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DEVELOPMENT OF *IN VITRO* AND *IN VIVO* MODELS FOR TESTING THE EFFICACY OF MYCOTOXIN DETOXIFYING AGENTS AND THEIR POSSIBLE INTERACTION WITH ORAL ABSORPTION OF VETERINARY DRUGS

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"If you can't explain it simply, you don't understand it enough"

A. Einstein

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3a-DON	3 acetyl-deoxynivalenol
15a-DON	15 acetyl-deoxynivalenol
Ab	antibody
AC	activated carbon
ACAT	acyl-coenzyme A:cholesterol acyl transferase
ACN	acetonitrile
ADME	absorption, distribution, metabolisation and excretion
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
AFM1	aflatoxin M1
AFQ1	aflatoxin Q1
AFL	aflatoxicol
ANOVA	analysis of variance
AST	aspartate aminotransferase
ATA	alimentary toxic aleukia
au	arbitrary units
AUC_{0-inf}	area under plasma concentration-time curve from time 0 to infinite
AUC_{0-t}	area under plasma concentration-time curve from time 0 to t
BEA	beauvericin
BEN	Balkan endemic nephropathy
BW	body weight
C_{ads}	concentration adsorbed
C_{aq}	concentration in solution
CK	creatine kinase
C_{max}	maximal plasma concentration
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DOM-1	de-epoxydeoxynivalenol
DON	deoxynivalenol
dp	diameter particle
EC	European Commission
EFSA	European Food Safety Authority
ELEM	equine leukoencephalomalacia
ELISA	enzyme-linked immunosorbent assay
ENN	enniatin
ENNs	enniatins
ESI	electrospray ionization
EU	European Union
FASFC	Federal Agency for the Safety of the Food Chain
FB1	fumonisin B1
FB2	fumonisin B2

FCS	fetal calf serum
FDA	Food and Drug Administration
FEEDAP	EFSA panel on additives and products or substances used in animal feed
FHB	*Fusarium* head blight
FWHM	full width at half maximum
g	g-force
g	goodness-of-fit coefficient
GAP	Good Agricultural Practices
GC	guanine-cytosine
GGT	γ-glutamyltransferase
GLDH	glutamate dehydrogenase
GMA	glucomannan
HATs	histone acetyltransferases
HBSS	Hank's buffered saline solution
HFB1	hydrolyzed fumonisin B1
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
HSCAS	hydrated sodium calcium aluminosilicate
HT-2	HT-2 toxin
IAF	immuno-affinity columns
IARC	International Agency for Research on Cancer
i.d.	internal diameter
Ig	immunoglobulin
IPEC	intestinal porcine epithelial cell
IPEC-J2	intestinal porcine epithelial cell line derived from the jejunum
IS	internal standard
K_a	affinity constant
k_a	absorption rate constant
k_{el}	elimination rate constant
K_F	Freundlich distribution coefficient
LAB	lactic acid bacteria
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD_{50}	lethal dose 50 value
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
MAD	maduramicin
MAPK	mitogen-activated protein kinase
mM	millimolar
MPN	mycotoxic porcine nephropathy
MRL	maximum residue limit
MS	mass spectrometry
m/z	mass over charge
n	capacity
N_2	nitrogen
NOAEL	no observed adverse effect level

NR	neutral red
ND	not detected
OBB	oral bioavailability
OD	optical density
OTA	ochratoxin A
OTα	ochratoxin alpha
PBS	phosphate buffered saline
PPE	porcine pulmonary oedema
r	correlation coefficients
R_E	extraction recovery
RNA	ribonucleic acid
rpm	rotations per minute
RSD	relative standard deviation
RSD_{max}	maximal relative standard deviation
T-2	T-2 toxin
TA	thymine-adenine
TIM	TNO intestinal model
Sa	sphinganine
SD	standard deviation
SEM	standard error of the mean
S/N	signal-to-noise
So	sphingosine
sp.	species
SPE	solid phase extraction
spp.	subspecies
SRM	selected reaction monitoring
SSE	signal suppression/enhancement
TDI	tolerable daily intake
TEER	transepithelial electrical resistance
TSQ	triple stage quadrupole
$T_{1/2a}$	absorption half-life
$T_{1/2el}$	elimination half-life
T_{max}	time to maximal plasma concentration
UDPGT	uridine diphosphate glucuronyl transferases
UHPLC	ultra-high performance liquid chromatography
UV	ultraviolet
V	volt
v/v	volume/volume
ZON	zearalenone
ZAN	zearalanone
ZOL	zearalenol
ZAL	zearalanol

GENERAL INTRODUCTION

1. Mycotoxins

1.1. The mycotoxin issue

A toxin can be defined as a substance that is synthesized by a plant species, an animal, or by micro-organisms, which is harmful to another organism. Mycotoxins are secondary metabolites produced by fungi, mostly by saprophytic moulds growing on a variety of feed- and foodstuffs (Turner et al., 2009). The name mycotoxin is a combination of the Greek word for fungus '*mykes*' and the Latin word '*toxicum*' meaning poison. Mycotoxin producing fungi can be divided in two classes, namely field and storage fungi. Field fungi, such as *Fusarium* species, produce mycotoxins during their growth in the field whereas storage fungi, such as *Aspergillus* and *Penicillium* species, produce mycotoxins after crop harvesting. Many factors can influence mycotoxin production, but temperature and humidity are commonly accepted as the most determining factors in the field, as during storage (Filtenborg et al., 1996). Mould contamination does not necessarily implicate mycotoxin production. Furthermore, some mycotoxins are produced by only a limited number of fungal species, while others may be produced by a relative large range of genera. The prevalence of different fungal species is region dependent. *Fusarium* produced mycotoxins are more likely to occur in moderate regions, such as Western-Europe and North-America, whereas *Aspergillus* and *Penicillium* species are more prevalent in (sub-)tropical regions. Nevertheless, ochratoxin A, produced by *P. verrucosum*, can also occur in more moderate regions (Duarte et al., 2010).

Contamination of feedstuffs with mycotoxins occurs worldwide at a higher level than generally assumed. In 2001, the Food and Agricultural Organization (FAO) stipulated that 25% of feedstuffs worldwide are contaminated (FAO, 2001). In a more recent paper, it was shown that one or more mycotoxins could be detected in 82% of European crop samples (Monbaliu et al., 2010). In general, more than 50% of European samples are contaminated with deoxynivalenol at low levels and 75-100% of the samples are contaminated with one or more mycotoxins (Streit et al., 2012).

Up until now, over 400 mycotoxins with toxic potential have already been described (Kabak et al., 2006), however only few of them have distinct toxic effects. The toxicological risk has deemed to exist since the beginning of organized agriculture. In the Old Testament, some references to ergotism have been found (Schoental, 1984) as well as to T-2 toxin (T-2) and

zearalenone (ZON). ZON is thought to be responsible for the decline of the Etruscan civilization (Schoental, 1991). Egyptian farao tombs would have contained ochratoxin A (OTA), held responsible for the death of archeologists (Pittet, 1998). In the Middle Ages, ergotism, due to ergot alkaloids from *Claviceps purpureae* fungi, was spreading. Ergotism was called Saint Anthony's fire, named after the monks of the Order of St. Anthony who would have been successful in treating the symptoms of the ergot alkaloids. It was not until the 1960's that the first cases of mycotoxicosis were demonstrated. In Great Britain, more than 100 000 turkeys died due to liver necrosis and biliary hyperplasia (Turkey 'X' disease), where the etiological agents were aflatoxins (Nesbitt et al., 1962). Since then, a tremendous amount of research has been conducted to investigate a variety of mycotoxins, their potential toxic effects and how to counteract their effects.

1.2. *Fusarium* mycotoxins

Fusarium fungi are field fungi, commonly occurring in Western-Europe due to its moderate climate. They can produce a variety of mycotoxins, however the most toxicological important are trichothecenes (including deoxynivalenol (DON) and T-2), ZON and fumonisin B1 (FB1).

Trichothecenes are generally produced by *Fusarium spp.*, however not limited as they can also be produced by other unrelated genera, such as *Stachybotrys*, *Trichoderma*, *Cephalosporium* and others (Ueno, 1985). Many trichothecene producing *Fusarium spp.* are causal agents of Fusarium Head Blight (FHB), root rot and foot rot in cereals. These include *F. graminearum*, *F. poae* and *F. culmorum* (Rocha et al., 2005). The most common ZON producing *Fusarium* fungi are *F. graminearum* and *F. culmorum*, but also *F. cerealis*, *F. crookwellense*, *F. semitectum* and *F. equisiti* (Zinedine et al., 2007). Fumonisins are produced by *F. verticillioides* (formerly *F. moniliforme*), *F. proliferatum* and other minor species including *F. nygamai* (Thiel et al., 1991).

Enniatins (ENNs) and beauvericin (BEA) are 'emerging' *Fusarium* mycotoxins which have only recently been of interest (Jestoi, 2008). The most important ENNs are enniatin (ENN) A, A1, B and B1. They are primarily produced by *F. avenaceum* but also by other species such as *F. tricinctum*, *F. poae* and *F. culmorum* (Logrieco et al., 2002).

Next to these *Fusarium* mycotoxins, also other compounds can be produced by several *Fusarium spp*. A synergistic effect between DON and fusaric acid (FA) has been demonstrated by Smith et al. (1997). Acute dosing of swine with FA causes vomiting, lethargy and neurochemical changes by altering the serotonin synthesis in the brain, as does DON (Smith and MacDonald, 1991). Bacon et al. (1995) observed synergistic effects between FA and FB1 (both produced by *F. verticillioides*) when administered simultaneously to developing chicks *in ovo*. However, FA is not commonly analyzed in feed samples as it is not considered a major contaminant by various research labs. At the time being it is still unclear what the contribution of FA is in feed-borne mycotoxicosis.

1.2.1. Deoxynivalenol and T-2 toxin

<u>Chemical structure</u>

DON and T-2 are both trichothecenes. They are sesquiterpenoids, consisting of an alkene group at C-9, 10, an epoxy at C-12, 12, which is essential for its toxicity (Desjardins et al., 1993), and a variable number of acetoxy and hydroxyl groups. They have been classified into A, B, C and D toxins, depending on their functional groups (Ueno, 1977). Members of group A (e.g. T-2 and HT-2 toxin) do not contain carbonyl on C-8. Hydrolysis of ester groups leads to the formation of a basic trichothecene moiety with one to five hydroxyl groups. Group B (e.g. DON) differs from group A by the presence of a carbonyl group on C-8. Group C members (e.g. crotocine) have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively. Compounds in group D, also called macrocyclic trichothecenes, (e.g. satratoxin G) include a macrocyclic ring between C-4 and C-15 (Wu et al., 2010).
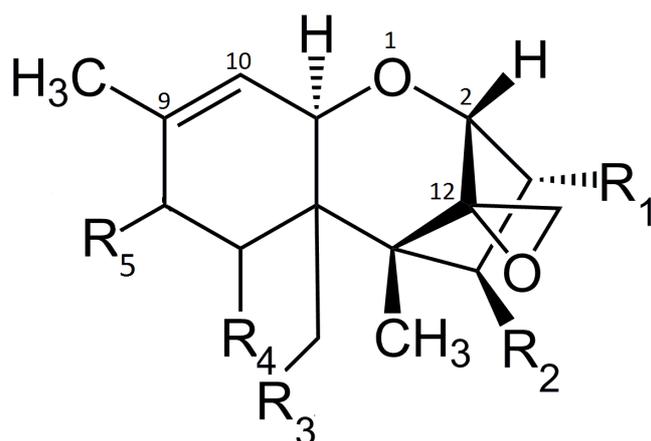
	T-2	HT-2	DON
R_1	-OH	-OH	-OH
R_2	-OAc	-OH	-H
R_3	-OAc	-OAc	-OH
R_4	-H	-H	-OH
R_5	$-OCOCH_2CH(CH_3)_2$	$-OCOCH_2CH(CH_3)_2$	=O

Figure 1. Chemical structure of T-2 toxin (T-2) , HT-2 toxin (HT-2) and deoxynivalenol (DON)

Mode of action

The most prominent molecular target of trichothecenes is the 60S ribosomal unit, where they prevent polypeptide chain initiation (T-2) or elongation-termination (DON) (Ueno, 1984). Thompson and Wannemacher (1986) demonstrated that T-2 is the most potent protein synthesis inhibitor, whereas DON is less potent. Addition of an acetyl chain (3a- or 15a-DON) further decreases its inhibitory potential. Furthermore, the de-epoxy metabolite of DON, de-epoydeoxynivalenol (DOM-1), has almost no inhibitory capacity. The main metabolite of T-2, namely hydrolyzed T-2 (HT-2), is also less potent than the parent compound. Trichothecenes also inhibit DNA and RNA synthesis, which is a secondary effect due to protein synthesis inhibition (Ueno, 1985). Furthermore, they inhibit mitosis and cause loss of membrane function via mitochondrial or non-mitochondrial pathways (Rocha et al., 2005). Finally, they activate mitogen-activated protein kinases (MAPKs) and induce apoptosis in a process called 'ribotoxic stress response' (Pestka, 2007). As a consequence of MAPK

activation, DON increases the expression and stability of cyclooxygenase-2 (COX-2) mRNA and hence protein content in leucocytes, confirming its role in the inflammatory process (Moon and Pestka, 2002). Recently it has been demonstrated that 15a-DON would be a more potent activator of MAPK *in vitro* compared to DON or 3a-DON (Pinton et al., 2012), in contrast to what is generally accepted.

Toxicity in pigs and poultry

The first symptoms of trichothecene intoxication were observed in the USSR in 1930s where consumption of overwintered moldy feed resulted in massive outbreaks of alimentary toxic aleukia (ATA) in pigs. Symptoms included vomiting, diarrhea, leucopenia, hemorrhage, shock and death (Joffe and Palti, 1974).

Pigs are the most sensitive species for DON as well as T-2, mainly due to their limited metabolic activity (Wu et al., 2010). High exposure of DON or 'vomitoxin' to pigs elicits abdominal distress, malaise, diarrhea, emesis and even shock or death (Pestka, 2010). The emetic effect is thought to be mediated through affection of the serotonergic activity in the central nervous system or via peripheral action on serotonin receptors (SCF, 1999). T-2 is one of the most acute toxic mycotoxins. Acute mycotoxicosis in pigs is characterized by multiple hemorrhages on the serosa of the liver and along the intestinal tract (Weaver et al., 1978).

Poultry are less susceptible to trichothecenes than pigs, however LD_{50} values, the acute dose at which 50% of the tested animals die within 24 h, are moderate. The LD_{50} value is 5 mg/kg BW for T-2 (compared to 1.2 mg/kg BW for pigs) and 140 mg/kg BW for DON (Chi et al., 1977; Huff et al., 1981; Schuhmacher-Wolz et al., 2010). Acute intoxication in broiler chickens has several consequences including internal hemorrhage, mouth and skin lesions (necrohemorrhagic dermatitis), impaired feather quality and neural disturbances (Sokolovic et al., 2008).

Chronic exposure to lower doses of DON and T-2 induces growth retardation, weight gain suppression and feed refusal in pigs and poultry. The immune system is very sensitive to trichothecenes and can be either stimulated or suppressed depending on the time, duration

and dose of exposure (Pestka, 2008; Sokolovic et al., 2008). Low concentrations induce pro-inflammatory gene expression at mRNA and protein levels, while high concentrations promote leukocyte apoptosis. Trichothecenes, especially DON and T-2, can provoke reproductive and teratogenic effects, however they do not exert carcinogenic effects. The International Agency for Research on Cancer (IARC) has listed them as group 3 substances (non-carcinogenic) (IARC, 1993).

1.2.2. Zearalenone

Chemical structure

ZON is a resorcylic acid, and was given the trivial name zearalenone as a combination of *Giberella zeae* (now *Fusarium graminearum*), resorcyclic acid lactone, -ene (for the presence of the C-1,2 double bond) and -one (for the presence of C-6 ketone) (Urry et al., 1966) (Figure 2).
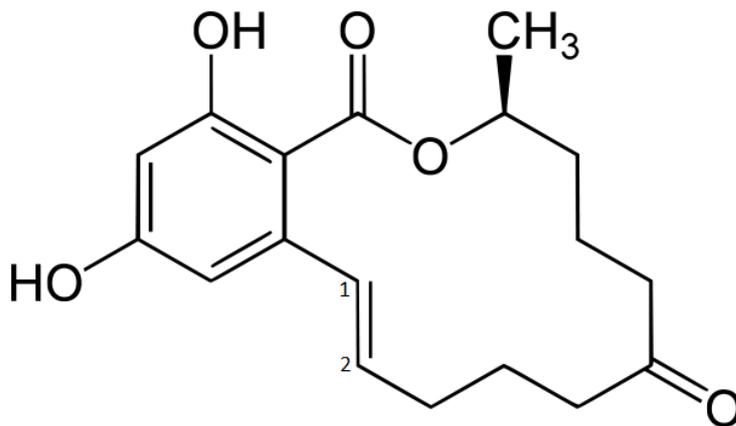
Figure 2. Chemical structure of zearalenone (ZON)

Mode of action

ZON can be listed as a non-steroidal or myco-estrogen (Tiemann and Dänicke, 2007). It resembles 17β-oestradiol, the principal hormone produced by the ovary, to allow ZON to bind estrogen receptors in target cells (Greenman et al., 1979). Estrogenic compounds diffuse in and out cells but are retained with high affinity and specificity by estrogen receptors. Once the estrogen receptor is bound, it undergoes a conformational change

allowing the receptor to interact with chromatin and to modulate transcription of target genes (Kuiper et al., 1998). Not all compounds have the same affinity for estrogen receptors. It has been shown that the metabolites of ZON can express lower or even higher affinities to estrogen receptors than the parent compound. Metabolisation of ZON occurs primarily in the liver, but a variety of organs show metabolisation activity such as intestine, kidney, ovary and testis. ZON is metabolized by 3α- and 3β-hydroxysteroid dehydrogenase (HSD) into α- and β-zearalenol (ZOL), respectively. β-ZOL has a 2.5 times lower affinity for the estrogen receptor, whereas α-ZOL has a 92 times higher binding affinity compared to ZON. The metabolisation to β-ZOL can therefore be regarded as an inactivation pathway, whereas the metabolisation to α-ZOL can be seen as an bioactivation pathway (Malekinejad et al., 2006). The rate of α- or β-ZOL production, and consequently the susceptibility, is species dependent. Pigs are the most sensitive species, which has been confirmed by *in vitro* data demonstrating that pig liver microsomes dominantly convert ZON into α-ZOL. Poultry and cattle metabolize ZON to a large extent into β-ZOL, confirming their relative resistance (Malekinejad et al., 2006; Zinedine et al., 2007).

Following or contemporary to these hydroxylation reactions, phase II metabolisation reactions take place. ZON and its metabolites are conjugated with glucuronic acid, catalyzed by uridine diphosphate glucuronyl transferases (UDPGT) (Olsen et al., 1981). Glucuronidation enhances the water solubility of compounds, thus enhancing renal elimination. On the other hand, it prolongs the total body residence time due to enterohepatic circulation, which has been demonstrated for ZON (Biehl et al., 1993).

Toxicity in pigs and poultry

The acute toxicity of ZON is rather low. Oral LD_{50} values in mice and rat vary from 4000 to >20000 mg/kg BW (Hidy et al., 1977). The specific manifestations of ZON in pigs are dependent of the dose, age, stage during estrus cycle and pregnancy or not. ZON intoxication leads to an estrogenic syndrome and affects primarily the reproductive tract and mammary gland. In young gilts, 1-5 mg/kg feed induces clinical signs such as hyperemia, oedemateus swelling of vulva and even vaginal or rectal prolaps (Minervini and Dell'Aquila, 2008). At lower doses (0.05 mg/kg feed) ZON induces vulva redness, swelling of the mammary gland and numerous vesicular follicles and some cystic follicles on the ovaries

(Bauer et al., 1987). In cyclic animals, nymphomania, pseudopregnancy, ovarian atrophy and changes in the endometrium are reported. During pregnancy, ZON can induce embryonic death, decrease fetal weight and induce teratogenic effects in piglets characterized by various genital abnormalities (D'Mello et al., 1999). In boars, ZON can suppress testosterone levels, testes weight and spermatogenesis, while inducing feminization and suppressing the libido (Zinedine et al., 2007).

ZON has little effect on poultry reproduction due to their well developed metabolisation pathways. Feeding mature chickens a diet contaminated with ZON up to 800 mg/kg did not affect their reproductive performance (Allen et al., 1980; Allen et al., 1981a). Moreover, this contamination level does not have negative effects on performance of mature broiler chickens or young turkey poults either (Allen et al., 1981b). However, feeding 100 mg ZON/kg feed to mature female turkeys, reduced the egg production by 20% (Allen et al., 1983).

Next to their major effects on the reproduction and hormone system, ZON and its metabolites may also affect other organ systems. ZON was shown to be hemotoxic. It disrupts the blood coagulation process, alters hematological parameters (such as hematocrit count, mean cell volume and number of platelets) as well as some serum biochemical parameters (such as aspartate aminotransferase, alanine aminotransferase, serum creatine and bilirubin) (Maaroufi et al., 1996). ZON is also hepatotoxic, shown by altered serum biochemical parameters (Zinedine et al., 2007). Genotoxic and immunotoxic effects of ZON have also been demonstrated *in vitro* and in mice (JECFA, 2000). The IARC has classified ZON as a non-carcinogenic component (group 3) (IARC, 1999). Nevertheless, more recent data demonstrate that ZON stimulates growth of mammary tumors containing estrogen receptors, indicating that it can play a role in tumor development upon chronic exposure (Ahamed et al., 2001; Yu et al., 2005).

