete absence of porcine liver microsomal metabolism of DON. The enzymes necessary for glucuronidation, although predominantly present in the liver, can also be found in tissues such as GI tract, lungs, kidney and skin. On the other hand, after oral administration of DON to pigs, the formed DON3GlcA accounts for approximately 35% (Dänicke et al. 2005). In chickens no DON-glucuronide was formed after IV and PO administration, in contrast to turkey poults (Devreese et al. 2015). Additionally, it needs to be noted that DOM-1 may also be conjugated with glucuronic acid.

Similar to glucuronidation, conjugation with a sulfate molecule may also occur. This has been described for chickens and turkeys, and to a lesser extent for sheep (Devreese et al. 2015; Prelusky et al. 1987; Wan et al. 2013). Deoxynivalenol-3α-sulfate (DON3S) accounted for approximately 88% of the orally administered dose of DON in chickens (Wan et al. 2013). The DON3S/DON ratios after single IV and PO administration to turkeys were 1.3-12.6 and 32.4-140.8, respectively, compared to 243-453 and 1,365-29,624 in broiler chickens (Devreese et al. 2015). Consequently, the possibility to form DON3S has also been suggested as one of the reasons why chickens are less sensitive to DON than several other species.

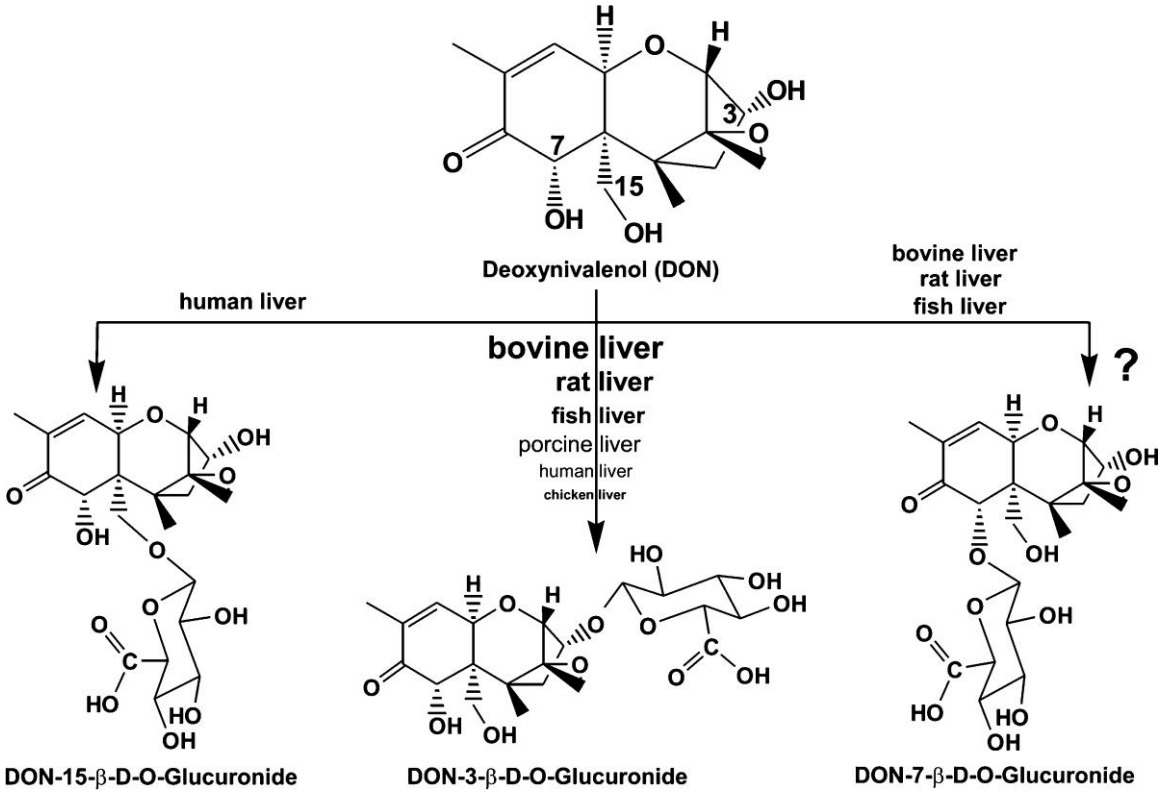


***Figure 6**. Species-dependent hepatic glucuronidation pattern of deoxynivalenol (adopted from Maul et al. 2012).*

Plasma elimination half-life ($t_{1/2el}$) of DON for chickens and pigs is situated between 0.4-6 h and 5-9.8 h, respectively (Dänicke and Brezina 2013; Osselaere et al. 2013). In chickens, DON is rapidly excreted. At 48 h p.a. the DON content in various tissues decreased tenfold, with kidney and GI tract exhibiting the fastest clearance (Cl). Within 48 h, DON was almost completely excreted. Fat tissue, having only a limited blood flow, exhibited the slowest clearance. High concentrations of DON have been detected in bile, exceeding concentrations in plasma, indicating that biliary excretion has a significant role in the elimination of DON (De Baere et al. 2011; Osselaere et al. 2013; Prelusky et al. 1986). Because of the low oral F, rapid and extensive biotransformation, chickens are relatively resistant towards the toxic effects of DON. For pigs, although a fast excretion can also be observed, higher oral bioavailability and a less extensive degree of metabolism make this species less resistant to the detrimental effects of DON.

2.5. Legislation on feed

In 2002, a provisional tolerable daily intake (pTDI) for DON exposure to humans was set by the Scientific Committee for Food (SCF) at 1 µg/kg body weight (bw) per day. Two years later, in 2004, the importance of the mycotoxin DON as a chronic contaminant of feed for farm animals has been recognised by EFSA (EFSA 2004). Based on this EFSA opinion, the guidance values for critical concentrations of DON in feed materials and complete feed were established (European Commission 2006). These guidance values are intended to protect farm animals from possible detrimental effects caused by the consumption of contaminated feed, and to ensure the awareness of all economic parties and supervisory authorities if these critical concentrations are exceeded, in order to identify the sources and take appropriate measures. The DON guidance values for complete feed reflect the species-specific sensitivity. The lowest guidance value of 0.9 mg/kg complete feed was established for all categories of pigs as the most sensitive farm animal species. Calves (<4 months of age), lambs and kids are considered to be protected if complete feed concentrations do not exceed 2 mg/kg, while 5 mg/kg applies for other farm animal species and categories, including ruminants, horses and poultry (**Table 3**).

Table 3. *The guidance values on the presence of deoxynivalenol in products intended for animal feed as determined in the Commission Recommendation of 17 August 2006 (2006/576/EC).*

Products intended for animal feed	**Guidance value in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12%**
Feed materials	
- Cereals and cereal products with the exception of maize by-products	**8**
- Maize by-products	**12**
Complementary and complete feedingstuffs with the exception of:	**5**
- Complementary and complete feedingstuffs for pigs	**0.9**
- Complementary and complete feedingstuffs for calves (<4 months), lambs and kids	**2**

A correct evaluation of mycotoxin contamination in food and feed is of utmost importance in determining the compliance with the guidance values. Because of the often highly heterogeneous distribution of mycotoxins in so-called 'hot-spots' or 'mycotoxin pockets' in feed, the most critical stage is taking representative samples. It needs to be noted that for

field fungi, such as *Fusarium* and *Alternaria* spp., the heterogeneity of distribution is usually of less concern than for storage fungi, such as *Aspergillus* and *Penicillium* spp. Appropriate sampling together with validated analysis is essential to ensure that the analytically derived (mean) concentration of a sample is representative for the true mean concentration of a batch (Baker et al. 2014; Chaytor et al. 2011; Wagner 2015).

3. Acetylated and modified forms of deoxynivalenol

Occasionally, manifested clinical symptoms due to intake of mycotoxin-contaminated feed (mycotoxicosis) are significantly greater than what would be expected based on the feed contamination level. This has led to the discovery of modified or masked mycotoxins, which owe their name to the 'ability' to escape detection by routine analytical methods (Gareis et al. 1990).

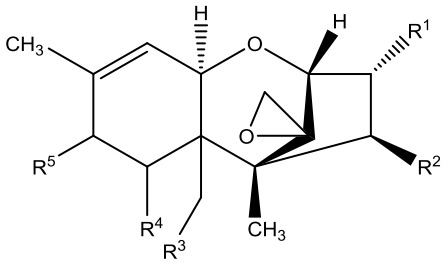
Due to the ambiguous use of the term 'masked mycotoxin', Rychlik et al. have recently systematically defined all modified mycotoxins according to four hierarchic levels, as depicted in **Table 4** (Rychlik et al. 2014). First, a distinction is made between 'free', 'matrix-associated' and 'modified' mycotoxins. Next, modified mycotoxins are divided into 'biologically' and 'chemically' modified derivatives, the latter is subclassified into 'thermally formed' and 'non-thermally formed'. Biologically modified compounds can be further distinguished into 'functionalised', 'conjugated' or 'differently modified'. Finally, for the biologically conjugated mycotoxins a distinction between 'plant', 'animal' and 'fungal' conjugates is made. Some mycotoxins, however, can be classified in more than one of the above defined categories. For instance, 3ADON, which is a precursor in the biosynthesis of DON and occurs as such in contaminated feed commodities, is regarded as a free mycotoxin. However, in an attempt to detoxify DON, plants have been engineered by implementing a 3-O-acetyltransferase, which allows the acetylation of DON to 3ADON, a trait which plants do not possess naturally. *In planta* produced 3ADON should therefore be classified as biologically conjugated by plants, or as a masked mycotoxin. Although these types of plants are not yet commercially available, this has been described for wheat, rice and barley (Karlovsky 2011; Manoharan et al. 2006; Ohsato et al. 2007; Okubara et al. 2002).

In 2002, the presence of a modified mycotoxin, zearalenone-14-glucoside (ZEN14G), was demonstrated for the first time in naturally contaminated wheat (Schneweis et al. 2002). This was an important breakthrough since its presence had only been hypothesised before by *in vitro* studies, with maize suspension cultures (Engelhardt et al. 1988; Zill et al. 1990), and by indirect methods such as enzymatic hydrolysis (Gareis et al. 1990). For DON, the production of modified forms will be highlighted in section 3.1.

Table 4. *Systematic definition of four hierarchic levels for (modified) mycotoxins (adopted from Rychlik et al. 2014); ILSI: International Life Science Institute.*

1st level	2nd level	3rd level	4th level	Example
Free mycotoxins				Deoxynivalenol, 3/15-acetyl-deoxynivalenol
Matrix-associated mycotoxins	Complexes, physically dissolved or trapped			
	Covalently bound			Deoxynivalenol-oligosaccharides
Modified mycotoxins	Biologically modified	Functionalised (phase I metabolites)		Aflatoxin B_1-epoxide
		Conjugated (phase II metabolites)	Conjugated by plants (ILSI: 'masked')	Deoxynivalenol-3-glucoside
			Conjugated by animals	Deoxynivalenol-3/7/15-glucuronide
			Conjugated by fungi	Zearalenone-14-sulfate
		Differently modified		De-epoxy-deoxynivalenol
	Chemically modified	Thermally formed		Nordeoxynivalenol A-C
		Non-thermally formed		Deoxynivalenol-sulfonate, Nordeoxynivalenol A-C (under alkaline conditions)

Despite demonstration of the natural occurrence of modified mycotoxins for over a decade, there are no directives, regulations nor recommendations in food and feed taking these modified derivatives into account. In addition, little is known about the toxicity, toxicokinetics and potential *in vivo* hydrolysis of these modified mycotoxins. The chemical structures of the main modified derivatives of DON are shown in **Figure 7**.



Mycotoxin	R^1	R^2	R^3	R^4	R^5
DON	OH	H	OH	OH	O
DON3G	$C_6H_{11}O_6$	H	OH	OH	O
15ADON	OH	H	OAc	OH	O
3ADON	OAc	H	OH	OH	O
DON3GlcA	$C_6H_9O_7$	H	OH	OH	O
DON15GlcA	OH	H	$C_6H_9O_7$	OH	O

Figure 7. *Structures of DON and its modified forms. OAc= acetoxy group.*

3.1. Production

At the level of food and feed, mycotoxins can be subjected to biological modification through conjugation by plants and fungi, or through chemical modification, either thermally or non-thermally, e.g. by food processing. These modified mycotoxins can contribute to the degree of contamination and may escape detection methods, causing an underestimation of the mycotoxin exposure and risk.

3.1.1. Plant conjugates

Many xenobiotics, among which mycotoxins, are readily absorbed by plants and could accumulate to toxic levels without efficient detoxification. The defense mechanisms of plants include biosynthesis pathways that can be divided into three phases, as depicted in **Figure 8**. During phase I, the transformation or activation phase, xenobiotics are subjected to hydrolysis, reduction or oxidation, resulting in the formation of reactive groups in the chemical structure of the xenobiotic. This phase typically affects lipophilic compounds by making them more hydrophilic. The main goal of these reactions is to create reactive groups necessary for phase II biotransformation. If a xenobiotic already has a reactive or functional group suitable for phase II, then detoxification can omit phase I, which is often the case for more hydrophilic compounds, such as DON. During phase II, also called solubilisation or conjugation phase, (activated) xenobiotics are conjugated with endogenous hydrophilic

molecules such as glutathione, sugars, sulfate or amino acids. The resulting conjugated xenobiotics have an increased polarity and are therefore more water soluble. This facilitates their transport mediated by adenosine triphosphate (ATP)-dependent glutathione-conjugate transporters to the vacuole or apoplastic space outside the cell, also known as compartmentalisation or phase III (Coleman et al. 1997). These conjugated xenobiotics are refrained from interacting with plant components and consequently unable to exert harmful effects to the plant. In contrast to similar phase I and II biotransformation pathways in mammals, residues may persist in plants for considerable periods of time and can have important toxicological consequences for their consumers. Furthermore, after oral intake, some of the conjugates formed in plants could be susceptible to hydrolysis by digestive enzymes upon ingestion, thereby releasing the unconjugated xenobiotic, e.g. free mycotoxins (Sandermann 1992, 1994).

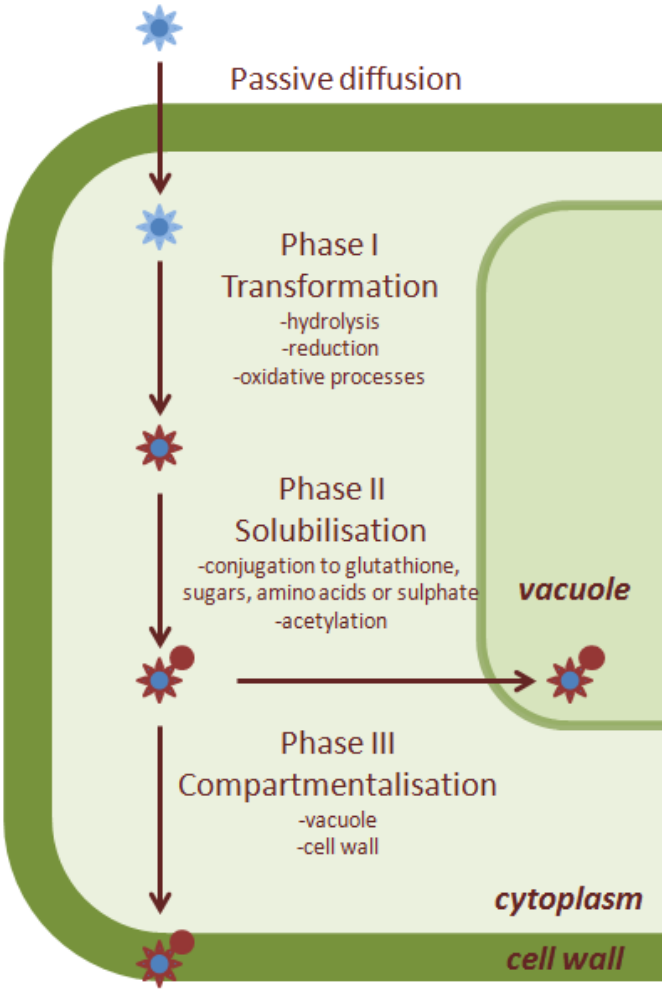


Figure 8. *Three stages of a plant biosynthesis pathway: transformation, solubilisation and compartmentalisation (based on Coleman et al. 1997).*

For mycotoxins, *in planta* transformation has been predominantly described for *Fusarium* toxins (Diana di Mavungu 2011). After the detection of ZEN14G in 2002, Berthiller *et al*. demonstrated, in 2005, the presence of deoxynivalenol-3-β-D-glucoside (DON3G) in wheat (Berthiller et al. 2005). The resistance trait of some FHB resistant wheat cultivars, e.g. Sumai 3 and Nyubai, is *inter alia* caused by a more pronounced ability to detoxify mycotoxins by conjugation, solubilisation and compartmentalisation. However, this appears to be an incomplete story, evidenced by a similar conjugated fraction of total DON in resistant as well as susceptible cultivars. A more efficient mechanism of resistance is caused by lignins (deposition in the secondary cell wall thereby thickening it, reducing pathogen spread), hydroxycinnamic acid amides (acting as phytoalexins and cell wall strengthening agents) and flavonoids (antimicrobial properties and elimination of produced reactive oxygen species) (Gunnaiah and Kushalappa 2014).

Next to the naturally occurring DON3G, DON can be converted to 3ADON by transgenic plants carrying the *FsTRI101* gene. This gene encodes for an enzyme that transfers an acetyl moiety to DON thereby biosynthesising 3ADON. 3ADON can be listed as a modified mycotoxin and is deemed to be less toxic than DON (Kimura et al. 1998; Manoharan et al. 2006; Ohsato et al. 2007). Although currently there are no commercially available plants carrying the *FsTRI101* gene, greenhouse resistance tests have shown that the inoculated wheat heads are partially protected against the spread of *F. graminearum* and its associated FHB disease (Okubara et al. 2002).

Besides ZEN14G and DON3G, also T-2 toxin-glucoside (T2-G) and HT-2 toxin-glucoside (HT2-G) were recently identified and characterised in natural samples by Lattanzio et al. (2012). Based on current literature data, these four modified mycotoxins represent the major occurring plant produced modified mycotoxins in natural samples.

In literature, several other *Fusarium* mycotoxin conjugates have been described, although they have only been reported to occur in artificially contaminated samples and/or are formed in *in vitro* studies. An engineered yeast expressing the *HvUGT14077* gene, encoding for a barley UDP-glucosyltransferase, produced an approximate 1:1 mixture of ZEN14G and zearalenone-16-glucoside (ZEN16G) after incubation with ZEN. *In planta* production of ZEN glucosides has also been investigated for barley seedlings. A significant amount of the

administered dose (66-87%) was recovered in the root extracts. In these extracts, a minimal amount of ZEN was detected next to 3−5% ZEN14G and a 16-18 fold highervlevel of ZEN16G (Kovalsky Paris et al. 2014). Besides barley, the model plant *Arabidopsis thaliana* was able to transform ZEN into 17 different compounds, including glucoside, dihexoside and pentosylhexoside conjugates, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (Berthiller et al. 2006). For T-2 and HT-2, the presence of diglucosides has also been described by Veprikova et al. (2012).

Nakagawa *et al*. reported glucoside forms of other trichothecenes, such as fusarenon-X (FUS-X) and NIV in artificially contaminated wheat (Nakagawa et al. 2013). Furthermore, glycosylation has been described for sporadically occurring trichothecenes such as verrucarin A and roridine A, D and E (Jarvis et al. 1996; Rosso et al. 2000). Next to conjugation to sugar moieties, the covalent attachment of fatty acids, such as palmitic acid is also known to occur for ZEN, T-2 tetraol, scirpentriol and trichothecolone (Chakrabarti and Ghosal 1986).

Although *in planta* conjugation of mycotoxins to amino acids, glutathione, sulfate or fatty acids can occur, these phase II reactions appear to be of subordinate importance compared to glycosylation based on available literature.

3.1.2. Fungal precursors

Both 3ADON and 15ADON are fungal biosynthetic precursors of DON and classified as free mycotoxins. The final step in the biosynthesis of ADONs is the removal of an acetyl group from 3,15-diacetyl-DON at either the C-3 or C-15 position, mediated by an esterase encoded by *TRI8*. Differential activity of this esterase determines production of either 3ADON or 15ADON chemotypes in *F. graminearum* (Alexander et al. 2011; Grove 2007). **Figure 9** represents a schematic overview of the trichothecene biosynthetic pathway in *Fusarium* with known genes included (adopted from McCormick et al. 2011). The genes responsible for deacetylation of the ADONs to DON have not been discovered yet.

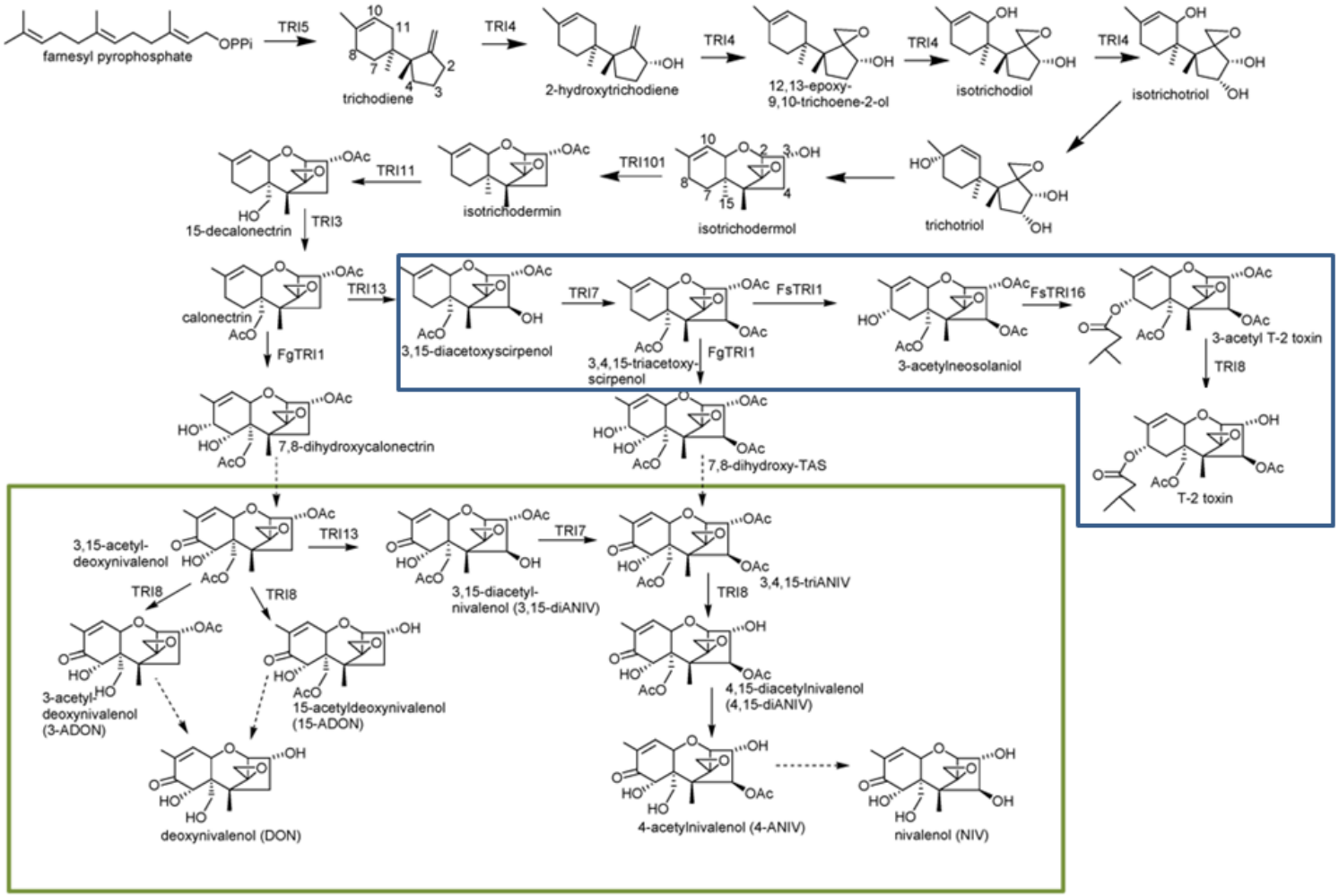


Figure 9. *Proposed trichothecene biosynthetic pathway in* Fusarium*. Identified genes encoding an enzymatic step are shown near the arrow indicating the step. Dashed arrows indicate steps for which a gene has not yet been assigned. Blue and green boxes identify type A and B trichothecenes, respectively (adopted from McCormick et al. 2011).*

Apart from fungal precursors, fungi can also metabolise existing mycotoxins. When fungi capable of producing mycotoxins (mycotoxigenic fungi) occur together with fungi that do not have that capability (non-mycotoxigenic fungi), they actively compete for space and nutrients from the host plant. This can result in a reduction of the growth and metabolism of the mycotoxigenic fungi and consequently a diminished production of mycotoxins. In ecology this is known as the competitive exclusion principle or Gause's law (Bacon et al. 2001). This technique has been used to reduce e.g. *A. flavus* contamination (Cotty and Bayman 1993). Next to this mycotoxigenic fungal growth antagonism, the non-mycotoxigenic fungus may also metabolise mycotoxins produced by the toxigenic fungus. Such metabolism has already been described for ZEN to ZEN14G and zearalenone-14-sulfate (ZEN14S) with the saprophytic *Rhizopus* fungus. This fungus has frequently been found on plants, fruits and vegetables (el-Sharkaway et al. 1991; Kamimura 1986).

To our knowledge, there are only two cases reported in which a fungus itself produces a glucoside-conjugate. Three decades ago, Gorst-Allman *et al.* reported a strain of *F. sambucinum* that produced monoacetoxyscirpenol-4-α-glucopyranoside, exhibiting a decreased toxicity compared to the free mycotoxin (Gorst-Allman et al. 1985). This compound has recently been detected in corn together with the free mycotoxin monoacetoxyscirpenol using high resolution mass spectrometry (HRMS) (Nakagawa et al. 2013). The second case, by Busman et al., reported the ability of liquid cultures of *F. sporotrichioides* to produce T-2, HT-2 and HT2-G, although in low amounts at a ratio of 100, 0.9 and 0.1, respectively (Busman et al. 2011).

3.1.3. Effects of food processing

The influence of food processing on the fate of free mycotoxins in food or feed has been extensively investigated and reviewed (Bullerman and Bianchini 2007; Hazel and Patel 2004; Jackson and Bullerman 1999; Milani and Maleki 2014; Trigo-Stockli 2002). The (thermo)stability of trichothecenes allows them to withstand most food and feed processes (Malachova et al. 2010). In contrast, the fate of acetylated and modified mycotoxins during food processing is considerably less explored. There are indications that food processing plays an important role in the concentration of acetylated and modified forms of DON in certain commodities. The role of food processing in the *de novo* synthesis of compounds appears to be of subordinate importance, with a limited number of reported cases. The

commodities mainly affected are cereal-based products that undergo a fermentation step (introduction of yeast) or a malting process.

One such commodity is beer, which is thoroughly investigated due to its widespread and large scale production. During the malting process, a slight increase of DON3G was observed before germination, in contrast to a distinct decrease in DON and the ADONs. Free mycotoxins, such as DON and the ADONs, are easily extracted by the introduction of water, possibly DON3G resists this extraction due to binding to matrix biopolymers (Lancova et al. 2008a; Schwarz et al. 1995). During germination, levels of free and modified mycotoxins significantly increased with an up to eightfold increase for DON3G. Possible sources are the release of these compounds from their binding to macromolecules or biopolymers. Another possibility is the *de novo* synthesis of DON3G from germinating barley grains (Malachova et al. 2010). Similar to the release from bound forms, a release from acetylated and modified forms could also explain an increased amount of DON.

During drying of the malt, no significant changes took place, either for DON or for the ADONs. For DON3G, variable results were obtained. A decrease was observed for naturally contaminated samples whereas no significant change for artificially contaminated samples was observed (Kostelanska et al. 2011; Lancova et al. 2008a).

As a final step, formed rootlets are removed from the grain. This fraction contained concentrations of DON3G, ADONs and DON which were 100, 4 and 4 times higher, respectively, than the amounts measured in the initial barley grains. These amounts cannot be neglected as this fraction is often used as raw feed material for animals and food supplements for humans.

The obtained malt is then brewed to beer (mashing, boiling and fermentation). During this brewing process, DON3G has been reported to increase up to sixfold (Lancova et al. 2008a). Regarding DON levels, contradictory results have been reported in the literature. Lancova *et al*. and Schwarz *et al*. observed that DON concentrations remained more or less equal. In contrast, Niessen and Donhauser measured a fourfold increase in the concentration of DON during brewing (Lancova et al. 2008a; Niessen and Donhauser 1993; Schwarz et al. 1995). It has been hypothesised by Lancova et al. that the observed large differences between the results of these studies might be due to possible cross-reactions associated with the ELISA

method based on a polyclonal antibody, as was used in the study by Niessen and Donhauser (Lancova et al. 2008a; Niessen and Donhauser 1993). On the other hand, Lancova *et al*. and Schwartz *et al*. employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) as detection methods, respectively, and comparable results were obtained (Schwarz et al. 1995).

The increase of DON3G during the production of beer can be demonstrated by concentrations of DON3G in commercial beers often exceeding that of free DON. Varga *et al.* found a molar DON3G/DON ratio in beer of up to 1.25, whereas the DON3G/DON ratio in cereals is around 0.1 - 0.2 (Malachova et al. 2011; Varga et al. 2013). Concerning the ADONs, only small concentrations were found and no significant changes were observed during brewing (Lancova et al. 2008a; Varga et al. 2013). Interestingly, the production of some DON-oligo-glycosides was demonstrated by Zachariasova *et al*., indicating the role of food processing not only in concentrating already present (modified) mycotoxins but also in the formation of these compounds (Kostelanska et al. 2011; Zachariasova et al. 2012).

Another food commodity frequently contaminated with acetylated and modified mycotoxins is bread. During the processing of wheat to bread, Kostelanska *et al*. described that milling had little influence on the ratio (12%) of DON3G to DON. Due to fractionation, milling decreased the DON3G and DON content in white flour by 40% compared to initial unprocessed wheat (Kostelanska et al. 2011). These findings are supported by a previous study by Schollenberger *et al*., who investigated the fractionation of 16 *Fusarium* mycotoxins during dry milling of maize. It was observed that bran, the hard outer layers of cereal grain which are discarded for the production of white flour, contained the highest concentrations for all tested mycotoxins (Schollenberger et al. 2008).

During kneading, fermenting and proofing, no significant changes occurred for DON, DON3G and 3ADON. However, when bakery improvers, such as enzyme mixtures, were employed as a dough ingredient, a distinct increase of up to 145% of DON3G occurred in fermented dough (Kostelanska et al. 2011; Lancova et al. 2008b; Vaclavikova et al. 2013; Valle-Algarra et al. 2009; Vidal et al. 2014). It is hypothesised that this increase in DON3G is due to a release from starch-based, matrix-bounded forms. According to Kostelanska *et al*. a decrease of 10% and 13% of both DON3G and DON, respectively, took place during baking (240 °C, 14

min) when compared to fermented dough. Valle-Algarra *et al*. baked at 240 °C for 30 min and observed a reduction inside the bread of 46–83% and 43–64% for 3ADON and DON respectively, whereas in the crust an even higher reduction took place (Valle-Algarra et al. 2009).

In conclusion, due to changes in physico-chemical properties (increased polarity, deviating thermostability, etc), acetylated and modified forms of DON may behave differently than DON during food processing. Additionally, a *de novo* synthesis of conjugates is possible at several levels in the processes. First steps towards unravelling the influence of food processing on acetylated and modified mycotoxins have been taken as demonstrated by these publications, but many questions still remain. Questions that not only address the often well regulated and standardised processes of the industry, but also the influence of processes at domestic levels, such as boiling of contaminated pasta or rice and the use of flour in pastries, are currently poorly understood.

3.2. Occurrence in food and feed

To give a concise overview of the current occurrence of DON and its acetylated and modified forms, reports published between 2010 and 2014 were investigated. Only papers regarding unprocessed cereals and those including a random sampling strategy were included. This yielded a total of nine reports on wheat, maize, barley, oat and rye, originating from following countries: Belgium, China, Czech Republic, Denmark, Italy, Nigeria, Norway and Sweden (Adetunji et al. 2014; Bertuzzi et al. 2014; De Boevre et al. 2012; Juan et al. 2013; Malachova et al. 2011; Rasmussen et al. 2012; van der Fels-Klerx et al. 2012; Wei et al. 2012; Zhao et al. 2014). It needs to be noted that the obtained values for incidence (%) and mean concentration (µg/kg) are calculated as the average of the individual publications (without weighing for number of samples within a given study). Range maximum is the largest measured value in all of the studies combined.

As can be seen in **Table 5**, the following acetylated and modified forms of DON were detected in order of decreasing incidence as well as decreasing average concentrations: DON3G (55%, 85 µg/kg), 15ADON (31%, 37 µg/kg) and 3ADON (22%, 15 µg/kg).

Table 5. *Occurrence data for deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-glucoside (DON3G) in unprocessed cereals. The table is based on nine research articles published between 2010-2014.* $n_{samples}$*= total number of unprocessed cereal samples (unprocessed wheat, maize, barley, oat, rye and spelt) analysed for a given compound (countries of origin: Belgium, China, Czech Republic, Denmark, Italy, Nigeria, Norway and Sweden). Incidence (%) and mean (µg/kg) are calculated as the average of the reported values in the individual publications (without weighing for number of samples within a given study). Range maximum is the largest measured value in all of the studies. Based on (Adetunji et al. 2014; Bertuzzi et al. 2014; De Boevre et al. 2012; Juan et al. 2013; Malachova et al. 2011; Rasmussen et al. 2012; van der Fels-Klerx et al. 2012; Wei et al. 2012; Zhao et al. 2014).*

Mycotoxin	$n_{samples}$	Incidence (%)	Mean (µg/kg)	Range maximum (µg/kg)
DON	5,743	84	458	27,088
3ADON	2,227	22	14.7	1,500
15ADON	686	31	36.6	1,734
DON3G	529	55	85.0	1,070

For DON3G, 15ADON and 3ADON, concentrations exceeding 1,000 µg/kg have been reported (Gareis et al. 2003), amounts not to be neglected knowing that the tolerable daily intake (TDI) for DON in humans is 1 µg/kg bw. Sasanya *et al*. detected DON3G concentrations which were higher than those of free DON in some hard red spring wheat samples (Sasanya et al. 2008). The occurrence data for the free mycotoxin DON are in accordance with recently published European studies (Streit et al. 2013a; Streit et al. 2013b).

In conclusion, sufficient attention should be paid to collect data on several commodities (wheat, rye, maize, etc.) as these can exhibit distinct conjugation profiles. Differences due to a geographical spread should also be taken into consideration, mainly to assess the influence of climatological parameters. The discovery of modified mycotoxins coincides with the rapid development of analytical methodologies, as reviewed by following studies: Berthiller et al. 2009; Berthiller et al. 2013; Cirlini et al. 2012; Krska et al. 2008; Li et al. 2013. With the rise of untargeted metabolomic approaches, using HRMS, this will remain a fast evolving domain from which one can expect interesting developments in the near future. The research on toxicity and toxicokinetic data is often unable to follow the fast pace set by the analytical sector.

3.3. Toxicity

One of the major questions associated with the existence of acetylated and modified mycotoxins is what the disposition and fate of these compounds are in the animal and human body? In addition, it has to be taken into account that upon ingestion the acetylated or modified mycotoxins could be converted back into their corresponding unconjugated form after e.g. *in vivo* hydrolysis. Another question, which will be addressed in this section, is what the intrinsic toxicity of the acetylated and modified forms of DON is. An overview of *in vitro* and *in vivo* research on the toxicity of acetylated and modified forms of DON is given in **Table 6**. The majority of this toxicity research is dedicated to human *in vitro* toxicity (59%). Animal and plant toxicity studies account for 35 and 6%, respectively. Mice and pigs are the most investigated animal species. *In vitro* research on trichothecenes is mainly performed using intestinal epithelial cell lines, such as a heterogeneous human epithelial colorectal adenocarcinoma cell line (Caco-2) and intestinal porcine epithelial cell lines (IPEC-1, IPEC-J2). Furthermore, approximately 70% of the studies compare the toxicity of the investigated acetylated and modified mycotoxins with that of the corresponding unconjugated mycotoxin. However, these studies utilize different cell lines, test systems or animal species. Consequently, these differences in study design may result in a broad range of toxicity.

Table 6. *Overview of* in vitro *and* in vivo *toxicity data for 3ADON, 15ADON and DON3G. All* in vitro *data are given in µM with molecular masses of 15ADON 338.4 g/mol; 3ADON 338.4 g/mol; DON3G 458.5 g/mol. Factor fold toxicity=toxicity of the acetylated or modified mycotoxins expressed as times the toxicity of the free mycotoxin; PO=per os; IP=intraperitoneal; SC=subcutaneous; IV=intravenous; bw=body weight; EC_{50}=effective concentrations inhibiting cell viability by 50%; CD_{50}=50% cytotoxic dose; LD_{50}=median lethal dose for 50% of subjects; N/A=not available; >=larger toxicity compared to DON; <=lower toxicity compared to DON.*

Mycotoxin	Cell line/system/ animal species	Exposure dose	Exposure period	Effect	Factor fold toxicity	Reference
15ADON	IPEC-1	0-30 µM	24 h	69% inhibition cell proliferation	>	Pinton et al. 2012
15ADON	IPEC-1	10 µM	48 h	75% reduction of transepithelial electrical resistance	>	Pinton et al. 2012
15ADON	IPEC-1	0-150 µM	24 h	EC_{80} of 10.7 µM	1.6	Alassane-Kpembi et al. 2015
15ADON	Caco-2	0-30 µM	6 h	no significant effect on cell viability	N/A	Kadota et al. 2013
15ADON	Caco-2	3 µM	6 h	damage to tight junctions in luciferase yellow assay	≥	Kadota et al. 2013
15ADON	Caco-2	0-3 µM	72 h	significant dose-dependent increase of IL-8	>	Kadota et al. 2013
15ADON	Caco-2	0-7 µM	48 h	IC_{50} 1.47 (MTT assay)/1.10 (neutral red assay) µM	1.06/0.92	Alassane-Kpembi et al. 2013
15ADON	MIN-GL1/K-562	N/A	N/A	CD_{50} 6 µM/1.2 µM	0.2/0.75	Visconti et al. 1991
15ADON	3T3	0.5-14.8 µM	24 h	50% DNA synthesis inhibition at 1.51 µM	1.0	Sundstol Eriksen et al. 2004
15ADON	Mice	2.5 mg/kg bw PO	2 h/6 h	altered cytokines expression	N/A	Wu et al. 2014
15ADON	Piglets	1240 µg DON and 935 µg 15ADON kg feed	4 weeks	decreased villus height compared to solely DON equivalent	>	Pinton et al. 2012
3ADON	IPEC-1	0-30 µM	24 h	13% inhibition cell proliferation	<	Pinton et al. 2012
3ADON	IPEC-1	30 µM	48 h	transepithelial electrical resistance unaffected	<	Pinton et al. 2012
3ADON	IPEC-1	0-150 µM	24 h	EC_{80} of 126 µM	0.13	Alassane-Kpembi et al. 2015
3ADON	Caco-2	0-30 µM	6 h	no significant effect on cell viability	N/A	Kadota et al. 2013
3ADON	Caco-2	0-3 µM	72 h	significant dose-dependent increase of IL-8	<	Kadota et al. 2013
3ADON	Caco-2	0-7 µM	48 h	IC_{50} 2.94 (MTT assay)/1.99 (neutral red assay) µM	2.12/1.67	Alassane-Kpembi et al. 2013
3ADON	MIN-GL1/K-562	N/A	N/A	CD_{50} 21 µM and 6 µM	0.06/0.15	Visconti et al. 1991
3ADON	3T3	0.9–29.6 µM	24 h	50% DNA synthesis inhibition at 14.4 µM	0.1	Sundstol Eriksen et al. 2004
3ADON	Mice	34 mg/kg bw PO 49 mg/kg bw IP	bolus	LD_{50}	1.4	Ueno 1984
3ADON	Mice	2.5 mg/kg bw PO	2 h/6 h	altered cytokines expression	N/A	Wu et al. 2014
DON3G	Wheat ribosome	0-20 µM	25 min	8% inhibition of protein synthesis (luciferase activity)	<	Poppenberger et al. 2003
DON3G	Mice	2.5 mg/kg bw PO	2 h/6 h	reduced cytokines expression	N/A	Wu et al. 2014
DON3G	Caco-2	0-10 µM	8 days	unaltered viability and barrier function of cells	<	Pierron et al. 2015
DON3G	Intestinal explants	0-30 µM	4 h	no histomorphological alterations	<	Pierron et al. 2015

3.3.1. 3ADON and 15ADON

The toxicity of DON and its acetylated forms on intestinal morphology, barrier function, tight junction proteins, cell proliferation, MAPKs and cytokine expression in pigs has recently been investigated by Pinton *et al.* (Pinton et al. 2012). Porcine intestinal epithelial cells (IPEC-1) were incubated for 24 h with different concentrations of DON, 3ADON and 15ADON. A reduction in cell proliferation of 60% ($p<0.0001$), 13% ($p<0.05$) and 69% ($p<0.0001$) was demonstrated for DON, 3- and 15ADON, respectively. With regard to expression of claudins, which are important components of the tight junction protein complex, 15ADON displayed the most pronounced toxicity. Expression was decreased by 40% after exposure to 10 µM 15ADON, whereas identical doses of DON or 3ADON were unable to exert a significant reduction. Considering activation of MAPKs, 15ADON was also more toxic than DON and 3ADON, based on *in vitro* (IPEC-1)*, ex vivo* (porcine jejunal explants) and *in vivo* (jejunum from piglets) studies (Pinton et al. 2012). These MAPKs are responsible for the cytotoxic effects of the mycotoxin.

These findings were in accordance with the results from Alassane-Kpembi *et al*., who investigated the influence of DON, 3ADON and 15ADON on Caco-2 cells. Proliferating, non-transformed Caco-2 cells were exposed to increasing doses of these mycotoxins and the induced cytotoxicity was measured by means of an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. A similar toxicity was found for DON and 15ADON with effective concentrations inhibiting epithelial cell viability by 80% (EC_{80}) of 16.5 and 10.5 µM. 3ADON, for which a lower toxicity was expected, exhibited an EC_{80} value of 125 µM, which is approximately ten times higher (Alassane-Kpembi et al. 2015).

Kadota *et al.* compared the toxicity of these compounds by measuring their effect on IL-8 secretion and intestinal transport in Caco-2 cells. A significantly higher absorption of 15ADON compared to DON and 3ADON was demonstrated. 15ADON also had a more profound effect on intestinal integrity, facilitating absorption through passive diffusion. Furthermore, the production of IL-8 was the lowest after 3ADON exposure, followed by DON and 15ADON respectively (Kadota et al. 2013).

Visconti *et al.* evaluated the cytotoxicity on cultured human acute myelocytic leukemia and human lymphoid B cell lines (K-562 and MIN-GL1) (Visconti et al. 1991). Sundstol Eriksen *et*

al. focussed on DNA inhibition in Swiss mouse 3T3 fibroblasts, and revealed analogous results (Sundstol Eriksen et al. 2004). The last study included DOM-1. This metabolite was found to be at least 50 times less toxic than DON. Both studies demonstrate a distinct toxicity for these compounds, which can be ranked as follows, according to increasing toxicity: DOM-1 << 3ADON < DON ≤ 15ADON.

3.3.2. DON3G

The toxicity of DON3G remains largely unknown. To date, only one study investigated the toxicity of DON3G towards animals. More specifically, the effect on splenic pro-inflammatory cytokines and chemokines messenger RNA (mRNA) expression was investigated in mice. Compared to DON, DON3G proved to be ineffective in evoking cytokine or chemokine mRNA responses, indicating a reduced toxicity (Wu et al. 2014). The reduced cytokines induction of DON3G could be related to its inability to elicit a ribotoxic stress response. For plants, this has been demonstrated by an assay determining the inhibition of *in vitro* protein synthesis of wheat ribosomes by DON and DON3G, with a clear decrease in toxicity of the latter. For DON 100% inhibition was seen at 5 µM, whereas 20 µM DON3G was unable to block the protein synthesis (Poppenberger et al. 2003).

EFSA recently published a scientific opinion on the risks for human and animal health related to the presence of modified forms of ZEN, NIV and T-2 and HT-2 in food and feed. However, for the acetylated and modified forms of DON, the EFSA CONTAM working group is currently preparing a scientific opinion (EFSA CONTAM Panel 2014).

3.3.3. Co-contamination

Another point of concern is the co-contamination of DON with acetylated and modified forms of DON, and their potentially additive or synergistic effects. Alassane-Kpembi *et al*. demonstrated an order of magnitude of synergy ranging from 2 to 7 for several combinations of DON, 3ADON, 15ADON, NIV and FUS-X on epithelial cell toxicity (IPEC-1 and Caco-2). Binary or ternary mixtures often showed a dose-dependent effect, substantial enough to differ between antagonism and synergy (Alassane-Kpembi et al. 2013; Alassane-Kpembi et al. 2015). This matter has received limited attention although possibly profound effects may be suspected at observed co-contamination occurrence levels.

3.4. Toxicokinetics

In this section following questions are addressed: what is the disposition and fate of acetylated and modified mycotoxins in the animal and human body and is there a possibility that upon ingestion the acetylated and modified mycotoxins are converted back into their corresponding free form by e.g. *in vivo* hydrolysis of conjugated mycotoxins?

Many of the articles on this topic are *in vitro* studies using incubations with isolated bacteria or faecal samples. However, mycotoxins are mainly absorbed in the proximal part of the small intestine. Consequently, hydrolysis and biotransformation occurring in the colon will not significantly affect systemic concentrations of a given mycotoxin or its metabolites.

3.4.1. 3ADON and 15ADON

The toxicokinetic properties of free DON in several animal species have recently been reviewed (Dänicke and Brezina 2013; Maresca 2013). Regarding acetylated and modified DON, several *in vitro* and *in vivo* studies are available. To determine the toxicokinetic behaviour and degree of hydrolysis of acetylated and modified DON forms, Sundstol Eriksen and Pettersson incubated acetylated and modified mycotoxins with isolated human faecal samples *in vitro* under anaerobic conditions for 48 h. For 3ADON about 78 ± 30% of the administered dose was recovered as DON, clearly demonstrating the possibility that human intestinal microbiota hydrolyse 3ADON to DON (Sundstol Eriksen and Pettersson 2003). Furthermore, the study proved that, in contrast to pigs, rats, mice, cows, chickens and sheep, isolated human faecal samples were unable to form the de-epoxidated metabolite DOM-1, as was the case for dogs and horses too (He et al. 1992; Kollarczik et al. 1994; Swanson et al. 1988; Swanson et al. 1990; Yoshizawa and Morooka 1975).

A similar *in vitro* study with pig faeces and ileal digests indicated that deacetylation of 3ADON to DON does occur in pigs. As mentioned before, pigs are able to further metabolise DON to DOM-1. However, not all pigs included in the study possessed this ability, illustrating high inter-individual variability. Moreover, this ability was not acquired after a 7-week period in which these pigs were fed a diet with low quantities of DON. On the other hand, faeces from pigs that were able to de-epoxidise DON were spread out in the pens of the pigs that lacked this metabolic feature. After one week, four out of five pigs had also acquired the

capability to metabolise DON to DOM-1, indicating that this ability can be transferred between pigs (Eriksen et al. 2002).

In addition to the above *in vitro* studies, some *in vivo* studies on the metabolism of 3ADON have been published in rats and pigs. Versilovskis *et al*. investigated the metabolism of 3ADON and 15ADON after oral administration to rats. One hour p.a. of 3ADON and 15ADON, 12% of the dose could be detected in the stomach as free DON, indicating a likely hydrolysis of ADONs in the stomach. It was also observed that both ADONs can be glucuronidated in the stomach (5% of the administered dose) without prior deacetylation (Versilovskis et al. 2012). Eriksen *et al*. investigated the ADME profile of 3ADON in pigs. After oral administration of 3ADON, neither 3ADON nor the acetylated de-epoxide metabolite could be detected in plasma. The retrieved metabolites in plasma 3 h p.a. were DON (54%) and DON-glucuronide (42%). In faeces, no 3ADON was detected either, a mere 2% of the administered dose was recovered in faeces as DON and DOM-1. Approximately 50% of the dose was excreted in urine with a significant fraction (33%) as DON-glucuronide (Eriksen et al. 2003).

In some cases, 3- and 15ADON exhibit a clearly distinct species-dependent behaviour as demonstrated by an *in vitro* study concerning the degradation of trichothecene mycotoxins by chicken intestinal microbiota. For 15ADON, a large fraction proved to have both the acetyl and epoxy moiety removed (± 90%), whereas only small quantities showed either solely de-epoxidation or deacetylation (6%). However, for 3ADON, deacetylation was the predominant reaction (64-94%) with only minimal subsequent de-epoxidation (4-24%) (Young et al. 2007).

Due to a decreased polarity, ADONs might have an increased passive diffusion and intestinal transport to the portal blood circulation. Next, these compounds can be hydrolysed in this pre-systemic circulation. These two phenomena make it possible that the total amount of DON equivalents that enters an organism is higher for ADONs than for DON, when equimolar doses are considered.

To perform a risk assessment in broiler chickens and pigs, more *in vivo* studies will be needed to assess the degree of *in vivo* hydrolysis, oral bioavailability and toxicokinetic parameters of 3ADON and 15ADON.

3.4.2. DON3G

Unlike for the ADONs, degradation of DON3G in the human upper GI tract (mouth, stomach and proximal part of the small intestine, duodenum) seems unlikely, based on an *in vitro* study by Berthiller *et al.* Different treatments with acid (HCl), artificial stomach juice, artificial gut juice and human cytosolic β-glucosidase at 37 °C for 18 h were not able to hydrolyse significant amounts of DON3G (< 0.2%) (Berthiller et al. 2011). These results were confirmed by De Nijs *et al.,* in a study in which two *in vitro* models representing the human upper GI tract, a fed digestion model and a Caco-2 transwell system, were unable to transform DON3G into DON (De Nijs et al. 2012). A study by Dall'Erta *et al.* further supported these findings, as a salivary, gastric and duodenal step in their model proved unable to release the DON aglycone from DON3G (Dall'Erta et al. 2013). De Angelis *et al.* evaluated bread contaminated with DON and DON3G in an *in vitro* digestion model, and observed an increase of DON3G in the duodenal compartment, together with a distinct decrease of DON. The authors hypothesize that this increase of DON3G could either be attributed to the release from embedded forms, bound to matrix macromolecules, or to glycosylation of DON by interaction with glucose molecules made available by the enzymatic release of starch in the bread matrix (De Angelis et al. 2014).

Regarding the human lower GI tract, i.e. jejunum and ileum together with the large intestine, there are some indications that hydrolysis could take place there. Under semi-aerobic *in vitro* conditions, Berthiller *et al*. evaluated the hydrolytic capability of 47 isolated bacterial strains from intestinal content (Berthiller et al. 2011). Particular species of the genera *Lactobacillus*, *Enterococcus*, *Enterobacter* and *Bifidobacterium* efficiently cleaved DON3G to DON, releasing 6 to 62% of the dose as DON after 8 h incubation. These results indicate that the intestinal microbiota species composition and density could play an important role. Consequently, different cleavage rates can be expected in different animals or humans, or within one species between adults and infants as well (Abbott 2004; Hattori and Taylor 2009). An interesting observation in the study by Berthiller *et al*. was that the hydrolytic enzyme cellobiase caused a 73% hydrolysis of DON3G after 18 h incubation, indicating that DON could be released in the GI tract of plant-based cellulose-foraging ruminants (Berthiller et al. 2011).

Gratz *et al*. investigated the metabolism of DON3G by human faecal microbiota and confirmed the above findings (Gratz et al. 2013). After 6 h of anaerobic incubation, 100% of DON3G was hydrolysed in 4 out of 5 cases. Dall'Erta *et al*. found similar results since after 24 h of incubation a complete degradation of DON3G was observed with a recovery of 90% as DON (Dall'Erta et al. 2013).

In vitro models do not take into account important physiological factors within and between different animal species and humans, such as intestinal mucosa and content composition, enterohepatic recirculation and the influence of the immune system (Gonzalez-Arias et al. 2013). To estimate these factors and especially the interactions between them, species-specific *in vivo* trials remain mandatory. The first DON3G *in vivo* study was published by Versilovskis *et al*. in 2012. Two rats were fed 25 µg of DON3G by oral gavage followed by blood collection at 17 and 55 min p.a. DON was detected in the stomach albeit at only 2% of the applied dose. This confirms the findings of the described *in vitro* studies stating that the hydrolysis of DON3G in the stomach is negligible. Further down in the GI tract, DON3G could be detected at 2-3% of the applied dose in the small intestine and at 1-2% in the colon. This remarkable decrease of DON3G in the small intestine is probably due to the presence of tissue β-glucosidases. In contrast, Berthiller *et al*. did not detect any hydrolysis after *in vitro* incubation with human β-glucosidase (Berthiller et al. 2011; Versilovskis et al. 2012). Nagl *et al*. orally administered DON and an equimolar dose of DON3G to six rats followed by urine and faeces collection over a two-day period. After PO administration of DON3G, less than 4% of the administered dose was recovered in urine, one third as DON, one third as DON-GlcA, 10% as DON3G and 20% as DOM-1. The presence of urinary DON clearly demonstrates the hydrolysis upon ingestion. In comparison, after DON administration, 15% of the dose was recovered in urine, indicating a relatively low oral bioavailability for DON3G compared to DON in rats. This set-up, however, did not allow determination of where and how the hydrolysis took place. Analysis of faecal samples of the rats dosed with DON3G revealed that the vast majority of the metabolites of DON3G was excreted as DON and DOM-1 (sum: 99.5 ± 0.4%). Only traces of DON3G were detected, confirming that intestinal microbiota are effective in hydrolysing this modified mycotoxin (Nagl et al. 2012). Recently, the results in rat are supported by *in vivo* experiments performed in pig by the same research group. After IV administration of DON3G, no DON was detected in plasma, indicating that systemic

hydrolysis of DON3G is negligible. After oral administration of DON3G, 40.3% of the dose was recovered in urine as DON3G, next to DON (21.6%), DON15GlcA (6.8%), DOM-1 (5.9%) and DON3GlcA (3.4%). Consequently, it was concluded that *in vivo* cleavage of DON3G predominantly occurs in the (lower) digestive tract. Oral bioavailability was lower for DON3G than for DON, derived from the recovered DON equivalent amounts in urine, namely 84.8% for DON and 40.3% for DON3G. However, the oral bioavailability for both compounds in pig was remarkably higher than in rat (Nagl et al. 2014). The species composition and density of intestinal microbiota can contribute to the observed interspecies differences in oral bioavailability and degree of hydrolysis. These differences demonstrate the need for animal trials in species with a high exposure to these mycotoxins.

The pig has remarkable similarities to humans in terms of anatomy and physiology of the GI tract, renal and cardiovascular organ systems. These similarities make the pig a suitable animal model for oral toxicokinetic studies in humans (DeSesso and Williams 2008; Roth et al. 2013; Svendsen 2006; Witkamp and Monshouwer 1998). At the moment, literature reports regarding the toxicokinetics of DON3G in humans are scarce. Warth *et al*. performed a study with a human volunteer consuming a diet naturally contaminated with DON3G (7 µg DON3G/day). The modified mycotoxin could not be detected in urine (Warth et al. 2013).

Overall, the limited published studies tentatively demonstrate a limited oral bioavailability of DON3G, so it might be hypothesised that DON3G is less toxic to the tested animal species. Nevertheless, the hydrolysis of DON3G to DON might contribute to the overall toxicity after oral intake of DON3G. Determination of the oral bioavailability, degree of hydrolysis and toxicokinetic parameters of DON3G in relevant species may support legislative authorities to carefully take measure of all aspects of the risk assessment of DON3G, as well as 3ADON and 15ADON, and to potentially consider the establishment of a TDI for acetylated and modified mycotoxins or to include them in a group TDI. Additionally, the data obtained for pigs can serve as a model for humans, for which studies are difficult to set up from an ethical point of view.

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AIMS OF THE THESIS

The *Fusarium* mycotoxin deoxynivalenol (DON) is one of the most frequently occurring mycotoxins worldwide, contaminating 56% of feed and feed raw materials. DON is known for its harmful effects on animals worldwide, leading to great economic losses. Additionally to DON, feed can be contaminated with acetylated forms of DON, such as 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), and modified forms such as deoxynivalenol-*3*-β-D-glucoside (DON3G). One of the major questions associated with acetylated and modified forms of DON is whether *in vivo* hydrolysis may result in the release of DON and what their disposition in the body is. Little is known about the toxicodynamics and toxicokinetics of these acetylated and modified forms of DON, compared to the extensively investigated DON. Determination of the oral bioavailability, rate and extent of *in vivo* hydrolysis and toxicokinetic characteristics of 3ADON, 15ADON and DON3G in relevant animal species by means of a state-of-the-art toxicokinetic analysis, may support legislative authorities to carefully consider all aspects of the risk assessment and to eventually consider the establishment of a tolerable daily intake (TDI) for acetylated and modified mycotoxins or to include them in a group TDI.

The **general objective** of this research is to determine the disposition, i.e. oral bioavailability, rate and extent of *in vivo* hydrolysis and toxicokinetic characteristics, of 3ADON, 15ADON and DON3G in broiler chickens and pigs in order to contribute to the risk assessment of these mycotoxins. Additionally, the cytotoxicity of these mycotoxins towards intestinal epithelial cells is investigated, as these are the first target after consumption of contaminated feed.

Pigs are of particular concern for at least two reasons: (1) due to the cereal-rich diet, pigs can be exposed to a high level of toxins; and (2) the pig is one of the most sensitive species for several mycotoxins. In addition, pigs are anatomically and physiologically quite similar to humans, and because of the similarities in the intestinal tract, pigs can be considered as a good model for humans. In contrast, broilers are, together with ruminants, among the least sensitive species to DON, despite their high exposure to these mycotoxins, which makes this species interesting for comparative purposes. Additionally, poultry meat is worldwide the most consumed meat expressed in kg per capita. Moreover, within poultry consumption, 84% originates from the consumption of broiler meat, further increasing the study relevance of this species.

Comparative toxicokinetic analysis with determination of the *in vivo* hydrolysis can contribute to explain the large interspecies difference in susceptibility to DON. Therefore, sensitive and validated multi-mycotoxin analysis methods in animal plasma are needed.

The **specific aims** of this research were as follows:

- To develop and validate a sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify DON, 3ADON, 15ADON and DOM-1 in broiler chicken and pig plasma (Chapter I).
- To perform toxicokinetic *in vivo* trials to determine oral bioavailability, *in vivo* hydrolysis and toxicokinetic parameters of DON, 3ADON and 15ADON in broiler chickens and pigs (Chapter II).
- To determine oral bioavailability, *in vivo* hydrolysis and toxicokinetic parameters of DON3G in broiler chickens and pigs. For this, a validated LC-MS/MS method to quantify DON, DON3G and DOM-1 in broiler chicken and pig plasma is needed (Chapter III).
- To assess the *in vitro* cytotoxicity of 3ADON, 15ADON and DON3G on differentiated and undifferentiated intestinal epithelial cells. As pigs are among the most sensitive species to DON toxicity, a porcine intestinal epithelial cell line (IPEC-J2) is chosen (Chapter IV).

EXPERIMENTAL STUDIES

CHAPTER I

Development and validation of an LC-MS/MS method for the toxicokinetic study of deoxynivalenol and its acetylated derivatives in chicken and pig plasma

Adapted from:

Broekaert N., Devreese M., De Mil T., Fraeyman S., De Baere S., De Saeger S., De Backer P., Croubels S. (2014). Development and validation of an LC-MS/MS method for the toxicokinetic study of deoxynivalenol and its acetylated derivatives in chicken and pig plasma. Journal of Chromatography B, 971, 43-51.

Abstract - This study aims to develop an LC-MS/MS method allowing the determination of 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, deoxynivalenol and an *in vivo* metabolite, de-epoxy-deoxynivalenol, in plasma of broiler chickens and pigs. These species have a high exposure to these toxins, given their mainly cereal based diet. Several sample cleanup strategies were tested and further optimised by means of fractional factorial designs. A simple and straightforward sample preparation method was developed consisting of a deproteinisation step with acetonitrile, followed by evaporation of the supernatant and reconstitution in water. The method was single laboratory validated according to European guidelines and found to be applicable for the intended purpose, with a linear response up to 200 ng/mL and limits of quantitation of 0.1 to 2 ng/mL. As a proof of concept, biological samples from a broiler chicken that received either deoxynivalenol, 3- or 15-acetyl-deoxynivalenol were analysed. Preliminary results indicate nearly complete hydrolysis of 3-acetyl-deoxynivalenol to deoxynivalenol; and to a lesser extent of 15-acetyl-deoxynivalenol to deoxynivalenol. No de-epoxy-deoxynivalenol was detected in any of the plasma samples. The method will be applied to study full toxicokinetic properties of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in broiler chickens and pigs.

Keywords - LC-MS/MS - plasma - deoxynivalenol - 3-acetyl-deoxynivalenol - 15-acetyl-deoxynivalenol - de-epoxy-deoxynivalenol - poultry - pig

1. Introduction

Fusarium is regarded as one of the most important mycotoxigenic fungi genera. Dean *et al.* (2012) proposed a top ten for fungal pathogens based on scientific and economic importance. *Fusarium graminearum* and *F. oxysporum* got nominated for places four and five, respectively. *Fusarium spp.* can produce several toxins of which the trichothecenes, zearalenone and fumonisins are the most important based on occurrence and toxicity. Of these trichothecenes, deoxynivalenol (DON) is the most prevalent. It is typically found in wheat, barley, corn, rye and consequently also in compound feeds (Döll and Dänicke 2011; Pestka 2010).

The research regarding the toxicity, toxicokinetics and occurrence of DON (and mycotoxins in general) has made significant progress during the last years. As the information and insight on native (or parent) mycotoxins increases, the focus of this research domain tends to shift partially towards the gathering of occurrence and toxicity data for masked and modified mycotoxins. The use of the term 'masked mycotoxin' is deliberately avoided throughout this manuscript. Masked mycotoxins owe their name to the fact that these mycotoxin conjugates or derivatives are undetectable by conventional techniques due to changes in their chemical strucutre and physico-chemical properties. Due to the ambiguous use of the term 'masked mycotoxin', Rychlik *et al*. (2014) systematically defined all potential modified mycotoxins. This nomenclature consists of a four hierarchic level definition and comprises modifications such as by plant biosynthesis pathways, animal conjugation, fungal production or by certain processing techniques (Rychlik et al. 2014). The acetylated derivatives of DON, 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), are intermediaries in its biosynthesis, hence they are often described as fungal precursors, or by Rychlik *et al*. as free mycotoxins.

Conversion of these toxin derivatives or conjugates back to their native form by *in vivo* hydrolysis in humans and animals cannot be excluded. Consequently, this would imply an underestimation of the degree of contamination upon analysis. De Boevre *et al.* (2012) reported the presence of 3ADON and 15ADON in 87% and 73% of different cereals and cereal derived products, respectively. In the latter study, the highest contamination levels were detected in maize with concentrations up to 305 ± 216 ng/g for 3ADON and 334 ± 270 ng/g for 15ADON (De Boevre et al. 2012).

The toxicological importance of 3ADON and 15ADON has recently been highlighted by Pinton *et al*. (2012) in a study regarding the toxicity of DON and its acetylated derivatives on intestinal morphology, barrier function, tight junction proteins and mitogen-activated protein kinases. A general conclusion was that these compounds have a distinct toxicity on the porcine intestine which can be ordered as follows, according to increasing toxicity: 3ADON < DON < 15ADON (Pinton et al. 2012).

Several methods have been developed to determine DON and its acetylated and/or modified forms in matrices such as wheat, maize, oats and derived products e.g. cornflakes, bread, flour and beer (De Boevre et al. 2012; Escrivá et al. 2014; Lancova et al. 2008; Suman et al. 2013; Varga et al. 2013). Untargeted analysis by means of high resolution mass spectrometry (HRMS) of modified and acetylated mycotoxins is increasingly used for food and feed commodities (Di Mavungu et al. 2011; De Boevre et al. 2015). The goal of this study however, was to develop a method which allows to study the toxicokinetic properties of DON and the *in vivo* hydrolysis of its derivatives 3ADON and 15ADON in chickens and pigs, as these species have a high exposure to these toxins, given their mainly cereal based diet. Therefore, a method determining these compounds in plasma is needed. This enables to study whether or not 3ADON and 15ADON are hydrolysed in the gastro-intestinal (GI) tract of the animal and are consequently absorbed in the blood either as intact molecule or as the parent toxin DON.

With respect to *in vivo* monitoring of DON and/or its derivatives few methods are published. Dänicke *et al.* (2012) developed a high-performance liquid chromatography method with diode-array detection (HPLC-DAD) to determine DON and the *in vivo* metabolite, de-epoxy-deoxynivalenol (DOM-1); as well as an indirect method using β-glucuronidase to determine DON and DON-glucuronide (DON-GlcA) in pig plasma (Dänicke et al. 2012). The cleanup was based on immunoaffinity solid phase extraction (SPE), a relatively expensive and elaborate technique. Furthermore, the described methods lack a full validation, with given data restricted to the limits of detection (LOD) and recoveries.

Recently several fully validated liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have been described. De Baere *et al.* (2011) developed a method to determine DON and DOM-1 in pig and chicken plasma. The limits of quantitation (LOQ)

ranged from 1 to 2.5 ng/mL using a SPE cleanup (Oasis® HLB column) (De Baere et al. 2011). Devreese *et al*. (2012) published a comparable method to determine DON and DOM-1 in pig plasma. The method used a straightforward cleanup, consisting of a simple deproteinisation step with acetonitrile, and managed to obtain comparable LOQ values (Devreese et al. 2012). Brezina et al. (2014) developed and validated an LC-MS/MS method with SPE cleanup to determine DON, DOM-1 and zearalenone and its metabolites in pig serum. However, all the above mentioned methods do not allow the determination of acetylated derivatives of the native toxin.

Methods to determine DON and/or its acetylated derivative 3ADON are available for pig but not for chicken plasma; for chickens only a method determining DON in plasma is available (De Baere et al. 2011). Eriksen *et al*. (2003) have reported a gas chromatography electron capture detector (GC-ECD) method allowing to determine DON, 3ADON and nivalenol in pig plasma and urine. As is common with more polar compounds, in GC a derivatisation step is required prior to analysis (Eriksen et al. 2003). To our knowledge, no method to determine 15ADON in plasma, regardless of the animal species, is described in literature.

The goal of this study was to develop a method to monitor DON, 3ADON or 15ADON and an *in vivo* metabolite, DOM-1, in broiler and pig plasma. Their chemical structures are presented in **Figure 1.**

The described method was validated according to European guidelines (Commission Decision 2002; Heitzman 1994) and results for the evaluation of the linearity, accuracy, precision (repeatability, reproducibility), sensitivity (LOD and LOQ), matrix effect and carry-over are presented for DON, 3ADON, 15ADON and DOM-1.

As a proof of concept, the method was applied for the analysis of plasma samples from a broiler chicken that received either DON, 3ADON or 15ADON. In a further stage the method will be applied to gain insight into the toxicokinetic properties of the acetylated forms of DON.

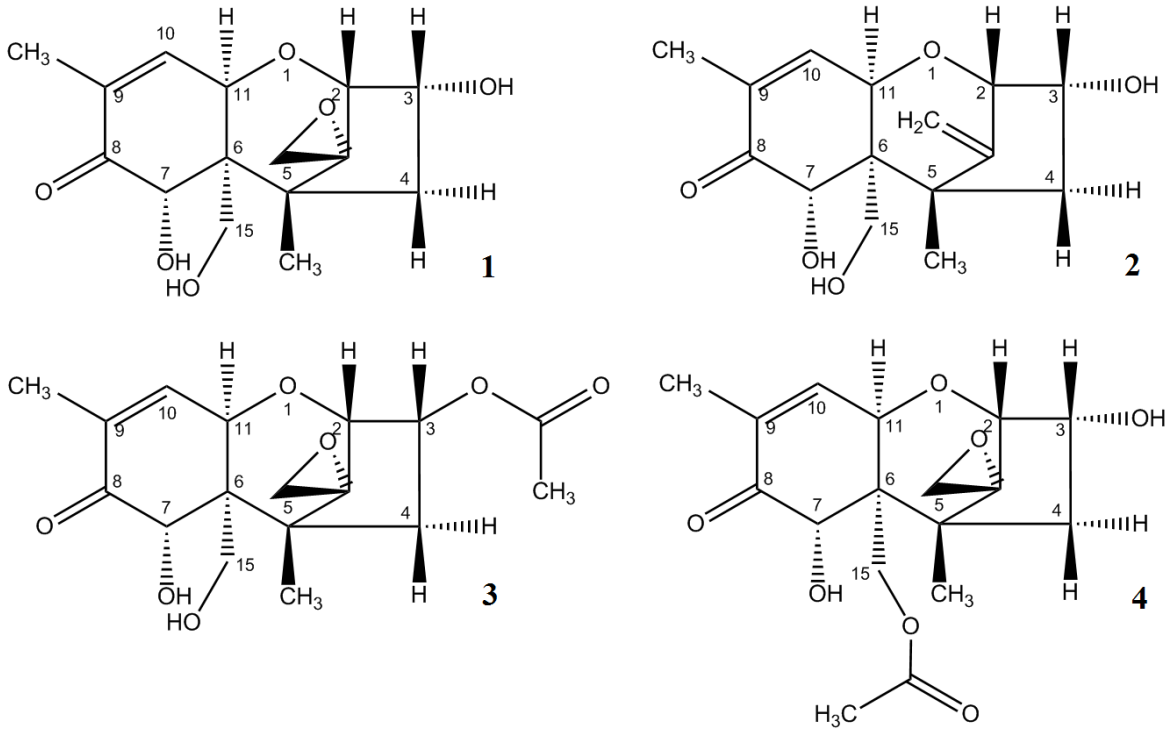

Figure 1. *Structure of 1: 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol or DON), 2: an* in vivo *metabolite, 3α,7α,15-trihydroxy-trichothec-9,12-dien-8-one (de-epoxy-deoxynivalenol or DOM-1), and two acetylated forms of DON, 3: 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetyl-deoxynivalenol or 3ADON) and 4: 15-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one (15-acetyl-deoxynivalenol or 15ADON).*

2. Experimental

2.1 Compounds, standards and solutions

DON was purchased from Fermentek (Jerusalem, Israel) and dissolved in acetonitrile yielding a stock solution of 1 mg/mL. DOM-1 (50 µg/mL), 3ADON (100 µg/mL), 15ADON (100 µg/mL) and $^{13}C_{15}$-DON (stable isotope labelled internal standard (IS), 50 µg/mL) stock solutions in acetonitrile were purchased from Romerlabs (Tulln, Austria). All the above mentioned stock solutions were stored at ≤−15 °C. Individual working standard solutions of 5 µg/mL were prepared by diluting the above stock solutions with HPLC-grade acetonitrile. All standard solutions were stored at ≤−15 °C. Standard mixture working solutions (containing DON, DOM-1, 3ADON and 15ADON) of 1 µg/mL and 100 ng/mL were prepared by mixing and diluting the individual working standard solutions in HPLC-grade acetonitrile and were kept at 2-8 °C. Acetonitrile solutions of DON have been reported stable for 24 months at room temperature, for 3ADON, 15ADON and DOM-1 no published stability data is available

(Widestrand and Pettersson 2001). However, the supplier claims a shelf-life of at least 6 months at 4 °C for a 100 ng/mL solution in acetonitrile.

2.2 Instrumentation, materials and reagents

Separation of the analytes of interest was achieved on a Hypersil Gold (reversed-phase) (50 x 2.1 mm i.d., 1.9 µm) column with a guard column (10 x 2.1 mm i.d., 5 µm) of the same type. Chicken plasma was analysed on a Surveyor® type MS pump Plus and Autosampler Plus HPLC in combination with a TSQ® Quantum Ultra™ mass spectrometer, operating in both the positive and negative heated electrospray ionisation (ESI) mode (Thermo Fisher Scientific, Breda, The Netherlands). Pig plasma was analysed on an Acquity UPLC system coupled to a Xevo® TQ-S mass spectrometer, operating solely in positive electrospray ionisation (ESI) mode (Waters, Zellik, Belgium). Microfilters Durapore PVDF 0.22 µm were obtained from Millipore (Overijse, Belgium). Water and methanol (ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Glacial acetic acid and ammonium acetate (both analytical grade) and acetonitrile (HPLC grade) were obtained from VWR (Leuven, Belgium) and ammonium acetate from Merck (Brussels, Belgium). One mL, 60 mg SPE cartridges Oasis® HLB were purchased from Waters (Zellik, Belgium) and Bond Elute C18, 100 mg, 1 mL from Agilent (Diegem, Belgium).

2.3 Plasma sample preparation

Blank plasma was obtained by the collection of heparinised blood from broiler chickens (Ross 308) and pigs (Landrace). The animals were fasted twelve hours prior to blood collection. Plasma was obtained by centrifugation (2851 x *g*, 10 min, 4 °C) of the blood. The blank plasma was pooled, homogenised and stored at ≤ – 15 °C until the moment of use for the preparation of matrix-matched calibrators and quality control samples.

Calibrator and quality control samples. To 250 µL of blank plasma, 5 µL of a 1 µg/mL internal standard working solution ($^{13}C_{15}$-DON) and appropriate volumes of the standard mixture working solutions (1 µg/mL and 100 ng/mL) were added to obtain calibrator samples with mycotoxin concentrations of 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL. After vortex mixing, acetonitrile was added up to a volume of 1 mL to precipitate plasma proteins. The samples were vortex mixed again, followed by a centrifugation step (10 min at 8517 x *g*, 4 °C). The

supernatant was transferred to a new tube and evaporated to dryness under nitrogen at 80 °C. The sample was then redissolved in 200 µL of UPLC-grade water, micro-filtrated and analysed by means of LC-MS/MS.

Incurred samples. To 250 µL of plasma, 5 µL of a 1 µg/mL internal standard working solution were added. After vortex mixing, the samples were subjected to the same sample preparation procedure as the calibrator samples.

Blank samples. After the addition of 750 µL of acetonitrile to 250 µL of blank plasma, the samples were extracted in the same way as the calibrator samples.

2.4 LC-MS/MS analysis

For chicken plasma all compounds were eluted with a gradient of UPLC-grade water + 0.1% glacial acetic acid (mobile phase A) and UPLC-grade methanol + 0.1% glacial acetic acid (mobile phase B) at a flow rate of 300 µL/min (**Figure 2 C**). The gradient started at 5% B for one minute, in six seconds the gradient increased to 20% B and this was maintained up to 5 minutes. From 5 minutes to 5.1 minutes the % B was augmented to 50 and was held up to 8 minutes from where it increased to 95% in six seconds which was maintained for one minute. Afterwards, the gradient was restored to its initial conditions. Column and autosampler temperatures were set to respectively 60 and 5 °C, injection volume (partial loop) was fixed at 10 µL. For pig plasma identical parameters were used, the sole difference was the use of 0.3% glacial acetic acid instead of 0.1%.

The mass spectrometers were operated in the multiple reaction monitoring (MRM) mode with two ion transitions for each target analyte. Instrumental and compound specific parameters were optimised by the direct infusion of either 1 µg/mL (TSQ Quantum Ultra) or 10 ng/mL (Xevo TQ-S) standard solutions in methanol/ultra-pure water (50/50; v/v) + 0.1% acetic acid at a flow rate of 10 µL/min. For the TSQ, the instrumental mass spectrometry parameters were set as follows: vaporizer temperature 50 °C, capillary temperature 350 °C and ion sweep gas pressure 2.0. The other parameters varied for ESI- and ESI+ respectively, spray voltage 3500 V (-) and 5000 V (+), sheath gas pressure 40 arbitrary units (au) (-) and 49 au (+), auxiliary gas pressure 10 au (-) and 25 au (+), source CID 5 (-) and -5 (+). Compound specific MS parameters, together with precursor and product ions used for quantification

and qualification, are given in **Table 1**. All compounds were detected in negative electrospray ionisation mode (ESI-) as $[M+Hac-H]^-$ adducts, except for 15ADON which exhibited better sensitivity when measured in ESI+, measured as the protonated precursor ion ($[M+H]^+$). For the Xevo TQ-S, the desolvation gas flow rate was fixed to 1000 L/h with a temperature of 600 °C, the cone gas flow rate was set at 150 L/h, the source temperature was set at 150 °C and the capillary voltage was optimised at 3.5 kV. Dwell times of 44 - 52 ms/transition were chosen. In **Table 1**, compound specific MS parameters such as cone voltage and collision energy are mentioned.