1.2.3. Fumonisin B1

<u>Chemical structure</u>

The chemical structure of fumonisins was first identified in 1988 (Gelderblom et al., 1988). Today, more than 28 fumonisin homologues have been identified. Fumonisin B1 is the most

thoroughly investigated because of its toxicological importance (Figure 3). Fumonisin B2, B3 and B4 are less prevalent and differ structurally from FB1 in the number and placement of hydroxyl groups, i.e. a loss of a hydroxyl group on C-10, C-5 and both C-5 and C-10, respectively (Voss et al., 2007). The primary amine function is necessary for the toxicological activity of fumonisins. Deamination leads to a significant reduction in toxicity (Lemke et al., 2001a). Cleavage of the tricarballylic acid side chains of FB1 leads to a less toxic metabolisation product, named hydrolyzed fumonisin B1 (HFB1) (Grenier et al., 2012).
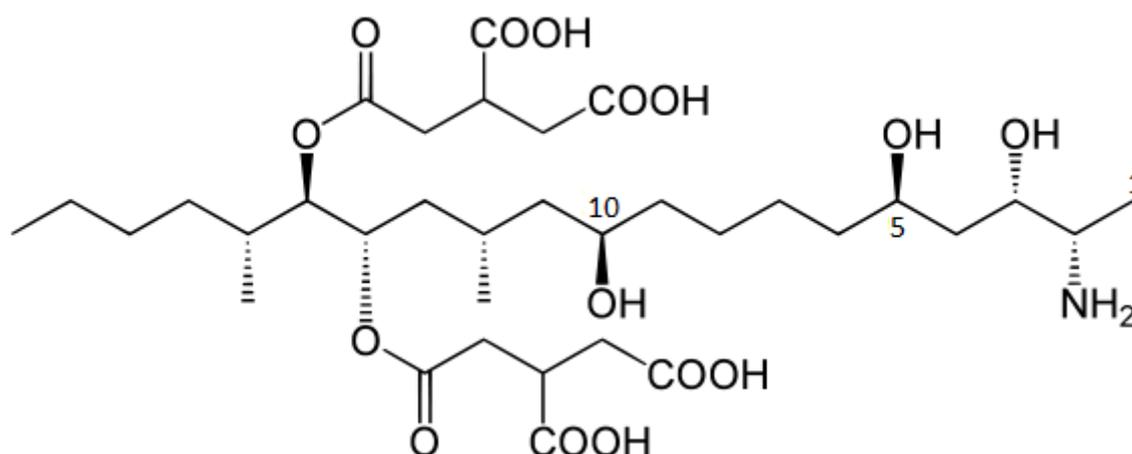
Figure 3. Chemical structure of fumonisin B1 (FB1)

Mode of action

Fumonisins competitively inhibit sphinganine N-acyl transferase (ceramide synthase) and consequently disrupt the ceramide and sphingolipid metabolism (Figure 4) (Merrill et al., 2001; Riley et al., 2001). The inhibition of ceramide synthase consequently leads to an accumulation of free sphinganine (Sa), and to a lesser extent of sphingosine (So), and to a decrease of complex sphingolipids formation. The increase of free Sa leads to an increased Sa:So ratio in tissues and body fluids, which has been demonstrated to be a suitable biomarker for fumonisin exposure in mammals and avian species (Haschek et al., 2001). This increase is dose and time dependent and is occurs rapidly and even at low levels (Voss et al., 2007). The increased concentrations of Sa and So, their phosphate adducts and a reduced ceramide concentration all contribute to the apoptotic, cytotoxic and growth inhibitory effects of fumonisins (Merrill et al., 2001). Moreover, the decrease of complex sphingolipids itself appears to contribute to the cellular effects of FB1 as well (Yoo et al., 1996).
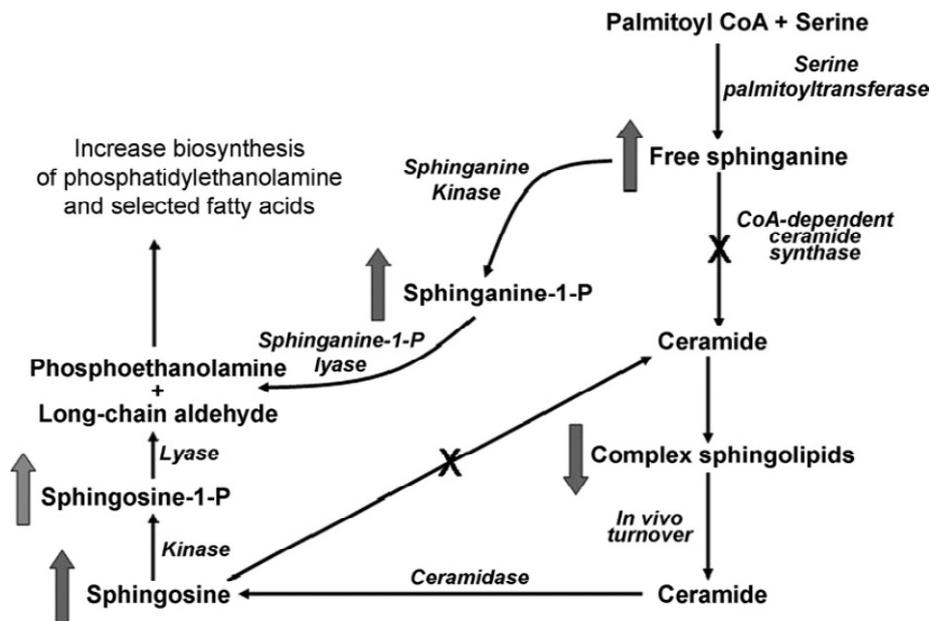
Figure 4. Mode of action of fumonisins (Voss et al., 2007)

Toxicity in pigs and poultry

Signs of acute fumonisin intoxication include non-species specific symptoms such as hepatotoxicity and renal failure, as well as species specific symptoms on target organs. The well described pathology in horses is called equine leukoencephalomacia (ELEM), where the brain is targeted. In pigs, primarily the heart tissue is affected, leading to cardiac insufficiency and consequently to pulmonary edema, called porcine pulmonary edema (PPE). FB1 as causal agent of PPE was first identified in 1992 (Osweiler et al., 1992). Thousands of pigs died in the USA due to consumption of corn contaminated with *F. verticillioides*. Symptoms of this intoxication are reduced feed intake at first, followed by respiratory distress and cyanosis a couple of days later and finally death caused by hydrothorax and acute pulmonary edema (Haschek et al., 2001). Poultry are quite resistant to fumonisin toxicity. Nevertheless, they are at risk because the major part of their diet consists of maize, which can be highly contaminated (Diaz and Boermans, 1994). High doses (up to 300 mg/kg feed) are needed to induce clinical toxicity including decreased weight gain and liver failure in broiler chickens (Ledoux et al., 1992). In general, high doses are needed to induce toxicity as fumonisins have a very low oral bioavailability (Martinez-Larranaga et al., 1999). Turkeys are also quite resistant to fumonisin toxicity, however they are still more susceptible compared to chickens (Weibking et al., 1994).

In mammals and poultry, immunosuppression has been demonstrated after chronic fumonisin exposure. This is economically important as adverse effects on the immune system can lead to increased pathogen susceptibility and lowered vaccinal response (Voss et al., 2007). Next to their effect on heart, liver and immune function, fumonisins exert reproductive, teratogenic and carcinogenic effects in laboratory animals (Howard et al., 2001; Riley et al., 2001; Voss et al., 1996a; Voss et al., 1996b). The IARC has classified FB1 as a group 2B compound (possibly carcinogenic to humans) (IARC, 1993).

1.2.4. Enniatins and Beauvericin

Chemical structure

BEA and ENNs are cyclic hexsadepsipeptides consisting of alternating D-α-hydroxy-isovaleryl and amino acid units. In BEA the three amino acid residues are aromatic N-methyl-phenylalanines, whereas in ENNs they are aliphatic N-methyl-valine and/or -isoleucine. The subunits are linked with peptide bonds and intra-molecular ester (lactone) bonds, forming a depsipeptide (Figure 5) (Jestoi, 2008).
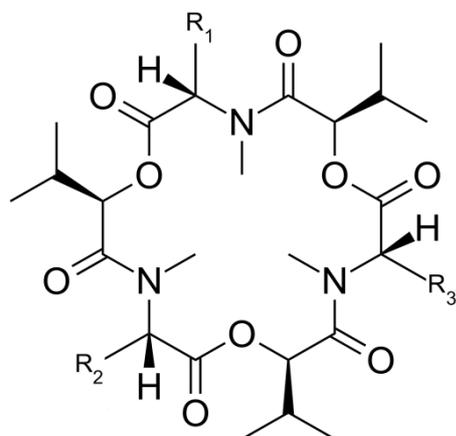
	R1	R2	R3
BEA	phenylmethyl	phenylmethyl	phenylmethyl
ENN A	*sec*-butyl	*sec*-butyl	*sec*-butyl
ENN A1	*sec*-butyl	*sec*-butyl	*iso*-propyl
ENN B	*iso*-propyl	*iso*-propyl	*iso*-propyl
ENN B1	*iso*-propyl	*iso*-propyl	*sec*-butyl

Figure 5. The chemical structures of beauvericin (BEA) and enniatin (ENN) A, A1, B and B1

Mode of action

ENNs and BEA are of interest because they can exert antibacterial, antifungal and phytotoxic effects (Jestoi, 2008; Meca et al., 2010; Meca et al., 2011). On the other hand, they can also be toxic for mammalian cells. Their main mode of action is based on their ionophoric properties (Ivanov et al., 1973). They are capable of transporting cations through the cell membrane, leading to toxic actions by disturbing the normal physiological level of cations in the cell (Jestoi et al., 2009). Next to their ionophoric properties, ENNs exhibit other effects, such as inhibiting acyl-coenzyme A:cholesterol acyl transferase (ACAT) and 30,50-cyclo-nucleotide phosphodiesterase enzymes (Tomoda et al., 1992), causing mitochondrial dysfunction (Tonshin et al., 2010), and the inhibition of multidrug resistance associated protein-1 (ABCG2) and P-glycoprotein (ABCB1) efflux pumps (Dornetshuber et al., 2009).

Toxicity in pigs and poultry

ENNs and BEA have not been thoroughly investigated because they have not been associated with clinical mycotoxicosis yet. Therefore, most research focused on other, more toxic compounds as those described in this thesis. Nowadays, the role of mycotoxins in subacute or subclinical toxicology is of greater concern in animal nutrition. Subclinical mycotoxicosis can lead to reduced feed consumption and growth, poorer reproductive capacities and altered immunological response which causes increased susceptibility for infectious disease (CAST, 2003). ENNs and BEA have been found to be common co-contaminants of feedstuff, the reason why they became of interest. In a study on Finnish grains, BEA, ENN A, A1, B and B1 were detected in 95, 95, 74, 100 and 100% of the samples, respectively, at concentrations ranging from trace levels up to 18.3 mg/kg (Jestoi et al., 2004).

Cytotoxicity of ENNs and BEA has been demonstrated *in vitro* on various cell types (for review see Jestoi, 2008). Data on their *in vivo* toxicity is, however, lacking. Feeding studies performed on chickens or turkeys did not show any negative effect of these mycotoxins (Leitgeb et al., 1999; Leitgeb et al., 2000; Zollitsch et al., 2003). However, contamination levels tested in these studies were rather low (1.4 -12.0 mg BEA/kg feed and 2.5 mg ENNs/kg feed), especially when one bears in mind that detected concentrations of BEA and ENNS are up to 520 and 18 mg/kg, respectively (Jestoi et al., 2004; Ritieni et al., 1997).

1.3. *Aspergillus* and *Penicillium* mycotoxins

Aspergillus and *Penicillium* fungi occur worldwide and are able to produce several mycotoxins. Toxicologically, the most important ones are OTA and aflatoxin B1 (AFB1). OTA was first isolated in 1965 from *A. ochraceus* (Van der Merwe et al., 1965). It is primarly produced during storage by *A. ochraceus*, in tropical and warmer regions, and by *P. verrucosum* in more temperate regions (Duarte et al., 2010). AFB1 is mainly produced by strains of *A. flavus* and *A. parasiticus*, but also by other minor species, such as *A. nomius*, *A. bombycis* and *A. pseudotamari,* all occurring in tropical climates (Bennett and Klich, 2003).

1.3.1. Ochratoxin A

Chemical structure

OTA consists of a dihydroisocoumarin subunit, linked to phenylalanine by a peptide bound (Mally and Dekant, 2009) (Figure 6). Cleavage of the dipeptide bound induces the formation of ochratoxin alpha (OTα), a nontoxic metabolite. Other major, but less toxic, ochratoxins are ochratoxin B and C, which vary from OTA by loss of the chlorine on C-5 or ethylester formation on the carboxyl function at C-11, respectively (Duarte et al., 2011; El-Khoury and Atoui, 2010; Wu et al., 2011).
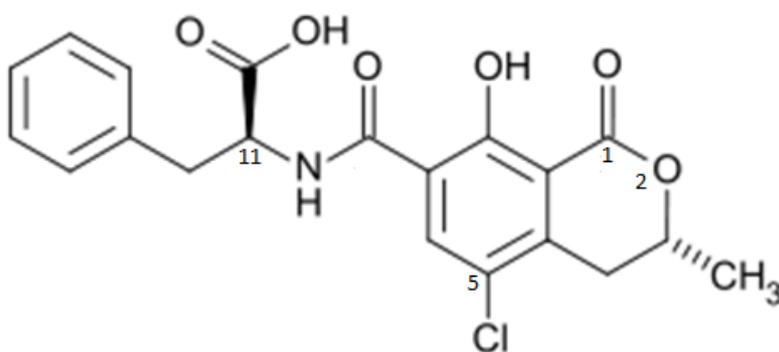
Figure 6. Chemical structure of ochratoxin A (OTA)

Mode of action

OTA does not act through a single well-defined mechanism but disturbs cellular physiology in multiple ways (Marin-Kuan et al., 2008). It seems that the primary effects are associated with inhibition of the enzymes involved in the synthesis of the phenylalanine tRNA-complex, thus interfering with phenylalanine metabolism. In addition, it stimulates lipid peroxidation (Bennett and Klich, 2003). OTA is also considered carcinogenic amongst laboratory animals, IARC Class 2B (IARC, 1993), although the mode of action has not been well described yet (Mally, 2012). The suggested molecular targets are histone acetyltransferases (HATs). These enzymes are critical in the regulation of a diverse range of cellular processes, including gene expression, DNA damage repair and mitosis through posttranslational acetylation of histone and nonhistone proteins (Czakai et al., 2011; Mally, 2012).

Toxicity in pigs and poultry

Dietary human exposure to OTA has long been suspected to be involved in Balkan endemic nephropathy (BEN) occurring in the 1950s although no direct proof can be put forward (Pfohl-Leszkowicz, 2009). The first report of OTA intoxication in animals was in the 1960s and 1970s in Denmark where mycotoxic porcine nephropathy (MPN) has been correlated with OTA ingestion (Krogh et al., 1973). The kidneys are the main target organ of OTA.

Considerable species differences in sensitivity towards acute OTA toxicity have been demonstrated (O'Brien and Dietrich, 2005). Pigs are particularly sensitive to OTA because of the long serum half-life and tissue accumulation. This is sustained by high protein affinity and enterohepatic and renal recirculation. Poultry species eliminate OTA faster than mammals, leading to a lower accumulation level. The half-life of OTA in pig plasma is 20-30 times longer than that in poultry plasma leading to a higher OTA contamination level and incidence in pigs (Duarte et al., 2011). This difference is also demonstrated by the difference of LD_{50} value in pigs and poultry: an oral LD_{50} value of 1.0 mg/kg BW for pigs versus 3.3 mg/kg BW for chickens and 5.9 mg/kg BW for turkeys (El-Sayed et al., 2009; Peckham et al., 1971).

Following chronic exposure to lower levels of OTA, the kidneys are again primarily affected, causing mycotoxic nephropathy in pigs as well as in chickens (Stoev et al., 2010). Several

pathological changes can be observed, varying from desquamation and focal degeneration of tubular epithelium cells to peritubular fibrosis and thickening of the basal membrane (O'Brien and Dietrich, 2005). This leads to renal insufficiency, but not to tumor promotion in poultry and mammals. In addition, OTA is hepatotoxic, teratogenic and immunotoxic (Duarte et al., 2011).

1.3.2. Aflatoxin B1

Chemical structure

Over a dozen different aflatoxins have been described. Based on their fluorescence under UV-light (blue or green) the four major aflatoxins are called aflatoxin B1, B2, G1 and G2, of which AFB1 is the most toxic (Squire, 1981). The structure of AFB1 was first elucidated by Asao et al. (1965), and it consists of a difuro-coumaro-cyclopentenone structure (Figure 7). Other aflatoxins have different substitutions but they all share the basic coumarine structure.
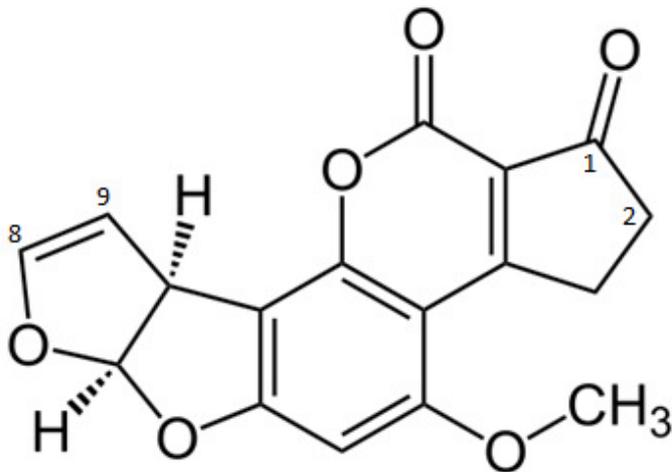
Figure 7. Chemical structure of aflatoxin B1 (AFB1)

Mode of action

Aflatoxins are converted by cytochrome P450 enzymes (phase I metabolisation) to the reactive 8,9-epoxide form, which is essential for the toxicity. The responsible converting enzymes in mammals are mainly CYP1A2 and CYP3A4 (Gallagher et al., 1996). In chickens and turkeys, the corresponding enzymes are CYP2A6 and to a lesser extent CYP1A1 orthologs (Diaz et al., 2010a, b). The epoxide metabolite can bind to both DNA (causing

genotoxicity) and proteins (causing cytotoxicity). More specifically it binds to guanine residues of nucleic acids (Doi et al., 2002). Moreover, aflatoxin B1-DNA adducts can result in guanine-cytosine (GC) to thymine-adenine (TA) transversions (Bennett and Klich, 2003). This both leads to irreversible DNA damage and causes hepatocellular carcinomas (Eaton and Gallagher, 1994).

The toxic epoxide metabolite can be detoxified by gluthatione conjugation (phase II metabolisation) or hydrolysis by an epoxide hydrolase to AFB1-8,9-dihydrodiol (AFB1-dhd) or by metabolisation to less toxic compounds such as aflatoxin M1 (AFM1) or Q1 (AFQ1) (Diaz et al., 2010b; Gallagher et al., 1996). This AFM1 for example is the main metabolite formed in cattle and is excreted in the milk. As this metabolite still possesses carcinogenic properties (10 times lower than AFB1), maximum limits in milk intended for human consumption have been established (0.05 µg/kg) (European Commission, 2010).

Toxicity in pigs and poultry

The main biological effects of aflatoxins are carcinogenicity, immunosuppression, mutagenicity and teratogenicity (Ramos and Hernandez, 1997). Because of its pronounced carcinogenic effects, even in humans, the IARC has classified AFB1 as a group 1 compound (IARC, 1993).

Acute aflatoxicosis in pigs has been long described (Coppock et al., 1989). Intake of contaminated feed (0.2 mg/kg) leads to reduced feed intake and body weight gain, impaired liver and immune functions and altered serum biochemical parameters (Harvey et al., 1990; Lindemann et al., 1993; Rustemeyer et al., 2010 and 2011).

Poultry species are the most susceptible production animals to AFB1. Feed contaminated with even small amounts of AFB1 results in significant adverse health effects, including death. On autopsy, generally a firm and pale liver is found, the target organ of aflatoxins. When chickens are chronically exposed to lower doses, growth retardation occurs, as well as immunological alterations and histological changes in the liver ('fatty liver') (Newberne and Butler, 1969). Turkeys are even more susceptible to aflatoxin intoxication than chickens, attributed to a combination of efficient AFB1 activation and deficient detoxification by phase II enzymes such as glutathione-S-transferase (Klein et al., 2000). Feeding a diet contaminated

with 1 mg/kg AFB1 to turkeys resulted in 88% mortality rate (Kubena et al., 1991). Lower concentrations induced poor performance, decreased organ weights, liver damage and changes in biochemical serum values (Coulombe, 1993; Kubena et al., 1991).

2. Methods to counteract mycotoxins

Because of the detrimental effects of mycotoxins, a number of strategies have been developed to reduce the growth of mycotoxigenic fungi and mycotoxin production, to detoxify contaminated feed and to lower the systemic availability once mycotoxins are ingested by the animal.

2.1. Pre- and post-harvesting strategies

Mycotoxin contamination may occur in the field, pre-harvesting, or during storage and processing, post-harvesting. Methods for preventing mycotoxicosis in animals may therefore be divided into pre- or post-harvesting strategies. Certain methods have been found to significantly reduce specific mycotoxin contamination although the complete elimination of mycotoxins is currently not achievable (Kabak et al., 2006).

The most important strategy to bear in mind for pre-harvesting is the application of Good Agricultural Practices (GAP). Appropriate GAP includes crop rotation, soil cultivation, irrigation and proper use of chemicals. Crop rotation is important and focuses on breaking the chain of infectious material, for example by wheat/legume rotations. Including maize in the rotation should be avoided, as it is very susceptible to *Fusarium spp.* infestations. Any crop husbandry that includes destruction, removal or burial of the infected crop is seen as good soil cultivation. The deeper the soil is inverted (ploughing), the less plausible fungi growth will be on the following crop (Edwards, 2004). Reducing plant stress by irrigation is also valuable to prevent fungi infestation. All plants in the field need an adequate water supply, however excess irrigation during flowering (anthesis) makes conditions favorable for *Fusarium* infection (Codex Alimentarius, 2002). Another factor which is known to increase the susceptibility of agricultural commodities to mould invasion is damage due to birds, insects or rodents. Insect damage and consequent fungal infection must by controlled by appropriate use of insecticides and fungicides. This should be integrated with adequate pest management control (Codex Alimentarius, 2002).

All these parameters can be controlled, however environmental conditions cannot. Relative humidity and temperature are known to have an important onset on mould infection and mycotoxin production. Drought damaged plants are shown to be more susceptible to

infection, so crop planting should be timed to avoid high temperatures and drought (Kabak et al., 2006). For *Fusarium spp.* infection on the other hand, sufficient moisture conditions at anthesis are critical for the onset of FHB (Aldred and Magan, 2004).

Post-harvest storage conditions are essential in preventing mould growth and mycotoxin production (Schrodter, 2004). For example, grains should be stored with less than 15% moisture to avoid hotspots with high moisture, favorable for mould growth (Jard et al., 2011). Before storage, visibly damaged or infected grains should be removed. This method is however not exhaustive or very specific (Jard et al., 2011) and multiple reduction strategies should be combined.

Several chemical detoxification methods have also been described. In all cases, they should destroy or inactivate mycotoxins, generate non-toxic products, warrant the nutritional value of the food or feed and do not induce modification to the technical properties of the product (Jard et al., 2011). The wide variety of chemical decontamination processes include radiation, oxidation, reduction, ammonization, alkalization, acidification and deamination (Kabak et al., 2006). These chemical methods are not allowed in the European Union (European Commission, 2001) as chemical transformation might lead to toxic derivatives. In the United States, only ammonization is licensed for detoxifying aflatoxins.

2.2. Mycotoxin detoxifying agents

The use of many of the previously described methods for the detoxification of agricultural commodities is restricted due to the associated problems including incomplete detoxification and inapplicability in practice. An alternative approach to reduce the exposure to mycotoxins in feed is to decrease the bioavailability by the inclusion of mycotoxin detoxifying agents (mycotoxin detoxifiers) in the feed. This method is the most commonly used today (Jard et al., 2011; Kolosova and Stroka, 2011). These detoxifiers can be divided into two different classes, namely mycotoxin binders and mycotoxin modifiers. An overview of the different products covered by both classes is given in Table 1. These two classes have different modes of action; mycotoxin binders adsorb the toxin in the gut, resulting in the excretion of toxin-binder complex in the faeces, whereas mycotoxin modifiers transform the toxin into non-toxic metabolites (EFSA, 2009). The extensive use of these additives has led, in 2009, to the establishment of a new group of feed additives: 'substances for reduction of

the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action' (European Commission, 2009). It should be pointed out that the use of such products does not mean that animal feed exceeding maximal regulatory limits may be used. Their use should rather improve the quality of the feed which is lawfully on the market, providing additional guarantees for animal health safety (Kolosova and Stroka, 2011).