Table 1. Compound specific MRM ion transitions and MS-parameters; Rt= retention time; IS= internal standard; [a] quantifier ion; for chicken plasma tube lens offset is mentioned, for pig plasma cone voltage.

		Measured form/adduct	Precursor ion (m/z)	Product ion (m/z)	Rt (min)	Tube lens offset /cone voltage	Collision Energy	ESI modus
Chicken	DON	$[M+Hac-H]^-$	355.2	265.1[a]	3.50	75	15	-
				295.1	3.50	75	10	-
	DOM-1	$[M+Hac-H]^-$	339.1	249.1[a]	4.75	85	15	-
				59.10	4.75	85	35	-
	3ADON	$[M+Hac-H]^-$	397.1	306.8[a]	7.05	95	15	-
				337.1	7.05	85	10	-
	15ADON	$[M+H]^+$	339.2	321.2[a]	7.00	80	15	+
				136.9	7.00	80	20	+
	$^{13}C_{15}$-DON (IS)	$[M+Hac-H]^-$	370.2	279.1[a]	3.50	75	15	-
				310.1	3.50	75	10	-
Pig	DON	$[M+H]^+$	297.1	249.1[a]	3.50	20	9	+
				203.4	3.50	20	14	+
	DOM-1	$[M+H]^+$	281.1	215.1[a]	4.75	20	12	+
				137.0	4.75	20	16	+
	3ADON	$[M+H]^+$	339.0	231.1[a]	7.05	30	10	+
				213.2	7.05	30	14	+
	15ADON	$[M+H]^+$	339.0	231.1[a]	7.00	30	10	+
				213.2	7.00	30	14	+
	$^{13}C_{15}$-DON (IS)	$[M+H]^+$	312.0	245.2[a]	3.50	20	10	+
				263.0	3.50	20	10	+

2.5 Validation

Given the unavailability of reference materials, validation was performed on spiked blank plasma samples. Both recommendations as defined by the European Community (Commission Decision 2002; Heitzman 1994) and the Veterinary International Conference on Harmonisation (VICH 2009) served as validation guidelines. The developed method was single laboratory validated.

Linearity of the response of the compounds was assessed by means of three matrix-matched calibration curves consisting of seven calibration points in the range of 1-200 ng/mL. The

correlation coefficients (r) and goodness-of-fit coefficients (gof) were determined, limits were set to ≥0.99 and ≤20%, respectively.

Within-day accuracy & precision were determined by analyzing six samples at a low concentration level (LOQ of the compounds) and at a high concentration level (100 ng/mL). Values for the relative standard deviation (RSD) could not exceed 2/3 of the $RSD_{max,}$ calculated according to the Horwitz equation (given below). The acceptance criteria for accuracy were: −30% to +10% and −20% to +10% for concentrations between 1 and 10 ng/mL, and ≥10 ng/mL, respectively. *Between-day accuracy & precision* were assessed by analyzing the low and high concentration levels in threefold on three consecutive days (n=3x3). The acceptance criteria for accuracy were identical to the values given above and RSD values could not exceed the RSD_{max}. The formula to determine RSD_{max} are given below.

$$\text{Within-day precision: } RSD_{max} = 2^{(1-0.5\log Conc)} \times 2/3$$

$$\text{Between-day precision: } RSD_{max} = 2^{(1-0.5\log Conc)}$$

The *LOQ* was calculated as the lowest concentration for which the method had acceptable results with regards to accuracy and precision. It was determined by spiking six plasma samples at 1 or 2 ng/mL. The LOQ was also established as the lowest point of the calibration curve. The *LOD* was calculated using the samples spiked at the LOQ level (n=6) corresponding to the lowest concentration that could be determined with a signal-to-noise (S/N) ratio of 3.

Carry-over was evaluated by analysing a mixture of mobile phase A and B (50/50; v/v) directly after the highest calibrator (200 ng/mL).

The *specificity*, the capability of the method to distinguish signals of the analytes from any other substances or interferences, was determined on six blank plasma samples. For an acceptable specificity the S/N ratio of possible interfering peaks with similar retention times in these samples could not exceed the S/N ratio of the analyte(s)' LOD.

Recovery and matrix effects. Two types of matrix-matched calibration curves (on pooled blank plasma derived from six different animals) were prepared, one by spiking the blank

calibrator samples before and one after extraction. A third calibration curve was prepared in standard solution. All curves consisted of seven calibration points in the range of 1-200 ng/mL. The slopes of these calibration curves (external calibration, without IS) were compared to calculate the apparent recovery (R_A = 100 × slope spiked before extraction/slope standard solution), the matrix effect denoted as signal suppression/enhancement (SSE = 100 × slope spiked after extraction/slope standard solution) and the recovery of the extraction step (R_E = 100 × slope spiked before extraction/slope spiked after extraction). Regarding SSE, values ≤ 1 indicate ion suppression due to matrix effect, values ≥ 1 are caused by ion enhancement (Matuszewski et al. 2003).

To test applicability of the method on plasma from other animals, the validation as described above was also executed on pig plasma.

2.6 Pilot study

Three male broilers (Ross 308, 20 days of age) were housed at the Faculty of Veterinary Medicine of Ghent University. Water and feed were present *ad libitum*. The stable was climate controlled with temperatures between 18 and 25 °C and a relative humidity between 40 and 80%. After one week of acclimatisation, the animals were treated with either DON, 3ADON or 15ADON by intravenous injection (IV) or *per os* (PO, by means of gavage) in a two-way crossover design with a washout period lasting 4 days. Twelve hours before administration of the compounds the animals were fasted. The administered doses were calculated to represent a worst case scenario within the legal framework of the European Union. The maximum EU guidance level in feed is 5 mg/kg DON (European Commission 2006) and broilers consume on average 100 g feed/kg body weight (bw)/day at three to four weeks of age. This resulted in the administration of 500 µg DON/kg bw. For the ADONs equimolar doses (571 µg/kg bw) were administered. The toxins were dissolved in ethanol at 25 mg/mL. The required amounts to administer per chicken were diluted with saline (0.9% NaCl, VWR, Leuven, Belgium) to a volume of one mL. Before the administration of the toxins and after 5, 10, 20, 30, 45, 60, 90, 120 and 240 min, blood (0.5 – 1 mL) was collected. Plasma was obtained by centrifugation of the blood samples (2851 x *g*, 10 min, 4°C) and stored at ≤−15 °C prior to analysis. All samples were analysed within one month after blood collection.

The animal trial was approved by the ethical committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering of Ghent University (EC2013/64).

3. Results and discussion

3.1 Method development

To optimize the LC-MS/MS method several HPLC columns were tested in a first stage. All the tested columns, including a Hypersil Gold column (50 x 2.1 mm i.d., 1.9 µm), did not provide a separation of both ADONs. However, there was no need for a simultaneous determination of both toxins, as the purpose of this study was to apply the method for determination of the toxicokinetic properties of the compounds after separate administration to the animals. The Hypersil Gold column did however give a satisfactory resolution for the other compounds, as all analytes showed a baseline separation, in a relatively short analysis time, with an elution time of 7 minutes for the longest retained compound (**Figure 2)**.

Following the column choice, the mobile phase system was optimised. As organic solvent both acetonitrile and methanol were tested. The latter solvent gave more satisfactory results regarding peak shapes. Both ammonium acetate ($NH_4^+CH_3COO^-$) and acetic acid (CH_3COOH) concentrations were evaluated as modifiers. Best results, based on peak shapes and signal intensities, were obtained with mobile phases consisting of UPLC-grade water + 0.1% acetic acid and UPLC-grade methanol + 0.1% acetic acid for broiler chicken plasma. For pig plasma, 0.3% acetic acid was deemed optimal.

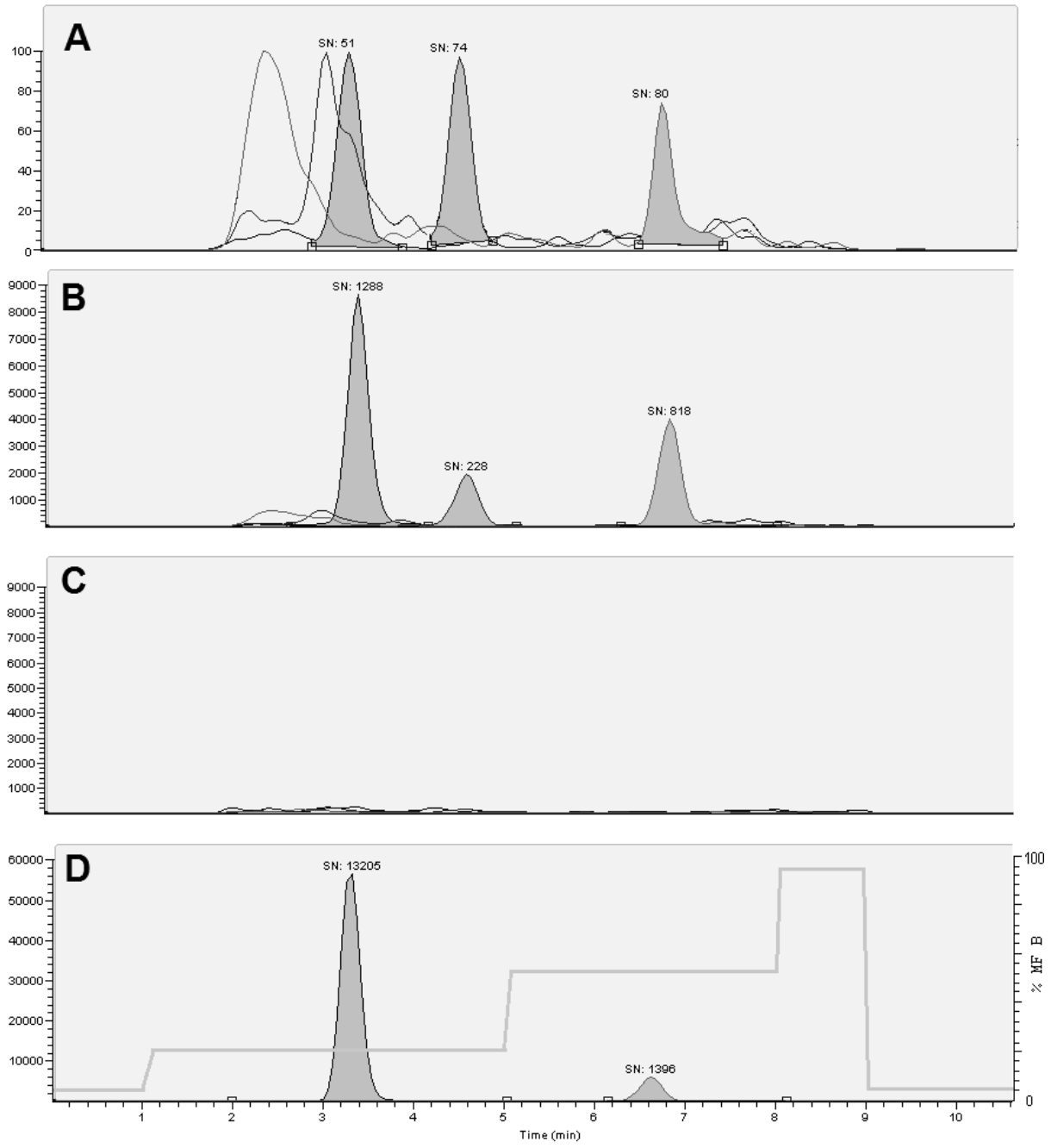

Figure 2. *LC-MS/MS chromatograms of A: DON (RT: 3.40 min), DOM-1 (RT: 4.50 min) and 3ADON (RT: 6.70 min) in broiler chicken plasma spiked at 1 ng/mL; B: DON, DOM-1 and 3ADON in broiler chicken plasma spiked at 10 ng/mL; C: a blank plasma sample; D: an incurred sample from a broiler chicken given an IV bolus injection of 571 µg 3ADON/kg body weight ten minutes after injection, DON (RT: 3.40, 73 ng/mL) and 3ADON (RT: 6.70, 21 ng/mL) together with the applied gradient, displayed as the grey line, % mobile phase B.*

Next, four sample cleanup strategies were evaluated for chicken plasma. The first one was a simple deproteinisation based on Devreese *et al.* (2012). Following the addition of 750 µL of acetonitrile to 250 µL of spiked plasma, the sample was centrifuged for 10 min at 8517 x *g* at 4 °C. Next, supernatant was transferred and dried by means of N_2 at 50 °C. After a

reconstitution in MeOH/H_2O (80/20; v/v) and a vortex step (15 sec) the extract was ultrafiltrated (PVDF, 0.22 µm) (Devreese et al. 2012). The second method was comparable to the first one but a liquid-liquid extraction step was added to remove lipophilic substances. After the addition of acetonitrile, 1 mL of hexane was added and the sample was vigorously vortex mixed for 15 sec. Hexane was removed and the supernatant transferred and treated as in the first method. The third method included an SPE step based on De Baere *et al*. (2011). A 250 µL sample of spiked plasma was mixed with 250 µL of MeOH and centrifuged (10 min, 8517 x *g*, 4 °C). Two mL of H_2O were added and this was loaded onto an SPE Oasis® HLB column (activated with 2 mL of MeOH and conditioned with 2 mL of H_2O/MeOH (90/10; v/v)). Analytes were eluted with 1 mL of MeOH, dried (N_2, 50 °C) and reconstituted (MeOH/H_2O; 80/20; v/v) (De Baere et al. 2011). The last method was a conventional C_{18} SPE cleanup, 250 µL of plasma were mixed with 750 µL of H_2O, vortexed (15 sec) and centrifuged (10 min, 8517 x *g*, 4 °C). The supernatant was loaded onto an SPE C_{18} column (activated with 2x2 mL of MeOH and conditioned with 2x2 mL of H_2O). The column was washed with 2x2 of mL H_2O and the analytes eluted with 2 mL of MeOH. The extract was dried (N_2, 50 °C) and reconstituted (MeOH/H_2O; 80/20; v/v). These four methods were executed in threefold on 100 ng/mL spiked chicken plasma. The results are depicted in **Figure 3**. Each bar represents the mean of three repeats, the whiskers represent the standard deviation. The graph was normalised by dividing the four means of the compounds by the mean of those means. The four methods all gave a comparable outcome, values ranged from 0.8 to 1.2 for all compounds. It was decided to further develop the first method as it has the best outcome for DOM-1 and 3ADON, it is the least time consuming and most economical. The method was further developed by means of fractional factorial designs.

These fractional factorial designs are experimental designs consisting of a specifically chosen subset or fraction of the experimental runs of a full factorial experiment. The latter is an experiment whose design consists of several factors, each with discrete possible levels, and whose experimental runs take on all possible combinations of these levels across all such factors. A three level full factorial design would require 3^y experimental runs, with y being the number of factors (variables). Three-level fractional factorial designs allow y factors to be evaluated on 3 levels with 3^{y-p} samples, where p is the number of factors or interactions that are confounded, *i.e.*, cannot be estimated independently. Nine experimental runs (or

samples) were executed to estimate three factors on three levels (3^{3-1}). This allowed to estimate the main effects, but these maybe confounded with two factor interactions. Five factors were tested in total, deproteinisation solvent (MeOH, acetonitrile and a 50/50; v/v mixture of both), water content of the deproteinisation solvent (0%, 25%, 50%), drying temperature (50, 65 and 80 °C), vortex time after reconstitution (15, 30 and 45”) and reconstitution solvent composition (MeOH/H_2O 50/50, 25/75 and 0/100; v/v).

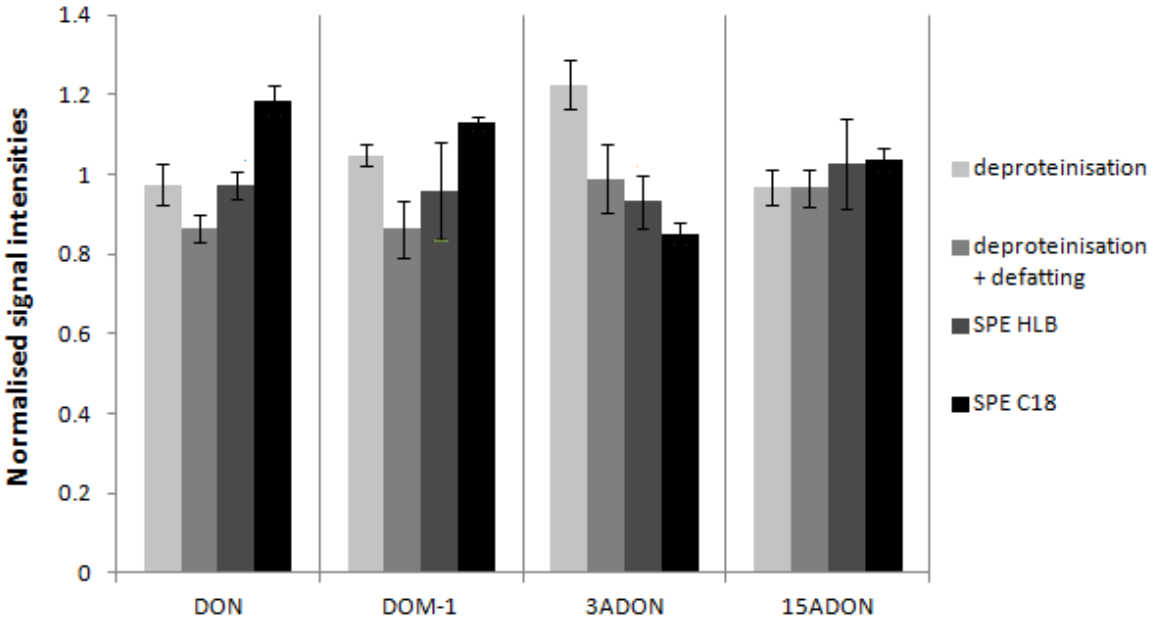

Figure 3. *Four sample cleanup strategies were tested for all the analytes. Each strategy was executed in threefold on chicken plasma spiked at 100 ng/mL. Each bar represents the mean of three repeats, the whiskers represent the standard deviation. The graph was normalised by dividing the four means for each compound by the mean of those means.*

Upon analysis by means of linear regression, three factors proved to be statistically significant ($p<0.05$), all three for the same compound 3ADON (results not shown). These were the reconstitution solvent, clearly better results were obtained with 100% water, and the deproteinisation solvent and its water content in which higher responses were obtained with acetonitrile without water. The statistically non-significant factors were set to a level that corresponds to the least time consuming, the most economical or the easiest to work cleanup strategy (drying temperature 80 °C, vortex time 15”). For pig plasma, the applicability of the developed extraction method for chicken was tested.

3.2 Validation

Validation results are based on peak area ratios with $^{13}C_{15}$-DON as IS. For the calibration model a linear, 1/x weighed, fitting was applied. The results for linearity (correlation coefficient (r) and goodness-of-fit (gof)) and sensitivity (LOD & LOQ) are given in **Table 2**. The results for linearity were all in accordance with the acceptance criteria, with r ≥ 0.99 and gof ≤ 20%. For chicken plasma the LOD varied from 0.04 (DON) to 0.70 (15ADON) ng/mL, whereas LOQ was either 1 (DON & 3ADON) or 2 (DOM-1 & 15ADON) ng/mL. For pig plasma LOD values ranged from 0.01 (DON and 3ADON) to 0.20 (15ADON) ng/mL, LOQ values were 0.1 (DON), 0.5 (DOM-1) and 1 (ADONs) ng/mL. **Table 3** displays the results for accuracy (%) and precision (RSD, %). All results for chicken as well as pig plasma were within the validation criteria.

Table 2. *Validation results for linearity (r & gof, 8 concentration points in the range LOQ-200 ng/mL), sensitivity (limit of detection (LOD) and limit of quantitation (LOQ); n=6), signal suppression and enhancement (SSE), apparent recovery (R_A) and extraction recovery (R_E).*

Animal	Compound	Correlation coefficient (r)	Goodness of fit (gof)	LOD (ng/mL)	LOQ (ng/mL)	SSE (%)	R_A (%)	R_E (%)
Chicken	DON	0.9988	13.22%	0.04	1.0	96.7	97.3	100.7
	DOM-1	0.9994	10.24%	0.51	2.0	75.3	70.2	93.3
	3ADON	0.9994	4.91%	0.13	1.0	79.0	72.4	91.6
	15ADON	0.9993	3.21%	0.70	2.0	73.1	67.1	91.8
Pig	DON	0.9996	12.08%	0.01	0.1	81.5	88.3	92.3
	DOM-1	0.9999	3.79%	0.07	0.5	97.0	69.3	71.5
	3ADON	0.9989	11.26%	0.01	1.0	80.3	53.0	66.1
	15ADON	0.9911	17.05%	0.20	1.0	44.4	28.2	64.8

Table 3. *Validation results for within-day precision (n=6) and between-day precision (n=3x3) with corresponding accuracy at low (limit of quantitation, LOQ) and high (100 ng/mL) concentration level.*

		Within-day				Between-day			
		Accuracy (%)		Precision (RSD, %)		Accuracy (%)		Precision (RSD, %)	
Animal	Compound	LOQ	100 ng/mL	LOQ	100 ng/mL	LOQ	100 ng/mL	LOQ	100 ng/mL
Chicken	DON	-4.3	-4.0	6.5	1.3	6.2	0.5	17.6	5.4
	DOM-1	5.5	-2.2	14.3	3.7	7.2	1.7	32.8	6.6
	3ADON	-28.4	0.4	22.6	6.0	5.8	1.9	28.3	12.1
	15ADON	2.0	-5.7	6.0	6.6	0.9	-0.9	12.1	16.7
Pig	DON	-19.6	1.4	14.5	2.8	11.1	7.3	21.3	11.4
	DOM-1	18.2	0.8	8.6	4.8	10.2	-0.3	12.4	11.3
	3ADON	17.4	1.4	9.5	5.9	7.5	1.4	12.2	6.0
	15ADON	18.5	4.2	16.4	14.2	-1.0	6.9	11.9	12.4

The outcome of specificity tests demonstrated that no interfering peaks were detected in the chromatographic elution zone of the analytes with S/N values ≥ 3, as depicted in **Figure 2 C**.

The results for matrix effects (SSE), apparent recovery (R_A) and extraction recovery (R_E) are depicted in **Table 2**. For chicken and pig, a clear case of ion suppression, SSE values from 73 to 96% and from 44 to 97%, respectively, could be demonstrated for the compounds, which demonstrated the need for matrix-matched calibration curves for a correct quantification. Matrix effect can be diminished by further optimizing the sample cleanup. However, a more elaborate cleanup tends to diminish the R_E. Because the developed method is straightforward, values for R_E are situated between 91 and 100% for chicken and 65 and 92% for pigs. R_A values range from 67 to 97% for chicken and from 28 to 88% for pig, with the majority of the deviation caused by SSE.

Finally, carry-over was evaluated (results not shown). For none of the compounds, signals were detected that could interfere with the response/area of the analytes at their given retention time. In **Figure 2 A** and **2 B** LC-MS/MS chromatograms are shown for 1 and 10 ng/mL spiked plasma samples from broiler chicken.

3.3 Pilot study

The pilot study was set up as a proof of concept. Acceptable plasma concentration-time profiles are obtained, depicted in **Figure 4**, in order to determine the toxicokinetic parameters in a next phase. As these profiles were obtained in one broiler, no statistical analysis of the data can be performed. However, several interesting observations can be made, such as the absence (< LOD) of DOM-1, previously described for DON treatments (Osselaere et al. 2013). Furthermore, one may observe *in vivo* hydrolysis of 3ADON to DON and to a lesser extent of 15ADON to DON. Interestingly, this has been described before for 3ADON in pigs (Eriksen et al. 2003), but it is now for the first time demonstrated in poultry.

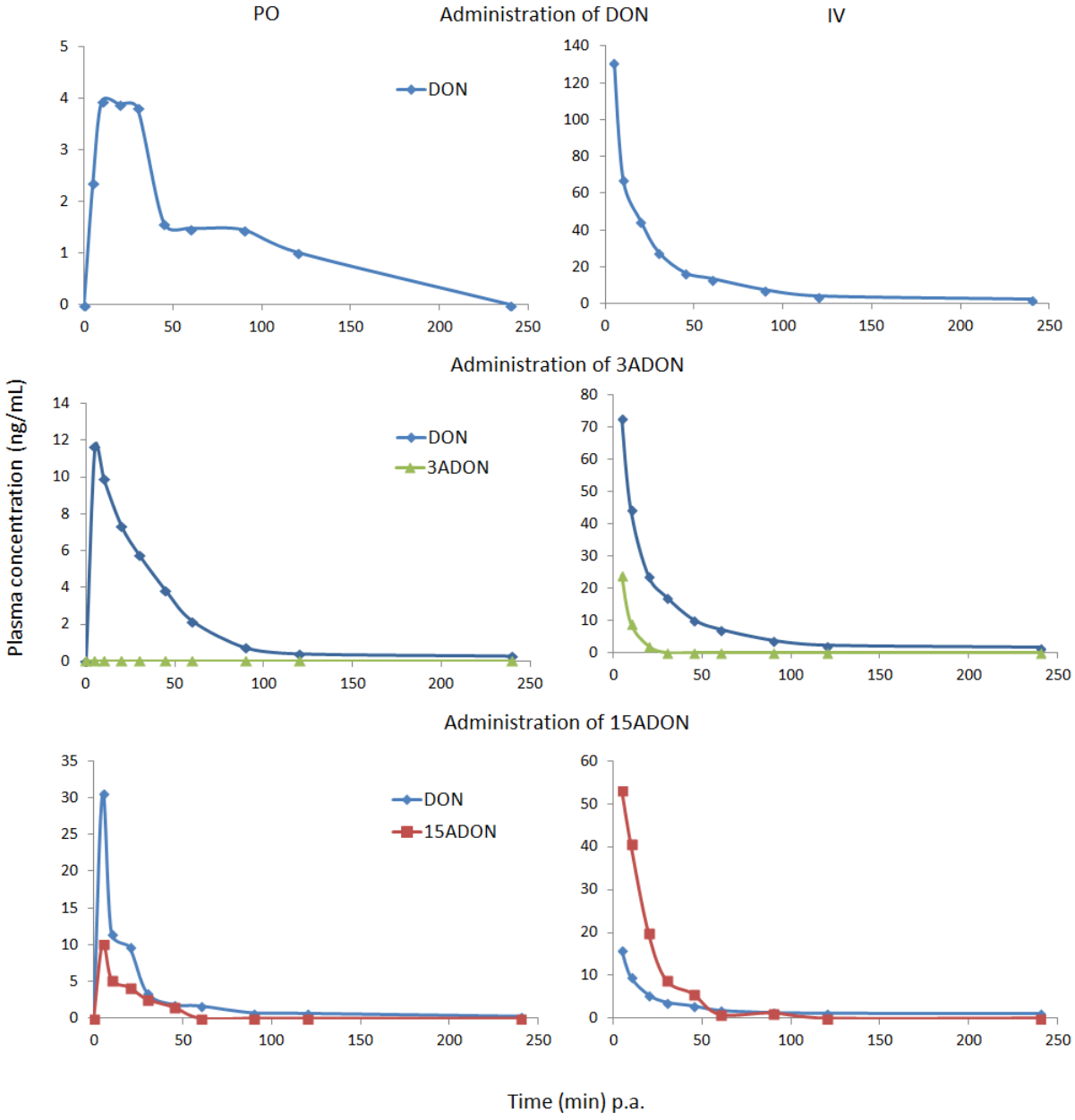

Figure 4. *Plasma concentration-time profiles in one broiler chicken after the PO (by means of gavage) and IV administration of DON (500 µg/kg body weight) or an equimolar dose of 3ADON or 15ADON (571 µg/kg body weight); p.a.= post administration.*

4. Conclusions

The aim of this study was to develop an LC-MS/MS method allowing to determine DON, 3ADON, 15ADON and DOM-1 in plasma of broiler chickens and pigs. After testing several sample cleanup strategies, a simple deproteinisation step with acetonitrile was favored. This was further optimised by the use of fractional factorial designs. Briefly, 250 µL of plasma was deproteinated by the addition of acetonitrile followed by centrifugation. The supernatant was evaporated to dryness, and the dry residue was reconstituted in 200 µL of water. The method was single laboratory validated and judged to be fit for the intended purpose. It is the first developed method to our knowledge to determine 3ADON and 15ADON in poultry and pig plasma.

As a proof of concept, a pilot study was set up. A few interesting observations were made, such as the absence of DOM-1 and the release of DON due to hydrolysis of 3ADON and, apparently to a lesser extent, of 15ADON. This method together with an *in vivo* trial, will allow to study the hydrolysis of 3ADON and 15ADON to DON and to determine the toxicokinetic parameters of these mycotoxins in broiler chickens as well as pigs, two species with a high exposure to these toxins.

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CHAPTER II

Oral bioavailability, hydrolysis and comparative toxicokinetics of 3-acetyl-deoxynivalenol and 15-acetyl-dexoynivalenol in broiler chickens and pigs

Adapted from:

Broekaert N., Devreese M., De Mil T., Fraeyman S., Antonissen G., De Baere S., De Backer P., Vermeulen A., Croubels S. (2015). Oral Bioavailability, Hydrolysis and Comparative Toxicokinetics of 3-Acetyl-deoxynivalenol and 15-Acetyl-deoxynivalenol in Broiler Chickens and Pigs. *Journal of Agricultural and Food Chemistry*, 63, 8734-42.

Abstract - The goal of this study was to determine the absolute oral bioavailability, (presystemic) hydrolysis and toxicokinetic characteristics of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in broiler chickens and pigs. Cross-over animal trials were performed with intravenous and oral administration of deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol to broilers and pigs. Plasma concentrations were analysed by using liquid chromatography-tandem mass spectrometry and concentrations were processed via a tailor-made compartmental toxicokinetic model. The results in broiler chickens showed that the absorbed fraction after oral deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol administration was 10.6%, 18.2% and 42.2%, respectively. This fraction was completely hydrolysed presystemically for 3-acetyl-deoxynivalenol to deoxynivalenol, and to a lesser extent (75.4%) for 15-acetyl-deoxynivalenol. In pigs, the absorbed fractions were 100% for deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, and both 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol were completely hydrolysed presystemically. The disposition properties of 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol demonstrate their toxicological relevance and consequently the possible need to establish a tolerable daily intake.

Keywords - plasma - deoxynivalenol - 3-acetyl-deoxynivalenol - 15-acetyl-deoxynivalenol - pig - broiler chicken - *in vivo* hydrolysis - toxicokinetics

1. Introduction

Among the more than 400 known mycotoxins, the trichothecene 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol or DON, **Figure 1**), typically found in wheat, barley, corn and rye, is one of the most prevalent mycotoxins. DON is known to cause significant economic losses in animal husbandry due to reduced body weight (bw) gain and feed conversion (Döll and Dänicke 2011; Pestka 2010). In addition to the free mycotoxin DON, other forms that are plant derived, such as deoxynivalenol-3-glucoside, and/or fungal derived, such as 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetyl-deoxynivalenol or 3ADON, **Figure 1**), and 15-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one (15-acetyl-deoxynivalenol or 15ADON, **Figure 1**), are frequently detected in food and feed as well (Van Asselt et al. 2012).

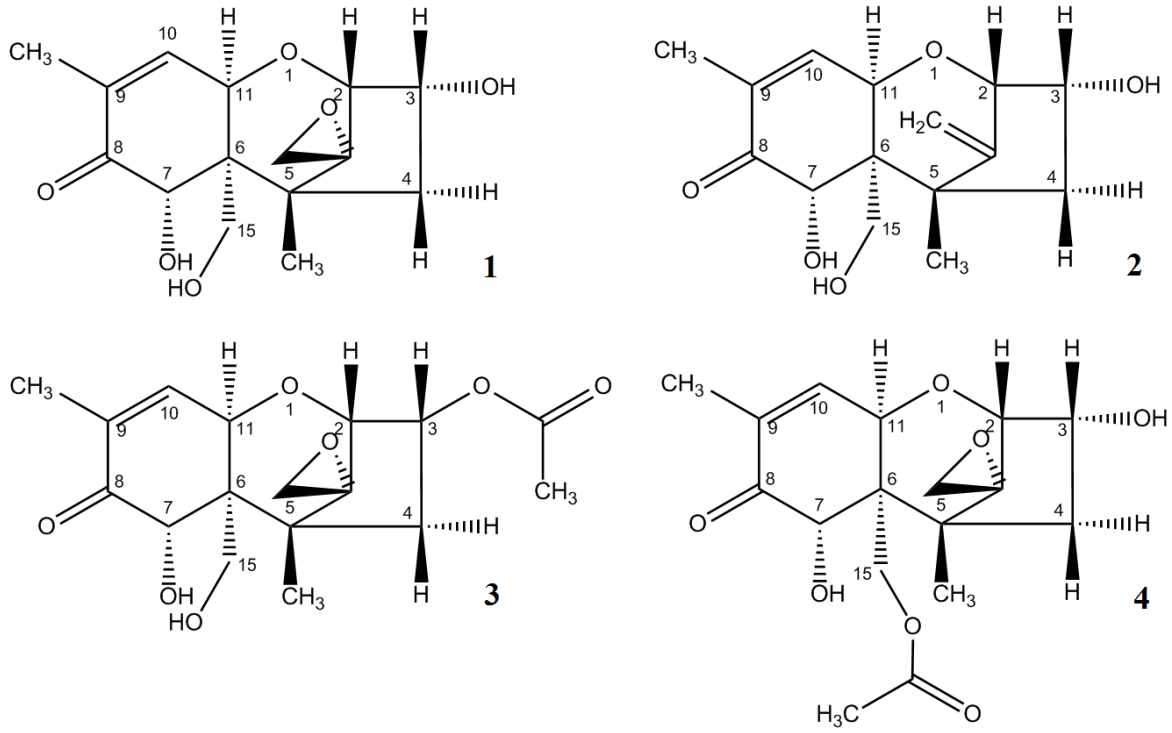

Figure 1. *Structure of 1: 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol or DON), 2: an* in vivo *metabolite, 3α,7α,15-trihydroxy-trichothec-9,12-dien-8-one (de-epoxy-deoxynivalenol or DOM-1), and two acetylated forms of DON, 3: 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetyl-deoxynivalenol or 3ADON) and 4: 15-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one (15-acetyl-deoxynivalenol or 15ADON).*

Due to the ambiguous use of the term 'masked' mycotoxin, all potential mycotoxin derivatives were systematically defined (Rychlik et al. 2014). The acetylated forms of DON are intermediaries in its biosynthesis and should be regarded as free mycotoxins (Yoshizawa and Morooka 1975).

The presence of both 3ADON and 15ADON has been investigated in 30 samples of cereals and cereal derived products. Both 3ADON and 15ADON were highly prevalent, contaminating respectively 87% and 73% of the samples, with the highest contamination levels being detected in maize at concentrations up to 305 ± 216 µg/kg for 3ADON and 334 ± 270 µg/kg for 15ADON (De Boevre et al. 2012). In a Dutch field survey, both ADONs were detected more frequently than DON itself, respectively in 21% and 7% of 42 maize samples (Van Asselt et al. 2012). Concentrations of 3ADON and 15ADON in corn exceeding 500 and 1000 µg/kg, respectively, are reported occasionally (Gareis et al. 2003). In risk assessments of DON, both ADONs have been considered, but neglected due to the lack of information on toxicity and toxicokinetics and given the lower concentration levels compared to DON.

The presence of mycotoxins gives rise to two important questions, as recently reviewed (Broekaert et al. 2015; De Boevre et al. 2015). First, what is the intrinsic toxicity of these contaminants? Second, what is the disposition and fate of these compounds in humans and animals? The toxicological importance of 3ADON and 15ADON has been previously highlighted by reporting the *in vitro*, *ex vivo* and *in vivo* toxicity of DON and its acetylated forms on intestinal morphology, intestinal epithelial cell proliferation and expression of intestinal epithelial cell tight junction proteins (Pinton et al. 2012). It was suggested that 3ADON is less toxic than DON and, in contrast, that the toxicity of 15ADON is higher or comparable to DON. A comparison of the effect of these compounds on the interleukin-8 (IL-8) secretion in Caco-2 cells, indicating activation of the innate immune system, and intestinal transepithelial transport over Caco-2 cells has been performed (Kadota et al. 2013). The study revealed a significantly higher intestinal absorption of 15ADON compared to DON and 3ADON. Furthermore, the authors showed that the ability to induce IL-8 secretion was ranked as follows: 3ADON < DON < 15ADON. Similar effects were previously reported for the cytotoxicity on cultured human myeloid and lymphoid cell lines (K-562 and MIN-GL1) (Visconti et al. 1991), and for DNA inhibition in Swiss mouse 3T3 fibroblasts (Sundstol Eriksen and Pettersson 2003). Furthermore, an *in vivo* metabolite 3α,7α,15-trihydroxy-trichothec-

9,12-dien-8-one (de-epoxy-deoxynivalenol or DOM-1, **Figure 1**), was included in the latter study and was found to be the least toxic compound, due to absence of the epoxide ring. In conclusion, these compounds have a distinct degree of toxicity, which can be ranked in order of increasing toxicity: DOM-1 << 3ADON < DON ≤ 15ADON.

A key question concerning the *in vivo* disposition is whether conversion of 3ADON and 15ADON to DON by *in vivo* hydrolysis in humans and animals would occur and possibly imply an underestimation of the degree of contamination and toxicity. Several *in vitro* studies described the hydrolysis of ADONs to DON in humans, pigs and poultry (Eriksen et al. 2002; Sundstol Eriksen and Pettersson 2003; Young et al. 2007). In contrast, only a limited number of *in vivo* studies are available on the hydrolysis and metabolism of ADONs. The hydrolysis of 3ADON and 15ADON after oral administration to rats has been investigated (Versilovskis et al. 2012), as well as the hydrolysis and toxicokinetics of 3ADON when orally administered to pigs (Eriksen et al. 2003). Both studies demonstrated that *in vivo* hydrolysis of ADONs to DON is possible. However, it was not reported if the hydrolysis occurs presystemically and/or systemically. No *in vivo* data are available for broiler chickens, although these animals have a high exposure to these mycotoxins, because of their mainly cereal-based diet.

The goal of this study was to determine the absolute oral bioavailability, the degree of hydrolysis (differentiating between presystemic and systemic hydrolysis) and toxicokinetic characteristics of 3- and 15ADON in broiler chickens and pigs. Species selection was based, as stated above, on the high exposure of these animals to the toxins, due to their mainly cereal-based diet and the high prevalence of mycotoxins in these diets. Additionally, pigs were included because of their sensitivity to DON. Animal species susceptibility to DON can be ranked as pigs > mice > rats > poultry ≈ ruminants (Prelusky et al. 1994). Differences in absorption, distribution, metabolism and elimination of DON among animal species might account for this differential sensitivity (Pestka 2010). Furthermore, the data from pig studies are highly relevant to humans because of the anatomical and physiological similarities of the gastrointestinal tract, liver, kidneys and cardiovascular system in both species (Helke and Swindle 2013; Swindle et al. 2012).

2. Experimental

2.1 Chemicals, products and reagents

DON, 3ADON and 15ADON had all >99% purity (Fermentek, Jerusalem, Israel). The mycotoxins were dissolved in analytical-grade ethanol (EtOH) (Merck, Brussels, Belgium), yielding a stock solution of 10 mg/mL and were used for both animal and analytical experiments. As internal standard (IS), $^{13}C_{15}$-DON (50 µg/mL) stock solution in acetonitrile was used (Romer Labs, Tulln, Austria). All stock solutions were stored at ≤-15 °C. Individual working standard solutions of 10 µg/mL, used for the analytical experiments, were prepared by diluting the above stock solutions with ULC/MS grade acetonitrile (Biosolve, Valkenswaard, The Netherlands). All working standard solutions were stored at ≤-15 °C. Standard mixture working solutions containing DON, 3ADON and 15ADON of 1 and 0.1 µg/mL were prepared by mixing appropriate dilutions of the individual working standard solutions in ULC/MS acetonitrile and were stored at 2-8 °C. Solutions of DON in acetonitrile have been reported to be stable for 24 months at room temperature (Widestrand and Pettersson 2001), whereas the supplier reported a stability of at least 6 months at 4 °C for a 100 µg/mL solution of 3ADON, and 15ADON in acetonitrile.

Water, methanol and glacial acetic acid were of ULC/MS grade (Biosolve). Microfilters Millex GV-PVDF 0.22 µm were obtained to filter deproteinised plasma samples (Millex, Overijse, Belgium).

2.2 Animal trials

Eighteen broiler chickens (Ross 308) were purchased from the Institute for Agriculture and Fisheries Research (Melle, Belgium), at the age of 3 weeks, as hatched and had an average bw ± standard deviation (SD) of 1210 ± 172 g. The broilers were randomly allocated to three groups of six animals. Water and feed were given *ad libitum*. The pen was climate controlled with temperatures between 21 and 25 °C, a relative humidity between 40 and 60% and an applied light schedule similar to commercial installations (18 h light/6 h dark). After one week of acclimatisation, the animals were treated with either DON (group 1), 3ADON (group 2) or 15ADON (group 3) by intravenous bolus injection (IV, administration in *vena basilica*) (three animals/group) or *per os* (PO, by means of gavage in the crop) (three animals/group)

in a two-way cross-over design. The administered doses were based on the EU maximum guidance level of 5 mg DON/kg poultry feed (European Commission 2006). Since the average feed consumption of 4-week-old broiler chickens was 100 g feed/kg bw/day, 500 µg DON/kg bw was administered. For the ADONs, an equimolar dose of 571 µg/kg bw was administered. The calculated volume of stock solution (10 mg/mL in EtOH), was diluted with saline (0.9% NaCl) (VWR, Leuven, Belgium) up to a volume of 0.5 mL (IV) or 1 mL (PO). After oral administration of the toxins, 1 mL of tap water was administered to flush the gavage syringe. A washout period of 3 days was maintained between treatments. Feed was deprived 12 h before administration and till 3 h post administration (p.a.). Blood (0.5-1 mL) was sampled from the leg vein by venipuncture at 0 min (before administration) and at 5, 10, 20, 30, 45, 60, 90, 120 and 240 min p.a. Blood samples were centrifuged (2851 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis.

For the pig study, nine clinically healthy male pigs (11 weeks of age, 28.6 ± 2.8 kg bw) were individually housed with feed and water *ad libitum.* Natural lighting was provided, the enclosure was climate controlled to keep the temperature between 20 and 24 °C and the relative humidity between 20 and 40%. After a 1 week acclimatisation period, the animals were treated with either DON (n=3), 3ADON (n=3) or 15ADON (n=3) by IV bolus injection using a surgically placed jugular catheter as previously described (Gasthuys et al. 2009), or PO (by means of gavage in the stomach) in a cross-over design. For this design, pigs were randomly divided into groups of three pigs. Each group received four treatments (two IV and two PO treatments), resulting in a cross-over for each compound in six animals. A washout period of 24 h was maintained between treatments. 12 h before administration of the mycotoxins, the animals were fasted until 4 h p.a. The administered doses were calculated as for the broiler chicken trial. The EU maximum guidance level in pig feed is 0.9 mg/kg DON (European Commission 2006). Pigs at 12 weeks of age consume on average 40 g feed/kg bw/day. This resulted in the administration of 36 µg DON/kg bw. For the ADONs, an equimolar dose of 41 µg/kg bw was administered. The calculated amount of toxin for each animal, dissolved in EtOH at 10 mg/mL, was diluted with saline to a volume of 1 mL (IV) or 10 mL (PO). After oral administration of the toxins, 50 mL of tap water was administered to flush the gavage tube. Blood (1-2 mL) was sampled from the jugular vein at 0 min (before administration) and at 5, 10, 20, 30, 45, 60, 90, 120, 240, 360 and 480 min p.a. Blood

samples were centrifuged (2851 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis.

Both animal trials were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2013/64).

2.3. LC-MS/MS analysis

Sample pretreatment and LC-MS/MS analysis was performed as previously described (Broekaert et al. 2014). In brief, 250 µL of plasma was supplemented with IS, 5 µL of a 1 µg/mL $^{13}C_{15}$-DON working solution. After vortex mixing for 15 sec, acetonitrile was added up to a volume of 1 mL to precipitate plasma proteins. The samples were vortex mixed, followed by a centrifugation step (8517 x *g*, 10 min, 4 °C). The supernatant was transferred and evaporated to dryness under a (gentle) nitrogen stream at 80 ± 5 °C. The dry residue was redissolved in 200 µL of ULC/MS-grade water, micro-filtrated through a Millex GV-PVDF filter (0.22 µm) and transferred to an autosampler vial. A 10 µL aliquot was injected onto the LC-MS/MS instrument. Broiler plasma was analysed on a Surveyor type MS pump Plus and Autosampler Plus HPLC in combination with a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, Breda, The Netherlands). Pig plasma was analysed on an Acquity UPLC system coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Zellik, Belgium).

Chromatographic separation of the analytes was achieved on a 50 mm x 2.1 mm i.d., 1.9 µm, Hypersil Gold column with a guard column of the same type (Thermo Fisher Scientific). All compounds were eluted with a gradient of ULC/MS-grade water containing 0.1% or 0.3% glacial acetic acid, for the analysis of chicken and pig plasma respectively (mobile phase A) and ULC/MS-grade methanol containing 0.1% or 0.3% glacial acetic acid, for the analysis of chicken and pig plasma respectively (mobile phase B) at a total flow rate of 300 µL/min. Following gradient elution program was run: 0-1 min, 5% B; 1.0-1.1 min, linear gradient to 20% B; 1.1-5.0 min, 20% B; 5.0-5.1 min, linear gradient to 50% B; 5.1-8.0 min, 50% B; 8.0-8.1 min, linear gradient to 95% B; 8.1-9.0 min, 95% B; 9.0-9.1 min, linear gradient to 5% B; 9.1-12.0 min, 5% B. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with two ion transitions for each target analyte. Limits of quantitation (LOQs)

varied from 0.1 ng/mL (DON in pig), 1.0 ng/mL (ADONs in pig, DON and 3ADON in chicken) to 2.0 ng/mL (15ADON in chicken).

2.4. Toxicokinetic modeling and statistical analysis

Toxicokinetic analysis was performed using WinNonlin Professional version 5.2.1. (Pharsight, St-Louis, MO). Plasma concentrations below the LOQ were not taken into account. For the toxicokinetic analysis, all values were recalculated to their molar concentrations, expressed as nmol/mL. For the analysis of DON IV and PO data in chickens, a tailor-made two-compartmental model (central compartment (Vc) and peripheral compartment (Vp)) with first order absorption and first order elimination was applied. For the IV and PO data of ADONs in chickens, a one-compartmental model was used in combination with the two-compartmental model for DON. The fraction of the dose of DON, 3ADON or 15ADON that was absorbed after PO administration (in any form, thus as DON and/or ADON) was indicated as FRAC (%). BioADON was the fraction of the absorbed dose that entered the systemic circulation in its unchanged form, thus as ADON (%). Consequently, the product of FRAC and BioADON indicates the absolute oral bioavailability of ADON (F%), i.e. the fraction of ADON absorbed in the systemic circulation in its unchanged form. The product of FRAC and (1-BioADON) then indicates the absorbed fraction that was presystemically hydrolysed to DON and that entered the systemic circulation as DON (presystemic hydrolysis, %). Systemic hydrolysis was set at 100% for 3ADON and 15ADON in pigs and in broiler chickens, as the hydrolysis to DON was the only assumed clearance (CL) of ADON. In **Figure 2** a graphical representation of the constructed model is given for DON (IV & PO) and ADON (IV & PO) in chickens.

The constructed models for pigs were identical to those for chickens, except for the ADON PO model. Insufficient to no ADON data above the LOQ after PO administration of ADONs led to the exclusion of the ADON compartment for this administration route in pigs. This model therefore assumes a full presystemic conversion of ADON to DON.

A $1/\hat{y}$ weighing was applied for all calculations with both the chicken and pig data. For all the described models, the Gauss-Newton (Levenberg and Hartley) algorithm was used and a maximum of 50 iterations was allowed.

The following primary and secondary toxicokinetic parameters were calculated for IV and PO administration: absorption rate constant ka (identical for DON and ADONs), intercompartmental flow for DON (Q_{DON}), total body clearance of DON (Cl_{DON}), total body clearance of ADON to DON (CL_{ADON}), central volume of distribution for DON (Vc_{DON}), peripheral volume of distribution for DON (Vp_{DON}), central volume of distribution for ADONs (Vc_{ADON}) and disposition rate constant for DON (β_{DON}). Additionally, for PO data the descriptive toxicokinetic parameters maximal plasma concentration (C_{max}) and time to C_{max} (t_{max}) were calculated for DON.

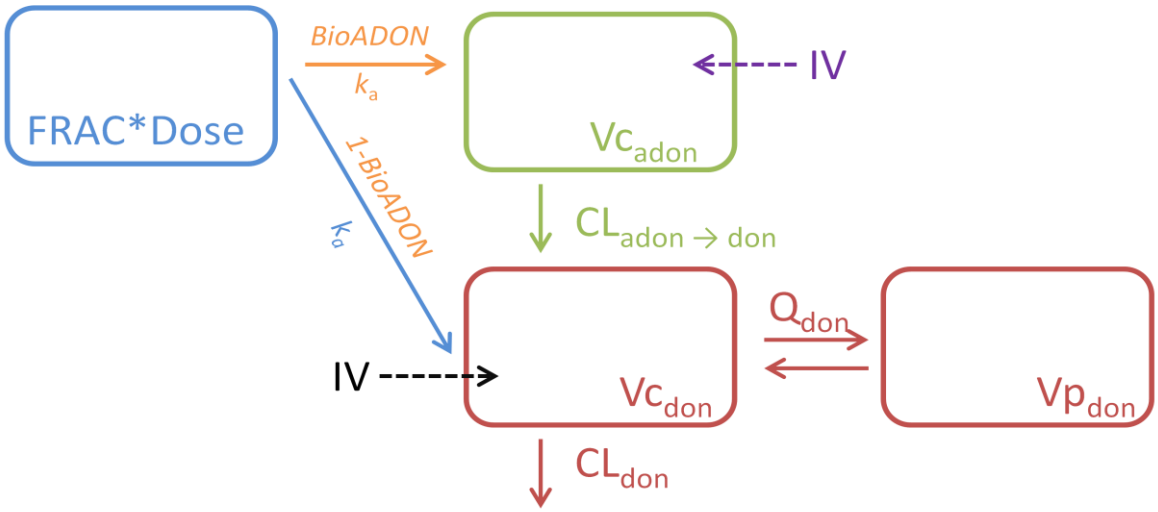

Figure 2. *Graphical representation of the applied models in broiler chicken for DON IV (black and red section), DON PO (blue and red section), ADON IV (purple, green and red section) and ADON PO (blue, orange, green and red section). IV=intravenous administration; ka=absorption rate constant (1/min); Cl_{DON}=clearance of DON (mL/min/kg); Q_{DON}=intercompartment flow for DON (mL/min/kg); Vc_{DON}=central volume of distribution for DON (mL/kg); Vp_{DON}=peripheral volume of distribution for DON (mL/kg); Cl_{ADON}=clearance of ADON to DON (mL/min/kg); Vc_{ADON}=central volume of distribution for ADON (mL/kg); FRAC=fraction of dose absorbed; BioADON=fraction of the absorbed dose that enters the systemic circulation as ADON; 1-BioADON=fraction of the absorbed dose that enters the systemic circulation as DON.*

Statistical analysis of these parameters consisted of one-way ANOVA with post-hoc Scheffé tests ($p < 0.05$) for both animal species (SPSS 20.0, IBM, Chicago, IL). All variables were log transformed in order to fulfil the equality of variances criterion as determined by the Levene's test for homogeneity of variances ($p > 0.01$).

3. Results and discussion

The goal of this study was to determine the absolute oral bioavailability of 3- and 15ADON, the degree of *in vivo* hydrolysis (differentiating between presystemic and systemic hydrolysis) and the toxicokinetic parameters in broiler chickens and pigs. The plasma concentration-time profiles were analysed by a tailor-made compartmental toxicokinetic model. This model has the advantage, compared to non-compartmental analysis, that for a given compound, PO and IV data can be fitted simultaneously for each animal. This is more accurate because certain toxicokinetic parameters, such as Cl and Vd, are independent of the administration route and thus more data is available to allow a reliable estimate. Furthermore, this model allows prediction of the plasma concentrations for scenarios that have not been studied yet, whereas non-compartmental analysis only uses observed values. For both ADONs, only a hydrolysis to DON is assumed in the model after IV and PO administration in broilers and pigs. It needs to be mentioned that also formation of glucuronide conjugates and possibly other metabolites can take place. Therefore, a study using high resolution mass spectrometry (HRMS) on pig and broiler chicken plasma after IV and PO administration was performed. The methodology was similar as the one used in a prior described study (Devreese et al. 2015). For both IV and PO administration in pigs, a mean peak area ratio of DON-glucuronide/DON of 4.98 and 4.95 was found, respectively (results not shown). Therefore, presystemic glucuronidation in pigs is considered neglectable. Consequently, the impact on estimated volumes of distribution, clearances and absorption and elimination rate constants for the studied compounds is expected to be very small. For chickens, only trace amounts of DON-glucuronide were detected (Devreese et al. 2015).

The developed model allows an estimation of the degree of presystemic and systemic hydrolysis after PO administration, which is not possible using non-compartmental analysis only.

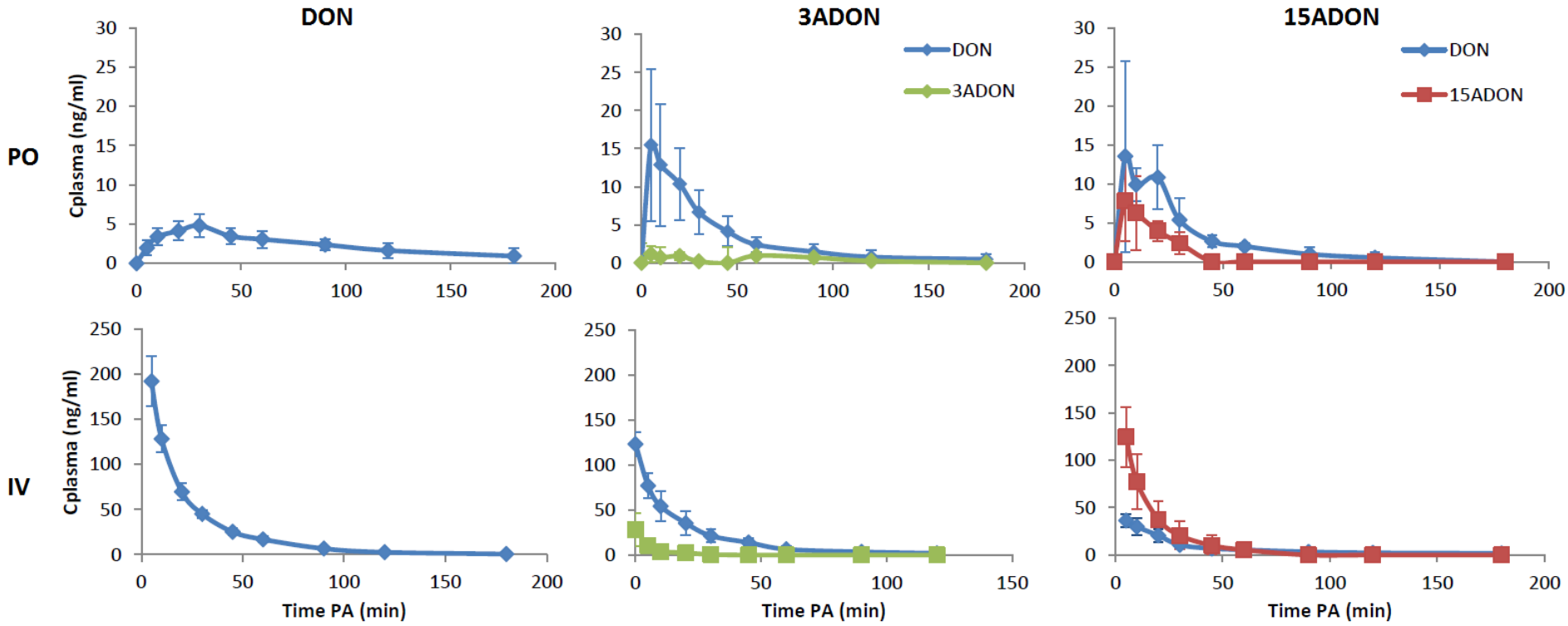

Figure 3. *Plasma concentration-time profiles of deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) after oral (PO) and intravenous (IV) administration (post administration = PA) of DON (dose=500 µg DON/kg body weight), 3ADON (dose=571 µg 3ADON/kg body weight) and 15ADON (dose=571 µg 15ADON/kg body weight) to broiler chickens (n=6 for each toxin). Values are presented as mean ± SD.*

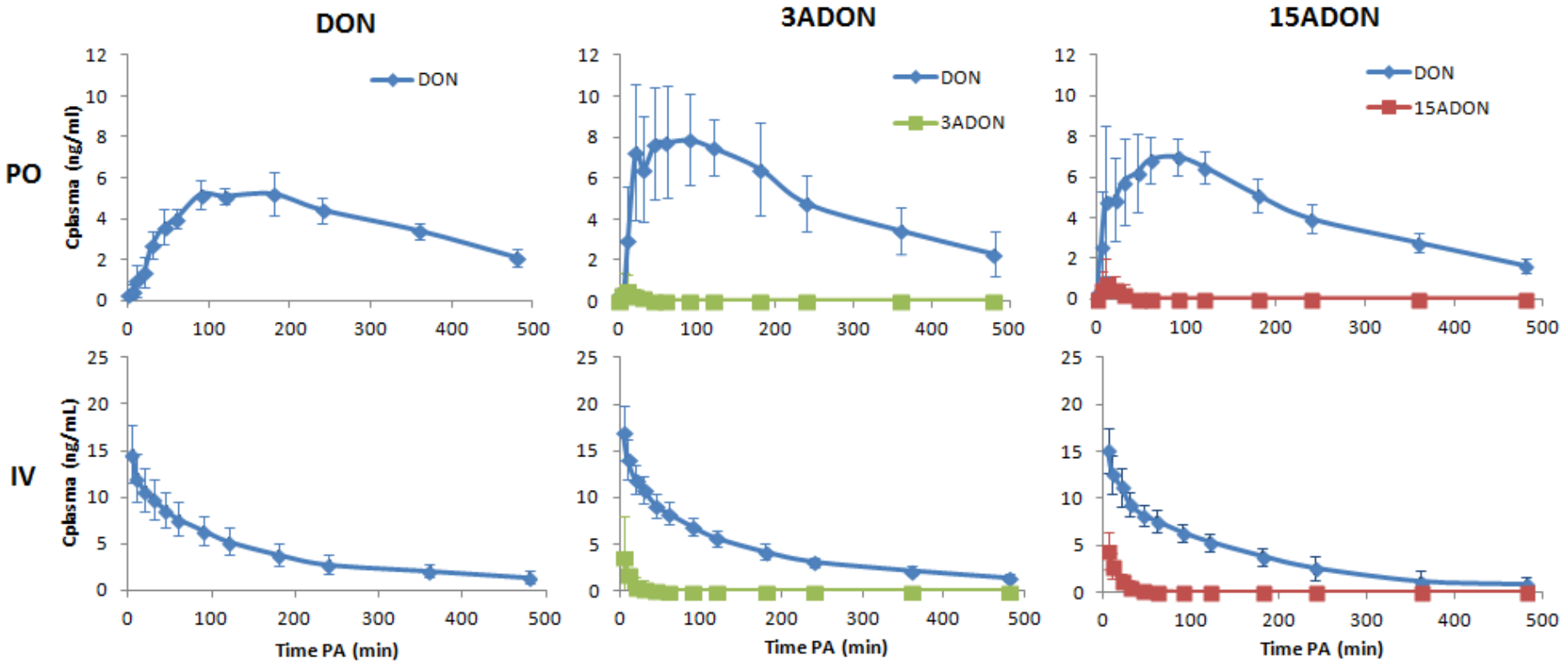

Figure 4. *Plasma concentration-time profiles of deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) after oral (PO) and intravenous (IV) administration (post administration=PA) of DON (dose=36 µg DON/kg body weight), 3ADON (dose=41 µg 3ADON/kg body weight) and 15ADON (dose=41 µg 15ADON/kg body weight) to pigs (n=6 for each toxin). Values are presented as mean ± SD.*

Plasma concentration-time profiles for DON, 3ADON and 15ADON are presented in **Figures 3** and **4**, respectively for broiler chickens and pigs. Each profile represents the mean of six animals ± standard deviation (SD). For broiler chickens, no adverse effects were observed during the animal trial following PO and IV bolus administration of the mycotoxins. Similarly, no adverse effects were observed during the pig trial following PO administration. Following IV dosing of DON, 3ADON and 15ADON respectively, three, two and two out of six pigs vomited within 0.5 h p.a. However, this had no observable influence on the results as these pigs were IV dosed.

The plasma concentration-time profiles were also expressed as DON equivalents (DEQ). For this, the ADON concentrations were converted by means of their molar mass (DON= 296.3 g/mol; ADONs=338.3 g/mol), multiplied with their respective volumes of distribution, added to the DON concentrations corrected for the volume of distribution and reported as DON equivalents in ng/kg bw. The results for pigs and chickens are shown in **Figure 5**. Clear differences can be seen for PO administration between DON, 3ADON and 15ADON in pigs as well as broiler chickens, while the IV profiles were similar, which can be regarded as a quality control of the utilised set-up.

Toxicokinetic parameters, absolute oral bioavailability (F%) and degree of presystemic hydrolysis (%) were calculated using compartmental modelling, the results of which are given in **Table 1**.

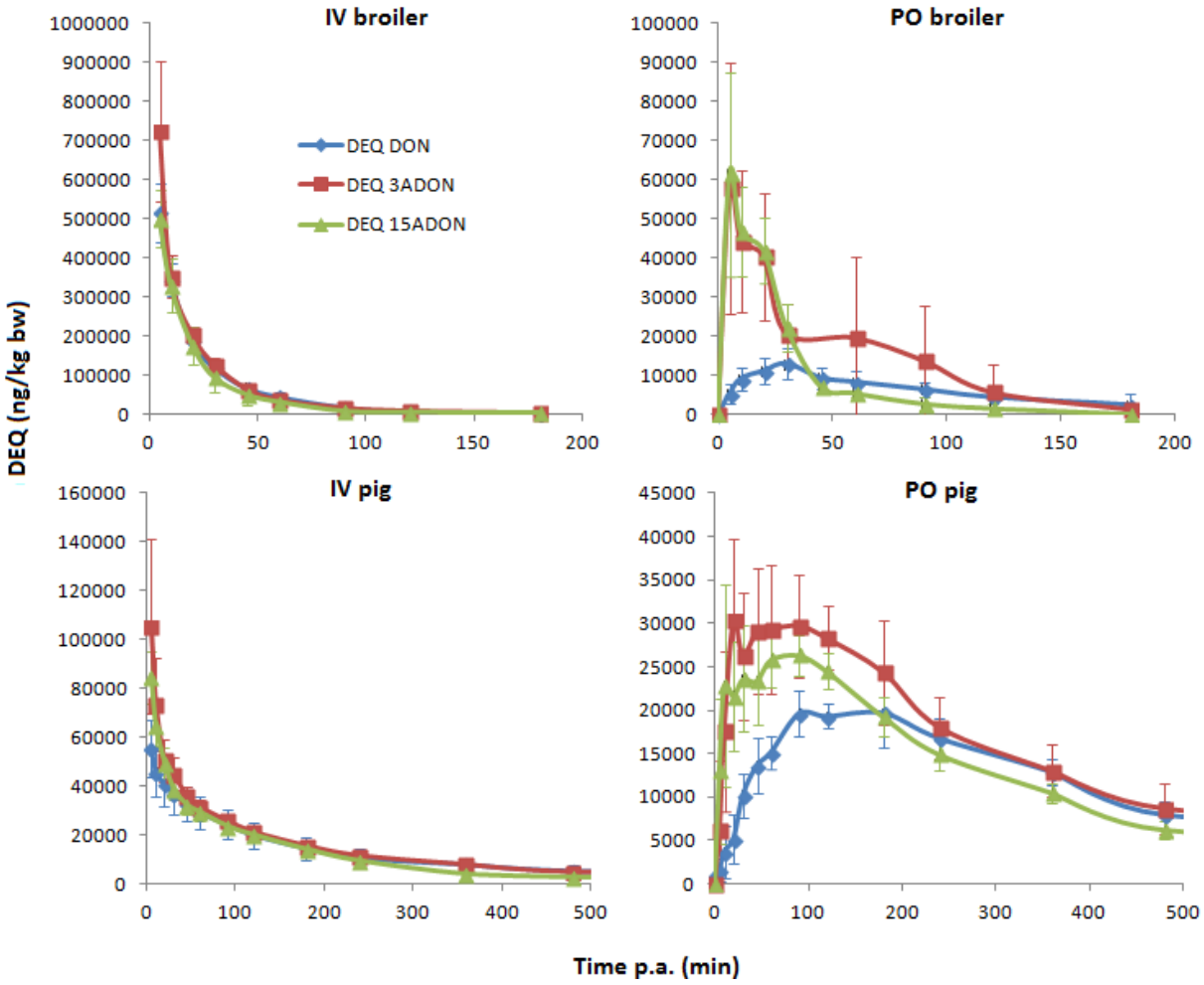

Figure 5. *DON equivalent (DONeq or DEQ) plasma concentration-time profiles after oral (PO) and intravenous (IV) administration of deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) in broiler chickens and pigs (n=6 animals for each toxin). DEQ is calculated by dividing the plasma concentration of the ADONs by their molar mass (338.3 g/mol) and by multiplying with that of DON (296.3 g/mol) and correcting for their volumes of distribution, and by adding this to DON plasma concentrations corrected for volume of distribution. Values are presented as mean ± SD.*

Table 1. Toxicokinetic parameters, presystemic hydrolysis of the bioavailable fraction (%) and absolute oral bioavailability (%) of DON, 3ADON and 15ADON after IV and PO administration to broiler chickens and pigs (n=6).

Animal species	Adm.	ka (1/min)	β_{DON} (1/min)	Cl_{DON} (mL/min/kg)	Q_{DON} (mL/min/kg)	Vc_{DON} (mL/kg)	Vp_{DON} (mL/kg)	Cl_{ADON} (mL/min/kg)	Vc_{ADON} (mL/kg)	FRAC (%)	BioADON (%)	Pres.Hydr. (%)	F (%)	C_{maxDON} (ng/mL)	t_{maxDON} (min)
Broiler chicken	DON	0.0203 ±0.0101[a]	0.0388 ±0.0062	102±12[a]	59.8±25.2[a]	1656 ±426[a]	1025 ±142[a]	-	-	10.6 ±4.6[a]	-	-	10.6 ±4.6[a]	4.2±0.9[a]	32±11[a]
	3ADON	0.0722 ±0.0497[a]	-	151±16[a]	155±65[a]	109 ±66[b]	3490 ±840[a]	1506±615[a]	15918 ±11620[a]	18.2 ±5.5[a]	0	18.2±5.5[a]	0[b]	15.0±8.8[b]	5.0±0[b]
	15ADON	0.0289 ±0.0216[a]	-	340±132[b]	287±118[b]	1861 ±1220[a]	18154 ±14573[b]	236±63[b]	3692 ±1707[b]	42.2 ±12.5[b]	24.6	31.9±14.8[b]	10.4 ±3.6[a]	16.2±7.6[a,b]	9.0±6.5[b]
Pig	DON	0.0164 ± 0.0161[a]	0.00405 ±0.00076	15.1±3.8[a]	21.1±12.7[a]	2605 ±570[a]	1174 ±600[a]	-	-	100[a]	-	-	100[a]	6.3±2.4[a]	109±41[a]
	3ADON	0.0179 ±0.0100[a]	-	14.0±2.4[a]	202±50[b]	175 ±96[b]	2579 ±406[b]	1428±1162[a]	13001 ±11625[a]	100[a]	0	100[a]	0[b]	7.5±1.7[a]	101±45[a]
	15ADON	0.0136 ±0.0044[a]	-	15.3±2.1[a]	113±6.0[c]	31.6 ±31.7[b]	2895 ±241[b]	426±231[a]	7151 ±2427[a]	100[a]	0	100[a]	0[b]	6.5±1.2[a]	78±39[a]

Values are presented as mean ± SD. Statistical analysis was performed within each animal species, results sharing the same letters in each column are significantly different ($p < 0.05$) compared to DON values in the same animal species. Adm.=administered mycotoxin; ka=absorption rate constant (1/min); β_{DON}=disposition rate constant for DON (1/min)(β_{DON} could theoretically also be calculated after administration of ADONs); Cl_{DON}=clearance of DON (mL/min/kg); Q_{DON}=intercompartmental flow for DON (mL/min/kg); Vc_{DON}=central volume of distribution for DON (mL/kg); Vp_{DON}=peripheral volume of distribution for DON (mL/kg); Cl_{ADON}=clearance of ADON to DON (mL/min/kg); Vc_{ADON}=central volume of distribution for ADON (mL/kg); FRAC=absorbed fraction; BioADON=fraction of the absorbed dose that entered the systemic circulation as ADON (%); Pres.Hydr.=percentage of the total dose that is hydrolysed presystemically to DON and absorbed as DON (%); F=absolute oral bioavailability (%); C_{maxDON}=maximum plasma concentration for DON (ng/mL); t_{maxDON}= time to maximum plasma concentration for DON (min).

The absorbed fractions of 3ADON (18.2%) and 15ADON (42.2%) in broiler chickens correspond to approximately double and quadruple that of DON (10.6%), respectively. However, no statistical difference ($p=0.061$) could be demonstrated between the absorbed fraction of DON and 3ADON in broiler chickens. The absorbed fraction of 15ADON on the other hand was found to be significantly higher than that of DON and 3ADON ($p<0.05$). An increase in absorbed fractions for the ADONs might be expected as these acetylated mycotoxins are less polar and have a more favourable log D value for absorption by passive non-ionic diffusion compared to DON (estimated log D_{ADON} and log D_{DON} at physiological pH are -0.5 and -1, respectively) (Maresca 2013). For pigs on the other hand, the absorbed fractions of DON, 3ADON and 15ADON were 100%. The relatively low absorbed fraction for chickens compared to pigs has been discussed (Prelusky et al. 1986) and may be partially due to the rapid transit time in the GI tract of chickens, leading to a reduced time for complete absorption. As another contributing factor, the high bacterial content in the GI tract of birds before the main site of absorption is proposed. These bacteria could metabolise DON and/or ADONs thereby decreasing their bioavailability and toxicity (Maresca 2013).

To correlate the absorbed fraction to the absolute oral bioavailability (F%), the degree of *in vivo* hydrolysis must be considered. As the ADONs can be hydrolysed presystemically to DON, their absorbed fraction may represent a combination of absorption as DON and as ADON, and can thus differ from their oral bioavailability, which is represented by the absorption as ADON. However, this is not the case for DON. For chickens, the F% value for DON is equal to its absorbed fraction, being 10.6 ± 4.6% in this study. For DON in broiler chickens, an oral F% of 19.3 ± 7.42% has been observed (Osselaere et al. 2013), which is in the same range. In contrast, in fasted pigs a complete absorbed fraction and complete oral bioavailability of DON was found. Prior studies in pigs observed an oral F for DON after chronic and acute exposure of 62.8% and 54.1%, respectively (Goyarts and Dänicke 2006; Prelusky et al. 1988). Differences in F for acute exposure can be attributed to either the prandial state (fed versus fasted) and/or the administration of a bolus (dissolved in e.g. ethanol) versus the administration of contaminated feed. The oral absorption as well as oral bioavailability of the ADONs in pigs and chickens has thus far not been investigated.

In broiler chickens, both ADONs have a significantly different degree of presystemic hydrolysis ($p<0.05$). While for 3ADON the fraction of the dose absorbed was completely

hydrolysed presystemically, the absorbed fraction of 15ADON that entered the systemic circulation as such (BioADON) was 24.6%, and was consequently 75.4% as DON. As the fraction of the dose absorbed for 3ADON was completely hydrolysed presystemically, an F% value of 0% was obtained, while for 15ADON an F% value of 10.4% was calculated. Differences in hydrolysis/metabolism between both ADONs in broiler chickens has already been demonstrated *in vitro* (Young et al. 2007). After anaerobic incubation of 3ADON for 72 h with microbiota from the GI tract of chickens, deacetylation was the predominant reaction (94%) with only minimal subsequent de-epoxidation (6%); there was no evidence for de-epoxidation only. However, for 15ADON, a large fraction proved to have both the acetyl and epoxy moiety removed (± 93%), whereas only small quantities showed either solely de-epoxidation (± 1%) or deacetylation (± 7%), leaving no 15ADON. The role of GI microbiota in our *in vivo* study has little to no influence on the presented results as the high degree of hydrolysis after IV administration indicates a systemic biotransformation rather than being the result of the action of GI microbiota. Additionally, the microbiota from the aforementioned *in vitro* study were isolated from the large intestine. At this site in the GI tract the absorption of the toxins will be negligible and transformation here will not be reflected by changes in plasma concentrations. Furthermore, the average GI transit time for chickens is 3 to 4 h, whereas the experimental set-up of this *in vitro* study consisted of a 72 h anaerobic incubation (Golian and Maurice 1992; Young et al. 2007).

In pigs, 3ADON and 15ADON exhibited a presystemic hydrolysis of >99% also leading to F% values of 0%. These findings for the *in vivo* hydrolysis of 3ADON in pigs correspond with an earlier study (Eriksen et al. 2003). Five castrated pigs were fed twice daily for three days 2.5 mg 3ADON/kg feed. No 3ADON, nor DOM-1, could be detected in plasma. The only detected metabolite in plasma was DON as such. After incubation of the plasma samples with β-glucuronidase, an increase of 72% DON was seen, indicating the presence of DON-glucuronide. The rapid deacetylation prior to reaching the systemic circulation, as suspected by Eriksen et al. 2003, has been confirmed in our study. These data can be related to several *in vitro* and *in vivo* studies in other species. One study investigated the hydrolysis and metabolism of 3ADON and 15ADON after oral administration to rats (Versilovskis et al. 2012). One h p.a. of 3ADON or 15ADON, 12% of the dose could be detected in the stomach as free DON, indicating a likely hydrolysis of both ADONs in the stomach. It was also

observed that both ADONs were glucuronidated in the stomach (5% of the administered dose) without prior deacetylation. Another *in vitro* study incubated human faecal samples under anaerobic conditions for 48 h with 3ADON (Sundstol Eriksen and Pettersson 2003). Of the administered dose, 78±30% was recovered as DON, clearly indicating the capability of human large intestinal microbiota to hydrolyse 3ADON to DON. Moreover, a rapid and complete hydrolysis of 3ADON and 15ADON to DON was also observed after IV administration.

The relatively higher absorbed fractions for ADONs compared to DON in broilers are also reflected by significantly higher observed maximal DON plasma concentrations after PO administration of 3ADON (C_{max}=15.0±8.8 ng/mL) and 15ADON (C_{max}=16.2±7.6 ng/mL) compared to PO administration of DON (C_{max}=4.2±0.9 ng/mL), as depicted in **Table 1** and **Figure 3**. When transforming to DONeq plasma concentrations this difference between DON and ADONs becomes even more explicit, as can be seen in **Figure 5**. Furthermore, in broiler chickens, the administration of ADONs appears to lead to a more rapid absorption, reflected by a shorter t_{max} for DON after 3ADON administration (t_{max}=5.0±0 min) and after 15ADON administration (t_{max}=9.0±6.5 min) compared to after administration of DON itself (t_{max}=32±11 min). For pigs, no significant changes were seen in C_{max} or t_{max} of DON after PO administration of DON, 3ADON or 15ADON.

In this study, an absorption rate constant ka for DON of 0.0164 ± 0.0161/min was found in pigs, compared to 0.00995 ± 0.00292/min (Eriksen et al. 2003) and 0.0310 ± 0.0238/min (Goyarts and Dänicke 2006). However, these results are difficult to compare since the aforementioned study utilised a contaminated feed strategy (chronic exposure), whereas this study was based on a single bolus model with fasted animals. The ka values in broiler chickens are approximately twice the values obtained for pigs, indicating indeed a fast presystemic hydrolysis of ADONs and a fast GI transit and absorption in poultry.