Table 1. Overview of different classes and subclasses of mycotoxin detoxifiers (adapted from EFSA (2009))

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 *Exophalia spinifera* *Rhinocladiella atrovirens* …		
	Enzymes	Epoxidase Lactonohydrolase or Lactonase Carboxypeptidase A α-Chymotrypsin Carboxylesterase		

2.2.1. Mycotoxin binders

Mycotoxin binders (adsorbing or sequestering agents) are large molecular weight compounds that should be able to bind the mycotoxins in the gastrointestinal tract of the animal. In this way the toxin-binder complex passes through the animal and is eliminated via the faeces. This prevents or minimizes exposure of animals to mycotoxins. Mycotoxin binders are divided in mainly silica-based inorganic compounds or carbon-based organic polymers (EFSA, 2009).

Inorganic binders

The efficacy of inorganic binders depends on the chemical structure of both the adsorbent and the mycotoxin. The most important feature is the physical structure of the adsorbent, i.e. the total charge and charge distribution, the size of the pores, and the accessible surface area. On the other hand, the properties of the adsorbed mycotoxins, such as polarity, solubility, shape and charge distribution, also play a significant role. Generally speaking, the binding capacity increases with surface area and chemical affinities between both (Avantaggiato et al., 2005; Huwig et al., 2001; Kabak et al., 2006).

Aluminosilicate minerals (clays) are the largest class of mycotoxin binders and most studies on the alleviation of mycotoxicosis by the use of adsorbents have focused on these clays. Within this group, there are two different subclasses: the phyllosilicate subclass and the tectosilicate subclass. Phyllosilicates include bentonites, montmorillonites, smectites, kaolinites and illites. The tectosilicates include zeolites (EFSA, 2009). Montmorillonite is primarily a layered phyllosilicate composed of layers of octahedral aluminium and tetrahedral silicon coordinated with oxygen atoms. Bentonite is generally an impure clay consisting mostly of montmorillonite. Zeolites are composed of tetrahedrons of SiO_4 and AlO_4 possessing an infinite three-dimensional cage-like structure. In these minerals, some of the tetravalent silicon are replaced by trivalent aluminium giving rise to a deficiency of positive charge, which is balanced by inorganic cations, such as sodium, calcium and potassium ions. Hydrated sodium calcium aluminosilicate (HSCAS) contains calcium ions and protons which are exchanged against the naturally occurring sodium ions (Huwig et al., 2001). This HSCAS is a heat processed and purified montmorillonite clay. It was developed by

Phillips et al. (1988) and commercialized as NovaSil®. Clay products, including bentonites, zeolites and HSCAS, are the most common feed additives effective in binding polar mycotoxins, such as aflatoxins (Kabak et al., 2006). *Fusarium* mycotoxins, such as fumonisins, ZON and trichothecenes, as well as OTA, are not bound to these clays because of the fairly non-polar properties of the toxins (Avantaggiato et al., 2005; Kabak et al., 2006; Phillips et al., 2008). HSCAS has a lamellar interlayer structure in which the planar AFB1 can be bound. The interaction is based on the negative charge of the clay with the partly positive charged dicarbonyls of AFB1 (Phillips et al., 2008). Although the mentioned clays have proven to be effective in preventing aflatoxicosis in various animal species, several disadvantages should be considered. They do not exert any binding potential towards other mycotoxins, they can adsorb vitamins and minerals and the risk of natural clays to be contaminated with dioxins should surely also be considered (Huwig et al., 2001; Jouany, 2007).

Another inorganic sorbent of interest is activated charcoal, also called active carbon (AC). AC is a non-soluble powder formed by pyrolysis of several organic compounds. It is manufactured by an activation proces to develop a highly porous structure (Galvano et al., 2001). The sequestrant properties of AC depend on many factors including pore size, surface area, structure of the mycotoxin and dose. The surface-to-mass ratio of AC varies from 500 to 3500 m^2/g. AC has been shown to be an effective binder of a wide variety of drugs and toxic agents. It has been commonly used as a medical treatment for severe intoxications since the 19th century (Huwig et al., 2001). AC has been proven an effective adsorbent of DON, ZON, AFB1, FB1 and OTA (Avantaggiato et al., 2004; Devreese et al., 2012; Huwig et al., 2001). Nevertheless, its unspecific binding is the major drawback in the practical use of AC as a feed additive. It diminishes nutrient absorption and impairs the nutritional value of feed (Avantaggiato et al., 2004; Ramos et al., 1996).

Synthetic or natural polymers are another group of inorganic mycotoxin binders. Several agents belong to this group, such as dietary fibre and polyvinylpyrrolidone (highly polar amphoteric polymer), but the most well-known is cholestyramine. Cholestyramine is an insoluble, quaternary ammonium anion exchange resin which strongly binds anionic compounds (Underhill et al., 1995). It has been used as drug in humans for absorbing bile acids in the gastrointestinal tract in order to reduce free cholesterol. This compound was proven to be an effective binder for FB1, OTA and ZON *in vitro* (Avantaggiato et al., 2003;

Avantaggiato et al., 2005; Döll et al., 2004; Ramos et al., 1996). The cost of polymers is high, limiting their practical use in animal feed (Kolosova and Stroka, 2011).

Organic binders

Organic mycotoxin binders which are commonly used are cell wall components from *Saccharomyces cerevisiae* yeasts. By using only yeast cell walls instead of the whole cell, mycotoxin binding can be enhanced. The fact that dead cells do not lose their binding ability shows that interaction of such products with mycotoxins is by adhesion to cell wall components rather than by covalent binding or metabolism (Shetty and Jesperson, 2006). It has been demonstrated that the β-D-glucan fraction of yeast cell wall is directly involved in the binding process with ZON, and that the structural organisation of β-D-glucans modulates the binding strength. Hydrogen and van-der-Waals bonds have been evidenced in the glucan-mycotoxin complexes (Jouany, 2007; Shetty and Jespersen, 2006; Yiannikouris et al., 2004; Yiannikouris et al., 2006). Based on *in vitro* assays, this glucomannan (GMA) binder has shown to effectively adsorb DON, T-2, ZON, OTA and AFB1 (Bejaoui et al., 2004; Freimund et al., 2003; Yiannikouris et al., 2004; Yiannikouris et al., 2006). Protective effects of GMA against the detrimental consequences of mycotoxins on animal production parameters have been demonstrated in several studies. Raju and Devegowda (2000) demonstrated that GMA has beneficial effects in broilers when included in feed contaminated with AFB1 (0.3 mg/kg), OTA (2 mg/kg) and T-2 (3 mg/kg). Individual and combined effects of these mycotoxins were examined. Significant interactions were observed between any two mycotoxins, such as additive effects on body weight or feed intake, or antagonistic effects on serum protein and cholesterol content. The GMA incorporation increased body weight and feed intake, decreased weight of liver, and improved some serum biochemical and hematological parameters which were negatively influenced by the mycotoxins in the feed (Raju and Devegowda, 2000). These binders also alleviate the adverse effects of AFB1 (1 mg/kg) on performance, liver weight and mortality in broiler chickens (Kamalzadeh et al., 2009). GMA counteracts most of the plasma parameter alterations caused by a DON contaminated diet (3 mg/kg) in chickens (Faixova et al., 2006). Aravind et al. (2003) showed a protective effect of GMA against anti-oxidant depletion in chicken livers caused by intake of a T-2 contaminated (8 mg/kg) diet. Some positive effects of these products have also been

demonstrated in pigs. In a study by Diaz-Llano and Smith (2007), GMA was able to counteract the alterations of serum biochemical parameters induced by DON (5.5 mg/kg) in sows, however no positive effect on feed intake and body weight gain was seen. Furthermore, Dänicke et al. (2007a) did not observe improved productive parameters in pigs due to GMA addition in a DON contaminated feed (4.4 mg/kg). On the other hand, Nesic et al. (2008) did observe improved performance of pigs when GMA was included in the diet compared to a diet only contaminated with ZON at 3.8 and 5.2 mg/kg.

Another group of organic mycotoxin binders, which have recently become of interest are lactic acid bacteria (LAB). LAB are Gram-positive, catalase-negative, non-sporulating, usually non-motile rods and cocci that utilize carbohydrates fermentatively and form lactic acid as major end product (Gerbaldo et al., 2012). These bacteria are mainly divided into four genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. They have been used in the food processing industry for decades because of their fermentative and food preserving abilities. They have also displayed mycotoxin binding abilities (Dalie et al., 2010). The interaction mechanism between LAB and mycotoxins is thought to be similar as the interactions involved in adsorption by GMA. It appears that the polysaccharide components (glucans and mannans) are common sites for binding, with different toxins having different binding sites. Several authors have concluded that the strength of the mycotoxin-LAB interaction was influenced by the peptidoglycan structure and, more precisely, by its amino acid composition (Dalie et al., 2010). The most extensively investigated mycotoxin binding LAB are strains of *Lactobacillus rhamnosus*. *L. rhamnosus* strains have an *in vitro* binding capability of DON, T-2, ZON, FB1, AFB1 and OTA (El-Nezami et al., 1998; El-Nezami et al., 2002a; El-Nezami et al., 2002b; Niderkorn et al., 2006; Piotrowska and Zakowska, 2005). The *in vitro* adsorption capacity is however, strain and dose dependent and it is a reversible process showing as well adsorption as desorption (Kankaanpaa et al., 2000; Lee et al., 2003). All the available literature on LAB-mycotoxin interactions are based on *in vitro* results. To date, no *in vivo* trials have been conducted to effectively demonstrate their mycotoxin binding potential and therefore prudency regarding their effectiveness is, in the mean time, recommended.

2.2.2. Mycotoxin modifiers

Another strategy to control mycotoxicoses in animals is the application of microorganisms and their enzymes, called mycotoxin modifiers or mycotoxin biotransforming agents. These products biodegrade or biotransform mycotoxins into less toxic metabolites. They can be divided into four classes: bacteria, yeasts, fungi and enzymes. They act in the intestinal tract of animals prior to the absorption of mycotoxins. It has to be pointed out that for effective use of mycotoxin modifiers as feed additives, certain prerequisites should be fulfilled. Those include rapid degradation, degradation into non-toxic (or far less toxic) metabolites under different oxygen conditions and in a complex environment, preserve the organoleptic and nutritive properties of the feed, safety of use and stability along the intestinal tract at different pH levels. In addition, the choice of the biodegradation approach depends on its practical and economical feasibility (Awad et al., 2010; Kolosova and Stroka, 2011). Anaerobic microorganisms isolated from animal gut contents are generally suitable for developing feed additives, which will act in the animals' intestines. Survival and adaptation of the microorganisms in the animal gut are key factors for successful detoxification (Zhou et al., 2008).

Bacteria

Mycotoxin degrading bacteria have been isolated from very different matrices such as rumen and intestinal flora, soil and even water. The most extensively investigated mycotoxin degrading microorganism is the *Eubacterium* BBSH 797 strain, originally isolated from bovine rumen fluid. This bacterial strain produces enzymes (de-epoxidases) that degrade trichothecenes by selective cleavage of their 12,13-epoxy group which is important for the toxicity of these mycotoxins. This detoxification was investigated for several trichothecenes (Fuchs et al., 2002) and the mode of action was proven *in vitro* and *in vivo* (Schatzmayr et al., 2006). During its manufacture, BBSH 797 is stabilized by freeze-drying and embedding into protective substances (mainly organic polymers) to guarantee stability when passing through the acidic gastric tract of animals. *Eubacterium* BBSH 797 is currently the only microorganism which has been developed into a commercial product for detoxifying trichothecenes (He et al., 2010).

A variety of other bacterial strains have shown mycotoxin degrading abilities *in vitro*. For example, *Nocardia asteroides*, *Corynebacterium rubrum*, *Mycobacterium fluoranthenivorans*, *Rhodococcus erythropolis*, *Flavobacterium aurantiacum* and *Pseudomonas fluorescens* (EFSA, 2009). However, none of them have been investigated already *in vivo*.

Yeast

Only one yeast, *Trichosporon mycotoxinivorans*, has been thoroughly investigated regarding its mycotoxin degrading abilities, which could result in commercial use. This yeast, derived from the hindgut of the termite *Mastotermes darwiniensis*, was isolated and characterized previously by Molnar et al. (2004). This yeast is able to modify ZON and OTA into non-toxic metabolites. ZON is detoxified by opening the macrocyclic ring of the ketogroup at C-6. The metabolite did not show any estrogenic effect in a yeast bioassay and did not interact with the β-estrogen receptor in an *in vitro* assay. Detoxification of OTA occurs by cleavage of the phenylalanine moiety from the isocoumarin derivate, producing OTα (Schatzmayr et al., 2006). The detoxification of OTA occurs fast, after 2.5 h a conversion of almost 100% was observed *in vitro*. For ZON on the other hand, the metabolisation requires a lot more time. Only after 24 h of incubation ZON was completely metabolized. This questions its practical use as detoxification should occur fast after ingestion (<8 h). The use of *T. mycotoxinivorans* as a mycotoxin modifier against OTA is promising. A study by Politis et al. (2005) demonstrated that inclusion of this yeast (10^5 CFU/g) in the diet alleviates the immunotoxic effects of OTA (0.5 mg/kg) in broiler chickens.

Other potential OTA degrading yeast are *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* (Peteri et al., 2007) but the responsible enzymes have not well been characterized and their practical application up to day is limited. Styriak and Conkova (2002) reported that two out of several tested *Saccharomyces cerevisiae* strains were able to degrade FB1, but only for 25 or 50% after 5 days of incubation, which is therefore unusable in practice.

Fungi

Fungi cannot only produce mycotoxins, but some of them are also able to degrade them. The fungal strains *Aspergillus niger*, *A. flavus*, *Eurotium herbariorum* and *Rhizopus sp*. are able to convert AFB1 to aflatoxicol (AFL) by reducing the cyclopentenone carbonyl of AFB1 (Wu et al., 2009). AFL has been reported to be 18 times less active than the parent compound, but it still has carcinogenic properties (Pawlowski et al., 1977), raising the question if this is an appropriate detoxification strategy. Other fungal strains showed AFB1 metabolizing properties as well, such as *Penicillium raistrickii*, although their metabolisation products have almost similar toxicity (AFB2) or were not all yet identified (Wu et al., 2009).

Next to their AFB1 degrading potential, *Rhizopus* isolates also showed ZON detoxifying abilities. The selected isolates include strains of *R. stolonifer*, *R. oryzae* and *R. microsporus* (Varga et al., 2005). Further studies are needed to identify the ZON degrading enzymes in the isolates. A preliminary study was performed to screen twelve black *Aspergillus* strains for their ZON transformation activity by incubation in contaminated culture medium. Analyses showed that ZON was removed after 48 h of incubation by two *A. niger* strains (EFSA, 2009). *Aspergillus niger* was also able to degrade OTA to the less toxic compound OTα. It was then further degraded into an unknown compound (Varga et al., 2000).

Fumonisin degrading fungi have been identified *in vitro* as well. *Exophalia spinifera* and *Rhinocladiella atrovirens* extensively metabolize fumonisin B1 to HFB1 and free tricarballylic acid *via* esterases (Blackwell et al., 1999).

Enzymes

An attractive alternative to the use of live microbes to counteract mycotoxins in animal feed is application of enzymes responsible for degradation of mycotoxins. Enzymatic reactions offer a specific, often irreversible, efficient and environmentally friendly way of detoxification that leave neither toxic residues nor any undesired by-products (Kolosova and Stroka, 2011). These mycotoxin degrading enzymes are primarily produced by microorganisms.

Epoxidases are enzymes which are able to detoxify trichothecenes by transforming their expoxy group into diene groups (Schatzmayr et al., 2006). For example, DON can be detoxified to its de-epoxy form, DOM-1.

Takahashi-Ando et al. (2002) reported that ZON was converted into a less estrogenic product by cleavage of the lactone structure. The responsible enzyme is a lactonohydrolase, originating from the fungus *Clonostachys rosea* IFO 7063.

Pitout (1969) presented the first *in vitro* hydrolysis of OTA by carboxypeptidase A and, in lower amounts, by α-chymotrypsin. Abrunhosa et al. (2006) reported the ability of several commercial proteases to hydrolyze OTA into OTα. After a long incubation period of 25 h, a significant hydrolytic activity was detected for protease A (87.3%) and for pancreatin (43.4%).

Recently, two genes of *Sphingopyxis sp*. MTA 144 responsible for detoxification of FB1 were identified and recombinant enzymes were produced (Heinl et al., 2010). The degradation of FB1 consisted of two consecutive pathways. FB1 is first metabolized to HFB1 by a carboxylesterase, followed by an aminotransferase which deaminates HFB1, leading to an even less toxic compound.

3. EC regulations on maximum levels of mycotoxins in animal feed

The growing awareness that mycotoxins are a great concern to animal health, has led to regulations of maximum allowed contamination levels in feed in many countries (van Egmond et al., 2007). In Europe, these levels have been defined by European Commission (EC) Regulations and Recommendations. The maximum levels set are influenced by several factors. One of the most important factors is species susceptibility. As mentioned, pigs are the most susceptible species for several major mycotoxins, including DON, ZON and OTA. Therefore the maximum levels in feed intended for pigs will be lower compared to less susceptible species, such as poultry. Maximum levels can also differ within one species. Piglets and gilts are more susceptible to the estrogenic effects of ZON, compared to sows and fattening pigs, resulting in lower maximum ZON contamination levels for those subcategories. Of course, the toxicity of the mycotoxin itself is a key determining factor. AFB1 has long been known to be acutely toxic and even carcinogenic upon chronic exposure, resulting in very low guidance values. Next to the toxicodynamic properties, the kinetic properties of mycotoxins play a role as well. Fumonisins for example have a very low oral bioavailability, ± 3.5% in pigs (Martinez-Larranaga et al., 1999), and consequently, only low concentrations reach the target tissues. Hence this allows higher contamination levels in feed. Maximum limits have been established for complete feed as well as for main feed materials (i.e. maize and cereals). In general, maximum set levels are higher for feed materials than for complete feedingstuffs. Nevertheless, it should be taken into account that when a component's composition in the daily ration of animals is higher than common practice, this should not lead to the animal being exposed to a higher level of these mycotoxins than normal.

EC Regulations for AFB1 in feed were established by Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed (European Commission, 2002). The guidance values for the presence of DON, ZON, OTA and fumonisins in products intended for animal feeding were determined in the Commission Recommendation of 17 August 2006 (European Commission, 2006). These limits are presented in Table 2.

In 2011, the European Food Safety Authority (EFSA) has determined a tolerable daily intake (TDI) for the sum of T-2 and its major metabolite, HT-2, of 100 ng/kg BW (EFSA, 2011a). Just

recently, the EC has made a proposition regarding these toxins in grains and complete feedingstuffs (European Commission, 2013) (Table 3). For the *Fusarium* mycotoxins, BEA and ENNs, which have recently become of interest, no maximum levels nor TDIs has been put forward. The EFSA is currently establishing its opinion on the risks to human and animal health related to the presence of BEA and ENNs in food and feed. The results are expected at the latest in September 2014.

A correct evaluation of mycotoxin contamination in food and feed is of utmost importance in determining the compliance with the acceptable safety standards. Because of the often highly heterogeneous distribution of mycotoxins in so-called 'hot-spots' in feed, the most critical stage is taking a representative sample. Appropriate sampling is essential to ensure that the analytically derived mean concentration of a sample is representative to the true mean concentration of a lot (Chaytor et al., 2011).

Table 2. The guidance values on the presence of deoxynivalenol, zearalenone, ochratoxin A and fumonisins in products intended for animal feeding as determined in the Commission Recommendation of 17 August 2006 (2006/576/EC), maximally allowed values on the presence of aflatoxin B1 in products intended for animal feeding as determined by EC Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 (2002/32/EC).

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12%
Deoxynivalenol	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
Zearalenone	Feed materials (*)	
	- Cereals and cereal products (**) with the exception of maize by-products	2

	- Maize by-products	3
	Complementary and complete feedingstuffs	
	- Complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
	- Complementary and complete feedingstuffs for sows and fattening pigs	0.25
	- Complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lambs) and goat (including kids)	0.5
Ochratoxin A	Feed materials (*)	
	- Cereals and cereal products (**)	0.25
	Complementary and complete feedingstuffs	
	- Complementary and complete feedingstuffs for pigs	0.05
	- Complementary and complete feedingstuffs for poultry	0.1
Fumonisin B1+B2	Feed materials (*)	
	- Maize and maize products (***)	60
	Complementary and complete feedingstuffs for:	
	- Pigs, horses (*Equidae*), rabbits and pet animals	5
	- Fish	10
	- Poultry, calves (<4 months), lambs and kids	20
	- Adult ruminants (>4 months) and mink	50
Aflatoxin B1	All feed materials (*)	0.02
	Complete feedingstuffs for cattle, sheep and goat with the exception of:	0.02
	- Complete feedingstuffs for dairy animals	0.005
	- Complete feedingstuffs for calves and lambs	0.01
	Complete feedingstuffs for pigs and poultry (except young animals)	0.02
	Other complete feedingstuffs	0.01
	Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs)	0.02
	Complementary feedingstuffs for pigs and poultry (except young animals)	0.02
	Other complementary feedingstuffs	0.005

(*) Particular attention has to be paid to cereals and cereal products fed directly to the animals that their use

in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration.

(**) The term 'Cereals and cereal products' includes not only the feed materials listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 on the circulation and use of feed materials (OJ L125, 23.5.1996, p. 35) but also under feed materials derived from cereals in particular cereal forages and roughages.

(***) The term 'Maize and maize products' includes not only the feed materials derived from maize listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in the Annex, part B of Directive 96/25/EC but also other feed materials derived from maize in particular maize forages and roughages.

Table 3. Indicative levels for the sum of T-2 and HT-2 toxin in cereals and cereal products, as determined in the Commission Recommendation of 27 March 2013 (2013/165/EC).

	Indicative levels for the sum of T-2 and HT-2 (µg/kg) from which onwards/above which investigations should be performed, certainly in case of repetitive findings (*)
1. Unprocessed cereals (**)	
1.1. barley (including malting barley) and maize	200
1.2. oats (with husk)	1000
1.3. wheat, rye and other cereals	100
2. Cereal products for feed and compound feed (***)	
2.1. oat milling products (husks)	2000
2.2. other cereal products	500
2.3. compound feed, with the exception of feed for cats	250

(*) The levels referred to in this Annex are indicative levels above which, certainly in the case of repetitive findings, investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin or on the effects of feed and food processing. The indicative levels are based on the occurrence data available in the EFSA database as presented in the EFSA opinion. The indicative levels are not feed and food safety levels.

(**) Unprocessed cereals are cereals which have not undergone any physical or thermal treatment other than drying, cleaning and sorting.

(***) The indicative levels for cereals and cereal products intended for feed and compound feed are relative to a feed with a moisture content of 12%.

4. Efficacy and safety testing of mycotoxin detoxifiers

In 2010, the EFSA panel on additives and products or substances used in animal feed (FEEDAP) released a scientific opinion on the establishment of guidelines for the assessment of additives from the functional group 'substances for reduction of the contamination of feed by mycotoxins' (EFSA, 2010). The main terms of reference were how to evaluate the efficacy of the additive at the existing maximum levels of mycotoxins (efficacy testing) and presence and characterization of possible interactions with nutrients, coccidiostats or veterinary medicinal products (safety testing).