The Cl of DON after DON administration in broilers is approximately 10 times higher compared to that in pigs. The Cl of DON in pigs, approximately 15 mL/min/kg, is comparable to previous reported values for acute DON administration, 9.3 mL/min/kg (Goyarts and Dänicke 2006) and 5.0 mL/min/kg (Devreese et al. 2014). The Cl of 3ADON in broilers and pigs is remarkably higher compared to that of DON and 15ADON. In contrast, compared to

DON, 15ADON showed a faster Cl in pigs and a slower clearance in broiler chickens. The intercompartmental flow Q for DON is similar in both animal species. The Vc for ADONs are in the same order of magnitude for pigs and broilers. The model had difficulties in estimating the central and peripheral volumes of distribution for DON separately. However, when making the sum of both volumes of distribution, the values are found to be in a range of 2.6-3.8 L/kg, except for 15ADON in chickens where the Vp was estimated to be approximately 18 L/kg, but with a higher uncertainty. It was therefore attempted to fix the value of this parameter to a lower one but this led to a poorly fitted model. For 15ADON administration to broilers, DON as well as 15ADON where clearly measurable for a relatively long period of time, unlike for 15ADON in pigs and 3ADON in broilers as well as pigs. This possibly increased the difficulty for the model to correctly fit all parameters. The disposition rate constant (β) was calculated for pigs and chickens by dividing the value obtained for clearance by that of the sum of the central and peripheral volume of distribution, and was found to be 0.0388±0.0062/min and 0.00405±0.00076/min, respectively. This corresponds to elimination half-lives for DON in broilers and pigs, calculated as 0.693/β, of 17.9 and 171 min, respectively. For pigs elimination half-lives of 15.2±12.9 h (IV) and 5.3±2.4 h (acute PO) (Goyarts and Dänicke 2006), and 2.39±0.71 h (PO) (Eriksen et al. 2003) were observed.

When combining the above mentioned results for absorbed fraction, oral bioavailability and presystemic hydrolysis of 3ADON and 15ADON in pigs, it can be concluded that the administered dose of both ADONs is completely absorbed and both show a complete presystemic hydrolysis. Therefore, both ADONs can be regarded as toxic as DON itself in pigs regarding systemic toxicity. Moreover, the ADONs may also exert a local toxic effect in the intestine as previously proposed (Pinton et al. 2012). For broiler chickens, the absorbed fractions of 3ADON and especially 15ADON are higher than or at least equal to that of DON. Furthermore, 3ADON, which is reported to be less toxic than DON, is completely hydrolysed presystemically to DON whereas 15ADON is reported more toxic and not fully hydrolysed. This results in a 'worst case scenario' for broilers where each mole of 15ADON could be as toxic as 4 moles of DON, and where the less toxic 3ADON can be regarded equally toxic to DON since it is completely hydrolysed presystemically.

In conclusion, both pigs and broiler chickens are exposed to DON after oral intake of ADONs. Combining these findings with the frequent co-contamination of ADONs and DON in food

and feed, this demonstrates the possible need to consider the establishment of a TDI for 3ADON and 15ADON or the inclusion of the ADONs in a group TDI for DON related substances.

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5. Supporting information

The dosing design for the pig trial (**S1**), and the written code of the utilised model are described (**S2**), together with the fits of the median plasma concentration-time profiles of DON, 3ADON and 15ADON in broiler chickens and pigs (semi-log); circles=PO; diamonds=IV; blue=DON; green=3ADON; red=15ADON (**S3**).

Supplementary material S1. *Schematic overview of the utilized cross-over design for the pig trial. By subjecting each animal to 4 treatments a cross-over in six animals was obtained for each mycotoxin. This design allowed the use of half the animals needed for a traditional two-way cross-over experiment.*

pigs	1st treatment	2nd treatment	3rd treatment	4th treatment
1-3	DON IV	15ADON IV	DON PO	15ADON PO
4-6	3ADON PO	DON IV	3ADON IV	DON PO
7-9	15ADON IV	3ADON PO	15ADON PO	3ADON IV

Supplementary material S2. *Model code for toxicokinetic analysis of DON, 3ADON and 15ADON after PO and IV administration in broiler chickens, using WinNonlin Professional version 5.2.1.*

```
Model 1
remark ***************************************************
remark Developer:      Nathan Broekaert
remark Model Date:   18-04-2014
remark Model Version:          1.0
remark ***************************************************
remark
remark - define model-specific commands
COMMANDS
NFUNCTIONS 4
NDERIVATIVES 7
NPARAMETERS 9
```

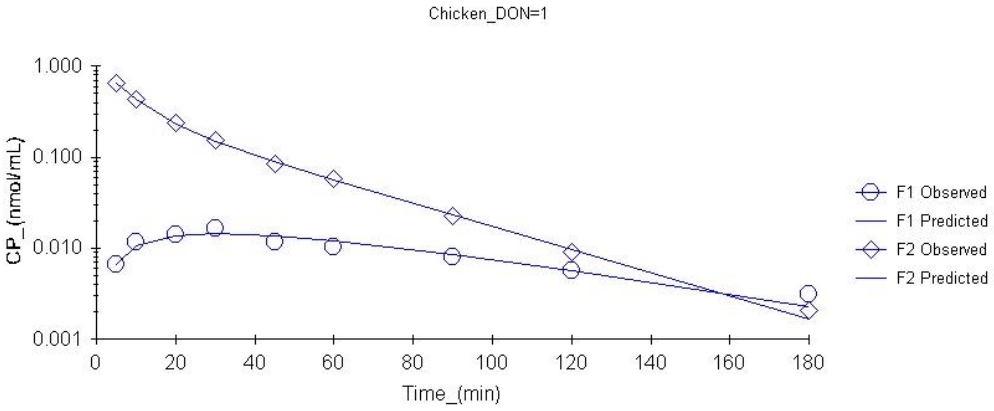
```
PNAMES 'Cldon', 'Cladon', 'Qdon', 'VCdon', 'VCadon', 'ka', 'FRAC', 'BioADON', 'VPdon'
END

remark - define temporary variables
TEMPORARY
TIME=X
DOSPO=1689
DOSIV=1689

END

remark - define differential equations starting values
START
Z(1) = DOSIV/VCadon
Z(2) = 0
Z(3) = 0
Z(4) = DOSPO*(1-FRAC)
Z(5) = 0
Z(6) = 0
Z(7) = 0
END

remark - define differential equations
DIFFERENTIAL
remark IV

DZ(1) = (-Cladon*Z(1))/VCadon
DZ(2) = (Cladon*Z(1) -Cldon*Z(2) -Qdon*Z(2) +Qdon*Z(3))/VCdon
DZ(3) = (Qdon*Z(2) -Qdon*Z(3))/VPdon

remark PO

DZ(4) = -ka*BioADON*Z(4) -(ka*(1-BioADON)*Z(4))
DZ(5) = (BioADON*ka*Z(4) -CLadon*Z(5))/VCadon
DZ(6) = ((1-BioADON)*ka*Z(4) +CLadon*Z(5) -CLdon*Z(6) -Qdon*Z(6) +Qdon*Z(7))/VCdon
DZ(7) = (Qdon*Z(6) -Qdon*Z(7))/VPdon
END

remark - define algebraic functions
FUNCTION 1
F= Z(1)
END

FUNCTION 2
F= Z(2)
END
```

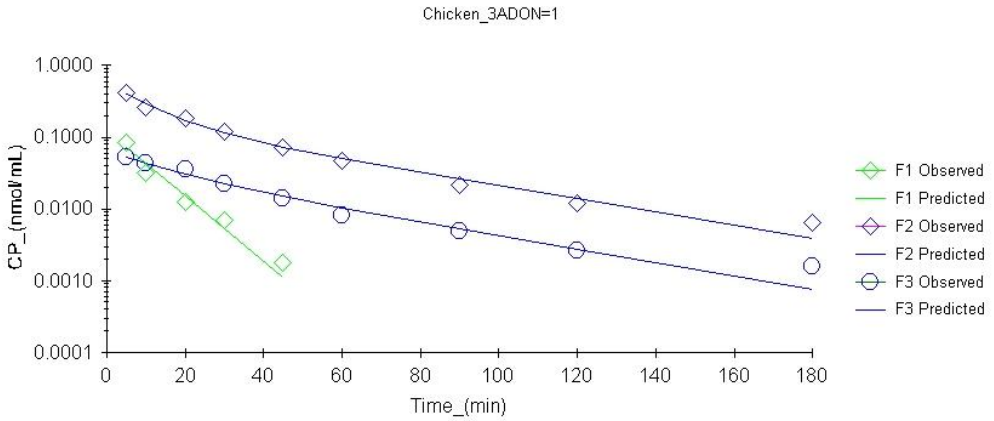
FUNCTION 3
F= Z(5)
END

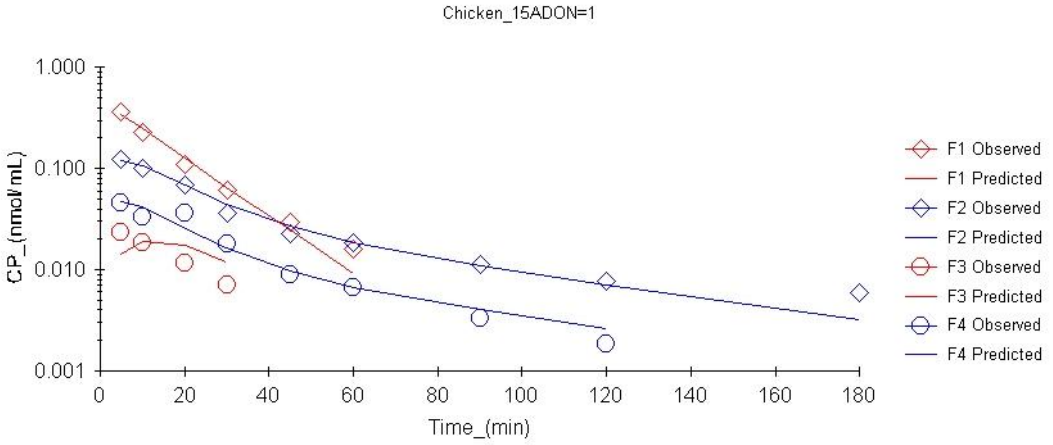
FUNCTION 4
F= Z(6)
END

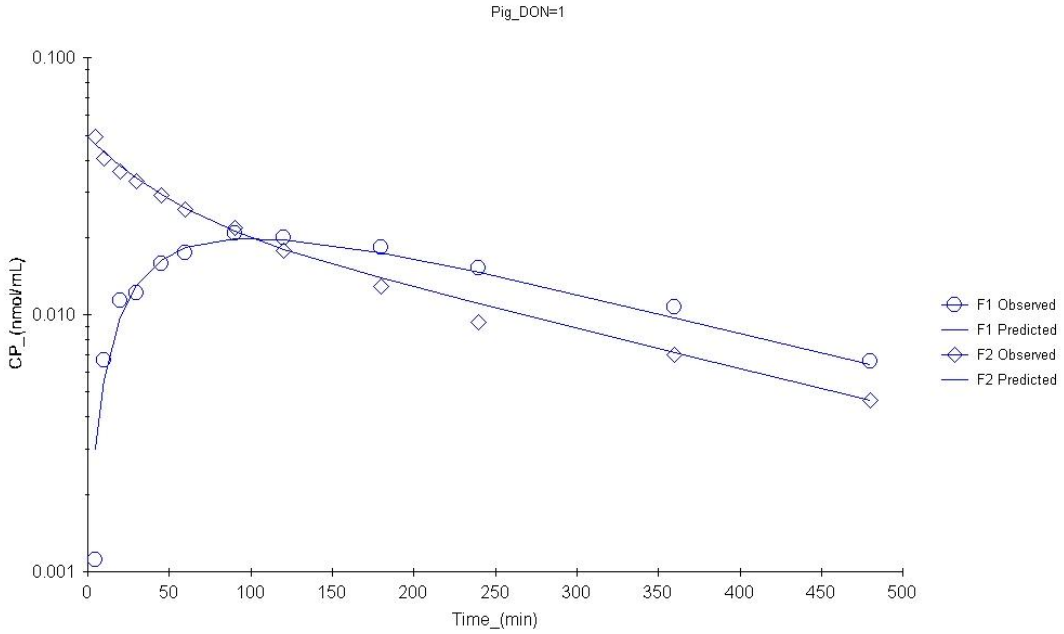
remark - define any secondary parameters
remark - end of model
EOM

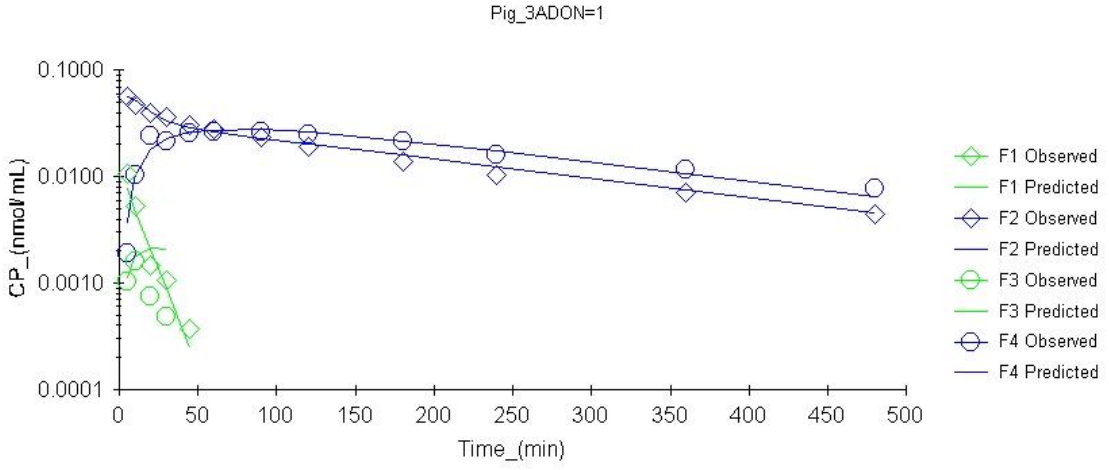
Supplementary material S3. *Fits of the median plasma concentration-time profiles of DON, 3ADON and 15ADON in broiler chickens and pigs after IV and PO administration using a tailor-made toxicokinetic model in WinNonlin Professional version 5.2.1 (semi-log); circles=PO; diamonds=IV; blue=DON; green=3ADON; red=15ADON.*

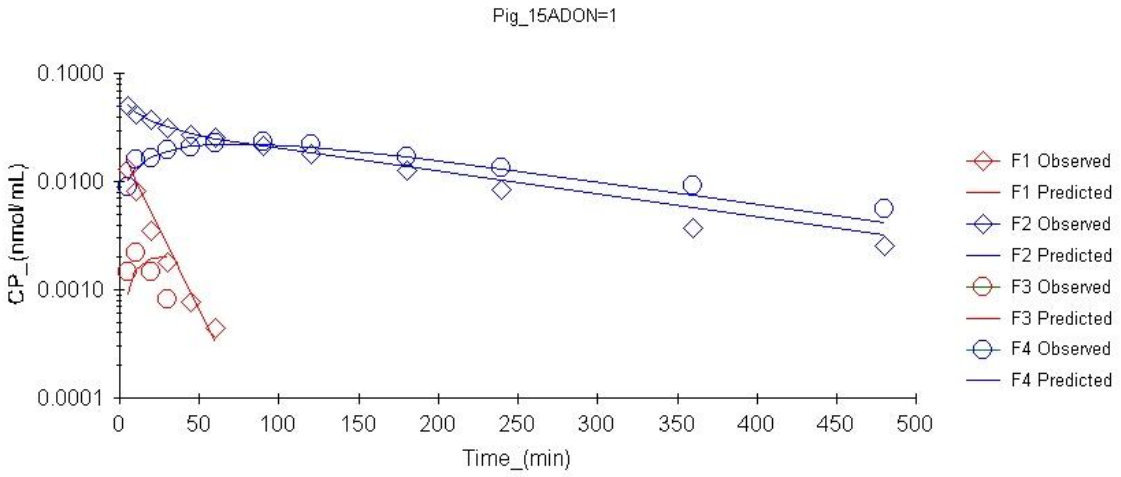





Chicken_15ADON=1
CP_(nmol/mL)
1.000
0.100
0.010
0.001
0
20
40
60
80
100
120
140
160
180
Time_(min)
F1 Observed
F1 Predicted
F2 Observed
F2 Predicted
F3 Observed
F3 Predicted
F4 Observed
F4 Predicted


Pig_DON=1
CP_(nmol/mL)
0.100
0.010
0.001
0
50
100
150
200
250
300
350
400
450
500
Time_(min)
F1 Observed
F1 Predicted
F2 Observed
F2 Predicted


Pig_3ADON=1
CP_(nmol/mL)
0.1000
0.0100
0.0010
0.0001
0
50
100
150
200
250
300
350
400
450
500
Time_(min)
F1 Observed
F1 Predicted
F2 Observed
F2 Predicted
F3 Observed
F3 Predicted
F4 Observed
F4 Predicted



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CHAPTER III

In vivo contribution of deoxynivalenol-3-β-D-glucoside to deoxynivalenol exposure in chickens and pigs: oral bioavailability, hydrolysis and toxicokinetics

Adopted from:

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Abstract - The goal of this study was to determine the absolute oral bioavailability and toxicokinetic characteristics of the modified mycotoxin deoxynivalenol-3-β-D-glucoside (DON3G) and its possible (presystemic) hydrolysis to deoxynivalenol (DON) in broiler chickens and pigs. Cross-over animal trials were performed with intravenous (IV) and oral (PO) administration of DON3G and DON to broilers and pigs. Systemic plasma concentrations of DON, DON3G and de-epoxyDON were quantified using liquid chromatography-tandem mass spectrometry. Liquid chromatography coupled to high-resolution mass spectrometry was used to unravel phase II metabolism of DON. In pigs, also portal plasma was analysed to study presystemic hydrolysis and metabolism. Data were processed via tailor-made compartmental toxicokinetic models. The results in broiler chickens indicate that DON3G is not hydrolysed to DON *in vivo*. Furthermore, the absolute oral bioavailability of DON3G in broiler chickens (3.79±2.68%) was low and comparable to that of DON (5.56±2.05%). After PO DON3G administration to pigs, only DON was detected in plasma, indicating a complete presystemic hydrolysis of the absorbed fraction of DON3G. However, the absorbed fraction of DON3G, recovered as DON, was approximately 5 times lower than after PO DON administration, 16.1±5.4% compared to 81.3±17.4%. Analysis of phase II metabolites revealed that biotransformation of DON and DON3G in pigs mainly consists of glucuronidation, whereas in chickens conjugation with sulfate predominantly occured. The extent of phase II metabolism is notably higher for chickens than for pigs, which might explain the relatively higher sensitivity of the latter species to DON. Although *in vitro* studies demonstrate a decreased toxicity of DON3G compared to DON, the species dependent toxicokinetic data and *in vivo* hydrolysis to DON illustrate the toxicological relevance and consequently the need for further research to establish a tolerable daily intake.

Keywords - deoxynivalenol (DON) - deoxynivalenol-3-β-D-glucoside (DON3G) - pig - broiler chicken - *in vivo* hydrolysis - toxicokinetics - oral bioavailability

1. Introduction

Mycotoxin contamination of food and feed has been classified by several authors as the most important chronic dietary risk for human and animal health. The *Fusarium* mycotoxin deoxynivalenol (DON) is one of the most frequently occurring mycotoxins, contaminating 56% of feed and feed raw materials worldwide (Schatzmayr and Streit 2013). Additionally to DON, feed can be contaminated with modified forms of DON, such as deoxynivalenol-3-β-D-glucoside (DON3G), causing a possible underestimation of the degree of contamination.

Following a request from the European Commission, the risks to human and animal health related to modified forms of the *Fusarium* mycotoxins zearalenone, nivalenol, T-2 and HT-2 toxin and fumonisins were evaluated. The European Food Safety Authority Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) considered it appropriate to assess human exposure to modified forms of the various toxins in addition to the parent compounds, because modified forms may be hydrolysed into the parent compounds or released from the matrix during digestion. However, no risk assessment for modified forms of DON is currently available (EFSA CONTAM Panel 2014). Concerning acetylated forms of DON, 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), both ADONs were completely absorbed and presystemically hydrolysed to DON in pigs. In chickens, for both ADONs a higher absorbed fraction was observed compared to DON, as well as a presystemic hydrolysis of 3ADON and 15ADON to DON of 100% and 75.4%, respectively (Broekaert et al. 2015b).

Recent occurrence data (2010-2014) for DON and DON3G have been reviewed for unprocessed cereals, such as wheat, maize, barley, oat, rye and spelt (Broekaert et al. 2015a). Observed incidence, average concentration and maximum detected concentration for DON and DON3G were 84%, 458 µg/kg, 27,088 µg/kg and 55%, 85 µg/kg, 170 µg/kg, respectively. DON3G concentrations exceeding those of free DON have been detected in some hard red spring wheat samples (Sasanya et al. 2008), which indicate its high prevalence.

The susceptibility of animal species to DON can be ranked in the following decreasing order: pigs > mice > rats > poultry ≈ ruminants (Pinton and Oswald 2014). The toxicity of DON depends on its ability to efficiently cross biological barriers in humans and animals, such as

the intestinal and the blood-brain barrier. At the cellular level, DON primarily affects protein synthesis. Consequently, fast dividing cells such as intestinal epithelial and immune cells will be more susceptible to the detrimental effects of DON (Maresca 2013; Pestka 2010; Rotter et al. 1996). The cellular toxicity is mainly attributed to the presence of the epoxide moiety (Pestka 2010; Ueno et al. 1973). This functional group allows DON, and trichothecenes in general, to bind to ribosomes, and to cause what is known as the 'ribotoxic stress response'.

It is expected that glucosylation of DON greatly reduces its toxicity. The phytotoxicity of DON and DON3G was studied by means of a wheat germ extract-coupled *in vitro* transcription/translation system. One µM of DON significantly decreased translation by 63% compared to a control. In contrast, 5 µM and 20 µM of DON3G resulted in a translation decrease of only 3.1% and 8.0%, respectively (Poppenberger et al. 2003). In a growth test with algal cells, 80 µM of DON3G, unlike an equimolar amount of DON, did not significantly alter growth compared to a control (Suzuki and Iwahashi 2015). Decreased toxicity has also been observed in mice, where DON3G was largely incapable of evoking cytokine or chemokine responses in the spleen of mice orally gavaged with 2.5 mg/kg body weight (bw), in contrast to DON, except for slight elevations in IL-1β mRNA expression (Wu et al. 2014). Recently, an *in silico* analysis suggested that DON3G, unlike DON, was unable to bind to the ribosome peptidyl transferase centre. Additionally, comparative cytotoxicity of DON and DON3G was assessed on both proliferative and differentiated Caco-2 cells by means of quantitation of the ATP present. After a 48 h exposure to DON, an IC_{50} of 1.3 µM (0.384 µg/mL) was observed in proliferative Caco-2 cells. In contrast, no cytotoxicity was observed with 0-10 µM of DON3G. For differentiated Caco-2 cells, no cytotoxicity was observed for 0-10 µM of DON nor DON3G (Pierron et al. 2015).

Regarding the hydrolysis of DON3G to DON, different *in vitro* studies reported that DON3G was not hydrolysed to DON in the human upper gastrointestinal (GI) tract by means of acid, stomach and gut juice, or cytosolic β-glucosidase (Berthiller et al. 2011; Dall'Erta et al. 2013; De Angelis et al. 2014; De Nijs et al. 2012). Regarding the human lower GI tract, some *in vitro* experiments indicate that hydrolysis can take place and that the intestinal microbiota composition and their abundance can play an important role (Abbott 2004; Berthiller et al. 2011; Dall'Erta et al. 2013; Gratz et al. 2013; Hattori and Taylor 2009). *In vitro* models do not take into account important physiological and anatomical factors within and between

different animal species and humans, such as intestinal mucosa and luminal content composition (enzymes, microbiota), splanchnic blood flow and enterohepatic recirculation (Gonzalez-Arias et al. 2013). To estimate these factors and especially the interactions between them, species-specific *in vivo* trials remain mandatory. Versilovskis *et al*. reported a first DON3G *in vivo* study in which two rats were fed 25 µg of DON3G by oral gavage, followed by blood collection at 17 and 55 min post-administration. DON was detected in the stomach albeit at only 2% of the applied dose. This confirms the conclusions of the *in vitro* studies, i.e. hydrolysis of DON3G in the stomach is negligible. Further down in the GI tract, DON3G could only be detected at 2-3% of the applied dose in the small intestine and at 1-2% in the colon. This remarkable decrease of DON3G in the small intestine is probably due to the presence of intestinal β-glucosidases (Versilovskis et al. 2012). Nagl *et al*. orally administered DON and an equimolar dose of DON3G to six rats, followed by urine and faeces collection over a two-days period. After PO administration of DON3G, less than 4% of the administered dose was recovered in urine, of which 35% as DON, 33% as DON-glucuronide (DON-GlcA), 24% as de-epoxy-deoxynivalenol (DOM-1) and 8% as DON3G. The presence of urinary DON clearly demonstrates the hydrolysis upon ingestion. In comparison, after PO DON administration, 15% of the dose was recovered in urine as DON-GlcA, DON and DOM-1, indicating a relatively low oral bioavailability for DON3G compared to DON in rats. Analysis of faecal samples of rats dosed with DON3G revealed that the vast majority of the metabolites of DON3G were excreted as DON and DOM-1 (sum: 99.5 ± 0.4%). Only traces of DON3G were detected, indicating that intestinal microbiota are effective in hydrolysing this modified mycotoxin in rats (Nagl et al. 2012). Recently, the results in rat were supported by *in vivo* experiments performed in pig by the same research group. After intravenous (IV) administration of DON3G, no DON was detected in plasma, indicating that systemic hydrolysis of DON3G is negligible. After oral administration of DON3G, 40.3% of the dose was recovered in urine as DON3G, next to DON (21.6%), DON-15-glucuronide (DON15GlcA) (6.8%), DOM-1 (5.9%) and DON-3-glucuronide (DON3GlcA) (3.4%) (Nagl et al. 2014). Consequently, it was concluded that *in vivo* cleavage of DON3G predominantly occurs in the (lower) digestive tract. Oral bioavailability was lower for DON3G than for DON, deduced from the recovered DON equivalent amounts in urine, namely 84.8% for DON and 40.3% for DON3G (Nagl et al. 2014). Moreover, the oral bioavailability for both compounds in pig was remarkably higher than in rat.

However, both study designs in rat and pig do not allow determination of the site of hydrolysis (GI microbiota, GI tissues, portal blood, liver, systemic blood). Sampling portal blood would offer a huge advantage as it would allow to differentiate between presystemic and systemic hydrolysis and therefore significantly contribute to the understanding of the (pre)systemic hydrolysis of DON3G. Transsplenic portal vein catheterisation in pigs has previously been described (Gasthuys et al. 2009) and was used to study the presystemic metabolisation of xenobiotics (Reyns et al. 2009). Therefore, portal vein catheterisation offers a valuable tool to study possible presystemic hydrolysis of DON3G in pigs.

Currently, literature reports regarding the toxicokinetics of DON3G in humans and other animal species, such as broiler chickens, are scarce. Warth *et al*. performed a study with a human volunteer consuming a diet naturally contaminated with 138 µg DON/day, 7 µg DON3G/day and 20 µg 3ADON/day. DON3G, in contrast to DON, could not be detected in urine (Warth et al. 2013). For broiler chickens no data is available, although poultry have a high exposure to DON3G given their mainly cereal based diet. The GI anatomy, physiology and distinct microbiota species composition and abundance of poultry further contribute to the relevance of performing research in these species.

The goal of this study was to determine the absolute oral bioavailability, the degree of hydrolysis and the main toxicokinetic parameters of DON3G in broiler chickens and pigs. Additionally portal vein catheterisation in pigs allowed determination of the site of presystemic hydrolysis.

2. Materials and methods

2.1. Standards, reagents and solutions

DON (>99% purity) was commercially obtained (Fermentek, Jerusalem, Israel). DON3G was enzymatically synthesised, purified and verified using nuclear magnetic resonance (NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)(<0.05% DON) by the Christian Doppler Laboratory for Mycotoxin Metabolism Center for Analytical Chemistry (Vienna, Austria). DON and DON3G were dissolved in analytical-grade ethanol (EtOH) (Merck, Brussels, Belgium), yielding a stock solution of 10 mg/mL and were used for animal trials and analytical experiments. DOM-1 (50 µg/mL) and $^{13}C_{15}$-DON (stable isotopically labelled internal standard, IS, 50 µg/mL) stock solutions in acetonitrile were purchased

(Sigma-Aldrich, Diegem, Belgium). All stock solutions were stored at ≤-15 °C. Individual working standard solutions of 10 µg/mL, used for the analytical experiments, were prepared by diluting the above stock solutions with ULC/MS grade acetonitrile (Biosolve, Valkenswaard, The Netherlands). All working standard solutions were stored at ≤-15 °C. Standard mixture working solutions containing DON, DON3G and DOM-1 of 1 and 0.1 µg/mL were prepared by mixing appropriate dilutions of the individual working standard solutions in ULC/MS acetonitrile, stored at 2-8 °C. Solutions of DON in acetonitrile have been reported to be stable for 24 months at room temperature (Widestrand and Pettersson 2001). Water, methanol and glacial acetic acid were of ULC/MS grade (Biosolve). Microfilters (GV-PVDF 0.22 µm) were obtained from Millipore (Overijse, Belgium).

2.2. Animal trials

2.2.1. Broiler chickens

Six broiler chickens (Ross 308) were purchased from the Institute for Agriculture and Fisheries Research (Melle, Belgium) at the age of 3 weeks, as hatched, and after one week acclimatisation the animals had an average bw ± standard deviation (SD) of 1400 ± 131 g. Water and feed were given *ad libitum*. The pen was climate controlled with temperatures between 21 and 25 °C, a relative humidity between 40 and 60% and an applied light schedule similar to commercial installations (18 h light/6 h dark). After a one week acclimatisation period, three broiler chickens were treated with DON3G by IV bolus injection in the *vena basilica*, and three broiler chickens received DON3G PO by means of gavage in the crop. After a wash-out period of 1 day, a cross-over design was applied which resulted in a PO and IV administration of DON3G in each of the six broiler chickens. Next, a second wash-out period of three days was respected, and the same broiler chickens were subjected to an identical two-way cross-over (IV and PO) study with DON. The administered doses were based on the EU maximum guidance level of 5 mg DON/kg poultry feed (European Commission 2006). Given an average feed consumption of 4-week-old broiler chickens of 100 g feed/kg bw/day, 500 µg DON/kg bw was administered. For DON3G, an equimolar dose of 774 µg/kg bw was administered. The calculated volume of stock solution (10 mg/mL in EtOH) was diluted with saline (0.9% NaCl) (VWR, Leuven, Belgium) up to a volume of 0.5 mL (IV) or 1 mL (PO). Feed was deprived 12 h before administration and until 3 h post administration (p.a.). After oral administration of the toxins, 1 mL of tap water was

administered to flush the gavage syringe and tube. Blood (0.5-1 mL) was sampled from the leg vein (*vena metatarsalis plantaris superficialis*) by venipuncture at 0 min (before administration) and at 5, 10, 20, 30, 45, 60, 90, 120 and 180 min p.a. Blood samples were centrifuged (2851 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis.

2.2.2. Pigs

For the pig study, six clinically healthy male pigs (11 weeks of age, 26.3 ± 1.8 kg bw) were individually housed and were provided feed and water *ad libitum.* Natural lighting was applied, the enclosure was climate controlled to keep the temperature between 20 and 24 °C and the relative humidity between 20 and 40%. After a one week acclimatisation period, double lumen catheters were surgically placed into the *vena jugularis* of all six pigs. Additionally, in four of the six pigs a single lumen catheter was placed into the *vena porta* to study presystemic hydrolysis and biotransformation. The surgical procedure was as previously described (Gasthuys et al. 2009). After a two-days period, the animals were treated with DON3G by IV bolus injection using one lumen of the double lumen jugular catheters, or PO by means of gavage in the stomach, in a two way cross-over design. After DON3G administration, an identical cross-over administration with DON was set up in the same animals and respecting a wash-out period of 24 h between treatments. Twelve h before administration of the mycotoxins, the animals were fasted until 4 h p.a. The administered doses were calculated as for the broiler chicken trial. The EU maximum guidance level in pig feed is 0.9 mg/kg DON (European Commission 2006). Pigs at 12 weeks of age consume on average 40 g feed/kg bw/day. This resulted in the administration of 36 µg DON/kg bw. For DON3G, an equimolar dose of 55.7 µg/kg bw was administered. The calculated amount of toxin for each animal, dissolved in EtOH at 10 mg/mL, was diluted with saline to a volume of 1 mL (IV) or 10 mL (PO). After oral administration of the toxins, 50 mL of tap water was administered to flush the gavage syringe and tube. Blood (1-2 mL) was sampled via the other lumen of the jugular catheter and the *vena porta* catheter at 0 min (before administration) and at 5, 10, 20, 30, 45, 60, 90, 120, 240, 360 and 480 min p.a. Blood samples were centrifuged (2851 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis.

The chicken and pig animal trials were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2013/64 and EC2015/14, respectively).

2.3. Plasma sample preparation

Blank plasma was obtained by the collection of heparinised blood from six different broiler chickens and pigs. The animals were fasted 12 h prior to blood collection. Plasma was obtained by centrifugation (2851 x *g*, 10 min, 4 °C) of the blood. The blank plasma was pooled, homogenised and stored at ≤ -15 °C until use for the preparation of matrix-matched calibrators and quality control samples.

Calibrator and quality control samples. To 250 µL of blank plasma, 25 µL of a 100 ng/mL IS working solution ($^{13}C_{15}$-DON) and appropriate volumes of the standard mixture working solutions (1, 10 100 and 1000 ng/mL) were added to obtain calibrator samples with mycotoxin concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 and 500 ng/mL. After vortex mixing, acetonitrile was added up to a volume of 1 mL to precipitate plasma proteins. The samples were vortex mixed again, followed by a centrifugation step (10 min at 8517 x *g*, 4 °C). The supernatant was transferred to a new tube and evaporated to dryness under nitrogen at 45 °C. The dry residue was then redissolved in 200 µL of ULC/MS grade water, micro-filtrated and 10 µL was injected onto the LC-MS/MS instrument.

Incurred samples. To 250 µL of plasma, 25 µL of a 100 ng/mL IS working solution were added. After vortex mixing, the samples were subjected to the same sample preparation procedure as the calibrator samples.

Blank samples. After the addition of 750 µL of acetonitrile to 250 µL of blank plasma, the samples were extracted in the same way as the calibrator samples.

2.4. LC-MS/MS analysis and validation

Separation of DON, $^{13}C_{15}$-DON, DON3G and DOM-1 was achieved on a Hypersil Gold (reversed-phase) (50 x 2.1 mm i.d., 1.9 µm) column with a guard column (10 x 2.1 mm i.d., 5 µm) of the same type. Chicken and pig plasma were analysed on an Acquity UPLC® system coupled to a Xevo® TQ-S MS instrument (Waters, Zellik, Belgium).

All compounds were eluted with a gradient of water containing 0.3% glacial acetic acid (mobile phase A) and methanol containing 0.3% glacial acetic acid (mobile phase B), at a flow rate of 300 µL/min. The following gradient elution program was run: 0-1 min, 5% B; 1.0-1.1 min, linear gradient to 12% B; 1.1-5.0 min, 12% B; 5.0-5.1 min, linear gradient to 50% B; 5.1-8.0 min, 50% B; 8.0-8.1 min, linear gradient to 95% B; 8.1-10.0 min, 95% B; 10.0-10.1 min, linear gradient to 5% B; 10.1-12.0 min, 5% B. The MS was operated in the multiple reaction monitoring (MRM) mode with two ion transitions for each target analyte as presented in **Table 1**. Instrumental and compound specific parameters were optimised by the direct infusion of 10 ng/mL standard solutions in methanol/ water (50/50, v/v) + 0.3% glacial acetic acid with a flow rate of 10 µL/min. For the Xevo TQ-S, the desolvation gas flow rate was fixed to 800 L/h with a temperature of 550 °C, the cone gas flow rate was set at 150 L/h, capillary voltage was optimised at 3.0 kV and a collision gas flow of 0.15 mL/min was used. Dwell times of 44 - 52 ms/transition were chosen. Compound specific MS parameters, together with precursor and product ions used for quantification and qualification, are given in **Table 1**. DON, $^{13}C_{15}$-DON and DOM-1 were detected in positive electrospray ionisation mode (ESI+) as the protonated precursor ion $[M+H]^+$, DON3G was measured in ESI- as $[M+CH_3COO]^-$ adduct.

Given the unavailability of reference materials, validation was performed on fortified blank pig and broiler plasma samples. Both recommendations as defined by the European Community (Commission Decision 2002; Heitzman 1994) and the Veterinary International Conference on Harmonisation (VICH 2009) served as validation guidelines. The developed method was single laboratory validated.

Table 1. *Compound specific MRM ion transitions and MS-parameters for the analysis of DON, DON3G, DOM-1 and $^{13}C_{15}$-DON in broiler and pig plasma; Rt= retention time; IS= internal standard; [a] quantifier ion.*

	Precursor ion (m/z)	Product ion (m/z)	Rt (min)	Cone voltage (V)	Collision Energy (eV)	Dwell time (ms)	ESI modus
DON	297.1 $[M+H]^+$	249.1[a] 203.4	2.5	20	9 14	0.25 0.25	+
DON3G	517.0 $[M+CH_3COO]^-$	427.2[a] 247.0	2.8	14	16 15	0.25 0.25	-
DOM-1	281.1 $[M+H]^+$	215.1[a] 137.0	4.0	20	12 16	0.81 0.81	+
$^{13}C_{15}$-DON (IS)	312.0 $[M+H]^+$	245.2[a] 263.0	2.5	20	10 10	0.25 0.25	+

Linearity of the response of the compounds was assessed by means of three matrix-matched calibration curves consisting of eight calibration points in the range of 0.1-500 ng/mL. The correlation coefficients (r) and goodness-of-fit coefficients (gof) were determined, limits were set at ≥0.99 and ≤20%, respectively.

Within-day accuracy & precision were determined by analyzing six samples fortified at a low concentration level (limit of quantitation (LOQ) of the compounds) and at a high concentration level (100 ng/mL). Values for the relative standard deviation (RSD) could not exceed 2/3 of the $RSD_{max,}$ calculated according to the Horwitz equation, i.e. $RSD_{max} = 2^{(1-0.5\log Conc)} \times 2/3$. The acceptance criteria for accuracy were: −30% to +10% and −20% to +10% for concentrations between 1 and 10 ng/mL, and ≥10 ng/mL, respectively.