At the time, several *in vitro* and *in vivo* models were already available to evaluate the efficacy and safety of mycotoxin detoxifiers. In this section, an overview of the different methods and models is given, in relation to EFSA's point of view.

4.1. Efficacy testing of mycotoxin detoxifiers

Methods used to evaluate the efficacy of mycotoxin detoxifiers range from simple *in vitro* tests to extensive *in vivo* investigations.

In vitro analysis is a powerful tool for screening potential mycotoxin detoxifiers. If a sequestering agent does not adsorb a mycotoxin *in vitro*, it has little or no chance to act *in vivo*. These laboratory techniques can be very useful in identifying and ranking potential mycotoxin detoxifiers and determining the mechanisms and conditions favorable for adsorption to occur (Lemke et al., 2001b). The experimental *in vitro* studies range from single-concentration studies to classical isotherm studies (binder concentration fixed, toxin concentration increasing) and beyond to more elaborate set-ups (gastro-intestinal tract models).

The single-concentration method is the simplest to perform and therefore most widely used. It measures adsorption of purified toxin preparations in aqueous medium, where a known amount of mycotoxin is added to a known amount of test product. The results are usually reported as '%ads', the fraction of toxin bound to the adsorbent.

When using adsorption isotherms, the amount of mycotoxin adsorbed per unit of weight is plotted against the concentration of mycotoxin in solution at a constant temperature and under stable conditions (Figure 8). This system takes into account that sequestering of

mycotoxins is a reversible process that can be characterized as a chemical equilibrium. The results from isotherm studies are usually interpreted by empirical models (such as Freundlich) based on the available dataset.
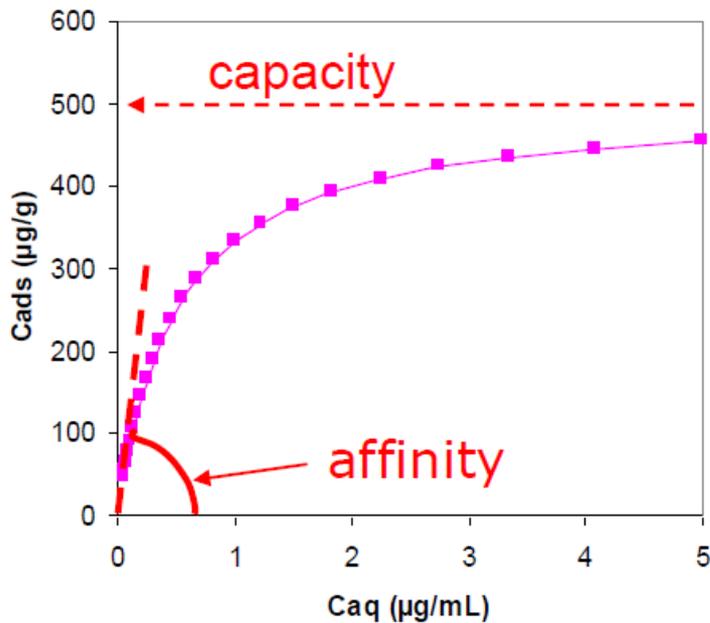
Figure 8. Ideal isotherm adsorption curve, showing the two regions of the curve mainly dominated by affinity and capacity (EFSA, 2009)

Next to these simple *in vitro* adsorption studies, more sophisticated models have been developed simulating the gastrointestinal tract. A 'dynamic gastrointestinal model' was developed by Minekus et al. (1995) and optimized by Avantaggiato et al. (2003, 2004). This so called TIM system (TNO Intestinal Model), compromises four compartments connected by peristaltic valves, simulating the kinetic digestive processes in respectively the stomach, duodenum, jejunum and ileum of humans and monogastric animals. Parameters include the secretion of saliva, gastric juice, pancreatic juice and bile for the simulation of realistic pH values, electrolyte concentrations, and digestive enzyme activities as well as body temperature and peristaltic movements for mixing and gastrointestinal transit. Hollow-fiber semi-permeable membranes are connected to the jejunum and ileum compartments for continuous dialysis of the digested and released compounds and absorption of water (Avantaggiato et al., 2007). Some other simulating models have been developed before, although they have not been applied to evaluate the binding or modifying capacity of mycotoxin detoxifiers. The Simulator of Human Intestinal Microbial Ecosystem (SHIME) is a validated dynamic model to study physicochemical, enzymatic and microbial parameters in

the gastrointestinal tract (Molly et al., 1993, 1994). The model consists of five reactors which sequentially simulate the stomach (acid conditions and pepsin digestion), small intestine (digestive processes) and the 3 regions of the large intestine, i.e. the ascending, transverse and descending colon (microbial processes). Control of the environmental parameters in these reactors allows to obtain complex and stable microbial communities which are highly similar in both structure and function to the microbial community in the different regions of the human colon. Recent evolutions of the SHIME involve TWINSHIME and SPIME. The TWINSHIME consists of two SHIME systems run in parallel, allowing direct comparison between two different conditions. The SPIME is similar to SHIME except for the microbiota, which is derived from pigs.

Although these dynamic systems resemble the *in vivo* situation far more than other available *in vitro* tests, they are costly and labor intensive which are compromising factors. Simple adsorption studies on the other hand, are too rudimentary to represent the actual *in vivo* situation. Therefore, there is a need for *in vitro* models which resemble the *in vivo* situation, but are still high-throughput in order to allow screening for potential mycotoxin detoxifiers in a timely manner.

In vitro studies can be used to select the most potent detoxifiers, however *in vivo* trials are still mandatory to fully proof the efficacy of the product (EFSA, 2009). Mycotoxin adsorption *in vivo* is complicated by physiological variables and the composition of feed, factors which are rarely accounted for *in vitro* (Lemke et al., 2001b).

Most of the *in vivo* studies on the efficacy of mycotoxin detoxifiers assess the effects of the products on recording so-called 'unspecific parameters' of the animals. These include:

- production parameters (growth rate, feed consumption, feed conversion ratio);
- organ weight, e.g. liver, kidney, intestine;
- serum biochemistry, e.g. total protein, albumin, globulins, glucose, uric acid, cholesterol, billirubin, phosphate, magnesium, calcium, iron, glutamate dehydrogenase, γ-glutamyl transferase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine kinase;
- hematology measurements, e.g. hematocrit, hemoglobin, red blood cell count;
- analysis of immunoglobulin (IgG, IgM and IgA) in serum and bile;

-   determination of antibody titers for an infectious disease after vaccinal challenge;

-   histological evaluation of liver, intestine or other target tissues;

When performing such experiments consisting of different animal groups fed different diets, a proper validated experimental design is mandatory. As unspecific parameters are not only influenced by the specific effect of the detoxifying agent, four different groups should be included in the trial. Namely, contaminated, contaminated with detoxifier, uncontaminated and uncontaminated with detoxifier. The latter is necessary to distinguish between specific and unspecific effects of the detoxifier (Figure 9) (Dänicke et al., 2007b; Döll and Dänicke, 2004).
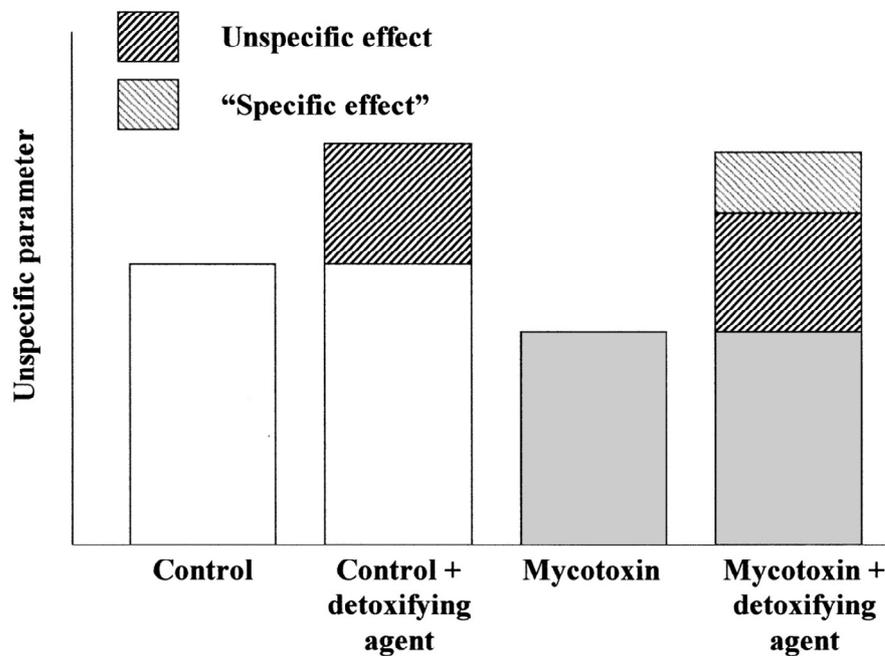
Figure 9. Investigation of the efficacy of mycotoxin detoxifiers using mycotoxin unspecific parameters (Döll and Dänicke, 2004)

In their scientific opinion, the EFSA states that unspecific parameters may be recorded although they are not sufficient to prove the efficacy of a detoxifier. The EFSA proposes other end-points, mainly based on specific toxicokinetic parameters (Table 3). These toxicokinetic parameters are derived from the ADME (absorption, distribution, metabolisation and excretion) approach. After oral intake, the target compound reaches the blood stream *via* uptake through the intestinal mucosa and submucosa (absorption). Next, it is distributed to different organs where it can take effect and/or be metabolized in other compounds, metabolites. These metabolites are generally more polar, facilitating their

excretion through bile or urine, and less toxic compared to the parent compound (some exceptions exist, such as AFB1). In general, mycotoxin/metabolites excretion in faeces/urine, concentration in blood/plasma/serum, tissues or products (milk or eggs) or other relevant biomarkers should be taken as end-points for demonstration the efficacy of mycotoxin detoxifiers. In order to do so, sensitive and specific bioanalytical techniques allowing the detection and quantification of mycotoxins in animal tissues are mandatory.

Table 3. Most relevant end-points for substances reducing the contamination of feed by mycotoxins (EFSA, 2010)

Mycotoxin(s) against which the additive is intended to act	Most relevant end-points
Aflatoxin B1	Aflatoxin M1 in milk/egg yolk
Deoxynivalenol (DON)	DON/metabolites in blood serum
Zearalenone (ZON)	ZON + α- and β-zearalenol in plasma
	Excretion of ZON/metabolites
Ochratoxin A (OTA)	OTA in kidney or blood serum
Fumonisin B1 + B2	Sphinganine/sphingosine ratio in blood, plasma or tissues

Regarding the *in vivo* efficacy trials, the EFSA proposed several other guidelines. A minimum of three *in vivo* studies showing significant effects should be provided to demonstrate efficacy at the lowest recommended dose. Those should be carried out at least at two different locations. Any extrapolation of data obtained with one animal species to other species is limited because of differences in intestinal mycotoxin absorption and potential mycotoxin degradation by the gastro-intestinal microbiota and different maximum mycotoxin contamination levels in feed. For additives intended to be used in all animal species except fish, studies should be performed in at least three major species, a poultry, a monogastric mammal and a ruminant. The mycotoxin content in feed used in studies should not exceed the values given in Commission Directive 2002/32/EC for AFB1 (European Commission, 2002) and in Commission Recommendation 2006/576/EC for DON, ZON, OTA and FB1+B2 for complete feedingstuffs for the respective animal species/category (European Commission, 2006). For mycotoxins without a maximum content established at EU level (e.g. BEA and ENNs), the dietary levels chosen should not exert adverse effects in the target

animals. As a source of mycotoxins, naturally contaminated feed materials are preferred. Alternatively, feed supplemented with mycotoxins could be used, if properly justified. However, because some mycotoxins regularly occur in nature associated with others, diets with more than one added mycotoxin may be used in the relevant studies. In any case, detailed analysis of mycotoxins present in feed should be provided for each trial. All these *in vivo* efficacy studies are considered short-term. Any measurement of end-points should not be started before metabolic steady-state of mycotoxin(s) in tissues/products is reached. In any case, the pre-sampling period should not be shorter than seven days. If long-term studies are performed, the sampling period (faeces and urine) should be at least five days. Blood samples should also be collected over a five-day period. Finally, tissues should be sampled without withdrawal of the mycotoxin from the diet.

4.2. Safety testing of mycotoxin detoxifiers

Not only requirements regarding the efficacy testing of mycotoxin detoxifiers have been put forward by the EFSA, but also in relation to possible negative effects of these additives.

For mycotoxin modifiers, the main concern is the toxicity of the mycotoxins' metabolites. As the mode of action of these products is modifying the chemical structure of mycotoxins, all degradation products should be identified. Next, analytical methods should be developed to determine these metabolites in the appropriate tissues. This would allow to characterize all metabolites formed, with special emphasis on the possible toxicity of the metabolites.

For mycotoxin binders, unspecific binding is the major consideration. Due to lack of specificity, mycotoxin binders may also adsorb nutrients, micronutrients and/or veterinary medicinal products. Very limited data are available on the interaction between mycotoxin binders and veterinary drugs. Aluminosilicates for example, have shown binding potential towards feed minerals, decreasing their utilization by the animal (EFSA, 2009). In 2011, EFSA's FEEDAP published a scientific opinion on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species (EFSA, 2011b). In this report it was stated that bentonite should not be mixed in feed concurrently with additives from the 'antibiotics', 'growth promoters', 'coccidiostats and other medical substances' groups, except in the case of monensin-sodium, narasin, lasalocid-sodium, flavophospholipol, salinomycin sodium and robenidine. Literature available on this topic is mainly based on

clinical symptoms, which are unspecific. For macrolide antibiotics for example, the Canadian Bureau of Veterinary Drugs reported that lack of efficiency was observed when tylosin was fed concurrently with bentonite clay to bovines (Canadian Bureau Of Veterinary Drugs, 1992). Shryock et al. (1994) reported that tilmicosin added to the diet in broiler chickens prevented development of airsacculitis after inoculation with *Mycoplasma gallisepticum*, whereas a combined use of tilmicosin and bentonite did not prevent air-sac lesions or clinical symptoms, indicating a possible negative interaction between the binder and the antibiotic.

Few studies used pharmacokinetic parameters to study the interaction between mycotoxin binders and the oral bioavailability of veterinary drugs. For instance, lowered plasma concentrations of lincomycin have been described when a clay-based mycotoxin binder was added to broiler feed (Amer, 2005). Recently, experiments were conducted to investigate possible interactions between mycotoxin detoxifiers and oral bioavailability of oxytetracycline and amoxicillin in broiler chickens and doxycycline in pigs.

After 28 days of feeding broiler chickens either uncontaminated feed or uncontaminated feed supplemented with one out of two evaluated mycotoxin detoxifiers, an oral bolus of either oxytetracycline (100 mg/kg BW) or amoxicillin (20 mg/kg BW) was administered (Osselaere et al., 2012). Remarkably, birds receiving feed supplemented with one of the mycotoxin detoxifiers showed higher area under the plasma concentration-time profile (AUC_{0-t}) and maximal plasma concentration (C_{max}) of oxytetracycline compared to the controls. Kidney tissue concentrations of oxytetracycline were also significantly increased related to addition of the mycotoxin detoxifier to the feed. These higher plasma and tissue concentrations could be due to the observed increase of villus height and villi absorption area caused by the detoxifier (Osselaere et al., 2013). Addition of the detoxifier also resulted in higher DON levels in the distal intestinal tract, causing damage to the intestinal tissues and consequently to an increased oral absorption of the antibiotic (Osselaere et al., 2013).

A more complex interaction between mycotoxins, mycotoxin binder and antibiotics was observed in pigs by Goossens et al. (2012). Animals were administered one of the following diets: control (uncontaminated), control + GMA, contaminated (0.1 mg T-2/kg) or contaminated + GMA. After one-week feeding, animals were administered a single oral bolus of either doxycycline (10 mg/kg BW) or paromomycin (100 mg/kg BW), both frequently used as mass medication practices in pig and poultry industry.

The group which received feed supplemented with T-2 and GMA showed a significantly higher AUC_{0-t} compared to the control group, but not to the other experimental groups. No significant differences in oral absorption of paromomycin were observed. A consecutive experiment was performed with the same experimental design but with DON contaminated feed (0.8 mg/kg, fed for 2 weeks). Again, higher plasma concentrations of doxycycline were observed in the contaminated + GMA group compared to the control, but not to the other groups. As stated above, addition of the binder could result in prolonged persistence of the toxins in the gastro-intestinal tract and consequently lead to promoted drug absorption. Similar to the previous experiment, no differences in oral absorption of paromomycin were demonstrated. These findings might be important for the withdrawal time of veterinary drug formulations containing doxycycline, and the occurrence of undesirable residues (above the Maximum Residue Limit, MRL) of the antibiotic in edible tissues. On the other hand, if the mycotoxin binder leads to increased plasma concentrations, this could, from a theoretical point of view, be compensated by reducing the dosage of the antibiotic.

There is an urgent need for *in vitro* and *in vivo* models for interaction testing of mycotoxin detoxifiers with veterinary medicinal products based on pharmacokinetic parameters, especially since some interactions between mycotoxin detoxifiers and oral bioavailability of veterinary drugs has been described.

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AIMS OF THE THESIS

Mycotoxins are considered a major issue worldwide because of their harmful effects on animals. This leads to great economic losses, especially in pig and poultry industry. The most commonly used method to counteract the negative impact of mycotoxins on animals is adding mycotoxin detoxifying agents (mycotoxin detoxifiers) to feed. These products, so-called mycotoxin binders or mycotoxin modifiers, either bind or biotransform mycotoxins in the gastrointestinal tract, respectively.

These products should be tested on their ability to bind or modify mycotoxins (efficacy) *in vitro* as well as *in vivo*. Also the possible interaction with oral absorption of veterinary drugs (safety) should be evaluated.

At the start of the research described in this thesis, no reliable *in vitro* models were available which are both high-throughput and capable of mimicking the gastrointestinal tract. The first goal was therefore to develop such an *in vitro* model able to evaluate the efficacy and safety of mycotoxin detoxifiers.

In vivo efficacy testing of mycotoxin detoxifiers is generally based on unspecific parameters, such as animal performance, blood biochemistry and/or histological changes in organs. As these parameters are unspecific and only indirectly proof the adsorbing or biotransforming effect of mycotoxin detoxifiers, a more direct approach should be followed, based on specific toxicokinetic parameters. At the time being, no *in vivo* models for efficacy testing of mycotoxin detoxifiers were available based on toxicokinetic parameters. In addition, to allow evaluating the effect of mycotoxin detoxifiers on oral bioavailability of mycotoxins, sensitive methods quantifying mycotoxins in animal tissues and body fluids were needed.

As mentioned, the possible interaction of mycotoxin binders with oral absorption of veterinary drugs is of great concern. Interactions between mycotoxin detoxifiers and several veterinary drugs (including lincomycin, tilmicosin, tylosin, doxycycline, oxytetracycline and amoxicillin) have already been described. However, no *in vitro* or *in vivo* models based on specific pharmacokinetic parameters were available to evaluate possible interactions.

The general objective of this research was to develop *in vitro* and *in vivo* models for testing the efficacy of mycotoxin detoxifiers and their possible interactions with oral absorption of veterinary drugs.

The specific aims of this research were as follows:

- To develop an *in vitro* model evaluating the efficacy and safety of mycotoxin detoxifiers. This model should be high-throughput and mimic the gastrointestinal tract (Chapter I).

- To develop sensitive and specific methods for the quantification of mycotoxins in plasma of pigs and poultry (Chapter II & III).

- To develop *in vivo* bolus models for efficacy and safety testing of mycotoxin detoxifiers based on specific toxico- and pharmacokinetic parameters, respectively (Chapter IV & V).

- Determine a possible correlation between specific and unspecific parameters for efficacy testing *in vivo* (Chapter VI).

EXPERIMENTAL STUDIES

CHAPTER I

An *in vitro* model using the IPEC-J2 cell line for efficacy and drug interaction testing of mycotoxin detoxifying agents

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Abstract - An *in vitro* model simulating the intestinal barrier for efficacy and drug interaction testing of mycotoxin detoxifying agents was developed using Transwell® cell culture inserts. Intestinal porcine epithelial cells derived from the jejunum of piglets were exposed to deoxynivalenol and a mycotoxin binder (efficacy testing) or exposed to tylosin and a mycotoxin binder (drug interaction testing). Active carbon and bentonite were used in the efficacy and drug interaction trials, respectively, to validate the developed model. The evaluated parameters were passage of deoxynivalenol and tylosin through the epithelial monolayer, the integrity of the monolayer by measurements of the trans-epithelial electrical resistance and the viability of the monolayer using the neutral red assay. In the efficacy model it was shown that active carbon effectively bound deoxynivalenol at both non-cytotoxic and cytotoxic concentrations of deoxynivalenol, respectively 0.5 and 1 µg/mL. Moreover, the negative effects of deoxynivalenol at cytotoxic concentrations on cellular viability and integrity were completely offset. A commercially available modified glucomannan binder was also tested and it was able to partly reduce the negative effects on these latter parameter. Moreover, it reduced the transepithelial passage of deoxynivalenol with 37 to 57% compared to active carbon, at both cytotoxic and non-cytotoxic concentrations of deoxynivalenol. In our drug interaction model, the interaction between tylosin and mycotoxin binders was investigated as some authors suggest binding of macrolide antibiotics to bentonite clays. Indeed, a bentonite clay showed decreased passage of tylosin through the epithelial monolayer, indicating binding of tylosin by bentonite. This indicates that the combined use of bentonite and tylosin in the feed could lead to therapy failure. The modified glucomannan binder did not alter the passage of tylosin significantly, indicating safe combined use.

Keywords – *in vitro* model **-** IPEC-J2 - mycotoxin binders - deoxynivalenol - tylosin

1. Introduction

The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production (Wu, 2007). Consequently, a variety of methods for the decontamination of feed have been developed, but mycotoxin detoxifying agents (mycotoxin detoxifiers) supplemented as feed additive seem to be the most promising and are therefore most commonly used (Kolosova and Stroka, 2011). The extensive use of these feed additives has led, in 2009, to the establishment of a new group of additives: 'substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption of mycotoxins (mycotoxin binders) or modify their mode of action (mycotoxin modifiers)' (European Commission, 2009).

Obviously, the binding or biotransforming ability of these feed additives should be examined. In addition, the binding products could affect the oral bioavailability of essential nutrients or interact with oral absorption of veterinary drugs. *In vivo* feeding trials are close to the situation in practice but they are cost and labor intensive. Therefore, *in vitro* analysis is a powerful tool as screening method to select the most potent mycotoxin detoxifiers. Basic models are single-concentration studies, where a known amount of test product is mixed in buffer solution with a known amount of mycotoxin, and adsorption isotherms, where the amount of mycotoxin adsorbed per unit of weight is plotted against the concentration of the mycotoxin (EFSA, 2009). These set-ups are very rudimentary and do not mimic the *in vivo* situation by far. The most complex set-up is the 'dynamic gastro-intestinal model' developed by Minekus et al. (1995) and optimized by Avantaggiato et al. (2004). Although it closely mimics the *in vivo* situation, it is labor intensive and cannot be used as a high-throughput screening method.

The European Food Safety Authority (EFSA) stipulates that the efficacy and safety of mycotoxin detoxifiers should be investigated (EFSA, 2010). At first their efficacy, i.e. whether they actually bind or biotransform mycotoxins, should be evaluated, but also their safety aspect. Mycotoxin binders could affect the oral bioavailability of essential nutrients or interact with oral drug absorption. However, the aim of this study was only to investigate the possible interactions with veterinary drugs. In veterinary medicine, and especially in mass medication applied in the pig and poultry industry, drugs are often administered through the

feed or drinking water. If a mycotoxin detoxifier decreases or enhances the oral absorption of drugs, this might have significant consequences for animal health. In the drug interaction model, the interaction between the macrolide antibiotic tylosin and a bentonite binder was investigated. The Canadian Bureau of Veterinary Drugs (1992) reported that there was insufficient efficacy of tylosin when fed concurrently with bentonite to bovines. Moreover, Schryock et al. (1994) showed that a diet supplemented with tilmicosin, structurally closely related to tylosin, reduced the symptoms caused by *Mycoplasma gallisepticum* in broilers. However, a diet containing tilmicosin together with bentonite could not prevent airsacculitis and clinical symptoms caused by *M. gallisepticum*. These interactions have recently been documented by the EFSA (EFSA, 2011), and led to a negative advise for concurrent administration of macrolide antibiotics and bentonite to animals.

2. Materials and methods

2.1. Cell culture conditions

The porcine jejunal intestinal epithelial cell line IPEC-J2 is a non-transformed cell line continuously maintained in cell culture. IPEC-J2 cells were cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 mixture (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 5% (v/v) Fetal Calf Serum (FCS) (Hyclone, Cramlington, England, UK), 1% (v/v) insulin-transferrin-Na selenite mixture (Gibco, Life Technologies, Paisley, Scotland, UK), 1% (v/v) penicillin-streptomycin (Gibco) and 1% (v/v) kanamycin (Gibco), further called cell medium. Cells were incubated at 37°C in ambient atmosphere with 5% CO_2.

2.2. Chemicals, veterinary drugs and mycotoxin binders

Deoxynivalenol (DON) (Fermentek, Jerusalem, Israel) used for the cell culture experiments was prepared in anhydrous methanol as a stock solution of 1 mg/mL and stored at -20°C. Serial dilutions were made in cell medium. The maximal final concentration of 0.1 % methanol was not found to be cytotoxic based on the neutral red assay.

The standard of DON, used for the analytical experiments, was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in acetonitrile (ACN) to obtain a stock solution of 1 mg/mL. Working solutions were used to prepare matrix-matched calibrators and quality

control samples in cell medium. These working solutions were prepared by mixing appropriate volumes of the stock solution with ACN and water (50/50, v/v), both of HPLC grade. The internal standard (IS) ($^{13}C_{15}$-DON, 25 µg/mL ACN) was obtained from Biopure (Tulln, Austria). A working solution of 1 µg/mL IS was prepared in ACN and water (50/50, v/v).

A stock solution of 1 mg/mL of tylosin (Tylan® Soluble, Elanco Animal Health, Beerse, Belgium) was used for the cell culture and analytical experiments and was prepared in water of HPLC quality (high performance liquid chromatography). Serial dilutions were made in cell medium. IPEC-J2 cells were exposed to 20 µg/mL tylosin which resembles the exposure of intestinal mucosa when used according to the leaflet. Gamithromycin, the IS for the analytical experiments was obtained from Merial (Brussels, Belgium) and was dissolved in ACN (1 µg/mL).

Active carbon (AC) (NORIT Carbomix®, KELA Pharma, Sint-Niklaas, Belgium), a modified glucomannan (GMA) (Mycosorb®, Alltech Inc., Lexington, KY, USA) and a bentonite clay (Toxisorb Classic®, Süd-Chemie, Moosburg, Germany) were suspended in cell medium at a concentration of 1 mg/mL.

2.3. Transepithelial passage assay in 24-well plate with Transwell® cell culture inserts

Cells were seeded in Transwell® inserts, with a collagen-coated membrane, a pore size of 0.4 µm and membrane diameter of 6.5 mm (Corning, NY, USA), at a density of 5.10^5/mL and cultured for 21 days until reaching confluence. Every three days, cell medium was refreshed.

In the efficacy trials, inserts were exposed to DON (0.5 µg/mL or 1 µg/mL) and/or AC or GMA (1 mg/mL). In the drug interaction trials, inserts were exposed to tylosin (20 µg/mL) and/or bentonite or GMA (1 mg/mL). Control inserts were only exposed to cell medium. Before adding DON, tylosin and/or mycotoxin binder to the wells, mixtures were first incubated during 90 min on a roller mixer (Stuart Scientific® 2, IMLAB, Boutersem, Belgium), in order to mimic the gastric passage. Next, 1 mL of the mixture was added to the basolateral chamber of the insert, whereas in the apical chamber 200 µL fresh cell medium was added. During the 48 h during trial, the 24-well plates were incubated on a shaker at 200 rpm (IKA® MTS 2/4

digital, Staufen, Germany) in order to prevent the binder to sediment. All experiments were conducted in triplicate with three repeats per experiment.

2.4. Measurement of Transepithelial Electrical Resistance (TEER)

To evaluate the integrity of the monolayer, TEER measurements were performed. During the 21-day grow-up, TEER was measured weekly to evaluate the confluence and viability of the monolayer. The measurement was performed using an epithelial volthommeter (EVOM) with Endohm chambers (World Precision Instruments, Sarasota, FL, USA) and expressed as kOhm/well. Only inserts with a TEER value > 1 kOhm/well, indicating confluence, were used. During the efficacy and drug interaction trials, TEER was measured just before (0 h) and 12, 24, 36 and 48 h after exposure.

2.5. Neutral Red assay (NR)

To assay the cytotoxicity after 48 h of exposure, cell viability was measured using the neutral red assay. Two hundred µL of freshly prepared neutral red medium (33 mg/L in DMEM without phenolred) was added to each insert, and incubated for 2 h in the dark at 37°C. Next, cells were washed three times with Hanks Buffered Saline Solution (HBSS) (Invitrogen Life Technologies). Afterwards, 200 µL of extraction solution (ethanol/water/acetic acid, 50/49/1, v/v/v) were added to the apical compartment of each insert. The plate was shaken for 10 min at 350 rpm, and 150 µL of the medium of each insert was transferred into a 96-well IWAKI microplate (Asashi Corp., Tokyo, Japan). The absorbance was measured at 540 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). Viability was calculated according to the following formula:

$$\% \text{ viability} = (a-b) / (c-b) \times 100$$

In this formula a: $OD_{540\ nm}$ derived from the inserts incubated with DON, tylosin and/or mycotoxin binder, b: $OD_{540\ nm}$ derived from blank inserts without cells, c: $OD_{540\ nm}$ derived from untreated control inserts.

2.6. LC-MS/MS analysis to determine transepithelial passage of DON and tylosin

To determine the passage of DON through the IPEC-J2 monolayer in the efficacy trials, 25 µL of the apical cell medium of each insert was taken at 12, 24, 36 and 48 h after the start of

the experiment, and replaced by fresh medium. Samples were stored at ≤ -15°C until analysis. The concentration of DON in these samples was determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. The samples (25 µL) were spiked with 25 µL working solution (1 µg/mL) of internal standard ($^{13}C_{15}$-DON) and 50 µL of ACN was added. Next, the samples were vortexed (15 sec) and centrifugated (10 min, 8517 x *g*, 4°C). The supernatant was then evaporated using a gentle nitrogen stream (40 ± 5°C). The dry residue was reconstituted in 100 µL water of HPLC grade. After vortex mixing, the samples were transferred into an autosampler vial and an aliquot (10 µL) was injected onto the LC-MS/MS instrument.

To determine the passage of tylosin through the IPEC-J2 monolayer in the drug interaction trials, 25 µL of the apical cell medium of each insert was taken at 12, 24, 36 and 48 h after the start of the experiment, and replaced by fresh medium. Samples were stored at ≤ -15°C until analysis. The concentration of tylosin in these samples was determined using a validated LC-MS/MS method. The samples (25 µL) were diluted in 200 µL water/ACN of HPLC grade (50/50, v/v) after spiking with 25 µL of a working solution of the internal standard (gamithromycin, 1 µg/mL). Next, samples were vortexed (15 sec) and centrifugated (8517 x *g*, 10 min, 4°C) and an aliquot was transferred to an autosampler vial and injected (5 µL) onto the LC-MS/MS instrument.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MS pump Plus and an autosampler, type Autosampler Plus, both from Thermo Fisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type, both from Thermo Fisher Scientific (Louvain-la-Neuve, Belgium). For DON analysis, the mobile phase A consisted of 0.1 % glacial acetic acid in water of UHPLC quality (ultra-high performance liquid chromatography). Mobile phase B consisted of methanol of UHPLC quality. A gradient elution was performed: 0-1 min (95% A/5% B), 4 min (linear gradient to 80% B), 4-5.1 min (20% A/80% B), 5.6 min (linear gradient to 95% A), 5.6-8 min (95% A/5% B). For tylosin, the mobile phase A consisted of 10 mM ammonium acetate in water of UHPLC quality, acidified to pH 3.5 with glacial acetic acid. Mobile phase B was ACN of UHPLC quality. A gradient elution was performed: 0-0.5 min (80% A/20% B), 0.5-1 min

(linear gradient to 90% B), 1-4.5 min (10% A/90% B), 4.5-5 min (linear gradient to 80% A), 5-8 min (80% A/20% B).

For both analytical experiments, the flow rate was 300 µL/min. The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe (Thermo Fisher Scientific), operating in the negative ionization mode for DON and in the positive ionization mode for tylosin. Following selected reaction monitoring (SRM) transitions were monitored and used for identification and quantification (*): for DON m/z 355.1 > 265.1* and 355.1 > 295.1, for $^{13}C_{15}$-DON m/z 370.1 > 279.1* and 370.1 > 310.1, for gamithromycin m/z 777.5 > 157.8 and 777.5 > 619.3* and for tylosin m/z 916.5 > 173.8* and 916.5 > 772.4.

The method was validated according to an in-house protocol compliant with the recommendations and guidelines of the European Community and FDA (European Commission, 2002; Heitzman, 1994; VICH GL 49, 2009). Calibration curve samples (range 5-1000 ng/mL for DON and 10-20000 ng/mL for tylosin) were prepared by applying standard working solutions directly into IPEC-J2 cell medium, followed by a vortex mixing step. After 5 min equilibration, the calibration curve samples were treated in a similar way as the unknown samples. The correlation coefficients (r) and goodness-of-fit coefficients (g) had to be ≥ 0.99 and ≤ 20%, respectively. The values of r and g for DON were 0.9999 and 4.22%, respectively. For tylosin values of r and g were 0.9983 and 9.58%, respectively. Within-day precision and accuracy were determined by analyzing six blank samples spiked at low (10 ng/mL for DON and 200 ng/mL for tylosin) and high (500 ng/mL for DON and 2000 ng/mL for tylosin) concentration levels on the same day. The between-day precision and accuracy was determined by analyzing quality control samples (10 and 500 ng/mL for DON and 200 and 2000 ng/mL for tylosin) together with each analytical batch of samples, run on three different days (n=12). The acceptance criteria for accuracy were -20% to +10%. Within-run precision (relative standard deviation, RSD) and accuracy for samples spiked with DON at 10 ng/mL were 5.8% and 0.4%, respectively, and for samples spiked at 500 ng/mL were 0.4% and 0.1%, respectively. Between-run precision and accuracy was 9.6% and -3.2% and 0.4% and 0.2%, respectively. Within-run precision and accuracy for samples spiked with tylosin at 200 and 2000 ng/mL were 3.5% and -5.0% and 7.5% and -1.3%, respectively. Between-run precision and accuracy for samples spiked with tylosin at 200 and 2000 ng/mL were 7.4%

and -1.5%, 8.2% and -0.3%, respectively. The limit of quantification (LOQ) was the lowest concentration of the analytes for which the method was validated with an accuracy and precision that fell within the recommended ranges. The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing six spiked samples and was for DON and tylosin 5 and 10 ng/mL, respectively. The limit of detection (LOD) was defined as the lowest concentration of the respective analytes that could be recognized by the detector with a signal-to-noise (S/N) ratio of ≥3. The LOD values were calculated using samples spiked at the LOQ level. The LOD of DON and tylosin was 0.3 and 0.9 ng/mL, respectively. The specificity of the method was evaluated with respect to interferences from endogenous compounds. Therefore, one blank sample was analyzed using the above mentioned procedure. The S/N ratio of a possible interfering peak in the blank sample had to be below the S/N ratio of the analyt in the same elution zone at the LOD level. No interfering peak was found in the blank sample. The carry-over was evaluated by analyzing a water sample just after the highest calibrator sample. The eventual analyte concentration in the sample had to be below the LOD. No peak was found in the water sample above the LOD. Recovery experiments were performed according to Matuszewski et al. (2003). In summary, two types of matrix-matched calibration curves were prepared for each analyte in IPEC-J2 medium, namely by spiking the blank calibrator samples before (spiked) and after extraction (spiked extract). One calibration curve was prepared using standard solutions. The slopes of the resulting linear, $1/x$ weighted spiked calibration curves (spiked and spiked extracts) were compared with the related slope of the calibration curve with standard solutions in order to calculate the recovery of the extraction step (R_E) and the signal suppression/enhancement (SSE) due to matrix effects. R_E and SSE for DON in IPEC-J2 medium were 90.5 ± 11.6% and 108.4 ± 12.9%, respectively. For tylosin, R_E and SSE were 87.2 ± 9.8% and 111.7 ± 16.3%, respectively.

2.7. Statistical analysis

All experiments were conducted in triplicate (independent replicates) with three repeats per experiment (technical replicates). For each experiment, means of the technical replicates were calculated. These means (independent replicates) were analyzed using one-way ANOVA after determination of normality and homogeneity of variances (SPSS 19.0, IBM, Chicago, IL, USA). All data are expressed as mean (+ or ± SEM) of the three independent

replicates. Significant differences between treated inserts and controls are indicated by a different superscript.

3. Results

3.1. Efficacy testing

3.1.1. IPEC-J2 cellular viability

The viability of IPEC-J2 cells after 48 h exposure to DON at 0.5 and 1 µg/mL and with or without mycotoxin binder (GMA or AC) is shown in Table 1. A concentration of 0.5 µg/mL DON was not cytotoxic for IPEC-J2 cells, whereas 1 µg/mL induced cell death. AC completely counteracted the cytotoxic effect of 1 µg/mL DON. GMA partially (57%) alleviated the cytotoxic effect of DON.

Table 1. Cellular viability of IPEC-J2 cells after 48 h of exposure to 0.5 or 1 µg/mL DON with or without mycotoxin binder (1 mg/mL; glucomannan or GMA, active carbon or AC).

Cellular Viability (%)		DON concentration (µg/mL)		
		0	0.5	1
Mycotoxin binder	/	100.0 ± 1.44^a	99.8 ± 1.12^a	31.4 ± 0.94^b
	AC	106.3 ± 2.63^a	98.5 ± 2.04^a	102.0 ± 3.12^a
	GMA	114.4 ± 1.97^a	107.3 ± 1.43^a	69.4 ± 1.87^c

Results represent the means of three independent experiments conducted in triplicate and their SEM. Values with a different superscript are statistically different at $p < 0.001$

3.1.2. Transepithelial passage of DON

The concentration of DON in the apical compartment after addition of DON at a non-cytotoxic, 0.5 µg/mL, or cytotoxic concentration, 1 µg/mL, to the basolateral compartment with or without mycotoxin binder (AC or GMA) is shown in Figure 1. AC diminished the transepithelial passage of DON almost completely (>99%) at 0.5 and 1 µg/mL DON. GMA was able to lower the passage at both DON concentrations, but to a lesser extent than AC (37-57%).

(A)
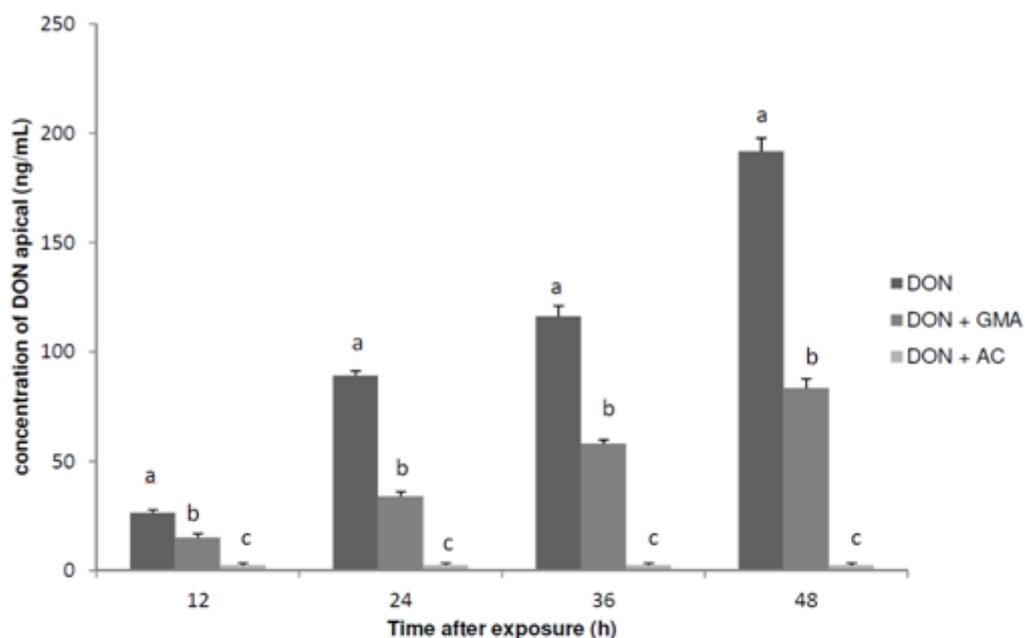
(B)
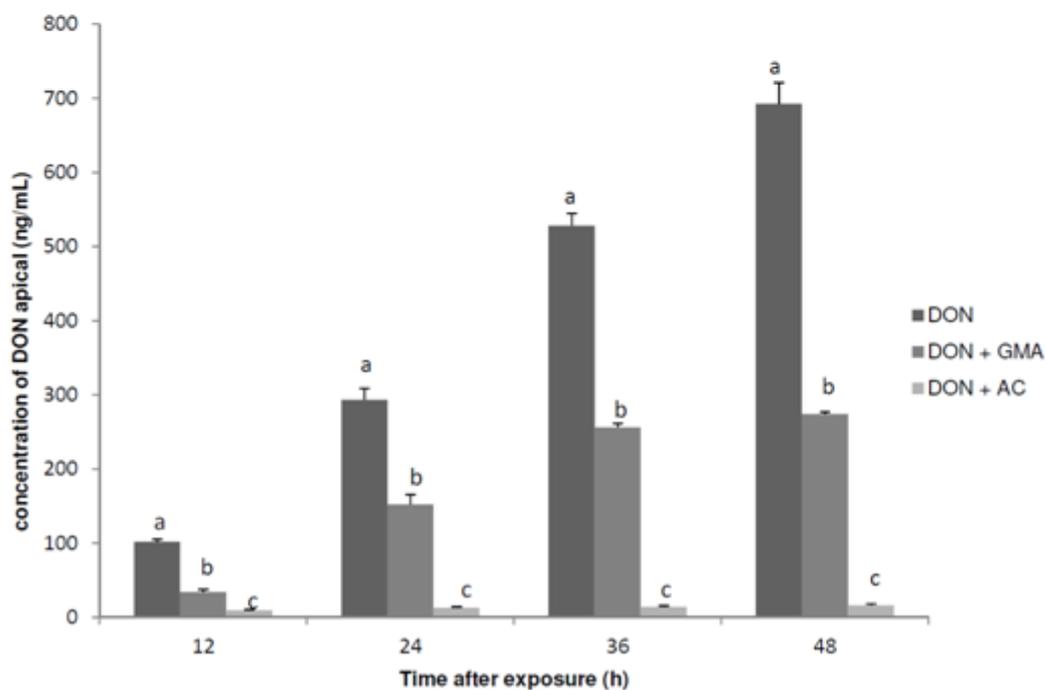
Figure 1. DON passage in the apical compartment after basolateral exposure of IPEC-J2 cells to 0.5 or 1 µg/mL DON ((A) and (B) respectively) with or without mycotoxin binder (1 mg/mL; glucomannan or GMA, active carbon or AC). For each data point, the mean and SEM are shown for three independent replicates, each independent replicate consisting of the mean of three technical replicates. Values with a different superscript within one time point are statistically different at p < 0.001

3.1.3. IPEC-J2 monolayer integrity

TEER measurements of the monolayer were performed before (0 h) and after 12, 24, 36 and 48 h addition of DON (1 µg/mL or 0.5 µg/mL) and/or mycotoxin binder (AC or GMA). The results of TEER measurements after exposure of IPEC-J2 cells to 0.5 and 1 µg/mL DON are shown in Figure 2. A concentration of 1 µg/mL DON decreased the TEER of IPEC-J2 cells. When AC was added, no decrease of TEER was observed, even at cytotoxic concentrations. GMA could partially (65%) prevent the decrease in TEER at 1 µg/mL DON.