Between-day accuracy & precision were assessed by analyzing the low and high concentration levels in threefold on three consecutive days (n=3x3). The acceptance criteria for accuracy were identical to the values given above and RSD values could not exceed $RSD_{max} = 2^{(1-0.5\log Conc)}$.

The *LOQ* was calculated as the lowest concentration for which the method had acceptable results with regards to accuracy and precision. It was determined by spiking six blank plasma samples. The LOQ was also established as the lowest point of the calibration curve. The *limit of detection* (*LOD*) was calculated using the samples spiked at the LOQ level (n=6) corresponding to the concentration that could be determined with a signal-to-noise (S/N) ratio of 3.

Carry-over was evaluated by analysing a mixture of mobile phase A and B (50/50; v/v) directly after the highest calibrator (500 ng/mL).

The *specificity*, the capability of the method to distinguish signals of the analytes from any other substances or interferences, was determined on six blank plasma samples. For an acceptable specificity the S/N ratio of possible interfering peaks with similar retention times in these samples could not exceed the S/N ratio of 3.

Recovery and matrix effects. Two types of matrix-matched calibration curves were prepared by spiking blank calibrator samples before and after extraction. A third calibration curve was prepared in standard solution. All curves consisted of eight calibration points in the range of

0.1-500 ng/mL. The slopes of these calibration curves (external calibration, without IS) were compared to calculate the apparent recovery (R_A = 100 × slope spiked before extraction/slope standard solution), the matrix effect denoted as signal suppression/enhancement (SSE = 100 × slope spiked after extraction/slope standard solution) and the recovery of the extraction step (R_E = 100 × *slope spiked before extraction/*slope spiked after extraction). Regarding SSE, values ≤ 1 indicate ion suppression due to matrix effect, values ≥ 1 indicate ion enhancement (Matuszewski et al. 2003).

2.5. High resolution mass spectrometry

To determine the presence of phase II metabolites, i.e. glucuronide and sulfate conjugates, high resolution mass spectrometry (HRMS) analysis was performed on pig and broiler chicken plasma samples from three animals after IV and PO administration for identification and semi-quantification of DON3G, DON and its metabolites. The methodology was similar as in a previously described study (Devreese et al. 2015). Sample treatment, HPLC column and mobile phases were identical to the LC-MS/MS analysis. However, a different gradient elution program was applied: 0-1.0 5% B, 1.0-4.0 linear to 80% B, 4.0-6.7 min 80% B, 6.7-7.0 linear gradient to 5% B, 7.0-10.0 5% B. The (U)HPLC consisted of an Accela type 1250 High Speed LC and autosampler coupled to an Exactive Orbitrap HR mass spectrometer, equipped with a heated electrospray ionisation (HESI) probe operating in the negative ionisation mode (all from Thermo Fischer Scientific, Breda, The Netherlands). Accurate masses for DON ($[M+CH_3COO]^-$), DOM-1 ($[M+CH_3COO]^-$), DON-3α-sulfate and DON3GlcA, 355.1414, 339.2016, 375.0755 and 471.1508, respectively, were based on Wan et al. and Devreese et al. (Devreese et al. 2015; Wan et al. 2014). Accurate mass of DON3G ($[M+CH_3COO]^-$) was determined to be 517.19250 by direct infusion of a 10 µg/mL solution in mobile phase A and B (50/50, v/v).

2.6. Toxicokinetic modeling and statistical analysis

Toxicokinetic analysis on the LC-MS/MS data was performed using WinNonlin Professional version 5.2.1. (Pharsight, St-Louis, MO). Plasma concentrations below the LOQ were not taken into account. For the toxicokinetic analysis, all values were recalculated to their molar concentrations, expressed as nmol/mL. For the analysis of DON IV and PO data in chickens and pigs, and for DON3G IV and PO in chickens, a tailor-made two-compartmental model

(central compartment (Vc) and peripheral compartment (Vp)) with first order absorption and first order elimination kinetics was applied. For the IV and PO administration of DON3G in pigs, a tailor-made one-compartmental model was fitted. Due to a lag time after PO DON3G administration to pigs, only terminal concentration points were above the LOQ. Consequently, it was not possible to calculate toxicokinetic parameters for the individual animals, and these parameters have been calculated simultaneously on all pooled pig PO DON3G data. As a result, no standard deviations could be calculated, instead coefficients of variation of the models' predicted versus observed concentrations are provided.

In **Figure 1** and **2** a graphical representation of the constructed model is given for DON (IV & PO) and DON3G (IV & PO) in broiler chickens and pigs, respectively.

A $1/\hat{y}$ weighing was applied for all calculations with both the chicken and pig data. For all the described models, the Gauss-Newton (Levenberg and Hartley) algorithm was used with a maximum of 50 iterations.

The following primary and secondary toxicokinetic parameters were calculated for DON and DON3G after IV and PO administration: absorption rate constant (ka*)*, intercompartmental flow (Q), total body clearance (CL), central volume of distribution (Vc), peripheral volume of distribution (Vp). FRAC is the absorbed fraction of the dose in either form (DON or DON3G). F indicates the absolute oral bioavailability, i.e. the fraction of DON or DON3G absorbed in the systemic circulation in its unchanged form. When both a central and peripheral volume of distribution were observed, the disposition rate constant (β) was calculated by dividing the Cl by the sum of Vc and Vp for each animal. When only a central volume of distribution was used, the elimination rate constant (ke) was calculated by dividing the Cl by the Vc for each animal. Presystemic hydrolysis (Pres. Hydr.) was the percentage of the total dose of DON3G that is hydrolysed presystemically to DON and absorbed as DON (%). Additionally, for PO data the descriptive toxicokinetic parameters maximal plasma concentration (C_{max}) and time to C_{max} (t_{max}) were given for DON. Elimination half lives ($t_{1/2el}$) were calculated as 0.693/β or 0.693/ke.

Statistical analysis of FRAC, F, C_{max} and t_{max} after DON3G administration compared to DON administration consisted of one-way ANOVA with post-hoc Scheffé tests (p value < 0.05) for

both animal species (SPSS 20.0, IBM, Chicago, IL). Equality of variances criterion was determined by the Levene's test for homogeneity of variances (p value > 0.01).

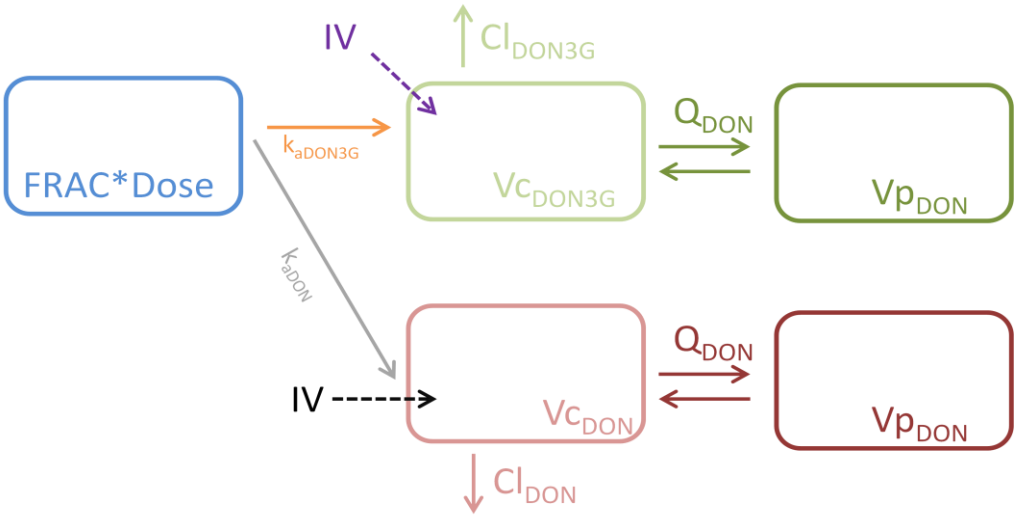

Figure 1. *Graphical representation of the applied model for broiler chicken: DON IV (black, light red and dark red section), DON PO (blue, grey, light red and dark red section), DON3G IV (purple, light green and dark green section) and DON3G PO (blue, orange, light green and dark green section), IV=intravenous administration; ka_{DON}=absorption rate constant of DON (1/min); ka_{DON3G}=absorption rate constant of DON3G (1/min); Cl_{DON}=clearance of DON (mL/min/kg); Q_{DON}=intercompartment flow for DON (mL/min/kg); Vc_{DON}=central volume of distribution for DON (mL/kg); Vp_{DON}=peripheral volume of distribution for DON (mL/kg); Cl_{DON3G}=clearance of DON3G (mL/min/kg); Q_{DON3G}=intercompartment flow for DON3G (mL/min/kg); Vc_{DON3G}=central volume of distribution for DON3G (mL/kg); Vp_{DON3G}=peripheral volume of distribution for DON3G (mL/kg); FRAC=fraction of dose absorbed.*

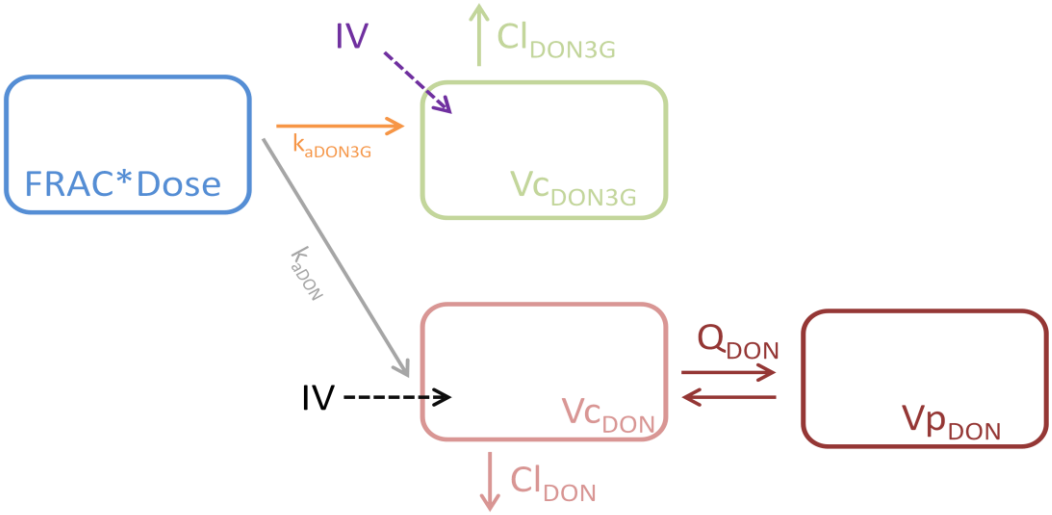

Figure 2. *Graphical representation of the applied model for pig: DON IV (black, light red and dark red section), DON PO (blue, grey, light red and dark red section), DON3G IV (purple and light green section) and DON3G PO (blue, grey and light red section + lag time). IV=intravenous administration; ka_{DON}=absorption rate constant of DON (1/min); ka_{DON3G}=absorption rate constant of DON3G (1/min); Cl_{DON}=clearance of DON (mL/min/kg); Q_{DON}=intercompartment flow for DON (mL/min/kg); Vc_{DON}=central volume of distribution for DON (mL/kg); Vp_{DON}=peripheral volume of distribution for DON (mL/kg); Cl_{DON3G}=clearance of DON3G (mL/min/kg); Vc_{DON3G}=central volume of distribution for DON3G (mL/kg); FRAC=fraction of dose absorbed.*

3. Results and discussion

3.1. LC-MS/MS method validation

For the calibration model a linear, 1/x weighed, fitting was applied. The results for linearity (*r* and *gof*) and sensitivity (LOD and LOQ) are given in **Table 2**. The results for linearity were all in accordance with the acceptance criteria, with $r \geq 0.99$ and $gof \leq 20\%$. For chicken plasma, the LOD varied from 0.11 (DOM-1) to 0.47 (DON) ng/mL, whereas the LOQ was 1 ng/mL for all compounds. For pig plasma, LOD values ranged from 0.01 (DON) to 0.28 (DOM-1) ng/mL, LOQ values were 0.1 (DON3G), 0.5 (DON) and 1 (DOM-1) ng/mL.

The results for matrix effects (SSE), apparent recovery (R_A) and extraction recovery (R_E) are also depicted in **Table 2**. SSE values for all mycotoxins varied from 44 to 112%, which demonstrated the need for matrix-matched calibration curves for a correct quantification. The matrix effects could be diminished by further optimizing the sample cleanup. However, a more elaborate cleanup tends to diminish the R_E, possibly limiting the sensitivity of the method. Values for R_E varied between 68.2 and 97.0% and R_A values ranged from 41.8 to 76.2%, with the majority of the latter deviation caused by SSE.

Table 2. *Validation results for linearity (r & gof, 8 concentration points in the range LOQ-500 ng/mL), sensitivity (LOD & LOQ; n=6), matrix effects (SSE), apparent recovery (R_A) and extraction recovery (R_E).*

Plasma	Compound	Correlation coefficient (r)	Goodness of fit (gof)	LOD (ng/mL)	LOQ (ng/mL)	SSE (%)	R_A (%)	R_E (%)
Chicken	DON	0.9998	4.92%	0.47	1.0	85.3	65.9	77.2
	DOM-1	0.9997	7.05%	0.11	1.0	112.0	76.2	68.2
	DON3G	0.9997	7.58%	0.38	1.0	58.1	43.9	75.6
Pig	DON	0.9997	5.94%	0.01	0.5	67.5	65.4	97.0
	DOM-1	0.9999	3.98%	0.28	1.0	44.0	41.8	95.0
	DON3G	0.9998	4.98%	0.03	0.1	73.1	64.8	88.7

Table 3 displays the results for accuracy (%) and precision (RSD, %). All results for chicken as well as pig plasma were within the acceptable ranges. The specificity test demonstrated that no interfering peaks were detected in the chromatographic elution zone of the analytes with S/N values ≥ 3 (results not shown).

Carry-over was evaluated and for none of the compounds signals were detected that could interfere with the response/area of the analytes at their given retention time (results not shown).

Table 3. *Validation results for within-day (n=6) and between-day precision (n=3x3) with corresponding accuracy at low (LOQ) and high (500 ng/mL) concentration level.*

		Within-day				Between-day			
		Accuracy (%)		Precision (RSD, %)		Accuracy (%)		Precision (RSD, %)	
Plasma	**Compound**	**LOQ**	**500 ng/mL**	**LOQ**	**500 ng/mL**	**LOQ**	**500 ng/mL**	**LOQ**	**500 ng/mL**
Chicken	DON	8.0	0.6	12.3	3.3	-9.5	-0.7	27.6	4.7
	DOM-1	15.6	0.7	24.1	4.6	-12.2	0.3	12.7	4.2
	DON3G	6.1	0.3	29.4	7.5	16.3	0.3	18.4	5.0
Pig	DON	-1.3	0.8	31.4	3.3	-5.7	-0.8	28.8	3.6
	DOM-1	-7.7	-0.6	6.4	2.0	-2.3	1.0	18.2	3.4
	DON3G	-11.4	-0.8	24.6	1.7	-1.1	-2.0	31.3	4.3

3.2. Toxicokinetic analysis

The goal of this study was to determine the absolute oral bioavailability of DON3G and DON, the degree of *in vivo* hydrolysis of DON3G to DON (differentiating between presystemic GI, presystemic portal, presystemic hepatic and systemic hydrolysis) and the toxicokinetic parameters of both toxins in broiler chickens and pigs. A tailor-made compartmental toxicokinetic model was developed which offers the advantage, compared to non-compartmental analysis, that for a given compound, PO and IV data can be fitted simultaneously for each animal allowing more reliable estimates as more data is available (Broekaert et al. 2015b).

For broiler chickens, no adverse effects were observed during the animal trial following PO and IV bolus administration of the mycotoxins. Similarly, no adverse effects were observed during the pig trial following IV and PO DON3G administration and PO DON administration. Following IV dosing of DON, two out of six pigs vomited within 0.5 h p.a. However, this had no observable influence on the results as these pigs were IV dosed.

Plasma concentration-time profiles for DON3G and DON in broiler chickens and pigs are presented in **Figure 3**. Each profile represents the mean of six animals ± SD for systemic plasma concentrations. Additionally for pigs, *vena porta* plasma concentration-time profiles are presented as mean of four animals ± SD. A first observation is the absence of DOM-1 in all analysed samples. The derived toxicokinetic results are shown in **Table 4**.

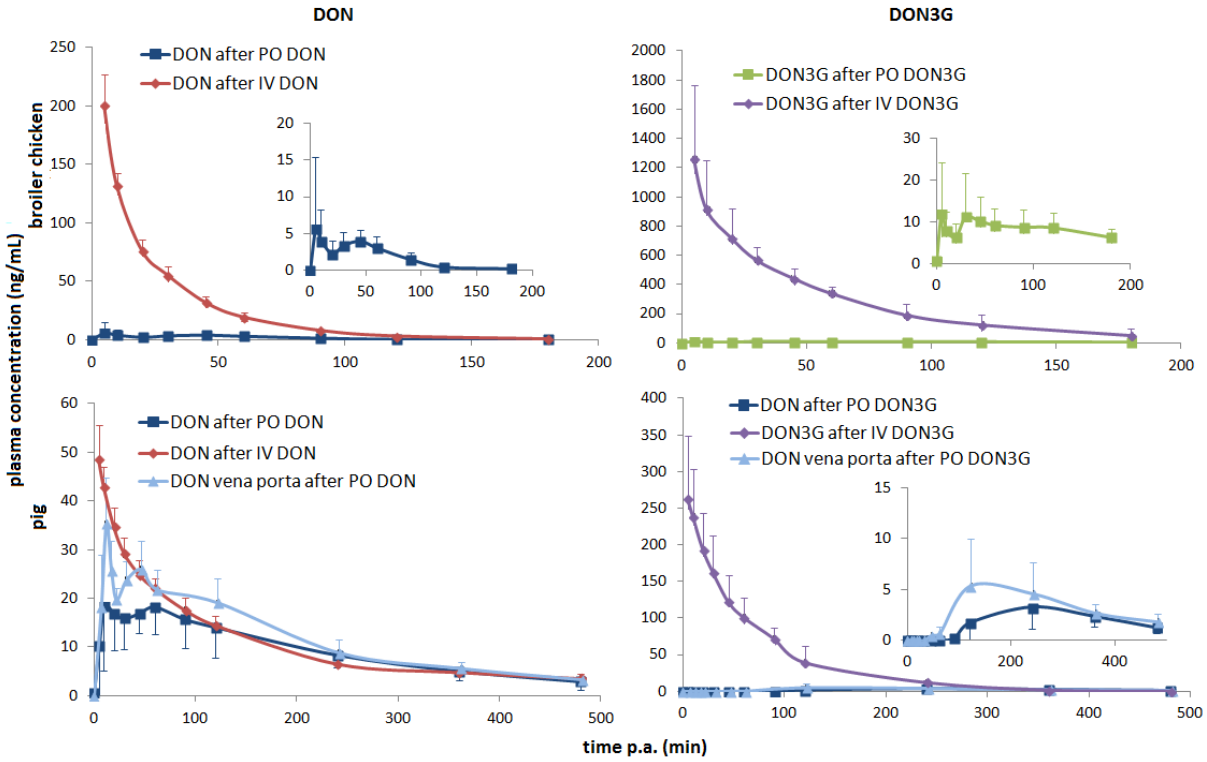

Figure 3. *Systemic plasma (chickens and pigs) and vena porta plasma (pigs) concentration-time profiles of deoxynivalenol (DON) and deoxynivalenol-3-β-D-glucoside (DON3G) after oral (PO) and intravenous (IV) administration (post administration=p.a.) of DON (dose=500 µg DON/kg body weight) and DON3G (dose=774 µg DON3G/kg body weight) to broiler chickens and of DON (dose=36 µg DON/kg body weight) and DON3G (dose=55.7 µg DON3G/kg body weight) to pigs (n=6 for systemic plasma, n=4 for plasma from vena porta). Values are presented as mean + or - SD. No DON was detected in chickens after DON3G administration. In pigs, no DON was detected after IV DON3G administration and no DON3G after PO DON3G administration. The inserts show PO administration data in more detail.*

Table 4. *Toxicokinetic parameters, presystemic hydrolysis of the bioavailable fraction (%) and absolute oral bioavailability (F%) of DON and DON3G after IV and PO administration to broiler chickens and pigs (n=6).*

	Broiler chicken		Pig		
	DON (IV&PO)	**DON3G (IV&PO)**	**DON (IV&PO)**	**DON3G IV**	**DON3G PO**
ka_{DON} (1/min)	0.0315±0.0173	-	0.0274±0.0107	-	0.00501±0.00160
Ke_{DON}/β_{DON} (1/min)	0.0369±0.0041	-	0.00425±0.00155	-	0.0217±0.0014
$t_{1/2elDON}$ (min)	18.8±2.1	-	163±59	-	31.9±2.1
Cl_{DON} (mL/min/kg)	88.8±12.4	-	5.25±1.25	-	25.9±2.8
Q_{DON} (mL/min/kg)	86.7±40.7	-	17.4±11.3	-	-
Vc_{DON} (mL/kg)	1350±578	-	614±127	-	1196±717
Vp_{DON} (mL/kg)	1087±211	-	789±486	-	-
t_{lag} (min)	-	-	-	-	83.6±5.6
ka_{DON3G} (1/min)	-	0.0238±0.0203	-	-	-
ke_{DON3G}/β_{DON3G} (1/min)	-	0.0204±0.0037	-	0.0128± 0.0007	-
$t_{1/2elDON3G}$ (min)	-	34.0±6.2	-	54.1±3.0	-
Cl_{DON3G} (mL/min/kg)	-	11.4±1.2	-	3.17±0.81	-
Q_{DON3G} (mL/min/kg)	-	27.7±25.8	-	-	-
Vc_{DON3G} (mL/kg)	-	290±188	-	249±67	-
Vp_{DON3G} (mL/kg)	-	284±167	-	-	-
FRAC (%)	5.56±2.05	3.79±2.60	81.3±17.4	-	16.1±5.4**
Pres.Hydr. (%)	-	0	-	-	100
F (%)	5.56±2.05	3.79±2.68	81.3±17.4	-	0*
C_{max} (ng/mL)	7.40±7.35	16.6±10.4	23.7±9.8	-	3.88±2.0**
t_{max} (min)	30.0±22.5	43.1±49.4	44.4±39.1	-	225±100**

*Values are presented as mean ± SD. For pigs, the DON3G PO parameters have been calculated simultaneously on all pooled pig PO DON3G data, as a result no SD could be calculated, instead coefficients of variation of the models' predicted versus the observed concentrations are given. ka_{DON}=absorption rate constant for DON (1/min); ke_{DON}=elimination rate constant for DON (1/min); β_{DON}=disposition rate constant for DON (1/min); $t_{1/2elDON}$=elimination half-life of DON (min); Cl_{DON}=clearance of DON (mL/min/kg); Q_{DON}=intercompartmental flow for DON (mL/min/kg); Vc_{DON}=central volume of distribution for DON (mL/kg); Vp_{DON}=peripheral volume of distribution for DON (mL/kg); t_{lag}=absorption lag time (min); ka_{DON3G}=absorption rate constant of DON3G (1/min); ke_{DON3G}=elimination rate constant for DON3G (1/min); β_{DON3G}=disposition rate constant for DON3G (1/min); $t_{1/2elDON3G}$=elimination half-life of DON3G (min); Cl_{DON3G}=clearance of DON3G (mL/min/kg); Q_{DON3G}=intercompartmental flow for DON3G (mL/min/kg); Vc_{DON3G}=central volume of distribution for DON3G (mL/kg); Vp_{DON3G}=peripheral volume of distribution for DON3G (mL/kg); FRAC=absorbed fraction; Pres.Hydr.=percentage of the total dose that is hydrolysed presystemically to DON and absorbed as DON (%); F=absolute oral bioavailability (%); C_{max}=maximum plasma concentration for DON (ng/mL); t_{max}= time to maximum plasma concentration for DON (min); * and ** statistically significant difference at $p<0.05$ and $p<0.01$, respectively, compared to DON values in the same animal species.*

The FRAC of PO administered DON and DON3G to chickens and PO administered DON to pigs was calculated using the tailor made model. However, the FRAC of DON3G to pigs could not be calculated by this model as after PO administration of DON3G only DON was recovered and after IV administration only DON3G. The FRAC was therefore calculated manually by correcting the area under the curve (AUC) of DON3G after IV administration by multiplying this value with its Cl, and by correcting the AUC of DON after PO DON3G dosing again by multiplying this value with its Cl and by correcting this ratio for the difference in molar masses, as shown in the formula below:

$$FRAC_{DON3G} = \frac{AUC_{DON\ after\ DON3G\ PO} * Cl_{DON\ after\ DON} * MM_{DON3G}}{AUC_{DON3G\ after\ DON3G\ IV} * Cl_{DON3G\ after\ IV\ DON3G} * MM_{DON}}$$

The mean (±SD) absorbed fractions of DON and DON3G in broiler chickens were 5.56±2.05% and 3.79±2.68%, respectively. Moreover, for DON and DON3G in chickens, F was equal to FRAC as DON and DON3G are absorbed in their unchanged form. The FRAC of DON in pigs, 81.3±17.4% was also identical to the F. The FRAC of DON3G in pigs was 16.1±5.4%. However, as after PO DON3G administration only DON was recovered, F equals 0, indicating complete hydrolysis of the absorbed fraction. The higher observed FRAC values for pigs compared to chickens is an important contributing factor to the decreased sensitivity of the latter species, and may be partially caused by its rapid GI transit time, leading to a reduced time for absorption in chickens.

After IV administration of DON3G to chickens and pigs, no hydrolysis to DON could be observed, indicating the absence of systemic hydrolysis for both species. For pigs, this confirms the findings of Nagl et al. (2014). After PO administration of DON3G to chickens, no hydrolysis was observed neither. In contrast, after PO DON3G administration to pigs, only DON was recovered, indicating a complete presystemic hydrolysis of the absorbed fraction. Presystemic hydrolysis can take place at the level of GI microbiota, GI tissues, portal blood or the liver. To further determine the site of presystemic hydrolysis, blood was collected from the porcine *vena porta*, which drains the venous intestinal blood to the liver first. Interestingly, only DON was recovered and no DON3G was detected in these portal plasma samples after PO DON3G administration. This absence of DON3G in portal plasma as well as

the absence of DON after IV DON3G administration, indicates that hydrolysis occurs at the site of the GI tract (microbiota or GI tissues) and not in portal blood nor in the liver. This is in accordance with previously reported *in vitro* results which reported that hydrolysis could be caused by intestinal microbiota and which have demonstrated the important role of microbiota composition and the abundance in the intestinal tract (Abbott 2004; Berthiller et al. 2011; Dall'Erta et al. 2013; Gratz et al. 2013; Hattori and Taylor 2009). The observed lag time for DON absorption after PO administration of DON3G to pigs, 83.6 min, further supports the hypothesised intestinal hydrolysis of DON3G. This is also reflected in the significant higher t_{max} value of DON after PO DON3G administration in pigs. DON is mainly absorbed in the proximal part of the small intestine by means of passive diffusion. A less effective passive non-ionic diffusion is expected for DON3G as it is more polar and has a less favourable log D value (-2.74) compared to DON (-0.97) at both physiological and acidic pH values (Maresca 2013). The pH in the stomach and duodenum of adult pigs is 4.4 and 6.4-7.4, respectively (Merchant et al. 2011). These rather low pH values are unfavourable for most GI commensal bacteria. When moving to more distal parts of the small intestine, the pH increases and a more abundant growth of microbiota is observed (Maresca 2013). Consequently, the probability of DON3G hydrolysis to DON is increased, resulting in the observed lag time of 83.6 min. As chickens have a high bacterial load at the crop, thus before the small intestine, which is the main site of absorption, hydrolysis of DON3G to DON by GI microbiota would be expected after PO administration to chickens. For pigs, where the high bacterial load is located after the site of absorption, only limited hydrolysis to DON is expected. Remarkably, our observations were the opposite of these theoretical expectations. This discrepancy likely finds its cause in the bacterial species composition and abundance in both the chicken crop and pig distal small and large intestine.

The mean (±SD) ka_{DON} values in chickens and pigs were comparable, 0.0315±0.0173/min and 0.0274±0.0107/min, respectively. For pigs ka_{DON} values of 0.00995±0.00292/min (Eriksen et al. 2003), 0.0310±0.0238/min (Goyarts and Dänicke 2006) and 0.0164±0.0161/min (Broekaert et al. 2015b) have been reported, and for chickens a ka_{DON} of 0.0203±0.0101/min (Broekaert et al. 2015b) was previously described. The ka_{DON3G} in chickens was slightly lower than that of DON, 0.0238±0.0203/min. For pigs no ka_{DON3G} could be calculated as it was fully hydrolysed to DON after PO administration. The resulting ka_{DON} after PO DON3G

administration was 0.00501/min, significantly lower than the ka_{DON} after DON administration. The ka_{DON} after PO DON3G administration is influenced by presystemic hydrolysis, consequently this value is also determined by biotransformation in addition to absorption.

Mean (±SD) Cl_{DON3G} values of 11.4±1.2 and 3.17±0.81 mL/min/kg were obtained for chickens and pigs, respectively. Cl_{DON} values in chickens and pigs were 88.8±12.4 and 5.25±1.25 mL/min/kg, respectively. For chickens, a comparable Cl_{DON} of 120 mL/min/kg (Osselaere et al. 2013) and 102±12 mL/min/kg (Broekaert et al. 2015b) have previously been described. Cl_{DON} values in pigs of 9.30±3.95 mL/min/kg (acute PO) (Goyarts and Dänicke 2006), 5.30±1.64 mL/min/kg (chronic PO) (Goyarts and Dänicke 2006), 15.1±3.8 mL/min/kg (acute PO) (Broekaert et al. 2015b) and a Cl_{DON}/F of 5.0±0.7 mL/min/kg (acute PO) (Devreese et al. 2014) have been reported, which are in the same range as the results in this study. The obtained mean (±SD) Vc_{DON} and Vp_{DON} values for chickens were 1350±578 and 1087±211 mL/kg, respectively. For pigs, Vc_{DON} and Vp_{DON} values were comparable, i.e. 614±127 and 789±486 mL/kg respectively. For chickens Vc_{DON3G} and Vp_{DON3G} values of 290±188 and 284±167 mL/kg were obtained. A Vd_{DON3G} in pigs of 249±67 mL/kg was obtained using a one-compartmental model. The observed lower Vd values for DON3G indicate that DON3G is more confined to intravascular fluids than DON, which can be theoretically explained by its increased polarity compared to DON, resulting in less effective passive non-ionic diffusion into tissues.

The mean (±SD) elimination half-lives ($t_{1/2el}$) for DON in broilers and pigs were 18.8±2.1 min and 163±59 min (2.71 h), respectively. For pigs, $t_{1/2el}$ of 5.3±2.4 h (acute PO, two-compartmental model) (Goyarts and Dänicke 2006), 2.4±0.7 h (acute PO, one-compartmental model) (Eriksen et al. 2003), 2.7±0.5 h (acute PO, one-compartmental model) (Devreese et al. 2014) and 2.9 h (acute PO, two-compartmental model) (Broekaert et al. 2015b) were observed. For broiler chickens comparable elimination half-lives of 27.9±6.9 min (IV) and 38.2±11.2 min (acute PO) (Osselaere et al. 2013) and 17.9 min (acute PO) (Broekaert et al. 2015b) were also reported. The $t_{1/2elDON3G}$ values for chickens and pigs were 34.0±6.2 min and 54.1±3.0 min, respectively. Remarkably, the $t_{1/2elDON3G}$ compared to that of DON was increased for chickens and decreased for pigs, highlighting the difference between

both species with respect to DON3G's elimination. For pigs, this is mainly attributed to a lower Vd. For broiler chickens, additional to a lower Vd a reduced Cl was observed.

3.3. HRMS

The plasma concentration-time profiles of phase II metabolites are depicted in **Figure 4** and **Figure 5** for pigs and chickens, respectively. Since only semi-quantitative analysis was performed, absolute peak areas obtained on the HRMS instrument are depicted. For pigs, a first observation is that glucuronidation patterns after PO administration do not differ between systemic (*vena jugularis*) and presystemic (*vena porta*) plasma. This indicates that glucuronidation does not occur presystemically to a significant rate and extent. This hypothesis is further supported by practically identical observed mean glucuronidation ratios (area of DON3GlcA/ area of DON) after IV and PO administration, 4.94 and 4.98, respectively. For pigs, no sulfate conjugates were detected, which is expected as this species' deficient phase II sulfation reaction is described (Riviere and Papich 2013). In contrast chickens predominantly formed DON-3α-sulfate. The mean DON-3α-sulfate/DON ratios in chickens after IV and PO DON administration were 392 and 9,890, respectively. The observed ratios are comparable to previously found values for IV and PO administration of DON to chickens of 243-453 and 1,365-29,624, respectively (Devreese et al. 2015). This remarkable contrast in ratio between IV and PO administration, points towards a presystemic sulfation of DON, either by GI microbiota, GI tissues or a presystemic liver effect. In chickens, no glucuronidation was observed. The extensive and rapid sulfation in chickens might partially explain the relative resistance of this species towards the detrimental effects of DON.

In conclusion, broiler chickens do not hydrolyse DON3G to DON *in vivo.* Additionally, DON3G has a low absolute oral bioavailability (3.79±2.68%) comparable to that of DON (5.56±2.05%). After PO DON3G administration to pigs only DON was recovered, indicating a complete presystemic hydrolysis of DON3G. However, the absorbed fraction is approximately 5 times lower than after PO DON administration, 16.1±5.4% compared to 81.3±17.4%. Analysis of phase II metabolites revealed that DON biotransformation in pigs consists mainly of glucuronidation, and in chickens sulfation predominantly occurs. Pigs are

Phase II biotransformation is much more extensive for chickens than for pigs, which can be a possible explanation for the differences in sensitivity of the latter species to DON. Furthermore, given the *in vivo* hydrolysis of DON3G in pigs, pigs are likely to be more sensitive to this modified mycotoxin compared to chickens. Although *in vitro* studies reported that DON3G is less toxic than DON, the demonstrated *in vivo* hydrolysis to DON in pigs, as well as the inter-species differences observed, indicate a decreased yet not neglectable toxicological relevance of DON3G and consequently the need for further research to possibly establish a tolerable daily intake.

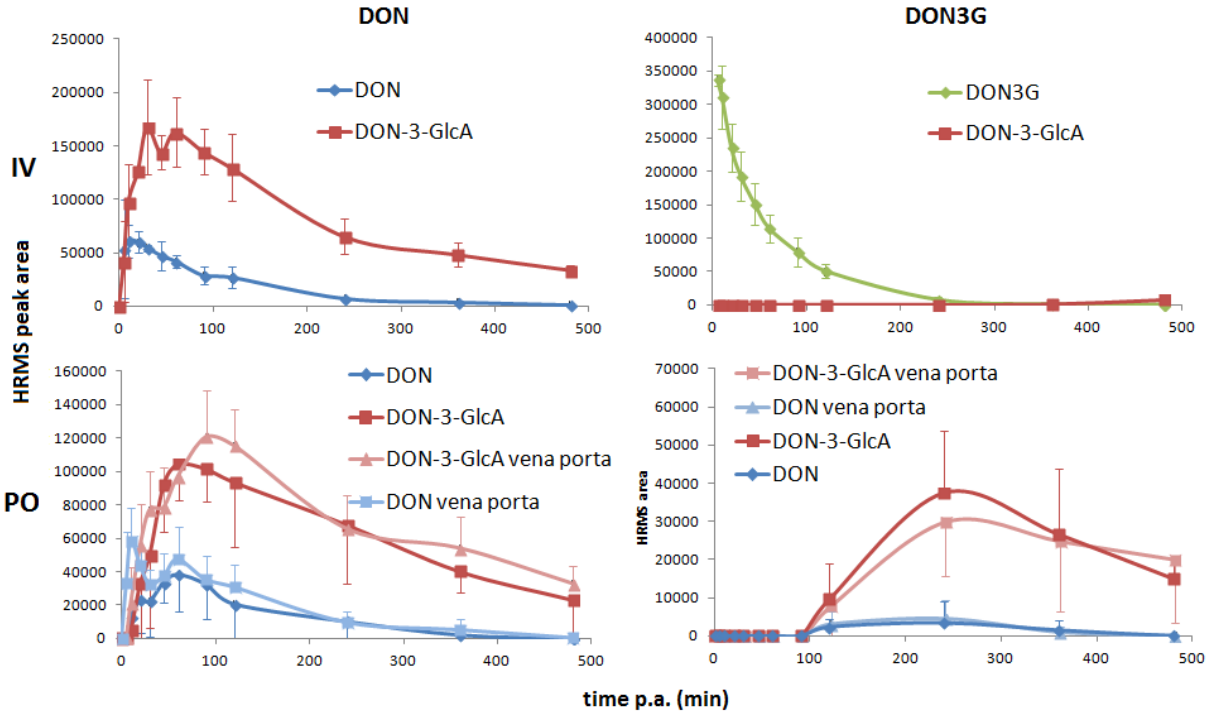

Figure 4. *Porcine plasma (systemic and vena porta) concentration-time profiles of deoxynivalenol (DON), deoxynivalenol-3-β-D-glucoside (DON3G) and deoxynivalenol-3-glucuronide (DON3GlcA) after oral (PO) and intravenous (IV) administration of DON (dose=36 µg DON/kg body weight) and DON3G (dose=55.7 µg DON3G/kg body weight) to pigs (n=3 for systemic plasma, n=3 for plasma from vena porta). Values are presented as mean + or - SD.*

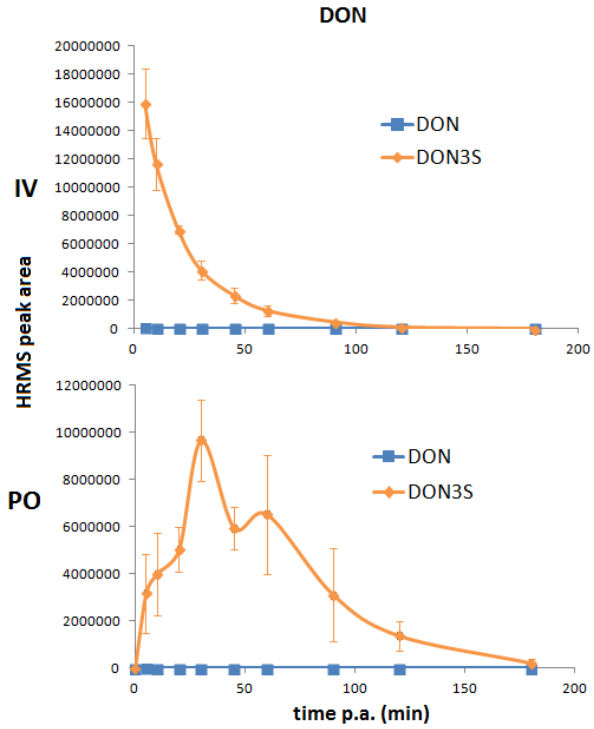

Figure 5. *Plasma concentration-time profiles of deoxynivalenol (DON) and deoxynivalenol-3α-sulfate (DON3S) after oral (PO) and intravenous (IV) administration of DON (dose=500 µg DON/kg body weight) to broiler chickens (n=3). Values are presented as mean + SD.*

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CHAPTER IV

In vitro cytotoxicity of acetylated and modified deoxynivalenol on a porcine intestinal epithelial cell line

Adapted from:

Broekaert N., Devreese M., Demeyere K., Meyer E., Croubels S. (2015). *In vitro* cytotoxicity of acetylated and modified deoxynivalenol on a porcine intestinal epithelial cell line. In preparation

Abstract - The gastrointestinal tract is the first target after ingestion of deoxynivalenol (DON) via feed and food. Deoxynivalenol is known to affect the proliferation and viability of animal and human intestinal epithelial cells. In addition to DON, feed and food is often co-contaminated with acetylated and modified forms of DON, such as 3-acetyl-deoxynivalenol (3ADON), 15- acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G). The goal of this study was to determine the *in vitro* intrinsic cytotoxicity of these acetylated and modified forms of DON towards differentiated and proliferative porcine intestinal epithelial cells by means of a flow cytometric technique. Cell death was assessed by means of dual staining with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), which allows the discrimination of viable cells ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$). Based on the data from the presented pilot study, it is concluded that cytotoxicity can be ranked as follows: DON3G << 3ADON < DON ≈ 15ADON.