(A)
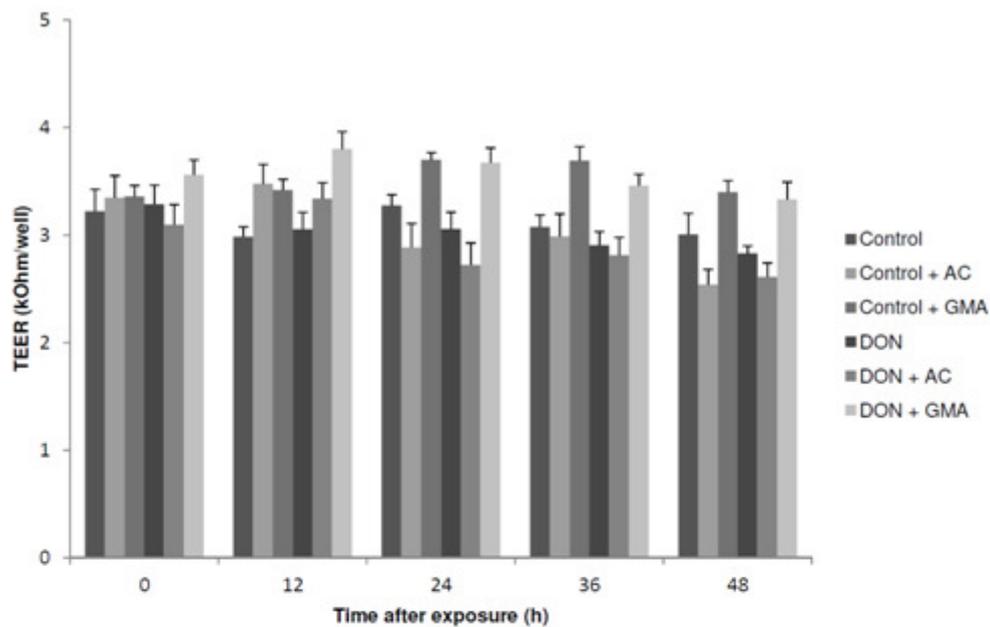
(B)
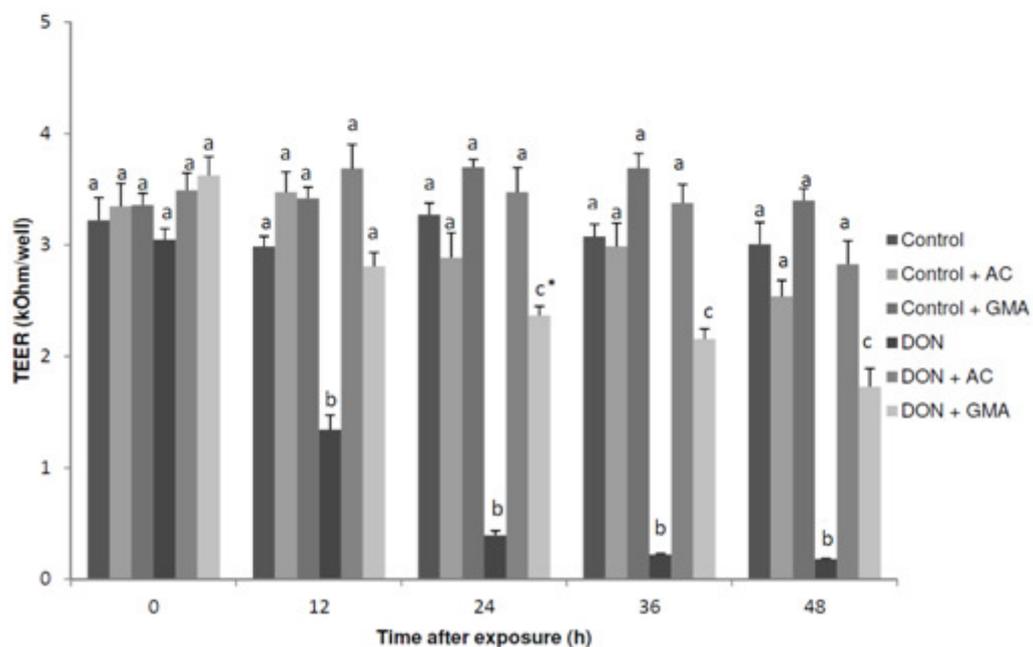
Figure 2. TEER of IPEC-J2 cells on different time points after exposure to 0.5 or 1 µg/mL DON ((a) and (b) respectively) with or without mycotoxin binder (1 mg/mL; glucomannan or GMA, active carbon or AC). For each data point, the mean and SEM are shown for three independent replicates, each independent replicate consisting of the mean of three technical replicates. Values with a different superscript within one time point are statistically different at $p < 0.001$. * indicates a significant difference of $0.01 < p < 0.05$ compared to control inserts

3.2. Drug interaction testing

3.2.1. IPEC-J2 cellular viability

Viability of IPEC-J2 cells after 48 h exposure to 20 µg/mL tylosin with or without mycotoxin binder (GMA or bentonite) was evaluated. No significant differences on cellular viability between different conditions was observed.

3.2.2. Transepithelial passage of tylosin

The concentration of tylosin in the apical compartment after addition of tylosin (20 µg/mL) to the basolateral compartment with or without mycotoxin binder (GMA or bentonite) is shown in Figure 3. Bentonite significantly reduced the transepithelial passage of tylosin (92%), whereas GMA did not.
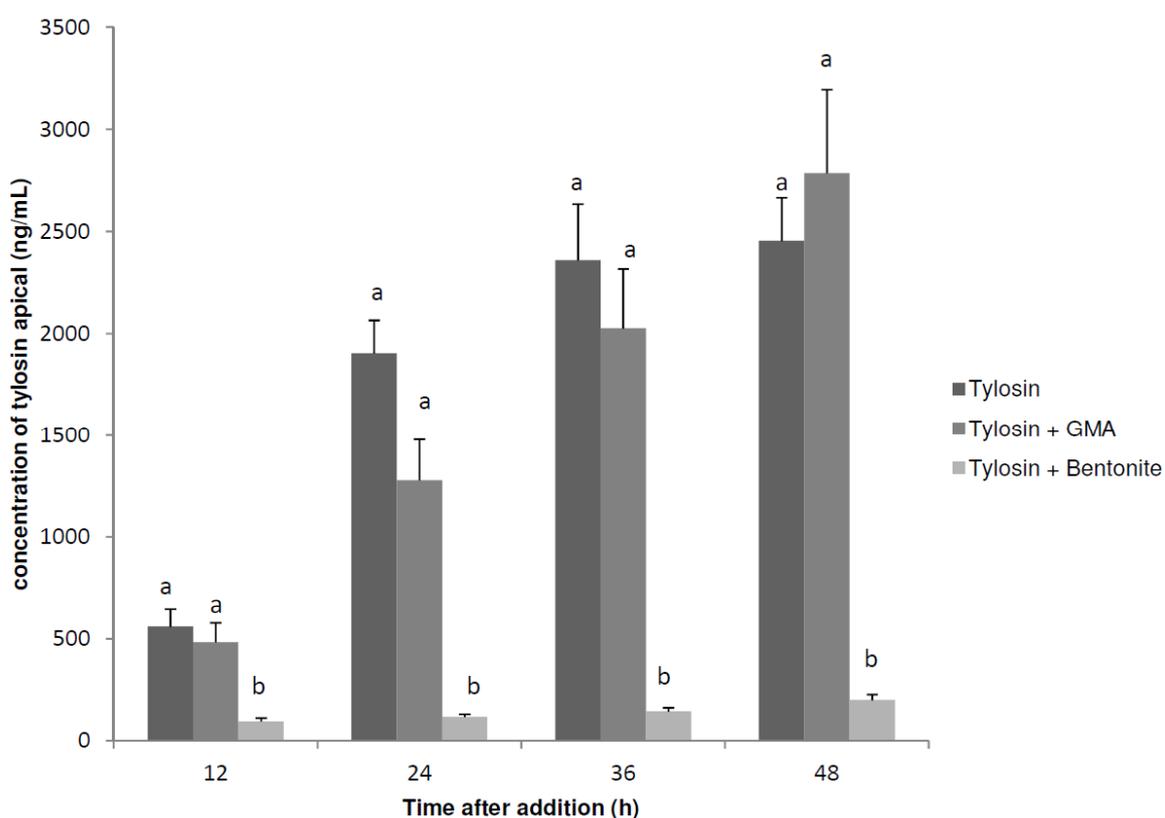
Figure 3. Tylosin passage in the apical compartment after basolateral exposure of IPEC-J2 cells to 20 µg/mL tylosin with or without mycotoxin binder (1 mg/mL; glucomannan or GMA, bentonite). For each data point, the mean and SEM are shown for three independent replicates, each independent replicate consisting of the mean of three technical replicates. Values with a different superscript within one time point are statistically different at $p < 0.001$

3.2.3. IPEC-J2 monolayer integrity

TEER measurements of the IPEC-J2 monolayer were performed before (0 h) and after 12, 24, 36 and 48 h addition of 20 µg/mL tylosin and/or mycotoxin binder (GMA or bentonite). No significant differences in TEER between the conditions was observed.

4. Discussion

The goal of this study was to develop an *in vitro* model for efficacy and drug interaction testing of mycotoxin detoxifiers. The model had to be high-throughput, in order to be useful as a screening method, but still reliable and more representative for the *in vivo* situation compared with single-concentration studies and adsorption isotherms.

Transwell® cell culture inserts with an intestinal epithelial cell monolayer allow the evaluation of the passage of mycotoxins and veterinary drugs through the monolayer and the effects on cellular viability and integrity (Dänicke et al., 2010; Diesing et al., 2011a; Diesing et al., 2011b). The collagen coated inserts are deemed to be most suited for *in vitro* research with IPEC-J2 (Geens and Niewold, 2011). At first, an efficacy model was developed using these inserts. Deoxynivalenol (DON) was used as it is the most common mycotoxin found in European commodities (Monbaliu et al., 2010). Pigs are the most susceptible to DON and therefore an intestinal porcine epithelial cell line derived from the jejunum of piglets (IPEC-J2 cell line) was used. Enterocytes are especially exposed to the effects of DON as this mycotoxin is almost completely absorbed in the proximal intestine in pigs (Dänicke et al., 2010; Eriksen et al., 2003). The intestinal epithelium is characterized by adherent and tight junctions, which restrict paracellular transport to small hydrophilic molecules. It was shown that DON disturbs epithelial cell layer integrity of IPEC-J2 cells (Diesing et al., 2011a; Sergent et al., 2006), leading to an increased paracellular passage of DON. Using this model, the absorption of mycotoxins through the monolayer, in the presence or absence of mycotoxin detoxifiers, can be evaluated. Two mycotoxin binders were tested, namely activated charcoal (AC) as positive control and modified glucomannan (GMA) as a commercially available binder. Suspensions of toxin and/or mycotoxin binder were first incubated on a roller mixer for 90 min to mimic the passage through the stomach. Moreover, pre-incubating the mycotoxin with the binder enhances the binding potency (Cavret et al., 2010). In contrast with Cavret et al. (2010), the mycotoxin binders were added

to the basolateral chamber of the Transwell® because addition to the apical chamber leads to damage of the epithelial cells due to friction and sinking of the particles (data not shown). This can be avoided by adding the binder to the basolateral chamber. However, this is not the physiological *in vivo* situation, where absorption through gut epithelia occurs from apical to basolateral. On the other hand, the DON absorption rate through the intestinal epithelial monolayer is independent of the route of application (Sergent et al., 2006). The same DON absorption rate from apical to basolateral as from basolateral to apical was observed in an intestinal epithelial model, because DON is absorbed through simple passive diffusion (paracellular transport) (Awad et al., 2007; Sergent et al., 2006). Therefore this route of application does not alter the realisticness of the model. What does depend of the route of application is the resistance of IPEC-J2 towards DON cytotoxicity (Diesing et al., 2011a). When added to the apical chamber, IPEC-J2 cells are more resistant than exposed from the basolateral side. Diesing et al. (2011a) demonstrated that 2 µg/mL of DON, added to the basolateral compartment, is cytotoxic for IPEC-J2 cells, whereas 0.5 µg/mL is not. We were able to reproduce these findings and refined them, as 1 µg/mL was also found to be cytotoxic. Both concentrations were used to evaluate the binding ability of mycotoxin binders at non-cytotoxic and cytotoxic concentrations. The passage was evaluated by measuring the concentrations of DON in the apical chamber (Figure 1). All inserts showed a passage increasing with time, suggesting adsorption mechanisms dependent on a thermodynamic equilibrium function of DON concentration in medium. The trend shown at the four different time points were the same (Figure 1). To even increase the high-throughput character of the model, time-points at 36 and 48 h can be excluded. AC was able to completely inhibit the passage through the monolayer at both DON concentrations, indicating complete adsorption of DON by AC. A lower binding capacity of GMA compared with AC was expected as AC was used as positive control and is preferably avoided in practice due to its non-specific binding (Avantaggiato et al., 2004; Ramos et al., 1996). The GMA added was able to decrease passage through the monolayer due to binding. This interaction is based on hydrogen bonds and Van der Waals forces between hydroxyl, ketone and lactone groups and β-1,3 and β-1,6 glucans (Yiannikouris et al., 2006). Other evaluated parameters in our model were cellular viability, using a NR assay, and cellular integrity, based on TEER measurements. DON can induce apoptotic and necrotic cell death of IPEC-J2 cells (Goossens et al., 2012), lowering the neutral red uptake by the lysosomes which is an

indicator for cell viability (Diesing et al., 2011b). As a part of cellular integrity, the loss of barrier function induced by DON is evoked by the decreased expression of claudin-4 protein, based on a MAPK-dependent mechanism (Pinton et al., 2010). No decrease in cellular viability and integrity was observed when AC was added, even at cytotoxic DON concentrations, confirming the complete adsorption of DON by AC. The cytotoxic effect of 1 µg/mL DON on IPEC-J2 cells is also important regarding the basolateral to apical passage of DON with and without binder. When no binder is present, the passage from basolateral to apical depends not only on absorption but mainly on diffusion due to tight junction damage and loss of viable cells. When DON is added with a mycotoxin binder two effects are involved. The direct effect is that the mycotoxin binder binds DON, leaving less free DON available for passage to the apical compartment. The indirect effect is that the cellular viability and integrity of the IPEC-J2 monolayer is less affected (Table 1 and Figure 2b), and an intact monolayer will also lower the passage to the apical compartment compared to a disrupted monolayer. When we look at the passage of DON in combination with GMA, Figure 1b, the concentration of DON in the apical compartment is around 50% or less compared to inserts treated with DON only. Based on the cytotoxicity and TEER measurements a passage of ≥ 50% would be expected. In essence, the fact that GMA only partly decreases the negative effects of cytotoxic DON concentrations, indicates that the bounded amount of DON is lower than but close to 50%, because 1 µg/mL DON severely affects cellular viability and monolayer integrity, whereas 0.5 µg/mL does not. As stated, the lower (≤ 50%) DON passage is due to the fact that GMA partly protects monolayer integrity and cellular viability, so the indirect binding effects play a role.

The interaction of mycotoxin binders with drugs, as a part of safety testing, was determined similarly as the efficacy model by using tylosin instead of DON. Tylosin was added to the basolateral chamber at a concentration of 20 µg/mL, corresponding with the exposure of epithelial gut cells when tylosin is used according to the leaflet. None of the conditions altered the cellular viability or integrity (data not shown), which was expected as in practice tylosin should not evoke cytotoxic effects on intestinal cells. To evaluate the tylosin binding of a bentonite clay and GMA, the passage through the epithelial monolayer was evaluated. Again, a similar trend was shown at all time points, therefore it is sufficient to evaluate the passage up to 24 h. Bentonite significantly reduced the tylosin passage and therefore binds

tylosin. These data now proof the suggestions made by other authors (Canadian Bureau of Veterinary Drugs, 1992; Shryock et al., 1994). Therefore, a combined use of bentonite in the feed with tylosin in the feed or drinking water, should be avoided as this could lead to therapy failure and eventually enhanced antibiotic resistance towards tylosin due to subtherapeutic plasma concentrations. With the proposed *in vitro* model, no interaction of tylosin with GMA was observed, indicating the safe combined use of tylosin and GMA.

In vitro models for efficacy and drug interaction testing should be used as a screening method in order to select the most potent mycotoxin detoxifiers and to determine significant interactions between detoxifiers and veterinary drugs. However, the EFSA stipulates that next to these *in vitro* trials, *in vivo* trials should be performed to fully prove the efficacy or potential interactions with veterinary drugs of mycotoxin detoxifiers (Devreese et al., 2012; EFSA, 2010). Nevertheless, *in vivo* trials are labor and cost intensive which emphasizes the need for high-throughput *in vitro* methods.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

6. Acknowledgements

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CHAPTER II

Quantitative determination of several toxicological important mycotoxins in pig plasma using multi-mycotoxin and analyte-specific high performance liquid chromatography-tandem mass spectrometric methods

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Abstract - A sensitive and reliable multi-mycotoxin method was developed for the identification and quantification of several toxicological important mycotoxins such as deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZON), zearalanone (ZAN), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1) in pig plasma using liquid chromatography combined with heated electrospray ionization triple quadrupole tandem mass spectrometry (LC-h-ESI-MS/MS). Sample clean-up consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in water/methanol (85/15, v/v). Each plasma sample was analyzed twice, i.e. once in the ESI + and ESI - mode, respectively. This method can be used for the assessment of animal exposure to mycotoxins and in the diagnosis of mycotoxicosis. For the performance of toxicokinetic studies with individual mycotoxins, highly sensitive analyte-specific LC-MS/MS methods were developed.

The multi-mycotoxin and analyte-specific methods were in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9974 - 0.9999 and 2.4% - 15.5%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification for the multi-mycotoxin and analyte-specific methods ranged from 2 - 10 ng/mL and 0.5 - 5 ng/mL, respectively, whereas limits of detection fell between 0.01 - 0.52 ng/mL and <0.01 - 0.15 ng/mL, respectively.

Keywords - multi-mycotoxin analysis – LC-ESI-MS/MS – pig plasma – validation – toxicokinetics

1. Introduction

Mycotoxins are secondary metabolites produced by different fungal species. Over 400 mycotoxins have been identified, although only a few of them present a significant toxic effect and are of major concern. These include deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZON), ochratoxin A (OTA), aflatoxin B1 (AFB1) and fumonisin B1 (FB1) (Lattanzio et al., 2011). The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production (Wu, 2007), especially for pigs as they are known to be one of the most susceptible species for the stated mycotoxins.

Diagnosing mycotoxicosis in animals and assessing animal exposure to mycotoxins is usually performed by analysis of commodities and feed. However, several disadvantages are present when estimating mycotoxin intake as such. In particular, no measurement of exposure at the individual level is possible, the variability of feed contamination is high and it does not provide a good dose response and specificity for the target mycotoxin. This can be overcome by measuring specific biomarkers or target compounds in body fluids, e.g. plasma or urine. Measuring the target compound in blood is also the most appropriate way for other purposes, including toxicokinetic studies with mycotoxins and evaluation of the efficacy of mycotoxin reduction strategies. Adding mycotoxin detoxifying agents (mycotoxin detoxifiers) to feed contaminated with mycotoxins is commonly used and seems to be the most promising way of counteracting the harmful effects of mycotoxins in animals (Kolosova and Stroka, 2011). Manufacturers have to prove the efficacy of these products, which is generally based on *in vitro* adsorption studies. However, the European Food Safety Authority (EFSA) has recently proposed guidelines for efficacy testing of mycotoxin detoxifiers (EFSA, 2010). In their guidelines, it is stated that *in vivo* efficacy trials should be performed based on absorption, distribution, metabolisation and excretion (ADME) studies. Devreese et al. (2012) has recently developed *in vivo* models for efficacy testing of mycotoxin detoxifiers based on these guidelines.

It is obvious that for assessing animal exposure to mycotoxins (screening) on the one hand and the investigation of mycotoxin toxicokinetics on the other hand, the availability of sensitive and specific validated analytical methods is mandatory. In the last decades, high-performance liquid chromatography (HPLC) has become the most important method for the analysis of mycotoxins in food, feed and other matrices. The mass spectrometer is a reliable detector for HPLC and it is an important analytical tool for routine analysis of mycotoxins in

complex matrices because of unambiguous identification and accurate quantification (Lattanzio et al., 2011). A huge amount of literature on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of several mycotoxins in food and feed is available (Malik et al., 2010; Songsermsakul and Razzazi-Fazeli, 2008), however, analytical methods for detection of mycotoxins in biological matrices, such as blood, are scarce. In addition, most methods are analyte-specific, which means that they allow the detection and/or quantification of only a limited number of mycotoxins at the same time. Until now, no multi-mycotoxin method for the analysis of plasma has been reported.

Therefore, the goal of this study was to develop such methods for the simultaneous determination and quantification of several toxicological important mycotoxins in animal plasma. The final methods should be applicable in two areas of expertise, i.e. screening for animal mycotoxin exposure and evaluation of the toxicokinetics of individual mycotoxins for the efficacy testing of mycotoxin detoxifiers. Since a large amount of samples had to be analyzed, special attention has been paid to the development of a high-throughput sample preparation procedure and LC-MS/MS analysis, but not at the expense of reliability and sensitivity.

2. Materials and Methods

2.1. Chemicals, products and reagents

The analytical standards of ZON, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, HT-2 and DOM-1 were obtained from Sigma-Aldrich (Bornem, Belgium). Standards of DON, T-2, OTA, FB1 and AFB1 were obtained from Fermentek (Jerusalem, Israel). Internal standards (IS) ($^{13}C_{15}$-DON, $^{13}C_{24}$-T-2, $^{13}C_{18}$-ZON, $^{13}C_{20}$-OTA, $^{13}C_{34}$-FB1, $^{13}C_{17}$-AFB1) were purchased from Biopure (Tulln, Austria). All standards were stored at ≤ -15°C. Water, methanol and acetonitrile (ACN) were of LC-MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, ethyl acetate and glacial acetic acid were of analytical grade and were obtained from VWR (Leuven, Belgium). Millex®-GV-PVDF filter units (0.22 µm) were obtained from Millipore (Brussels, Belgium).

2.2. Preparation of standard solutions

Standard stock solutions of DON, T-2, HT-2, ZON, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, T-2, OTA, FB1 and AFB1 were prepared in acetonitrile (analyte concentration: 1 mg/mL). Following standards were purchased as solutions: DOM-1 (50 µg/mL ACN), $^{13}C_{17}$-AFB1 (10 µg/mL ACN), $^{13}C_{15}$-DON, $^{13}C_{24}$-T-2, $^{13}C_{18}$-ZON, $^{13}C_{20}$-OTA and $^{13}C_{34}$-FB1 (all 25 µg/mL ACN). The stock solutions were stored at ≤ -15°C.

Working solutions of 100 µg/mL were prepared in ACN/water (50/50, v/v). Serial dilutions of separate working solutions were prepared to achieve analyte concentrations of 10, 1, 0.1 and 0.01 µg/mL. A combined working solution of 10 µg/mL of all analytes (except IS) was prepared by transferring 10 µL of each stock solution of 1 mg/mL and 200 µL of the stock solution of DOM-1 (50 µg/ml) to an eppendorf cup, followed by further dilution with ACN/water (50/50, v/v) up to a final volume of 1 mL. By appropriate dilution of this solution with ACN/water (50/50, v/v), combined working solutions of 1, 0.1 and 0.01 µg/ml were obtained. For the internal standards, individual and combined working solutions of 1 µg/mL were prepared in ACN/water (50/50, v/v). All working solutions were stored at 2-8°C.

2.3. Biological samples

Blank plasma samples were obtained from pigs (19.4 ± 1.8 kg BW) that received blank feed for a one week acclimatization period. The blank plasma samples were used for the preparation of matrix-matched calibrator and quality control (QC) samples.

After the acclimatization period, a bolus toxicokinetic study was performed. Six pigs received a single oral bolus of the following mycotoxins (Fermentek, Jerusalem, Israel): DON, T-2, ZON, OTA and AFB1 (all 0.05 mg/kg BW). Blood samples were drawn before (0 min) and at 15, 30 and 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h post-administration. Blood samples were taken in heparinized tubes and centrifugated (2851 x *g*, 10 min, 4°C). Aliquots (250 µL) of plasma samples were stored at ≤ -15°C until analysis.

This animal experiment was approved by the Ethical Committee of Ghent University (case number EC 2012_08).

2.4. Sample pretreatment

To 250 µL of plasma were added 12.5 µL of the combined IS working solution and 750 µL of ACN, followed by a vortex mixing (15 sec) and centrifugation step (8517 x *g*, 10 min, 4°C). Next, the supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (45 ± 5°C). The dry residue was reconstituted in 200 µL of water/methanol (85/15, v/v). After vortex mixing (15 sec), the sample was passed through a Millex® GV-PVDF filter (0.22 µm) and transferred into an autosampler vial. An aliquot (2.5 - 10 µL) was injected onto the LC-MS/MS instrument.

2.5. Liquid chromatography

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MS pump Plus and an autosampler with temperature controlled tray and column oven, type Autosampler Plus, from Thermo Fisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type (10 mm x 2.1 mm i.d., dp: 3 µm), both from Thermo Fisher Scientific. The temperatures of the column oven and autosampler tray were set a 45 and 5°C respectively.

For multi-mycotoxin screening analysis, two gradient elution programs were performed depending on the MS/MS detection mode, i.e. positive or negative electrospray ionization (ESI). The flow rate was set at 300 µL/min. An overview of the gradient programs is given in Table 1.

For the toxicokinetic studies, separate LC-MS/MS methods were run for each type of mycotoxin and its metabolites, i.e. DON and DOM-1, T-2 and HT-2, ZON and metabolites, FB1, OTA and AFB1. Instrument parameters (mobile phase, gradient programs and MS/MS conditions) were optimized in such a way that optimal chromatographic separation and sensitivity could be reached for all analytes of interest. An overview of the HPLC conditions for the analysis of the individual mycotoxins (and their metabolites) is shown in Table 1.

Table 1. Overview of the LC gradient programs that were used for the multi-mycotoxin and the analyte-specific LC-MS/MS methods.

Method	Analyte(s)	Mobile phase		Time (min)	MF A:B ratio (%)
		A	**B**		
Multi-mycotoxin	**DON, DOM-1, ZON, α/β-ZOL, α/β-ZAL, ZAN, OTA (ESI -)**	0.1 % acetic acid in water	methanol	0.0 - 1.0	95:5
				1.0 - 4.0	Linear to 20 % A
				4.0 - 5.1	20:80
				5.1 - 5.6	Linear to 95 % A
				5.6 - 8.0	95:5
	T-2, HT-2, FB1, AFB1 (ESI +)	0.1 % acetic acid in water	methanol	0.0 - 0.5	70:30
				0.5 - 5.0	Linear to 30 % A
				5.0 - 6.4	30:70
				6.4 - 6.5	Linear to 70 % A
				6.5 - 10.0	70:30
Analyte-specific	**DON, DOM-1**	0.1% acetic acid in water	methanol	0.0 - 1.0	95:5
				1.0 - 4.0	Linear to 20 % A
				4.0 - 5.1	20:80
				5.1 - 5.6	Linear to 95 % A
				5.6 - 8.0	95:5
	T-2, HT-2	5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v)	methanol/5 mM ammonium acetate in water/acetic acid (97/2/1, v/v/v)	0.0 - 0.1	60:40
				0.1 - 1.0	Linear to 10 % A
				1.0 - 4.0	10:90
				4.0 - 4.1	Linear to 60 % A
				4.1 - 8.0	60:40
	ZON, α/β-ZOL, α/β-ZAL, ZAN	0.01% acetic acid in water	ACN	0.0 - 0.5	70:30
				0.5 - 5.0	Linear to 30 % A
				5.0 - 6.4	30:70
				6.4 - 6.5	Linear to 70 % A
				6.5 - 10.0	70:30
	OTA	5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v)	ACN	0.0 - 1.0	65:35
				1.0 - 1.5	Linear to 10 % A
				1.5 - 3.5	10:90
				3.5 - 3.7	Linear to 65 % A
				3.7 - 6.0	65:35
	FB1	0.1% acetic acid in water	methanol	0.0 - 1.5	65:35
				1.5 - 2.0	Linear to 10 % A
				2.0 - 3.5	10:90
				3.5 - 3.7	Linear to 65 % A
				3.7 - 6.0	65:35
	AFB1	0.01% formic acid	methanol	0.0 - 0.5	90:10
				0.5 - 1.5	Linear to 20 % A
				1.5 - 5.1	20:80
				5.1 - 5.6	Linear to 90 % A
				5.6 - 8.0	90:10

2.6. Mass spectrometry

The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe operating in both the positive and negative ionization mode (all from Thermo Fisher Scientific). Instrument parameters were optimized by syringe infusion of working solutions of 1 µg/mL of each compound (flow rate 10 µL/min) in combination with its corresponding mobile phases (50% A, 50% B, flow rate: 200 µL/min).

The following general MS/MS parameters were used for the multi-mycotoxin screening method: ESI(+): spray voltage: 4000 V, vaporizer temperature: 300°C, sheath gas pressure: 40 au (arbitrary units), ion sweep gas pressure: 2.0 au, auxiliary gas pressure: 15 au, capillary temperature: 300 °C, tube lens offset: 100 V, source CID collision energy: 5 V, collision pressure: -1.5 mTorr and quad MS/MS bias: 3.0. The parameters for ESI(-) analysis were set at: spray voltage: -3500 V, vaporizer temperature: 300 °C, sheath gas pressure: 40 au, ion sweep gas pressure: 2.0 au, auxiliary gas pressure: 15 au, capillary temperature: 300°C, tube lens offset: -60 V, source CID collision energy: 5 V, collision pressure: -1.5 mTorr and quad MS/MS bias: 3.0. The resolution for Q1 and Q3 were set at 0.7 peak width half-height.

Acquisition was performed in the selected reaction monitoring (SRM) mode. For each compound, the two most intense product ions of the precursor ion were monitored in the SRM mode for quantification and identification, respectively. For the analyte-specific methods, MS/MS parameters were optimized for each individual mycotoxin (and its metabolites) so that an optimal sensitivity could be obtained and the results are shown in Table 2.

2.7. In-house method validation

Both the multi-mycotoxin and analyte-specific methods were validated according to a validation protocol previously described by De Baere et al. (2011). A set of parameters that were in compliance with the recommendations and guidelines defined by the European Community and with criteria described in the literature, were evaluated (European Commission, 2002; Heitzman, 1994; VICH GL 49, 2009).

Linearity was evaluated by preparing matrix-matched calibration curves. Calibration curve samples (range 1-200 ng/mL for ZON and metabolites and 1-1000 ng/mL for all other compounds) were prepared by applying standard working solutions directly onto the blank plasma samples, followed by a vortex mixing step. After 5 min of equilibration, the calibration curve samples were treated in a similar way as the unknown samples. The correlation coefficients (r) and goodness-of-fit coefficients (g) were calculated and had to be ≥ 0.99 and ≤ 20%, respectively.

Within-run precision (repeatability) and accuracy were determined by analyzing six blank samples spiked at low (1 and/or 10 ng/mL) and high (50, 100, 200 and/or 500 ng/mL) concentration levels in the same run. The between-run precision (reproducibility) and accuracy were determined by analyzing quality control samples (1, 10 and/or 100 ng/mL) together with each analytical batch of samples, run on three different days. 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. For the precision, the relative standard deviation (RSD) had to be below the RSD_{max} value with $RSD_{max} = 2^{(1-0.5logConc)}$ x 2/3 for within-run precision (RSD_r) and $2^{(1-0.5logConc)}$ for between-run precision (RSD_R) (European Commission, 2002; Heitzman, 1994). RSD_{max} values for the RSD_r were as follows: 1 ng/mL: 30.2%, 2 ng/mL: 27.2%, 5 ng/mL: 23.7%, 10 ng/mL: 21.3%, 50 ng/mL: 16.7%, 100 ng/mL: 15.1%, 200 ng/mL: 13.6% and 500 ng/mL: 11.8%. For the RSD_R the RSD_{max} values were: 10 ng/mL: 32.0%, 50 ng/mL: 25.1%, 100 ng/mL: 22.6% and 200 ng/mL: 20.4%.

The limit of quantification (LOQ) was the lowest concentration of the analytes for which the method was validated with an accuracy and precision that fell within the recommended ranges (see section accuracy and precision). The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing six spiked samples.

The limit of detection (LOD) was defined as the lowest concentration of the respective analytes that could be recognized by the detector with a signal-to-noise (S/N) ratio of ≥3. The LOD values were calculated using samples spiked at the LOQ level.

The specificity of the method was evaluated with respect to interferences from endogenous compounds. Therefore, one blank sample was analyzed using the above mentioned

procedure. The S/N ratio of a possible interfering peak in the blank sample had to be below the S/N ratio of the analyt(es) in the same elution zone at the LOD level.

The carry-over on the LC-MS/MS instrument was evaluated by analyzing an ACN/water sample (50/50, v/v) just after the highest calibrator sample. The eventual analyt concentration in the sample had to be below the LOD.

Recovery experiments were performed according to Matuszewski et al. (2003). In summary, two types of matrix-matched calibration curves were prepared for each analyte, namely by spiking the blank calibrator samples before (spiked) and after extraction (spiked extract). One calibration curve was prepared using standard solutions. The slopes of the resulting linear, 1/x weighted, spiked calibration curves with spiked and spiked extracts samples were compared with the related slopes of the calibration curves with spiked extracts and standard solution, in order to calculate the recovery of the extraction step (R_E) and the signal suppression/enhancement (SSE) due to matrix effects, respectively.

Table 2. Overview of the different MS/MS parameters that were used for the analyte-specific LC-MS/MS method.

Analyt	MM (g/mol)	Precursor ion (m/z)	Product ions (m/z)		RT (min)	CE (V)	DT (ms)	SV (V)	VT (°C)	SG (au)	ISG (au)	AG (au)	CT (°C)	TLO (V)	CGP (mTorr)	Quad MS/MS bias
DON	296	355.20 [M+CH$_3$COO]$^-$	265.10 295.10	*	3.49	15 10								-75		
DOM-1	280	339.10 [M+CH$_3$COO]$^-$	59.10 249.10	*	4.26	35 15	200	-3500	300	40	2.0	15	300	-85	-1.5	3.0
^{13}C$_{15}$-DON	311	370.20 [M+CH$_3$COO]$^-$	279.10 310.10	*	3.45	15 10								-75		
ZON	318	317.3 [M-H]$^-$	131.00 175.00	*	4.55	41 37								-80		
ZAN	320	319.2 [M-H]$^-$	205.00 275.10	*	4.55	32 28								-67		
α/β -ZOL	320	319.2 [M-H]$^-$	275.10 301.11	*	3.77/2.93	28 25	100	-3500	300	40	2.0	25	300	-67	-1.5	2.8
α/β -ZAL	322	321.2 [M-H]$^-$	277.13 303.11	*	3.59/2.80	30 32								-74		
^{13}C$_{18}$-ZON	336	335.3 [M-H]$^-$	169.10 185.06	*	4.50	38 30								-76		
OTA	403	402.20 [M-H]$^-$	166.96 358.04	*	6.53	36 28								-86		
^{13}C$_{20}$-OTA	423	422.25 [M-H]$^-$	175.03 377.10	*	6.54	46 32	200	-3500	300	39	2.0	20	300	-90	-0.5	3.7
T-2	466	484.08 [M+NH$_4$]$^+$	185.05 215.07	*	5.82	25 24								100		
HT-2	424	442.02 [M+NH$_4$]$^+$	263.10 215.07	*	5.21	15 14	200	4000	300	43	2.0	12	300	100	-0.5	2.6
^{13}C$_{24}$-T-2	490	508.18 [M+NH$_4$]$^+$	198.10 229.14	*	5.83	25 23								100		
FB1	721	722.3 [M+H]$^+$	333.90 352.37	*	3.96	44 35	250	4000	300	23	4.0	18	300	136	-0.5	2.1
^{13}C$_{34}$-FB1	755	756.4	356.17	*	3.97	34								125		

Compound	MM	Precursor ion	Product ion		RT	CE	SV	VT	SG	ISG	AG	CT	TLO		CGP
		[M+H]$^+$	374.19			31									
AFB1	312	312.90 [M+H]$^+$	240.92 284.89	*	3.65	46 35	300	4000	300	41	2.0	20	300	100 -0.5	2.6
$^{13}C_{17}$-AFB1	329	330.04 [M+H]$^+$	255.04 300.88	*	3.59	34 35								100	

MM : molecular mass; RT: retention time; CE: collision energy; ; DT: dwell time ; SV: spray voltage; VT: vaporizer temperature; SG: sheath gas; ISG: ion sweep gas; AG: auxiliary gas; CT: capillary temperature; TLO: tube lens offset; CGP: collision gas pressure; the asterix (*) indicates the quantifier ion.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation and extraction

The goal of this study was to develop a sensitive LC-MS/MS method for screening and quantitation of toxicological important mycotoxins in plasma. These plasma samples can be taken during assessment experiments to evaluate animal exposure to mycotoxins (screening) or as a part of toxicokinetic studies with mycotoxins (quantitation). Since these surveys or trials generate a large number of samples, the use of a simple and practical sample preparation procedure is advisable in order to reduce the time and cost of analysis. When using a sensitive and specific analytical technique such as LC-MS/MS, cleanup of raw extracts can be kept to a minimum (Van Pamel et al., 2011). However, the most simple procedure - dilute-and-shoot - was not found suitable for plasma analysis, as this could lead to clogging and serious contamination of the MS instrument. On the other hand, more sophisticated extraction procedures using solid phase extraction (SPE) and immuno-affinity columns (IAC) are not recommended, as they are generally time-consuming and expensive, which is a disadvantage if a large amount of samples have to be analyzed (i.e. for screening or toxicokinetical analysis). To achieve a good compromise between simplicity of extraction and acceptable sample clean-up, a generic extraction procedure was developed which consisted of a combination of protein precipitation with liquid extraction.

Organic solvents, such as methanol and acetonitrile, are commonly used for deproteinization of plasma samples (Corcuera et al., 2011). In our method, 750 µL acetonitrile was used for combined precipitation of plasma proteins and extraction of mycotoxins, as it showed high extraction recoveries (R_E, %) for all compounds (ranging from ± 73 to 110 %), as can be seen in Table 3. Acetonitrile gave more clear supernatants after centrifugation compared to methanol, indicating improved deproteinization efficiency compared to methanol. In order to concentrate the samples as much as possible, the supernatants were evaporated under nitrogen and reconstituted in 200 µL water/methanol (85/15, v/v).

Matrix effects (SSE, %) were evaluated and varied between ± 76 and 113 % for all compounds, as can be seen in Table 3. These results are acceptable taking into account that

a generic extraction procedure was used and correlate well with those reported by other authors (Corcuera et al., 2011; De Baere et al., 2011; Munoz et al., 2010). Hence, it can be stated that for the first time a generic extraction procedure was developed for the simultaneous extraction of mycotoxins (and their metabolites) belonging to different groups, from animal plasma.

3.1.2. Optimization of LC-MS/MS conditions

During preliminary experiments, three different reversed-phase C_{18} columns were tested (Nucleosil, Varian/Agilent; Alltima HP, Alltech; Hypersil Gold, ThermoScientific). The Hypersil Gold column was retained since the best results were obtained with respect to separation of analytes within a short analysis time (i.e. less than 10 min).

For the simultaneous detection of several mycotoxins, the mobile phases reported in the literature consist generally of a combination of water with the addition of a volatile organic acid (formic or acetic acid or ammonium acetate) and an organic solvent (methanol or acetonitrile) (De Baere et al., 2011; Monbaliu et al., 2010; Razzazi-Fazeli et al., 2003). In case of the multi-mycotoxin method, it was decided to perform two analytical runs, i.e. in the ESI + and ESI - mode, respectively. In both ionization modes, the best sensitivity for all investigated compounds was achieved using 0.1 % acetic acid in water (MF A) and methanol (MF B) as mobile phase. In addition, nearly all compounds could be separated, except ZAN and the α- and β-isomers of ZOL and the α- and β-isomers of ZAL, which eluted at the same retention time (Supplementary Material: Figure 1).

For quantitative purposes, it was necessary to achieve a baseline-separation between all analytes of interest, especially between ZAN and the isomers of ZOL and between the isomers of ZAL, and to reach a sensitivity that was as high as possible. Therefore, it was decided to optimize the chromatographic and MS/MS parameters for each individual mycotoxin and its metabolites (analyte-specific methods). For DON, T-2 and ZON analysis, the LC-MS/MS conditions were based on methods previously developed by our group (De Baere et al., 2011; De Baere et al., 2012). As can be seen from Table 1, all mobile phases consisted of a combination of acidified water and an organic solvent, except for T-2 and HT-2. For these compounds, mobile phases containing 5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v) and 5 mM ammonium acetate in methanol/water/acetic acid (97/2/1, v/v/v) were used. The inclusion of ammonium salts in

the mobile phase induced the formation of ammonium adducts ($[M+NH_4]^+$), which are easier to fragment compared to protonized ($[M+H]^+$) ions and sodium adducts ($[M+Na]^+$) (Heitzman, 1994). Fragmentation of the $[M+NH_4]^+$ ions enhanced the sensitivity of the LC-MS/MS method, shown by lower LOQ and LOD values obtained when using these mobile phases compared with those of the multi-mycotoxin method.

In case of ZON analysis, the organic modifier has been changed from methanol to acetonitrile, resulting in a baseline-separation of ZAN, α- and β-ZOL on the one hand and α- and β-ZAL on the other hand (Supplementary Material: Figure 2).

Each compound was analyzed in its most sensitive ESI mode: T-2, HT-2, FB1 and AFB1 were acquired in the positive ionization mode, whereas DON, DOM-1, OTA, ZON, ZAN, α/β-ZOL and α/β-ZAL were acquired in the negative mode. These data are compliant with other papers (Lattanzio et al., 2011; Soleimany et al., 2012).

Acquisition was performed in the SRM mode, which means that for each analyte the two most intense precursor ion > product ion transitions were monitored. The product ion with the highest intensity was selected as quantifier, while the other ion was used as qualifier. In Table 2 the MS/MS conditions for all target analytes are shown.

3.1.3. Internal standard

The use of an isotopically labeled internal standard for every single mycotoxin is preferable (Spanjer et al., 2008), as this leads to maximal compensation for losses during extraction, matrix effects during LC-MS/MS analysis, and general analysis errors. Moreover, isotopically labeled internal standards have similar physical and chemical properties as the target compound (European Commission, 2002). Disadvantages of the use of isotopically labeled internal standards are the high costs and the dependence on commercial availability. As an alternative only one isotopically labeled internal standard could be used per ionization mode. However, due to the diversity in chemical and physical properties of the different toxin classes, we preferred to use a ^{13}C labeled internal standard for all parent compounds in the presented methods (i.e. $^{13}C_{15}$-DON, $^{13}C_{24}$-T-2, $^{13}C_{18}$-ZON, $^{13}C_{20}$-OTA, $^{13}C_{34}$-FB1 and $^{13}C_{17}$-AFB1). Prior to use, all internal standards were analyzed using the described LC-MS/MS method in order to determine if any non-labeled mycotoxins were present in the solution, which was negative.

3.2. Method validation

Both the multi-mycotoxin and the analyte-specific methods were in-house validated. The following parameters were evaluated for each mycotoxin and its respective metabolite(s): linearity, within- and between-run precision and accuracy, LOQ, LOD and specificity. The results are shown in Table 3 and as Supplementary Material: Table 4 and 5.

Matrix-matched calibration graphs were linear over the working concentration range for all tested mycotoxins, with r values between 0.9974 and 0.9999 and g values between 2.4 and 15.5 %. The within- and between-run accuracy and precision were tested at two different concentration levels and fell within the acceptability ranges. The limits of quantification (LOQ) ranged from 2-10 ng/mL for the multi-mycotoxin method and from 0.5-5 ng/mL for the analyte-specific methods. These lower LOQ levels for the analyte-specific methods can be explained by the fact that optimal LC and MS/MS conditions were used for each individual mycotoxin, whereas the conditions for the multi-mycotoxin were the best compromise for all analytes combined. The limits of detection (LOD) were calculated taking into account a S/N ratio of 3 and ranged from 0.01 to 0.52 ng/mL for the multi-mycotoxin method and from <0.01 to 0.15 ng/mL for the analyte-specific methods. Several studies reported by other authors showed that the concentrations of the investigated mycotoxins in human and animal plasma fell within the lower ppb (ng/mL) range (De Baere et al., 2011; Munoz et al., 2009; Santini et al., 2010). When developing a method for assessing mycotoxin exposure in animals by analyzing plasma, it is therefore a prerequisite to reach these low LOQ and LOD levels. Moreover, for toxicokinetic studies, dosing high mycotoxin concentrations to animals is no longer ethically acceptable as it compromises animal welfare. The developed methods succeeded in the detection and quantitation of the various mycotoxins at these low levels. For the evaluation of the specificity of the method, a blank plasma sample was analyzed. No endogenous interferences were detected in the elution zone of the target analytes.

Carry-over of one sample to another on the LC-MS/MS instrument was evaluated by the analysis of a solvent sample after the highest calibrator sample. No carry-over was present, as there were no peaks detected in the same retention time zone of the compounds of interest.

Table 3. Results of the evaluation of linearity (goodness-of-fit coefficient (g), correlation coefficient (r)), extraction recovery (R_E), matrix effect (SSE), limit of quantification (LOQ), limit of detection (LOD) for all analytes using the multi-mycotoxin and the analyte-specific methods, respectively.

Method	Analyte	Calibration Range (ng/mL)	g (%)	r	R_E (%)	SSE (%)	LOQ (ng/mL)	LOD (ng/mL)
Multi-mycotoxin	DON	10-1000	2.95	0.9999	99.27	85.49	10	0.40
	DOM-1	5-1000	5.07	0.9990	101.58	95.39	5	0.22
	ZON	5-1000	5.15	0.9999	94.38	90.73	5	0.52
	ZAN	5-1000	5.83	0.9985	109.80	90.58	5	0.05
	α/β-ZOL	5-1000	6.63	0.9990	108.49	83.73	5	0.02
	α/β-ZAL	5-1000	6.73	0.9984	108.58	87.73	5	0.02
	OTA	2-1000	4.90	0.9995	77.57	87.89	2	0.02
	T-2	2-1000	2.39	0.9999	96.37	108.67	2	0.05
	HT-2	5-1000	6.79	0.9990	77.62	80.15	5	0.15
	FB1	2-1000	3.11	0.9997	102.85	98.77	2	0.01
	AFB1	5-1000	4.11	0.9996	99.36	98.74	2	0.05
Analyte-specific	DON	1-1000	7.37	0.9993	99.85	101.40	1	0.05
	DOM-1	2-1000	5.09	0.9992	93.86	86.49	2	0.04
	ZON	1-200	12.58	0.9993	83.46	100.60	1	0.03
	ZAN	1-200	8.28	0.9994	84.65	97.36	1	0.04
	α-ZOL	5-200	7.41	0.9989	85.14	112.86	5	0.05
	β-ZOL	1-200	15.52	0.9979	84.48	102.24	1	0.02
	α-ZAL	1-200	3.87	0.9996	85.97	105.06	1	0.02
	β-ZAL	1-200	6.32	0.9991	84.60	102.52	1	<0.01
	OTA	0.5-1000	4.88	0.9979	99.89	97.43	0.5	<0.01
	T-2	1-200	5.51	0.9981	86.52	80.03	1	0.05
	HT-2	1-200	6.70	0.9974	72.95	76.35	1	0.03
	FB1	1-1000	5.39	0.9995	96.10	89.31	1	0.08
	AFB1	1-1000	4.15	0.9993	96.68	96.93	0.5	0.15

Note: acceptance criteria: g ≤ 20 %, r ≥ 0.99

3.3. Biological samples

To demonstrate the applicability of the developed multi-mycotoxin and analyte-specific methods, six piglets were administered an intra-gastric bolus of DON, ZON, OTA, T-2 and AFB1. Fumonisin B1 was not included in the bolus, as the maximum allowed concentration in pig feed is high, up to 60 mg/kg feed (European Commission, 2006), which would have led to a serious increase of the mycotoxin purchase costs. Lowering the administered dose was not an option as FB1 has a low oral bioavailability in pigs (± 3.5%) (Martinez-Larranaga et al., 1999). Blood samples were drawn at different time points post-administration. All plasma samples were extracted once using the described sample preparation procedure. Thereafter, the extracted samples were injected several times, i.e. once for each ionization mode for the multi-mycotoxin method and once for each analyte-specific method. Following analysis, the correlation between the plasma concentrations obtained for both methods were set-up for each analyte and the correlation coefficients (R^2) were calculated.