Keywords - deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), deoxynivalenol-3-β-D-glucoside (DON3G), IPEC-J2, flow cytometry

1. Introduction

Small grain cereals, such as wheat, maize and oats are often infected by *Fusarium* species. These fungi may produce trichothecenes, a class of mycotoxins. Deoxynivalenol (DON) is the most prevalent trichothecene in cereals in Europe and North America (Gareis et al. 2003). In addition to its presence in raw cereals, DON is particularly stable towards most food-processing techniques and thus can persist in processed food and feed (Hazel and Patel 2004). After consumption of food/feed contaminated with DON, and mycotoxins in general, the gastrointestinal tract is the first target of these compounds.

DON inhibits the absorption of certain nutrients (e.g. glucose and aminoacids) in human (Maresca et al. 2002) and animal (Awad et al. 2007; Awad et al. 2014; Hunder et al. 1991; Marin et al. 2011) intestinal epithelial cells (IECs). Furthermore, DON is known to affect the tight-junctions of the intestinal epithelium, thereby compromising the intestinal barrier function (Antonissen et al. 2015a; Diesing et al. 2011a; Diesing et al. 2011b; Goossens et al. 2012; Maresca et al. 2002; Pinton et al. 2010; Pinton et al. 2012). Additionally, DON may cause intestinal inflammation (Maresca et al. 2008; Maresca and Fantini 2010), increased bacterial translocation (Maresca and Fantini 2010; Vandenbroucke et al. 2009; Vandenbroucke et al. 2011), a decrease in the number of goblet cells and a diminished mucus production (Antonissen et al. 2015b; Obremski et al. 2008; Pinton et al. 2015). All these processes may facilitate the crossing of microbial antigens indirectly affecting the IECs innate immunity (Maresca et al. 2008; Maresca and Fantini 2010). The IECs innate immunity may also be directly affected via the activation of signaling pathways by DON itself (Cano et al. 2013; Van De Walle et al. 2008; Maresca 2013; Van De Walle et al. 2010; Vandenbroucke et al. 2011). Finally, DON can alter the viability and proliferation of animal and human IEC. At low doses (IC_{50}= 0.3-1.5 mg/L) inhibition of cell proliferation is observed, at higher concentrations (IC_{50}= 3-15 mg/L) cytotoxic effects and apoptosis can be seen in human, pig and rat IEC (Bianco et al. 2012; Diesing et al. 2011a; Diesing et al. 2011b; Vandenbroucke et al. 2011). Factors influencing cytotoxicity such as status of the cells (differentiated vs undifferentiated) and exposure site (apical vs basolateral) have been investigated for pig IEC. It was observed that undifferentiated cells were 10-times more sensitive and that basolateral exposure resulted in a 4-times higher cytotoxicity compared to apical exposure (Diesing et al. 2011a; Diesing et al. 2011b; Diesing et al. 2012).

Food and feed containing DON is often co-contaminated with its modified forms. These modified mycotoxins predominantly consist of 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G). Both 3ADON and 15ADON are fungal precursors in the biosynthesis of DON. DON3G is produced *in planta* as phase II metabolite in an attempt to detoxify and eliminate DON. Concentrations exceeding 1000 µg/kg have been reported for 3ADON, 15ADON and DON3G (Gareis et al. 2003), amounts not to be neglected knowing that the tolerable daily intake (TDI) for DON in humans is 1 µg/kg bodyweight (bw). DON3G/DON ratios larger than 1 have been observed in some hard red spring wheat samples (Sasanya et al. 2008).

These modified mycotoxins have not been as extensively investigated as DON. Questions such as their potential *in vivo* hydrolysis and their intrinsic toxicity still remain. The goal of this study was to determine the *in vitro* cytotoxicity of 3ADON, 15ADON and DON3G towards differentiated and undifferentiated intestinal epithelial cells. As pigs are among the most sensitive species to DON toxicity, a porcine intestinal epithelial cell line (IPEC-J2) was chosen.

2. Experimental

2.1. Chemicals

DON, 3ADON and 15ADON (>99% purity) were purchased from Fermentek (Jerusalem, Israel). DON3G was enzymatically synthesised, purified and verified using nuclear magnetic resonance (NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (<0.05% DON) by the Christian Doppler Laboratory for Mycotoxin Metabolism Center for Analytical Chemistry (Vienna, Austria). DON, 3ADON, 15ADON and DON3G were dissolved in ethanol yielding a stock solution of 10 mg/mL. All the above mentioned stock solutions were stored at ≤−15°C.

2.2. Cell line and culture conditions

The IPEC-J2 cell line is a continuous intestinal cell line derived from the jejunal epithelium isolated from a neonatal piglet. The IPEC-J2 cells are unique as they are derived from the small intestine and are neither transformed nor tumourigenic in nature (Vergauwen 2015). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (1:1) medium (Invitrogen™ Life Technologies, CA, USA), supplemented with 5% fetal calf serum (FCS,

HyClone, UK), 1% (v/v) insulin/transferrin/Na-selenite (Gibco, Thermo Fischer Scientific, MA, USA), 1% (v/v) penicillin/streptomycin (Gibco), 1% (v/v) kanamycin (Gibco) and 0.1% fungizone (Bristol-Myers Squibb, Braine-l'Alleud, Belgium), further referred to as culture medium. The cells were routinely seeded at a density of 3×10^5 cells/mL with 40 mL of medium in plastic tissue culture flasks (150 cm^2, Nunc, Denmark), maintained in a humidified incubator at 37°C under 5% CO_2, and passaged twice weekly.

2.3. Cytotoxicity assay

For the cytotoxicity experiment, IPEC-J2 cells were seeded at 5×10^5 cells/well on 24-well plates in 1 mL of culture medium and allowed to grow for 2 or 21 days for proliferative and differentiated cells, respectively. The IPEC-J2 cell line in culture with addition of 5% FCS undergoes a process of spontaneous differentiation that leads to the formation of a polarised monolayer with high transepithelial electrical resistance (TEER) within 1–2 weeks (Vergauwen 2015). Each well was washed twice with 1 mL of sterile Hank's buffered salt solution (HBSS, Gibco) in order to remove dead cells caused by the trypsin treatment/seeding protocol. Monolayers were exposed in fivefold to DON, 3ADON, 15ADON or DON3G, all dissolved in ethanol and diluted with culture medium, for 72 h at following concentrations: 0, 1, 5, 10, 15 and 20 µg/mL. In order to exclude a position effect on the plate, the order of the toxins was different for each 24-well plate. After incubation, the IPEC-J2 cells were trypsinised and cells together with their culture medium (containing detached cells) were joined in a flow cytometric tube. In order to remove the mycotoxins and cellular debris, each tube was centrifuged (10 min, 524 x *g*, 4°C) after which the pellet was resuspended in 500 µL of HBSS. This was done three consecutive times. Next, the cells were centrifuged (10 min, 524 x *g*, 4°C) followed by an incubation for 10 min in the dark at room temperature with 100 µL of a solution containing 20 µL of a commercial Annexin-V-fluorescein isothiocyanate reagent (Annexin-V-FITC reagent, Sigma-Aldrich, Belgium) and 20 µL of a 50 µg/mL propidium iodide solution (PI, Sigma-Aldrich) dissolved in 960 µL of incubation buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 140 mM NaCl and 5 mM $CaCl_2$. Cells were analysed by a FACSCanto flow cytometer (Becton, Dickinson and Company, Belgium). A positive apoptosis control was included by staining the cells solely with Annexin-V-FITC after a 4 h treatment with 1.9 µM staurosporine. A positive necrosis control was obtained by single staining the cells with PI

after a 1 min incubation with RIPA buffer (PBS with 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1% NP-40). Additionally, positive controls were used to quantify and compensate for spectral bleed-through. Furthermore, unstained cells (no Annexin-V-FITC and no PI) were also included to determine autofluorescence of IPEC-J2 cells. To obtain viable cells as a negative control for Annexin-V-FITC staining and to determine the distinction between positive and negative stained cells, the culture medium was removed and the cells were washed twice with HBSS before trypsin treatment to exclude nonviable cells. Cell death was assessed using dual staining with Annexin-V-FITC and PI which allows the discrimination of viable cells ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$), see **Figure 1**. The mycotoxin concentration resulting in 50% reduction in viable cells (IC_{50}) was calculated using linear regression.

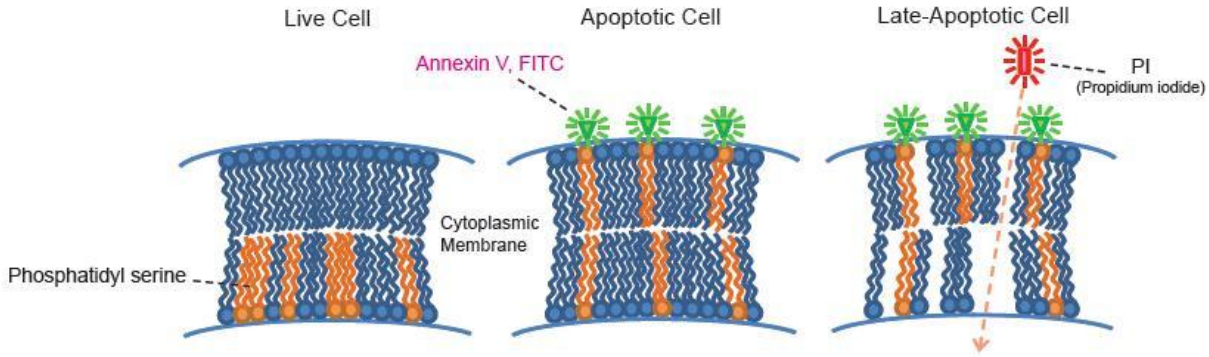

***Figure 1**. Dual staining with Annexin V and PI. In a live, intact cell, PI cannot enter the cell and label dsDNA/RNA and phosphatidylserine (PS) is maintained exclusively on the inner leaflet via flippase, resulting in negative staining for both dyes. Annexin V labels externalized PS- on the cell surface following the initiation of apoptosis. During the latter stages of apoptosis/necrosis both PS is exposed and PI can enter the cell due to the loss of membrane integrity (adopted from http://www.dojindo.com/store/p/847-Annexin-V-FITC-Apoptosis-Detection-Kit.html)*

2.4. Statistical analysis

Statistical analysis of mean Annexin-V-FITC intensity consisted of one-way ANOVA with post-hoc Scheffé tests (P value < 0.05) followed by Levene's test for homogeneity of variances (P value > 0.01).

3. Results and discussion

The goal of this study was to determine the *in vitro* intrinsic cytotoxicity of DON, 3ADON, 15ADON and DON3G towards differentiated and proliferative IPEC-J2 cells. By means of flow cytometry (FCM), distinction and quantification of viable cells ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$) was made. In **Figure 2** viable (blue), apoptotic (green) and necrotic (black) populations are depicted after a 72h exposure to different concentrations of DON.

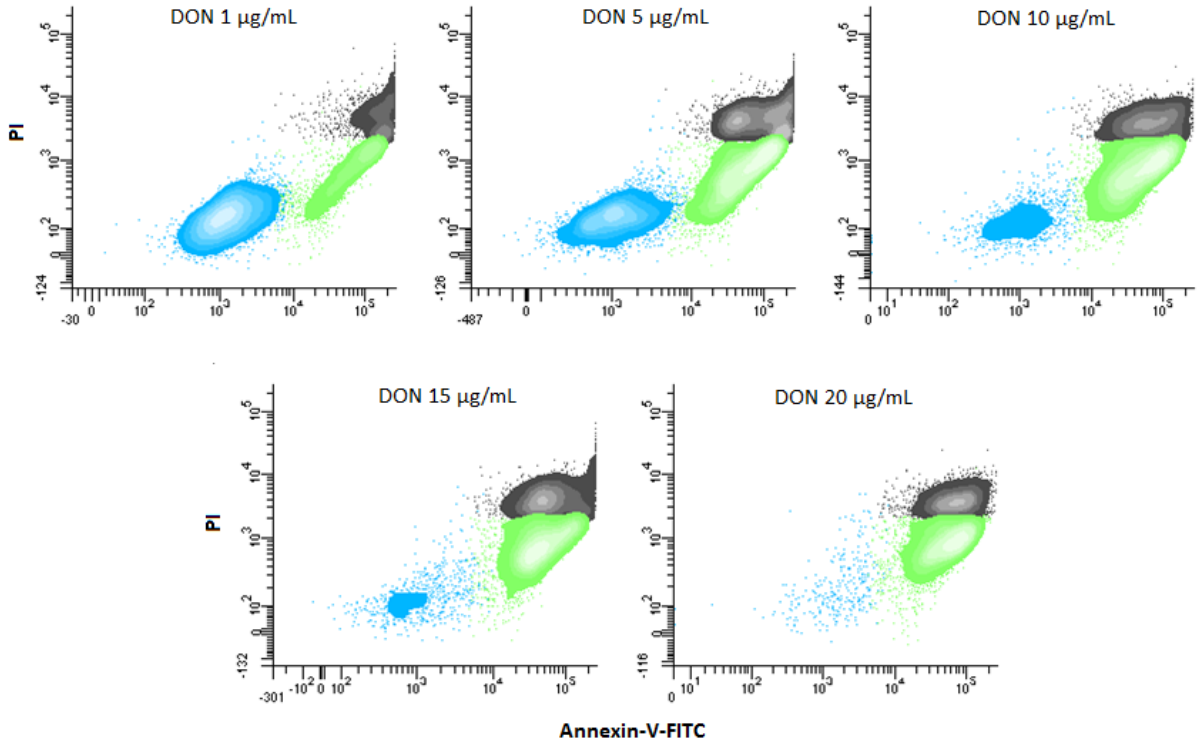

***Figure 2**: Population plots displaying viable (blue), apoptotic (green) and necrotic (black) populations of IPEC-J2 cells after a 72 h exposure of 1, 5, 10, 15 and 20 µg/mL of DON. Y-axis: PI intensity; X-axis: Annexin-V-FITC intensity*

Figure 3 shows viable, apoptotic and necrotic proliferative and differentiated IPEC-J2 cells after 72 h incubation with different concentrations of DON, 3ADON, 15ADON or DON3G and the corresponding IC_{50} values. A first observation is that differentiated IPEC-J2 cells are less susceptible to the evaluated mycotoxins compared to proliferative cells. Similar observations for DON have been made using IPEC-J2 cells (Vandenbroucke et al. 2011) and other intestinal epithelial cell lines such as Caco-2 (Bony et al. 2006; Pierron et al. 2015) and HT-29-D4 (Maresca et al. 2002). Regarding the cytotoxic effect of DON on IPEC-J2 cells as determined by a neutral red assay, the viability of proliferative cells was significantly decreased after a 24

h exposure to concentrations exceeding 0.1 µg/mL. The viability of differentiated cells was not significantly affected after a 24 h exposure to 10 µg/mL of DON (Vandenbroucke et al. 2011). A study by Goossens et al. demonstrated via FCM a decreased viability for proliferative IPEC-J2 cells after a 72 h exposure to 2.5 µg/mL of DON, with an IC_{50} value of 6.98 µg/mL (Goossens et al. 2012).

The higher susceptibility of proliferative cells could be attributed to the capacity of DON to inhibit protein synthesis and subsequently nucleic acid synthesis, mechanisms necessary for dividing cells. Another explanation is the well known cyclical fluctuation of many enzymatic activities, particularly those related to the metabolizing capacities that are increased at differentiation, as demonstrated for the Caco-2 cell line. Among the metabolizing enzymes, the UDP-glucuronosyltransferases (UGTs) for which DON can be a substrate (Maul et al. 2012) and glutathione S-transferases (GSTs) for which the 12–13 epoxide group characterizing most trichothecenes are possible targets (Bony et al. 2006; Meky et al. 2003), have been reported. Also, an increased expression of transporters/efflux systems of the ABC superfamily, i.e. P-glycoprotein (P-gp) and non-Pgp carriers, in differentiated cells has been described (Delie and Rubas 1997).

The IC_{50} values in proliferating IPEC-J2 cells calculated in this study were similar to prior published cytotoxicity results obtained with DON, 3ADON and 15ADON in proliferating Caco-2 cells using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A similar toxicity was found for DON and 15ADON with IC_{80} values of 16.5 µM (4.88 µg/mL) and 10.5 µM (3.55 µg/mL), respectively. 3ADON, for which a lower toxicity was expected, exhibited an IC_{80} value of 125 µM (42 µg/mL), which is approximately ten times higher (Alassane-Kpembi et al. 2015).

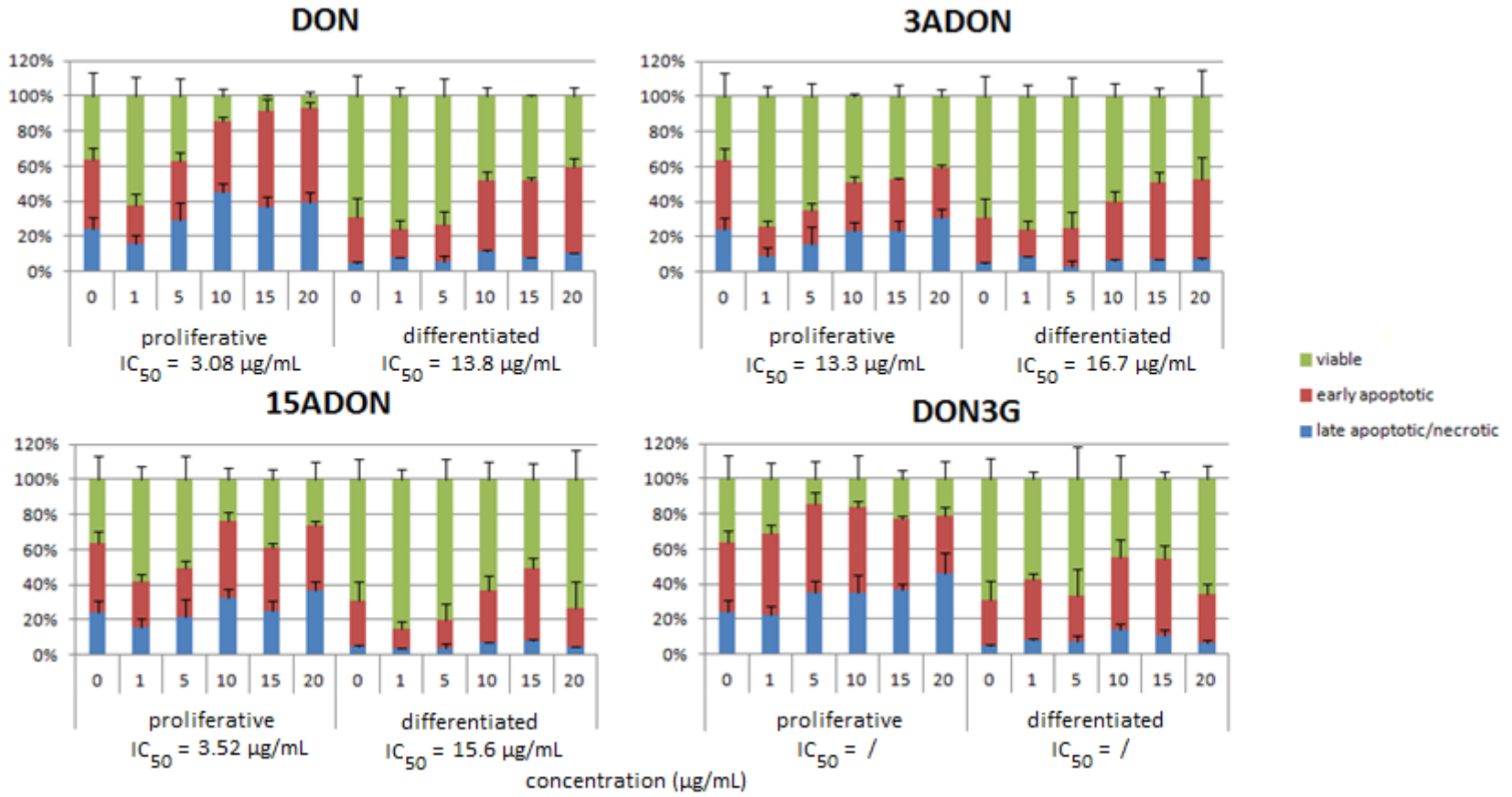

Figure 3: *Viable (green), apoptotic (red) and necrotic (blue) proliferative and differentiated IPEC-J2 cells after a 72 h incubation with 0, 1, 5, 10, 15 or 20 µg/mL DON, 3ADON, 15ADON or DON3G and the resulting IC_{50} values; Y-axis: percentage of cells (%); X-axis: concentration; /= not determined.*

The toxicity of DON3G in mammals remains largely unknown. Pierron et al. (2015) recently assessed comparative cytotoxicity of DON and DON3G on both proliferative and differentiated Caco-2 cells by quantitation of the present ATP. However, this set up does not allow distinction between viable and apoptotic cells since both cell types produce ATP. Nevertheless, this set up can discriminate between necrotic versus the sum of viable and apoptotic cells. After a 48 h exposure of proliferative Caco-2 cells to DON, an IC_{50} of 1.3 µM (0.384 µg/mL) was observed. In contrast, no cytotoxicity was observed with 0-10 µM of DON3G. For differentiated cells, no cytotoxicity was observed for 0-10 µM of DON or DON3G (Pierron et al. 2015). Our study could not calculate IC_{50} values for DON3G since the viability of cells incubated with DON3G appeared to be concentration independent, indicating absence of cytotoxicity in the concentration range tested. In contrast, the viability plots of DON, 3ADON and 15ADON were all characterised by a clear decreasing slope, indicating a concentration dependent cytotoxicity.

When assessing cytotoxicity from a population point of view, necrosis and apoptosis often result in a decrease in cell volume causing a population shift in the forward scatter (FSC) area versus side scatter (SSC) area plot. In **Figure 4 A** this population shift of apoptotic and necrotic cells (black) compared to viable cells (blue) is depicted after exposure to 5 µg/mL of DON. In contrast, for 10 µg/mL of DON3G no such population shift could be detected as depicted in **Figure 4 B**.

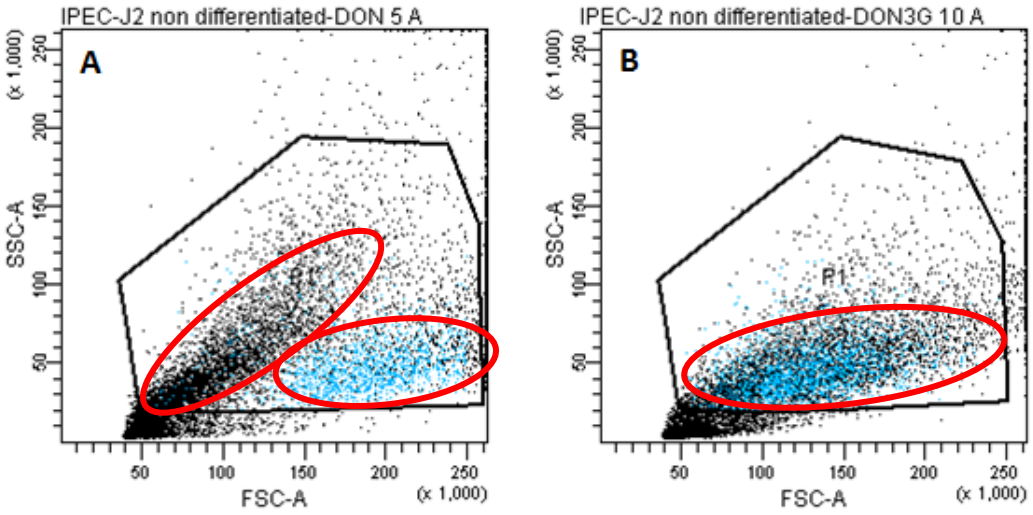

***Figure 4**: Side scatter (SSC) – forward scatter (FSC) area plots displaying necrotic and apoptotic cells (black) and viable proliferative IPEC-J2 cells (blue). **A**: 5 µg/mL DON causes a morphological shift of non-viable cells; **B**: 10 µg/mL DON3G causes no morphological shift.*

Another method to plot the FCM data is to determine mean FITC intensity for a given test condition. This is presented for apoptotic (Annexin-V-FITC positive, PI negative) proliferative cells in **Figure 5**. For each mycotoxin, mean Annexin-V-FITC intensities of the treated cells (1-20 µg/mL) were compared with their control (0 µg/mL). When regarding the apoptotic cells, DON, 3ADON as well as 15ADON exhibited a statistically significant increase in mean FITC intensity compared to control, from 5, 5 and 10 µg/mL, respectively, indicating an increase in apoptosis. In contrast, DON3G concentrations up to 20 µg/mL did not lead to a significant increase in FITC, indicating the absence of a DON3G induced apoptosis.

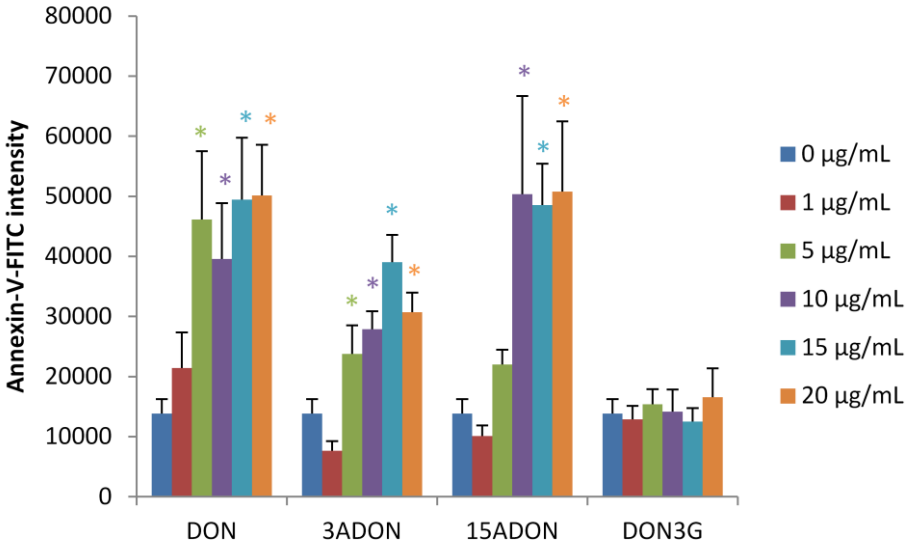

Figure 5: *Mean ± SD Annexin-V-FITC signal intensity for apoptotic proliferative IPEC-J2 cells. * Statistically significant compared to the control (0 µg/mL), p<0.05 determined by one-way ANOVA with post-hoc Scheffé tests.*

In conclusion, based on the data from the presented study, cytotoxicity can be ranked as follows: DON3G << 3ADON < DON ≈ 15ADON. However, it is essential to keep in mind that cytotoxicity to IECs is merely one toxicological endpoint. Other endpoints, such as effects on the immune system, may display significantly more or less sensitive dose-response curves. Furthermore, susceptibility to DON has been demonstrated to exhibit large interspecies differences. These differences can be partly attributed to a varying sensitivity of the animals cells, tissues and organs to the effects of DON or to a different exposure, caused by toxicokinetic differences such as distinct differences in absolute oral bioavailability or *in vivo* hydrolysis of acetylated and modified mycotoxins.

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GENERAL DISCUSSION

The *Fusarium* mycotoxin deoxynivalenol (DON) is one of the most frequently occurring mycotoxins, contaminating 56% of feed and feed raw materials worldwide (Schatzmayr and Streit 2013). In addition to its high prevalence in raw cereals, DON is particularly stable towards most food processing techniques and thus can persist in processed food and feed (Hazel and Patel 2004). DON is known for its harmful effects on animals, leading to great economic losses. Additionally to the native mycotoxin, feed can be contaminated with acetylated and modified forms of DON, such as 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G). Little is known about the toxicity and toxicokinetics of these acetylated and modified mycotoxins, compared to the well investigated free mycotoxin DON. One of the major questions associated with acetylated and modified mycotoxins is whether *in vivo* hydrolysis may result in the release of free mycotoxins and what their disposition in the animal's body is. This doctoral thesis contributes to this research gap, more specifically with respect to the oral bioavailability, (pre)systemic hydrolysis and main toxicokinetic characteristics of 3ADON, 15ADON and DON3G in broiler chickens and pigs. **Figure 1** shows an overview of the main methodologies and main results achieved in this doctoral thesis.

Determination of the oral bioavailability, rate and extent of *in vivo* hydrolysis and toxicokinetic characteristics of 3ADON, 15ADON and DON3G in relevant animal species by means of a state-of-the-art toxicokinetic analysis supports legislative authorities to carefully take measures with respect to their risk assessment. In turn, this risk assessment would assist in the establishment of a tolerable daily intake (TDI) for acetylated and modified mycotoxins or to include these compounds in a group TDI. The European Food Safety Authority (EFSA) recently published a scientific opinion on the risks for human and animal health related to the presence of modified forms of zearalenone (ZEN), nivalenol (NIV), T-2 toxin (T-2) and HT-2 toxin (HT-2) in food and feed. However, for the acetylated and modified forms of DON, no assessment is currently available due to their limited toxicity and toxicokinetic data (EFSA CONTAM Panel 2014). The research presented here may also support such assessment.

A more general discussion on (modified) mycotoxins is presented below, linked to the results obtained in this thesis.

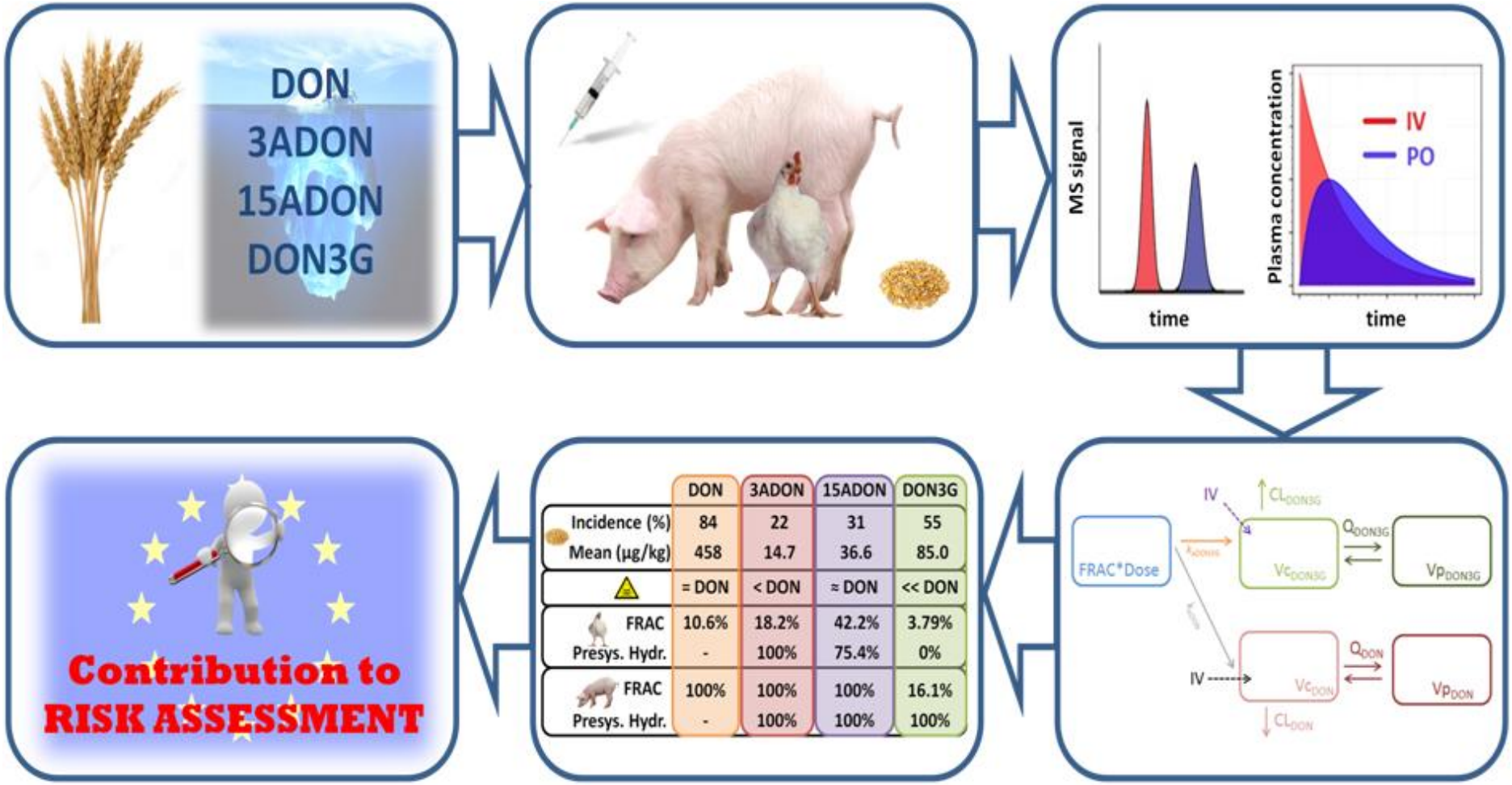

***Figure 1**. Overview of the main methodologies and main results achieved in this doctoral thesis. IV=intravenous; PO=per os; ka=absorption rate constant (1/min); Cl=clearance (mL/min/kg); Q=intercompartmental flow (mL/min/kg); Vc=central volume of distribution (mL/kg); Vp=peripheral volume of distribution (mL/kg); FRAC=absorbed fraction; Pres.Hydr.=percentage of the total dose that is hydrolysed presystemically to DON and absorbed as DON (%)*

Is there a need for risk assessment with respect to acetylated and modified deoxynivalenol?

"Wouldn't it be more practical to just lower the guidance level in feed and TDI for DON, thereby also accounting for the fraction of acetylated and modified mycotoxins rather than investing time and resources in research on acetylated and modified mycotoxins?"

At first glance this would seem an adequate solution to the acetylated and modified mycotoxin problem. However, several facts demonstrated in this doctoral thesis, make this statement flawed.

The first problem lies in the varying amounts of acetylated and modified mycotoxins in relation to DON. These ratios are highly dynamic and are significantly influenced by factors such as the producing fungi, geographical location and time of sampling/harvesting and the subsequent climatological conditions. Simply lowering the TDI and maximum guidance levels for DON cannot address these differences. If in a particular batch of food or feed only DON and no acetylated nor modified forms would be present, this would result in falsely discarding the batch, leading to economic losses. On the other hand, in case DON may be present in feed in low amounts but with high concentrations of modified forms, this would lead to acceptance of such a batch while adverse health effects may occur after consumption.

Another problem is that such an approach would imply the assumption that the intrinsic toxicity of acetylated and modified forms of DON is equal to that of DON. Although these acetylated and modified forms are structurally similar to DON, it has been demonstrated that these compounds may exhibit distinct toxicities: DON3G & DOM-1 << 3ADON < DON ≈ 15ADON (Pierron et al. 2015; Pinton et al. 2012). The decreased toxicity of DON3G and increased toxicity of 15ADON compared to DON are clear examples of the flaws of assuming equal toxicity.

Furthermore, this doctoral thesis showed that toxicokinetic characteristics can significantly differ between animal species, further complicating the matter. In general, when compared to DON, the more polar DON3G would result in a lower fraction absorbed (FRAC) after oral intake, whereas the less polar ADONs have more favourable physico-

chemical properties resulting in higher oral absorbed fractions. Indeed in pigs, DON, 3ADON and 15ADON are completely absorbed orally and both ADONs are completely hydrolysed to DON. Consequently, with respect to systemic toxicity, DON, 3ADON and 15ADON will have similar effects. In contrast, the absorbed fraction of DON3G is only 16.1±5.4% in pigs and is present in the systemic circulation as DON, hereby limiting the contribution of systemic toxicity with a factor of 5 compared to the ADONs.

In broiler chickens, which are deemed more resistant to DON than pigs, the less toxic 3ADON has an absorbed fraction of 18.2±5.5%, which is nearly double that of DON (10.6±4.6%), and it is completely hydrolysed presystemically. The more toxic 15ADON partially persists with a presystemic hydrolysis limited to 75.4%. Both ADONs may therefore exert more toxic effects than equal molar amounts of DON. Furthermore, the oral fraction absorbed of 15ADON is 42.2±12.5%, four times that of DON. In contrast, DON3G is not hydrolysed in broiler chickens, unlike in pigs, and has a limited oral bioavailability of 3.79±2.68%. Combined with its decreased toxicity, this results in a limited contribution of DON3G towards the toxicity in broiler chickens.

In conclusion, these data clearly demonstrate the complexity of the acetylated and modified mycotoxin problem including the inter-species variability and subsequently the need for risk assessment.

Do intestinal bacteria play a major role in presystemic hydrolysis?

The human and animal body contains roughly 10 times more bacteria than cells. Especially the intestinal microbiota of humans and animals contain a huge number of different bacterial species. The intestinal microbiota play an important role in the development of the intestinal systems, nutrient digestion but also in the protection of the host against hazardous xenobiotics, such as DON and its acetylated and modified forms (Antonissen et al. 2014; Antonissen et al. 2015; Maresca 2013). **Figure 2** shows regional pH and microbiota densities in the digestive tract of monogastric animals, ruminants and poultry. For both ruminants and poultry, the proximal part of the GI tract is less acidic than for monogastric animals, allowing a higher bacterial density already present before the absorption site of most mycotoxins, i.e. the small intestine.

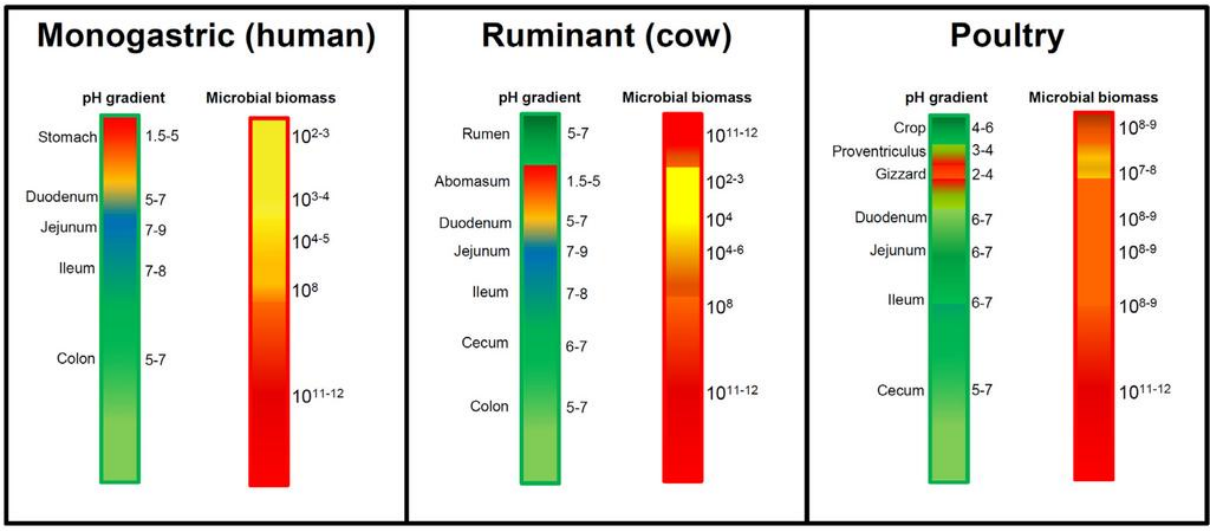

***Figure 2**. Regional pH and bacterial densities (per mL of intestinal fluid content) in the different segments of the digestive tract of humans, ruminants and poultry (adopted from Maresca 2013).*

The localisation of bacteria throughout the GI tract in relation to regional pH differences can lead to interspecies variability regarding the intestinal metabolism and hydrolysis of DON, 3ADON, 15ADON and DON3G. Based on these intestinal characteristics two groups can be distinguished. First, animals with a high bacterial load both before and after the small intestine such as polygastric animals and birds. Second, animals with only a high bacterial load after the small intestine, mainly in the colon, such as most of the monogastric species.

In pigs and chickens, the results of this thesis show that 3ADON and 15ADON are presystemically hydrolysed to DON. This suggests that either chemical hydrolysis (reaction with water catalysed by acid or reaction with diluted alkali) or gut bacteria, luminal intestinal lipases, IEC, blood esterases or the liver during first passage may convert ADONs to DON. If complete hydrolysis would occur in the intestinal lumen before absorption, one would expect identical plasma concentration-time profiles of DON, 3ADON and 15ADON after the administration of equimolar doses. In broiler chickens, we demonstrated clearly higher DON plasma levels after 3ADON or 15ADON administration. This indicates that the less polar ADONs are not hydrolysed in the lumen but are absorbed, more efficiently than DON, followed by hydrolysis in the IEC, portal blood or liver before entering the systemic circulation. Remarkably, since after IV administration both ADONs are also rapidly hydrolysed, it is most likely that liver enzymes, blood esterases or chemical hydrolysis are

responsible for the deacetylation of the ADONs rather than the intestinal microbiota. In contrast, DON3G shows no hydrolysis after IV administration in neither of the species studied. In humans, β-glucosidase is located in the brush border of the small intestine. Consequently, limited or no contribution to DON3G hydrolysis is expected after IV administration. Furthermore, β-glucosidase from humans and almonds proved incapable of DON3G hydrolysis to DON, a typical trait of micro-organisms (Berthiller et al. 2011).

In such case, hydrolysis of DON3G to DON would be expected after oral administration to chickens, given the high bacterial load before the small intestine is reached, the site of absorption. For pigs, where the high bacterial load is located after the site of absorption, no hydrolysis to DON would be expected. Our observations were however the exact opposite. The observed presystemic hydrolysis of DON3G to DON in pigs can be attributed to GI micriobiota or GI tissues, as no DON3G was present in portal plasma after PO administration. The hypothesis that GI microbiota play a major role is supported by the observed lag time of 84 min in DON plasma concentrations. The discrepancy between the absence and presence of hydrolysis for chickens and pigs, respectively, will likely find its cause in the bacterial species composition and abundance of both the chicken's crop and pig's distal small intestine and large intestine. The microbiota of pigs and chickens consist mainly of the phyla *Firmicutes* and *Bacteroidetes,* accounting for approximately 90% and 80% of the relative abundance, respectively (Kim and Isaacson 2015; Waite and Taylor 2015). The crop, anterior small intestine, duodenal and jejunal epithelial cells, and digesta of chicken are dominated by *Lactobacillus* spp. (Watkins and Kratzer 1983). The hydrolytic capacity of *Lactobacillus* spp. towards DON3G has been described (Berthiller et al. 2011). Of the tested *L. amylovorus*, *crispatus, fermentum, gasseri, paracasei, rhamnosus* and *plantarum,* only the latter was able to hydrolyse DON3G to DON, more specifically 34% after 4h incubation. This *L. plantarum* has been found in the GI tract of chickens, however it does not belong to the most abundant GI *Lactobacillus* species (Hilmi et al. 2007), in contrast, it is commonly found in human and other mammalian gastrointestinal tracts (de Vries et al. 2006). The hydrolytic capacities towards DON3G of *Enterococcus*, a large genus within *Firmicutes* species, has also been investigated. Of the seven tested species, *E. avium* was the only species unable to hydrolyse DON3G to DON. *E. casseliflavus, durans, faecalis, faecium, gallinarum* and *mundtii* all hydrolysed DON3G varying between 2% and 39% after a 4 h

incubation. The occurrence of both *Enterococcus* species has been described in the GI tract of both chickens and pigs. Possibly, the relative amounts of *Enterococcus* and *Lactobacillus* species in the GI microbiota could partially explain our *in vivo* findings. However, the GI microbiota of animals consists of a multitude of phyla and genus, demonstrating interspecies as well as intraspecies differences, and differing between intestinal sections, providing a multitude of options for the observed differences in hydrolysis between chickens and pigs.

The pig as an animal model for humans

The (mini)pig presents a favourable species as a non-rodent animal model in terms of applicability to different toxicological and pharmacological studies, due to anatomical, physiological and biochemical resemblances between (mini)pigs and humans. The (mini)pig as a model in toxicity testing of xenobiotics has been well accepted by EU, USA and Japan (Gasthuys et al. 2015; Ikeda et al. 1998). The resemblances between pigs and humans have been illustrated for several organ systems or physiological processes, especially the striking resemblance of the GI tract, hepatic and renal system are of major importance as these are the principle organs involved in ADME processes (Bode et al. 2010; Helke and Swindle 2013; Svendsen 2006). With respect to acetylated and modified mycotoxins, the results obtained for pigs in this thesis are twofold. First, the data contribute to the risk assessment for pigs as target species themselves. Second, the data may contribute to the risk assessment for humans by extrapolation of pig data. Indeed, the presystemic hydrolysis mechanisms responsible for the results obtained in this thesis are comparable in addition to the toxicokinetic characteristics with respect to intestinal absorption, phase I and II biotransformation and renal excretion. Nevertheless, species extrapolation should always be performed with caution since for instance, humans predominantly form deoxynivalenol-15-β-D-O-glucuronide and to a lesser extent deoxynivalenol-3-β-D-O-glucuronide (Heyndrickx et al. 2015), which is in contrast to pigs where the ratios of DON15GlcA to DON3GlcA varied between individual piglets (0.5–2.2) (Nagl et al. 2014). Another argument favouring the predictive value of pigs is that the porcine microbiota shows large similarities to the human intestinal microbial ecosystem (Heinritz et al. 2013), further contributing to the pigs' applicability to study human presystemic hydrolysis. Furthermore, the potential use of piglets as an animal model for paediatric toxicity testing has been described (Gasthuys et al.

2015), with the additional benefit that this requires smaller quantities of the often expensive test compounds compared to full grown animals.

Future perspectives for research on acetylated and modified mycotoxins

Animal trials are for the moment indispensable tools in toxicology. Nonetheless, animal trials are heavily debated from an ethical point of view. Ethical committees set ethical standards for animal experiments, evaluate and guarantee the quality of animal trials and prevent unnecessary use and suffering of animals, based on the three R's principle (Russell and Burch 1959): replacement, reduction and refinement. In light of ethical awareness, a first future perspective could be the **further application of the 3R principle** in the determination of mycotoxin toxicity and toxicokinetics. These alternatives include the use of *ex vivo* tests to study toxicity and to simulate GI permeability, such as the testing of intestinal passage through GI tissue explants mounted in Ussing chambers. Another alternative is *in vitro* batch incubations with faecal matter or microbiota to determine GI metabolism and where applicable hydrolysis. These incubations can be performed for each animal species of interest and also using human faecal material. Such incubations would be very valuable in this research domain to unravel which micro-organisms are responsible for hydrolysis of the modified and/or acetylated mycotoxins. The drawback of such a system is the discrepancy between the absorption site of most mycotoxins and the site from which GI bacteria are often sampled. Similar to batch incubations, but more efficiently resembling the *in vivo* situation, are dynamic gastrointestinal simulators such as the TNO (gastro-)intestinal models (TIM) (Minekus & Havenaar 1996), the simulator of human intestinal microbial ecosystem (SHIME) (Molly et al. 1994), or its porcine equivalent the SPIME. It is a dynamic model of the GI tract used to study physicochemical, enzymatic and microbial parameters. The model consists of five or six reactors which sequentially simulate the stomach (acid conditions and pepsin digestion), duodenum and jejunum (digestive processes), ileum (digestive processes) and the three regions of the large intestine, i.e. the ascending, transverse and descending colon (microbial processes) (Molly et al. 1994). Finally, *in silico* modelling also provides a possible alternative to animal experiments. Many different types of *in silico* methods have been developed to characterize and predict toxic outcomes in animals and humans, making use of structure-activity relationships (Combes 2012; Maltarollo et al. 2015).

Data from animal trials is often analysed by means of non-compartmental toxicokinetic analysis. However, analysis and interpretation of toxicokinetic results may benefit from a **model-based approach**. Advantages of compartmental over some non-compartmental analysis include the ability to predict the concentration at any time. Additionally, compartmental approaches are more easily extrapolated to other individuals, demonstrating increased predictive properties. The disadvantage is the difficulty in developing and validating a proper model. A more profound integration of model-based approaches will provide a valuable tool in the future research on mycotoxins.

In this thesis toxicokinetic research was limited to the analysis of plasma, systemic as well as portal. Analysis of other matrices from animal origin may provide new insights and/or opportunities. For instance, we have observed that the absorbed fraction of DON3G in pigs is completely presystemically hydrolysed. However, this leaves the question whether the unabsorbed fraction remaining in the GI tract is present as DON, and thus hydrolysed as well, and/or as DON3G, not hydrolysed, and what the relative amounts are, which is an important aspect with regard to local gastrointestinal toxic effects. Analysis of urine samples can be useful as an alternative method to determine bioavailability. Furthermore, analysis of faeces and urine samples may contribute to the research on biomarkers for mycotoxin exposure. Which phase I and II metabolites are formed? How long do they remain in these matrices after exposure? Can this data be quantitatively linked to the exposure? Several studies in this field indicated that deoxynivalenol-15-glucuronide was the main urinary DON biomarker for human DON exposure (Warth et al. 2013; Heyndrickx et al. 2015). In this respect, HRMS proves to be a very valuable tool, aiding in the search for phase I and II metabolites.

Throughout this thesis, LC-MS/MS and HRMS were used to determine the concentrations of mycotoxins and their metabolites in plasma to study the toxicokinetic properties. The developed LC-MS/MS methods were limited to selected compounds. For our purposes, a chromatographic separation of 3ADON and 15ADON was not required as the ADONs were not simultaneously administered during the animal trials. However, in practice both ADONs can co-occur in food and feed. In such cases, chromatographic separation would be a valuable tool to separately quantify both 3ADON and 15ADON as no specific ion transitions were found to distinguish between 3ADON and 15ADON. The HRMS section, which is an

untargeted approach and is not limited to prior selected compounds, however, only provides semi-quantitative results due to the lack of analytical reference standards. This opens a third future perspective, namely the **development of quantitative multi-mycotoxin methods in plasma** allowing the simultaneous determination of free and modified mycotoxins together with phase I and II metabolites, consequently saving analysis time and financial resources. Such methods in animal plasma (and other matrices of animal origin) are currently lacking, in contrast to food/feed and human urine (Njumbe Ediage et al. 2013; Rodriguez-Carrasco et al. 2014; Song et al. 2013; Warth et al. 2014; Zhao et al. 2015). This would also eliminate the need to deconjugate phase II metabolites, such as by using β-glucuronidase, during sample clean up, in order to determine the ratio between free and conjugated mycotoxins. The development of such quantitative methods requires the availability of **reference standards**. These can be isolated from naturally contaminated material or can be chemically or biochemically synthesised. This doctoral thesis demonstrates the applicability of appropriate toxicokinetic models and tools for blood collection (systemic as well as portal). These tools in combination with the production of analytical standards as well as suitable isotopically labelled internal standards, also provides a perspective to study other acetylated, modified and emerging mycotoxins, such as T-2 toxin-glucoside.

In toxicity studies compounds are often administered solely in order to attribute observations to one specific compound. In practice however, **co-contamination** is often observed with acetylated and/or modified forms of the toxin as well as with other classes of mycotoxins. This co-contamination and the potentially additive or synergistic effects are a point of concern and provide another future perspective. Alassane-Kpembi *et al*. demonstrated an order of magnitude of synergy ranging from 2 to 7 for several combinations of DON, 3ADON, 15ADON, NIV and fusarenon-X on epithelial cell toxicity (IPEC-1 and Caco-2) (Alassane-Kpembi et al. 2015; Wan et al. 2013). Binary or ternary mixtures often showed a dose-dependent effect, substantial enough to differ between antagonism and synergy (Alassane-Kpembi et al. 2013; Alassane-Kpembi et al. 2015; Huff et al. 1988; Ruiz et al. 2011).

Conclusions

Additionally to DON, food and feed are often co-contaminated with acetylated and modified forms of DON. This study and previously published studies have demonstrated that these compounds may exhibit distinct toxicities: DON3G & DOM-1 << 3ADON < DON ≈ 15ADON. This doctoral thesis clearly demonstrates that *in vivo,* most of these forms can be hydrolysed with release of DON (ADONs in pigs and chickens, DON3G in pigs), thereby contributing to the *in vivo* DON exposure of broiler chickens and pigs. Furthermore, due to the altered physico-chemical characteristics of the acetylated and modified forms, these compounds can display a significant increase (3ADON and 15ADON in pigs) or decrease (DON3G in pigs) in systemic absorbed fractions. For DON3G, the site of hydrolysis was investigated by collecting systemic as well as portal plasma. Results indicate that presystemic hydrolysis occurs at the site of GI tract, most likely by GI microbiota and possibly by GI tissues. Additionally, analysis of phase II metabolites revealed that DON metabolism in pigs consists mainly of glucuronidation, and in chickens sulfation predominantly occurs. Phase II biotransformation was much more extensive for chickens than for pigs, which can be a possible explanation for the differences in sensitivity of the latter species to DON. In conclusion, the global occurrence, species dependent toxicity and toxicokinetics for DON, 3ADON, 15ADON and DON3G demonstrated in this thesis, highlight the importance of research on mycotoxins and their acetylated and modified forms as chronic food and feed contaminants. Mycotoxigenic fungi will never be completely eradicated, but research will allow to minimize the risk associated with these fungi and their mycotoxins, thereby contributing to food and feed safety in humans and animals.

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SUMMARY

Mycotoxin contamination has been classified by several authors as the most important chronic dietary risk factor both in food and feed. The *Fusarium* mycotoxin deoxynivalenol (DON) is one of the most frequently occurring mycotoxins, contaminating 56% of feed and feed raw materials worldwide. In addition to DON, feed can be contaminated with acetylated and modified forms of DON, such as 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G), respectively. Both ADONs are produced by *Fusarium* fungi as well, whereas DON3G is formed by conjugation in plants.

Little is known about the toxicodynamics and toxicokinetics of these acetylated and modified forms of DON, compared to the well investigated free mycotoxin DON. One of the major questions associated with acetylated and modified mycotoxins is whether *in vivo* hydrolysis may result in the release of the free mycotoxin and what their *in vivo* fate and disposition in the body is. Determination of the oral bioavailability, rate and extent of *in vivo* hydrolysis and toxicokinetic characteristics of 3ADON, 15ADON and DON3G in relevant animal species by means of state-of-the-art toxicokinetic analysis may support legislative authorities to carefully take measures with respect to the risk assessment of these contaminants.

The **general introduction** of this doctoral thesis gives an overview of the mycotoxin issue and mycotoxin management in general. Next, the occurrence, toxicity and toxicokinetics of DON are described, followed by the established legislation in feed. Furthermore, the production of acetylated and modified forms of DON by plant, fungus and food processing is discussed, followed by an overview of their occurrence, toxicity and toxicokinetic properties.

The **general objective** of this research was to determine the absolute oral bioavailability, rate and extent of *in vivo* hydrolysis and toxicokinetic characteristics of 3ADON, 15ADON and DON3G in broiler chickens and pigs, in order to contribute to the risk assessment of these mycotoxins.

To achieve this goal analytical methods to detect and quantify mycotoxins and their metabolites in animal plasma are needed. In **chapter I**, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method allowing the quantification of 3ADON, 15ADON, DON and an *in vivo* metabolite, de-epoxy-deoxynivalenol (DOM-1), in broiler chicken and pig plasma was developed. Several sample cleanup strategies were evaluated and further

optimised by means of fractional factorial design. A simple and straightforward sample preparation method was developed consisting out of a deproteinisation step, followed by evaporation of the supernatant and reconstitution. The method was validated according to European guidelines and was applicable for the intended purpose, with a linear response up to 200 ng/mL and limits of quantitation ranging between 0.1 and 2.0 ng/mL.

In **chapter II**, the developed method was applied to study the disposition of DON, 3ADON and 15ADON in broiler chickens and pigs, more specifically the absolute oral bioavailability, (presystemic) hydrolysis and toxicokinetic characteristics. For this purpose, cross-over animal trials were performed with intravenous (IV) and oral (PO) administration of DON, 3ADON and 15ADON to broilers and pigs. Plasma concentration-time data were processed *via* a tailor-made compartmental toxicokinetic model. The results in broiler chickens demonstrate that the mean absorbed fraction after oral DON, 3ADON and 15ADON administration was 10.6%, 18.2% and 42.2%, respectively. For 3ADON, this fraction was completely hydrolysed presystemically to DON, but for 15ADON it was hydrolysed to DON to a lesser extent (75.4%). In pigs, the absorbed fractions were 100% for DON, 3ADON and 15ADON, and 3ADON and 15ADON were completely hydrolysed presystemically. Also after IV administration, both ADONs were remarkably fast and completely hydrolysed. When combining these results, both ADONs can be regarded as toxic as DON itself in pigs with respect to systemic toxicity. The results for broiler chickens demonstrate a 'worst case scenario' since both ADONs have a higher absorbed fraction, with a four- and twofold higher DON exposure after 15ADON and 3ADON consumption compared to DON itself, respectively. Moreover, 3ADON can be regarded as at least equally toxic to DON since it is completely hydrolysed presystemically.

The goal of **chapter III** was to investigate the absolute oral bioavailability and toxicokinetic characteristics of the modified mycotoxin DON3G and to unravel its possible (presystemic) hydrolysis to DON in broiler chickens and pigs. Again, cross-over animal trials were performed with IV and PO administration of DON3G and DON to broilers and pigs. Plasma concentrations of DON and DON3G were quantified using an in-house developed and validated LC-MS/MS method and phase II glucuronide and sulfate metabolites were semi-quantified by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). Data were processed by tailor-made compartmental toxicokinetic models. In

contrast to the ADONs, no hydrolysis of DON3G to DON after IV administration in both animal species was observed. After PO exposure, the results in broiler chickens indicate that DON3G is not hydrolysed to DON neither. Furthermore, the absolute oral bioavailability of DON3G was low (3.79%) and comparable to that of DON (5.56%). Interestingly, after PO DON3G administration to pigs, only DON was detected in plasma, indicating a complete presystemic hydrolysis of DON3G. However, the mean absorbed fraction of DON3G, recovered as DON, was approximately 5 times lower than after PO DON administration, i.e. 16.1% compared to 81.3%. Additionally, blood was sampled from the *vena porta* by surgically placed catheters in pigs, to further investigate the site of presystemic DON3G hydrolysis. Only DON but no DON3G was detected in portal plasma. The deficiency of systemic hydrolysis after IV dosing and the absence of DON3G in plasma from the *vena porta* indicate that the intestinal epithelial cells, or more likely, the porcine intestinal microbiota play a major role in the hydrolysis. Analysis of phase II metabolites revealed that biotransformation of DON and DON3G in pigs mainly consists of glucuronidation, whereas in chickens sulfate conjugation predominantly occurs. The extent of phase II biotransformation is notably higher for chickens than for pigs, which contributes to the relative sensitivity of the latter species to DON. Although *in vitro* studies demonstrate a decreased toxicity of DON3G compared to DON, the possibility of *in vivo* hydrolysis to DON in pigs demonstrates the toxicological relevance of DON3G.

Additionally to the toxicokinetic research, the toxicity of acetylated and modified forms of DON was investigated in **Chapter IV**. The gastrointestinal tract is the first target after ingestion of DON. Consequently, the goal of this chapter was to determine the *in vitro* intrinsic cytotoxicity of acetylated and modified forms of DON towards differentiated and proliferative porcine intestinal epithelial cells by means of a flow cytometric technique. Cell death was assessed by means of dual staining with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), which allows the discrimination of viable cells ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$). Based on the data from the presented study, it is concluded that cytotoxicity can be ranked as follows: DON3G << 3ADON < DON ≈ 15ADON.

In the **general discussion,** the need for a risk assessment regarding acetylated and modified forms of DON is discussed. Next, the undisclosed role of GI microbiota with respect to

hydrolysis is explored. Furthermore, in this thesis pigs were studied as target species but due to their comparative anatomy, physiology and biochemistry to humans, their role as non-rodent animal model in human (acetylated and modified) mycotoxin research is explored. This is followed by some future perspectives on the research towards acetylated and modified mycotoxins are given.

In **conclusion**, this doctoral thesis demonstrates the *in vivo* hydrolysis of 3ADON and 15ADON to DON in both broiler chickens and pigs, as well as of DON3G in pigs. In contrast, DON3G is not hydrolysed in broiler chickens. For the ADONs this is most probably a chemical hydrolysis, whereas for DON3G this hydrolysis takes place in the GI tract, most likely by GI microbiota and possibly by GI tissues. Results from HRMS analyses revealed that phase II biotransformation of DON in pigs consist mainly of glucuronidation, while in chickens sulfation predominantly occurs. Phase II biotransformation is more extensive for chickens than for pigs, which can be a possible explanation for the differences in sensitivity of the latter species to DON. These findings contribute to gain insight in the overall risks of acetylated and modified mycotoxins, thereby contributing to maintaining food and feed safety in humans and animals.

SAMENVATTING

Contaminatie van voedsel en voeder met mycotoxinen wordt door meerdere auteurs geclassificeerd als de belangrijkste chronische risicofactor voor (dieren)voeding. Het *Fusarium* mycotoxine deoxynivalenol (DON) is één van de meest voorkomende mycotoxinen, met een wereldwijde prevalentie van 56% in diervoeders en diervoedergrondstoffen. Naast DON kan voeder bijkomend verontreinigd zijn met geacetyleerde en gemodificeerde vormen van DON, zoals 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) en deoxynivalenol-3-β-D-glucoside (DON3G). Beide ADONs worden net zoals DON geproduceerd door *Fusarium* schimmels, terwijl DON3G wordt gevormd door conjugatie in de plant.

In vergelijking met het intensief bestudeerde DON is er weinig bekend over de toxicokinetiek en toxicodynamiek van deze geacetyleerde en gemodificeerde vormen. Een van de belangrijke vragen geassocieerd met deze derivaten van DON is of ze hydrolyse in het lichaam kunnen ondergaan met vrijstelling van het mycotoxine DON, alsook wat hun beschikbaarheid en dispositie in het lichaam is. Bepaling van de orale biologische beschikbaarheid, snelheid en mate van *in vivo* hydrolyse en toxicokinetische eigenschappen van 3ADON, 15ADON en DON3G in relevante diersoorten met behulp van geavanceerde toxicokinetische modellen, kan bijgevolg wetgevende instanties ondersteunen om maatregelen te nemen met betrekking tot de risicobeoordeling van deze contaminanten.

De **algemene inleiding** van dit doctoraatsproefschrift geeft een overzicht van de mycotoxine problematiek en mogelijke maatregelen om dit in te perken. Vervolgens wordt het voorkomen, de toxiciteit en toxicokinetiek van DON beschreven, gevolgd door de bestaande wetgeving omtrent maximale gehalten en richtwaarden voor diervoeding. Daaropvolgend wordt de productie van geacetyleerde en gemodificeerde vormen van DON in planten, schimmels en tijdens sommige voedselverwerkende processen besproken, waarna aansluitend een overzicht van hun voorkomen, toxiciteit en toxicokinetische eigenschappen gegeven wordt.

De **algemene doelstelling** van dit onderzoek was om de absolute orale biologische beschikbaarheid, snelheid en mate van *in vivo* hydrolyse en toxicokinetische eigenschappen van 3ADON, 15ADON en DON3G bij vleeskippen en varkens vast te stellen, teneinde bij te dragen tot de risicobeoordeling van deze mycotoxinen.

Om dit doel te bereiken waren analytische methoden om deze mycotoxinen en hun metabolieten te kwantificeren in plasma afkomstig van dieren een vereiste. In **hoofdstuk I**, werd een vloeistofchromatografische-tandem massaspectrometrische (LC-MS/MS) methode ontwikkeld en gevalideerd voor de bepaling van 3ADON, 15ADON, DON en een *in vivo* metaboliet, de-epoxy-deoxynivalenol (DOM-1), in plasma van vleeskippen en varkens. Verschillende staalvoorbereidingsstrategieën werden geëvalueerd en verder geoptimaliseerd door middel van fractionele factoriële designs. Een eenvoudige staalvoorbereidingsmethode werd uitgewerkt, bestaande uit een deproteïnisatie stap, gevolgd door indampen van het supernatans en heroplossen van het staal. De methode werd gevalideerd volgens de prestatiecriteria beschreven in de Europese richtlijnen en voldeed aan de beoogde voorwaarden met een lineaire respons tot 200 ng/mL en kwantificatielimieten tussen 0,1 en 2,0 ng/mL.

In **hoofdstuk II** werd de ontwikkelde methode toegepast om de dispositie van DON, 3ADON en 15ADON te bestuderen in vleeskippen en varkens, meer in het bijzonder de absolute orale biologische beschikbaarheid, de mogelijke (presystemische) hydrolyse en toxicokinetische eigenschappen. Hiervoor werden dierproeven uitgevoerd volgens een cross-over design met intraveneuze (IV) en orale (PO) toediening van DON, 3ADON en 15ADON. Plasmaconcentratie-tijdsprofielen werden geanalyseerd door middel van een in-huis ontworpen compartimenteel toxicokinetisch model. De resultaten bij vleeskippen toonden aan dat de gemiddelde geabsorbeerde fractie na orale DON, 3ADON en 15ADON toediening respectievelijk 10,6%, 18,2% en 42,2% bedroeg. Voor 3ADON werd deze fractie geheel presystemisch, dus voor het bereiken van de algemene systemische bloedcirculatie, gehydrolyseerd naar DON. Daarentegen werd 15ADON slechts gedeeltelijk gehydrolyseerd (75,4%). Bij varkens waren de geabsorbeerde fracties na orale toediening 100% voor DON, 3ADON en 15ADON, bovendien werden 3ADON en 15ADON volledig presystemisch gehydrolyseerd. Na IV toediening werden zowel 3ADON als 15ADON eveneens opmerkelijk snel en volledig gehydrolyseerd. Hieruit kan besloten worden dat beide ADONs met betrekking tot systemische toxiciteit bij het varken als even toxisch kunnen worden beschouwd als DON. De resultaten voor vleeskippen duiden op een ‘worst case scenario’ aangezien beide ADONs een hogere geabsorbeerde fractie vertonen dan DON, wat aanleiding geeft tot dubbele en viervoudige DON blootstelling na respectievelijk 3ADON en

15ADON toediening in vergelijking met DON zelf. Overigens kan 3ADON even toxisch worden beschouwd als DON vanwege de volledige presystemische hydrolyse ervan.

Het doel van **hoofdstuk III** was de absolute orale biologische beschikbaarheid, mogelijke (presystemische) hydrolyse en toxicokinetische kenmerken van het gemodificeerde mycotoxine DON3G te onderzoeken bij vleeskippen en varkens. Opnieuw werden cross-over dierproeven uitgevoerd met IV en PO toediening van DON3G en DON. Plasmaconcentraties van DON en DON3G werden gekwantificeerd met behulp van een ontwikkelde en gevalideerde LC-MS/MS methode. Bijkomend werden de fase II glucuronide en sulfaat metabolieten semi-kwantitatief bepaald door vloeistofchromatografie gekoppeld aan hoge resolutie massaspectrometrie (LC-HRMS). De resultaten werden geanalyseerd aan de hand van aangepaste compartimentele toxicokinetische modellen. In tegenstelling tot 3ADON en 15ADON, werd geen hydrolyse waargenomen na IV DON3G toediening. Na PO toediening bij vleeskippen werd eveneens de afwezigheid van *in vivo* hydrolyse van DON3G vastgesteld. Verder bleek de gemiddelde absolute orale biologische beschikbaarheid van DON3G laag (3,8%), en vergelijkbaar met die van DON (5,6%). Bij het varken werd na PO DON3G toediening opmerkelijk enkel DON gedetecteerd in plasma, hetgeen wijst op een volledige presystemische hydrolyse van DON3G. Echter, de geabsorbeerde fractie van DON3G, teruggevonden als DON, was ongeveer 5 keer lager dan na PO DON toediening, namelijk 16,1% in vergelijking met 81,3%. Teneinde de plaats van deze presystemische hydrolyse bij het varken te ontrafelen, werd aanvullend plasma geanalyseerd afkomstig van bloed uit de gekatheteriseerde *vena porta*, die zich situeert tussen darm en lever. Ook in het portale plasma werd enkel DON en geen DON3G gedetecteerd. Het ontbreken van systemische hydrolyse en de afwezigheid van DON3G in plasma van de *vena porta* wijzen op de betrokkenheid van darmepitheelcellen en/of de bijdrage van gastrointestinale microbiota van het varken. Tot slot bleek uit de analyse van de fase II-metabolieten dat biotransformatie van DON en DON3G bij varkens voornamelijk bestaat uit glucuronidatie, terwijl bij vleeskippen voornamelijk sulfatatie optreedt. De mate van fase II biotransformatie is beduidend hoger voor kippen dan voor varkens, hetgeen mogelijks bijdraagt aan de hoge relatieve gevoeligheid van varkens aan DON. Hoewel *in vitro* studies een sterk verlaagde toxiciteit van DON3G ten opzichte van DON rapporteren, toont de mogelijkheid van *in vivo* hydrolyse bij varkens de niet te verwaarlozen toxicologische relevantie van DON3G aan.

Aanvullend aan het toxicokinetisch onderzoek werd de cytotoxiciteit van geacetyleerde en gemodificeerde vormen van DON onderzocht in **hoofdstuk IV**. Het maagdarmkanaal is het eerst blootgestelde orgaan na consumptie van DON gecontamineerd voeder. Het doel van dit hoofdstuk was dan ook om de *in vitro* cytotoxiciteit van geacetyleerde en gemodificeerde vormen van DON op gedifferentieerde en proliferatieve porciene darmepitheelcellen na te gaan. Door middel van een flowcytometrische techniek werd celdood bepaald door middel van dubbele kleuring met Annexine-V-fluoresceïne-isothiocyanaat (FITC) en propidium jodide (PI). Zo werd een scheiding en kwantificering mogelijk van levensvatbare ($FITC^-/PI^-$) , apoptotische ($FITC^+/PI^-$) en necrotische cellen ($FITC^+/PI^+$). Op basis van de bekomen data werd geconcludeerd dat de cytotoxiciteit als volgt kan worden gerangschikt: DON3G << 3ADON < DON ≈ 15ADON.

In de **algemene discussie** wordt dieper ingegaan op de risicobeoordeling voor de geacetyleerde en gemodificeerde vormen van DON. Vervolgens werd de mogelijke rol van de gastrointestinale microbiota betreffende de presystemische hydrolyse meer in detail besproken. In de algemene discussie werd eveneens een korte bespreking vermeld over de geschiktheid van het varken als diermodel voor de mens voor wat onderzoek naar (geacetyleerde en gemodificeerde) mycotoxinen betreft, vanwege hun opvallende gelijkenissen in anatomie, fysiologie en biochemie. Vervolgens werden enkele toekomstperspectieven betreffende het onderzoek naar de risicobeoordeling van geacetyleerde en gemodificeerde mycotoxinen aangehaald.

Samengevat toont dit proefschrift aan dat *in vivo* hydrolyse van 3ADON en 15ADON naar DON optreedt bij zowel vleeskippen en varkens, alsook van DON3G bij varkens. Daarentegen wordt DON3G niet gehydrolyseerd in vleeskippen. Voor de ADONs betreft het vermoedelijk een chemische hydrolyse. Voor DON3G daarentegen vindt de hydrolyse plaats ter hoogte van het gastrointestinaal kanaal, zeer waarschijnlijk door gastrointestinale microbiota en mogelijks door gastrointestinale weefsels. Uit de resultaten van HRMS analysen blijkt dat fase II biotransformatie van DON bij varkens voornamelijk bestaat uit glucuronidatie, terwijl bij kippen overwegend sulfatatie optreedt. Fase II biotransformatie is beduidend belangrijker voor vleeskippen dan voor varkens, wat een mogelijke verklaring biedt voor de verschillen in gevoeligheid van deze diersoort aan DON. Deze bevindingen dragen bij tot het verwerven

van inzicht in de risico's van geacetyleerde en gemodificeerde mycotoxinen, hetgeen finaal bijdraagt tot het vrijwaren van de voedselveiligheid bij mens en dier.

CURRICULUM VITAE

Nathan Broekaert werd geboren op 10 maart 1985 te Gent. Na het beëindigen van het algemeen secundair onderwijs aan het Koninklijk Atheneum Voskenslaan te Gent, richting moderne talen - wetenschappen, startte hij in 2004 met de studies farmaceutische wetenschappen aan de Universiteit Gent. Hij behaalde in 2009 het diploma van Master of Science in de Geneesmiddelenontwikkeling. Aansluitend volgde hij de opleiding Master-na-Master Industriële Farmacie.

Vrijwel onmiddellijk na het afstuderen in 2010 begon hij als wetenschappelijk medewerker bij het laboratorium voor Bromatologie aan de faculteit Farmaceutische Wetenschappen in het kader van een éénjarig EFSA project. Geboeid door het wetenschappelijk onderzoek vatte hij in maart 2012 zijn doctoraatsonderzoek aan het laboratorium voor Farmacologie en Toxicologie van de faculteit Diergeneeskunde aan. Dit Associatieonderzoeksproject omtrent geacetyleerde en gemodificeerde vormen van deoxynivalenol werd gefinancierd door het Bijzonder Onderzoeksfonds van de Universiteit Gent.

Nathan Broekaert is auteur en mede-auteur van meerdere wetenschappelijke publicaties, gaf presentaties op verschillende nationale en internationale congressen en begeleidde master studenten van de Faculteiten Diergeneeskunde en Farmaceutische Wetenschappen bij het voltooien van hun thesis. Tot slot vervolledigde hij in 2015 het trainingsprogramma van de Doctoral Schools of Life Sciences and Medicine van de UGent.

Nathan Broekaert was born in Ghent on March 10, 1985. After he graduated from secondary school at the Royal Atheneum Voskenslaan in Ghent, he enrolled in the studies pharmaceutical sciences at Ghent University in 2004. In 2009 he obtained his Master of Science in Drug Development degree. One year later he obtained the degree of Master in Industrial Pharmacy.

After graduating in 2010, he started as a research assistant at the Laboratory of Food Analysis of the Faculty of Pharmaceutical Sciences under the supervision of Prof. S. De Saeger. This one-year project was funded by the European Food Safety Authority (EFSA). Fascinated by scientific research, he commenced his doctoral research in April 2012 at the Laboratory of Pharmacology and Toxicology of the Faculty of Veterinary Medicine. This Association Research project on acetylated and modified forms of deoxynivalenol was funded by the Special Research Fund (BOF) of Ghent University.

Nathan Broekaert is author and co-author of several scientific papers published in international peer reviewed journals. He gave presentations at various national and international congresses and supervised several students of the Faculty of Veterinary Medicine and the Faculty of Pharmaceutical Sciences in completing their master thesis. He successfully completed the doctoral training program of the Doctoral Schools of Life Sciences and Medicine of Ghent University in 2015.

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"Haast is de moeder van de mislukking."
Herodotus, Grieks geschiedkundige BC 484-425

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"if I were never drunk, than how would my friends know I love them"

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