Using the multi-mycotoxin method, DON and OTA could be detected and quantified, whereas AFB1 and ZON could be detected but not quantified as the concentrations were below the LOQ (2 and 5 ng/mL, respectively). No metabolites of these compounds, nor T-2 toxin could be detected. T-2 toxin is very rapidly metabolized, as demonstrated by a very short elimination half-life ($T_{1/2el}$), < 5 min in broiler chickens (Osselaere et al., 2013). This explains why this toxin was not detected in our biological samples.

The mean concentrations detected for DON, ranging from 0 – 30 ng/mL, and ZON, up to 2.1 ng/mL, correlate well with previous results from our group (De Baere et al., 2013; Devreese et al., 2012). Plasma concentrations for OTA, up to 135 ng/mL, and AFB1, ranging from LOD to 2.2 ng/mL, are in accordance with other literature (Corcuera et al., 2011).

The same analytes were detected and quantified using the analyte-specific methods, but AFB1 could also be quantified as the plasma concentrations were above the LOQ (0.5 ng/mL). The plasma concentrations for DON and OTA correlate very well between the multi-mycotoxin and analyte specific methods (R^2 = 0.98 and 0.97, respectively). The correlation between the two methods for AFB1 is lower, however still acceptable (R^2 = 0.90), because the concentrations fell below the LOQ in the multi-mycotoxin method. For ZON, no R^2 was

calculated as the plasma concentrations were below the LOQ for both methods. Results are presented in Supplementary Material: Table 6.

4. Conclusions

This paper describes a highly sensitive and specific LC-MS/MS method for the analysis of several toxicological important mycotoxins in pig plasma. All analytes were isolated from plasma by performing a generic, fast and low-cost sample preparation procedure. The extracted samples could be analyzed by two types of LC-MS/MS methods: a multi-mycotoxin method was used for the simultaneous analysis of all mycotoxins and can be applied in the field of animal exposure assessment to mycotoxins (screening). Analyte-specific methods were developed for the analysis of every mycotoxin (and its relevant metabolites) separately. These methods can be used for toxicokinetic studies, *in vivo* efficacy testing of mycotoxin detoxifiers based on toxicokinetic parameters and diagnosing mycotoxicosis in animals. Both the multi-mycotoxin and analyte-specific methods were successfully validated. In addition, the applicability of the method was shown by the analysis of real plasma samples that were taken from pigs that received an oral bolus of the investigated mycotoxins.

5. Acknowledgments

6. References

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7. Supplementary material

Table 4. Results of the within-run and between-run precision and accuracy evaluation for the analysis of toxicological important mycotoxins in pig plasma determined by a multi-mycotoxin LC-MS/MS method.

Analyt	Theoretical concentration (ng/mL)	Mean concentration ± SD (ng/mL)	Precision, RSD (%)	Accuracy (%)
DON	10	10.5 ± 0.69 [a]	6.6 [a]	4.7 [a]
	50	50.3 ± 4.73 [a]	9.4 [a]	0.5 [a]
	50	50.8 ± 2.74 [b]	5.4 [b]	1.6 [b]
	200	205.4 ± 8.28 [a]	4.0 [a]	2.7 [a]
	200	204.8 ± 5.95 [b]	5.4 [b]	2.9 [b]
DOM-1	10	10.7 ± 1.02 [a]	9.5 [a]	7.2 [a]
	50	49.8 ± 3.47 [a]	7.0 [a]	-0.2 [a]
	50	48.8 ± 4.49 [b]	9.2 [b]	-2.5 [b]
	200	194.0 ± 14.51 [a]	7.5 [a]	-3.0 [a]
	200	192.4 ± 11.59 [b]	6.0 [b]	-3.8 [b]
ZON	10	9.7 ± 1.70 [a]	17.4 [a]	-2.7 [a]
	50	54.2 ± 3.09 [a]	5.7 [a]	8.5 [a]
	50	53.7 ± 4.74 [b]	8.8 [b]	7.3 [b]
	200	210.3 ± 12.39 [a]	5.9 [a]	5.2 [a]
	200	209.1 ± 12.85 [b]	6.1 [b]	4.5 [b]
ZAN	10	9.8 ± 1.09 [a]	11.1 [a]	-2.1 [a]
	50	52.5 ± 3.53 [a]	6.7 [a]	4.9 [a]
	50	53.8 ± 3.42 [b]	6.4 [b]	7.5 [b]
	200	204.6 ± 9.02 [a]	4.4 [a]	2.3 [a]
	200	206.9 ± 6.54 [b]	3.2 [b]	3.5 [b]
α/β-ZOL	10	10.8 ± 0.65 [a]	6.1 [a]	7.7 [a]
	50	54.1 ± 3.93 [a]	7.3 [a]	8.3 [a]
	50	54.7 ± 3.64 [b]	6.7 [b]	9.3 [b]
	200	205.1 ± 14.22 [a]	6.9 [a]	2.5 [a]
	200	206.4 ± 14.69 [b]	7.1 [b]	3.2 [b]
α/β-ZAL	10	9.9 ± 0.87 [a]	8.8 [a]	-1.2 [a]
	50	52.6 ± 3.74 [a]	7.1 [a]	5.1 [a]
	50	54.5 ± 5.41 [b]	9.9 [b]	9.0 [b]
	200	203.3 ± 10.54 [a]	5.2 [a]	1.7 [a]
	200	208.3 ± 12.10 [b]	5.8 [b]	4.1 [b]
OTA	10	10.5 ± 0.38 [a]	3.6 [a]	4.7 [a]
	50	53.3 ± 3.95 [a]	7.4 [a]	6.5 [a]
	50	53.1 ± 3.16 [b]	6.0 [b]	6.2 [b]
	200	204.5 ± 6.82 [a]	3.3 [a]	2.3 [a]
	200	206.5 ± 6.37 [b]	3.1 [b]	3.3 [b]
T-2	10	10.2 ± 0.18 [a]	1.8 [a]	1.6 [a]
	50	51.7 ± 1.67 [a]	3.2 [a]	3.5 [a]
	50	51.3 ± 2.95 [b]	5.8 [b]	2.6 [b]
	200	205.3 ± 2.84 [a]	1.4 [a]	2.6 [a]
	200	200.2 ± 7.89 [b]	3.9 [b]	0.1 [b]
HT-2	10	9.6 ± 1.04 [a]	10.9 [a]	-4.1 [a]
	50	49.4 ± 5.58 [a]	11.3 [a]	-1.1 [a]
	50	50.2 ± 5.34 [b]	10.6 [b]	0.3 [b]
	200	193.6 ± 11.76 [a]	6.1 [a]	-3.2 [a]
	200	197.0 ± 11.56 [b]	5.9 [b]	-1.5 [b]
FB1	10	9.6 ± 0.77 [a]	8.0 [a]	-3.7 [a]
	50	51.4 ± 2.06 [a]	4.0 [a]	2.9 [a]
	50	50.5 ± 3.29 [b]	6.5 [b]	1.0 [b]
	200	204.5 ± 6.14 [a]	3.0 [a]	2.2 [a]
	200	200.7 ± 6.97 [b]	3.5 [b]	0.4 [b]
AFB1	10	9.8 ± 0.50 [a]	5.1 [a]	-2.2 [a]
	50	48.4 ± 2.45 [a]	5.1 [a]	-3.3 [a]

50	48.1 ± 2.49 [b]	5.2 [b]	-3.8 [b]
200	195.4 + 8.02 [a]	4.1 [a]	-2.3 [a]
200	199.9 ± 10.40 [b]	5.2 [b]	0.0 [b]

[a] Results of within-run accuracy and precision (n=6); [b] Results of between-run accuracy and precision (n=12); SD: standard deviation; RSD: relative standard deviation

Table 5. Results of the within-run and between-run precision and accuracy evaluation for the analysis of toxicological important mycotoxins in pig plasma determined by analyte-specific LC-MS/MS methods.

Analyte	Theoretical concentration (ng/mL)	Mean concentration ± SD (ng/mL)	Precision, RSD (%)	Accuracy (%)
DON	1	1.1 ± 0.25 [a]	21.5 [a]	14.6 [a]
	1	1.1 ± 0.18 [b]	14.5 [b]	12.2 [b]
	10	9.4 ± 0.55 [a]	5.8 [a]	-5.7 [a]
	10	9.5 ± 0.47 [b]	4.9 [b]	-4.6 [b]
	100	100.1 ± 1.83 [a]	1.8 [a]	0.1 [a]
	100	100.7 ± 1.71 [b]	1.7 [b]	0.7 [b]
DOM-1	1	0.9 ± 0.10 [a]	11.4 [a]	-8.6 [a]
	1	1.0 ± 0.15 [b]	14.9 [b]	-0.6 [b]
	10	9.8 ± 0.56 [a]	5.8 [a]	-2.0 [a]
	10	10.4 ± 0.83 [b]	8.0 [b]	4.4 [b]
	100	107.5 ± 2.62 [a]	2.4 [a]	7.5 [a]
	100	100.7 ± 5.44 [b]	5.4 [b]	0.7 [b]
ZON	1	1.2 ± 0.08 [a]	6.5 [a]	16.3 [a]
	10	10.8 ± 0.54 [a]	5.0 [a]	7.9 [a]
	10	10.4 ± 0.97 [b]	9.3 [b]	4.0 [b]
	100	100.9 ± 4.93 [a]	4.9 [a]	0.9 [a]
	100	104.2 ± 4.57 [b]	4.4 [b]	4.2 [b]
ZAN	1	1.0 ± 0.16 [a]	16.9 [a]	-3.2 [a]
	10	11.0 ± 0.77 [a]	7.0 [a]	2.9 [a]
	10	10.5 ± 0.84 [b]	7.9 [b]	5.3 [b]
	100	105.7 ± 3.06 [a]	2.9 [a]	5.7 [a]
	100	105.4 ± 3.07 [b]	2.9 [b]	5.4 [b]
α-ZOL	5	5.3 ± 0.33 [a]	6.1 [a]	6.9 [a]
	10	10.3 ± 0.93 [a]	9.0 [a]	3.0 [a]
	10	10.5 ± 0.42 [b]	4.0 [b]	5.0 [b]
	100	101.9 ± 3.19 [a]	3.1 [a]	1.9 [a]
	100	102.9 ± 7.87 [b]	7.6 [b]	2.9 [b]
β-ZOL	1	0.9 ± 0.15 [a]	16.0 [a]	-8.4 [a]
	10	9.6 ± 1.22 [a]	11.4 [a]	-4.1 [a]
	10	10.8 ± 0.84 [b]	7.8 [b]	-7.5 [b]
	100	102.3 ± 4.68 [a]	4.6 [a]	2.3 [a]
	100	105.8 ± 6.28 [b]	5.9 [b]	5.8 [b]
α-ZAL	1	1.2 ± 0.14 [a]	11.6 [a]	17.1 [a]
	10	10.1 ± 0.80 [a]	7.9 [a]	1.4 [a]
	10	10.5 ± 0.84 [b]	7.9 [b]	5.3 [b]
	100	103.0 ± 5.94 [a]	5.8 [a]	3.0 [a]

	100	107.4 ± 7.00 [b]	6.5 [b]	7.4 [b]
β-ZAL	1	0.8 ± 0.16 [a]	19.5 [a]	-20.3 [a]
	10	10.6 ± 0.64 [a]	6.0 [a]	6.2 [a]
	10	10.6 ± 0.39 [b]	3.6 [b]	5.8 [b]
	100	103.4 ± 3.09 [a]	3.0 [a]	3.4 [a]
	100	107.5 ± 5.47 [b]	5.1 [b]	7.5 [b]
OTA	10	10.5 ± 0.26 [a]	2.4 [a]	5.1 [a]
	10	11.0 ± 0.83 [b]	7.3 [b]	8.4 [b]
	100	100.4 ± 1.51 [a]	1.5 [a]	0.4 [a]
	500	514.6 ± 13.42 [a]	2.6 [a]	2.9 [a]
	500	512.3 ± 14.09 [b]	2.7 [b]	2.5 [b]
T-2	1	1.0 ± 0.29 [a]	22.1 [a]	3.1 [a]
	10	9.7 ± 1.13 [a]	11.6 [a]	-3.0 [a]
	10	9.4 ± 1.66 [b]	17.6 [b]	-5.9 [b]
	100	107.7 ± 4.18 [a]	3.9 [a]	7.7 [a]
	100	99.2 ± 5.35 [b]	5.4 [b]	-0.8 [b]
HT-2	1	0.8 ± 0.20 [a]	26.0 [a]	-22.3 [a]
	10	10.5 ± 1.25 [a]	11.9 [a]	4.9 [a]
	10	10.5 ± 1.18 [b]	11.2 [b]	5.1 [b]
	100	105.1 ± 2.32 [a]	2.2 [a]	5.1 [a]
	100	99.0 ± 10.0 [b]	10.1 [b]	-1.0 [b]
FB1	1	1.1 ± 0.08 [a]	8.0 [a]	5.1 [a]
	10	10.2 ± 0.84 [a]	8.3 [a]	2.1 [a]
	10	9.7 ± 0.90 [b]	9.3 [b]	-3.4 [b]
	100	101.9 ± 5.67 [a]	5.6 [a]	1.9 [a]
	100	103.7 ± 5.51 [b]	5.3 [b]	3.7 [b]
AFB1	1	1.0 ± 0.07 [a]	7.2 [a]	2.3 [a]
	10	10.1 ± 0.37 [a]	3.7 [a]	1.3 [a]
	10	10.0 ± 0.48 [b]	4.8 [b]	0.0 [b]
	100	101.2 ± 2.41 [a]	2.4 [a]	1.2 [a]
	100	100.6 ± 1.97 [b]	2.0 [b]	0.6 [b]

[a] Results of within-run accuracy and precision (n=6); [b] Results of between-run accuracy and precision (n=12)

Table 6. Slopes a and intercepts b of equation y = ax + b, their standard deviations and the correlation coefficient R^2 of deoxynivalenol (DON), ochratoxin A (OTA) and aflatoxin B1 (AFB1) of the comparison between analysis by the multi-mycotoxin method and analyte specific methods (n = 84)

Analyte	a	b	R^2
DON	1.030 ± 0.045	0.539 ± 0.082	0.9806
OTA	0.989 ± 0.049	-2.082 ± 0.389	0.9705
AFB1	1.033 ± 0.082	-0.156 ± 0.009	0.9058

Figure 1. LC-MS/MS chromatogram of a spiked plasma sample (concentration of all analytes: 10 ng/mL) analyzed using the multi-mycotoxin method, showing the SRM traces of (a) DON, DOM-1, $^{13}C_{15}$-DON, OTA, $^{13}C_{20}$-OTA, ZON, ZAN, $^{13}C_{18}$-ZON, (α and β)-ZOL and (α and β)-ZAL in the ESI - mode and of (b) T-2, HT-2, $^{13}C_{24}$-T-2, FB1, $^{13}C_{34}$-FB1, AFB1 and $^{13}C_{17}$-AFB1 acquired in the ESI + mode

(a)
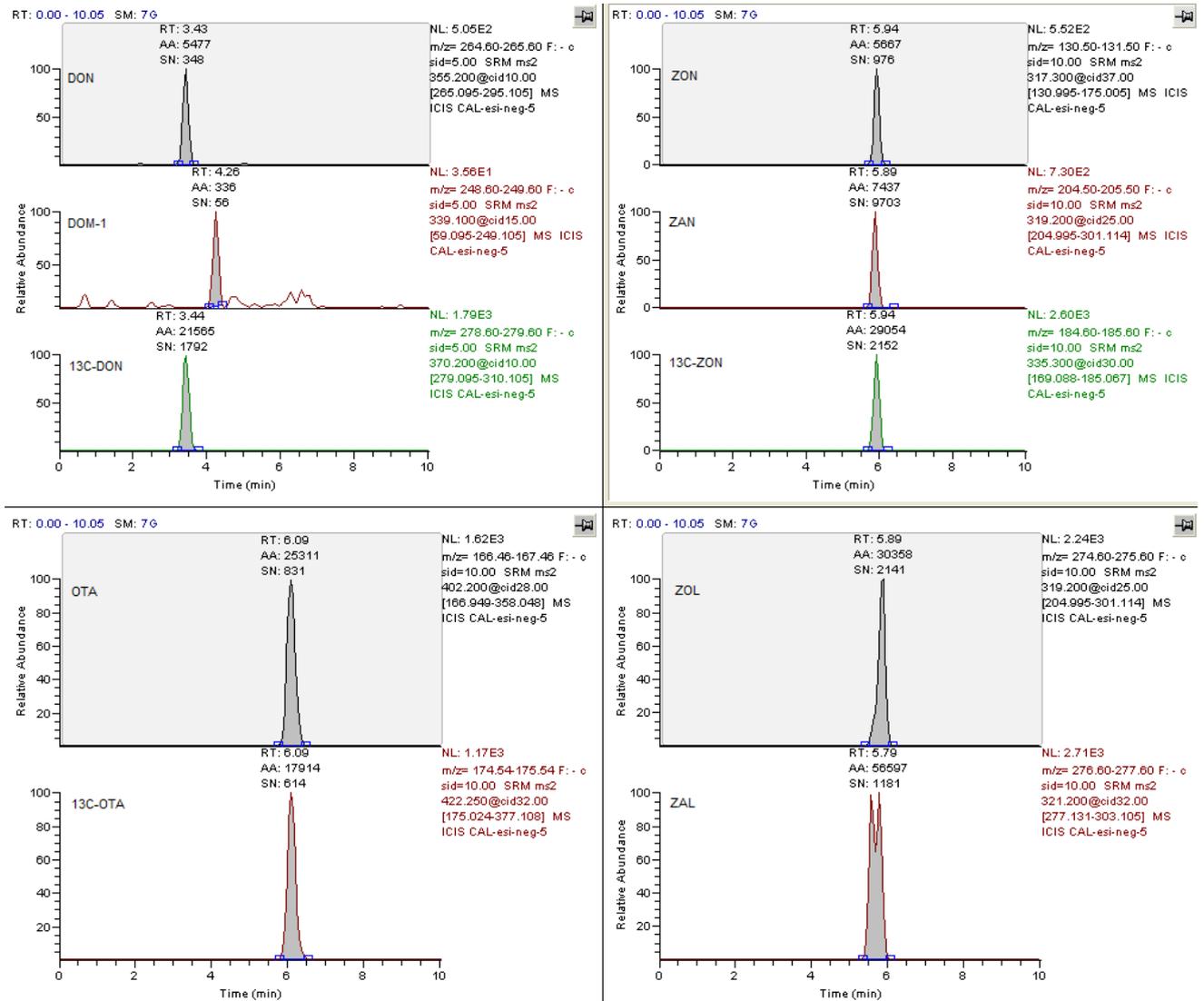
(b)
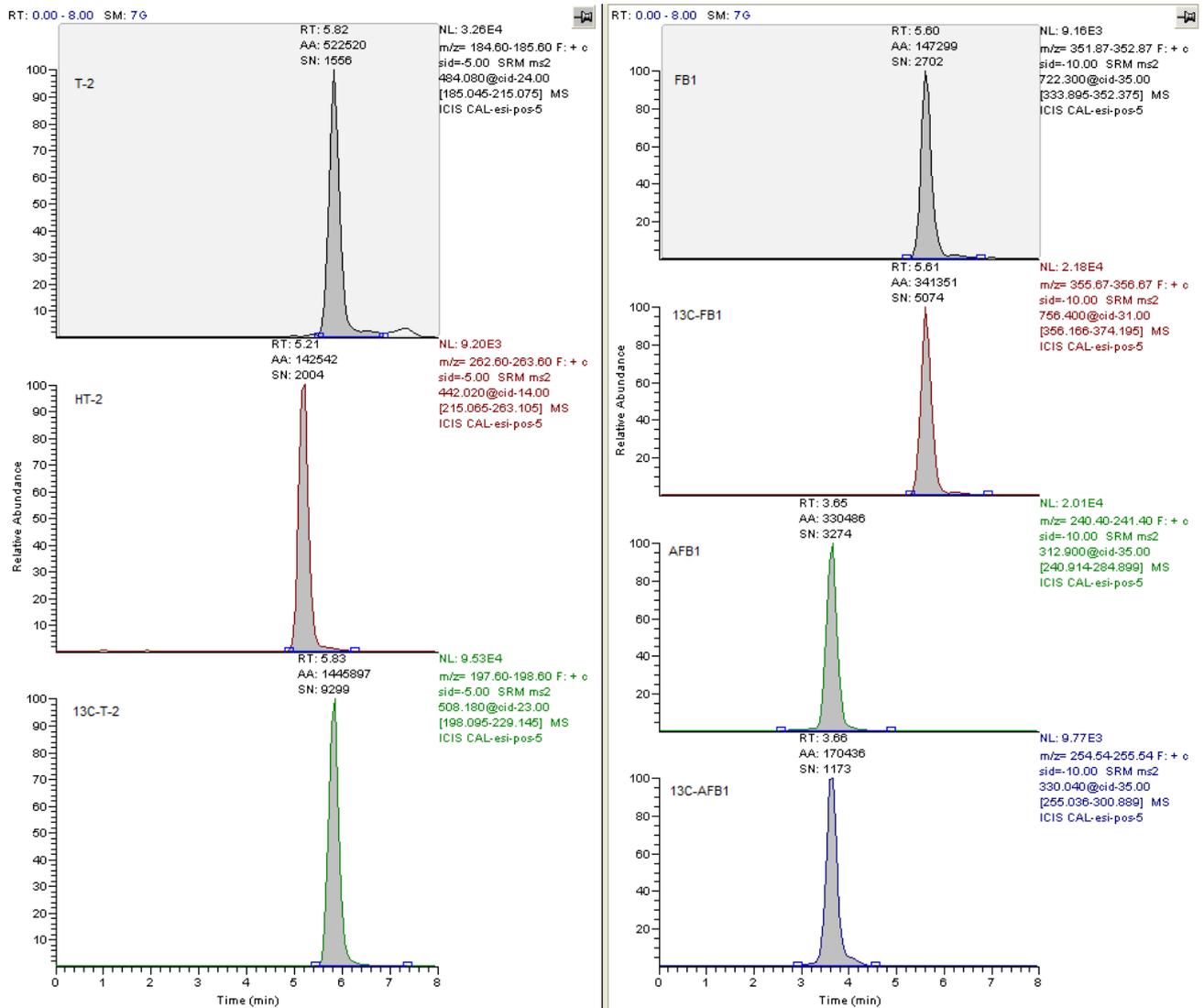
Figure 2. LC-MS/MS chromatogram of a spiked plasma sample of ZON and its major metabolites (analyte concentration: 10 ng/mL) using the analyte-specific method for ZON, showing the SRM traces of ZON, $^{13}C_{18}$-ZON and the separated metabolites β-ZOL, α-ZOL and ZAN and β-ZAL and α-ZAL
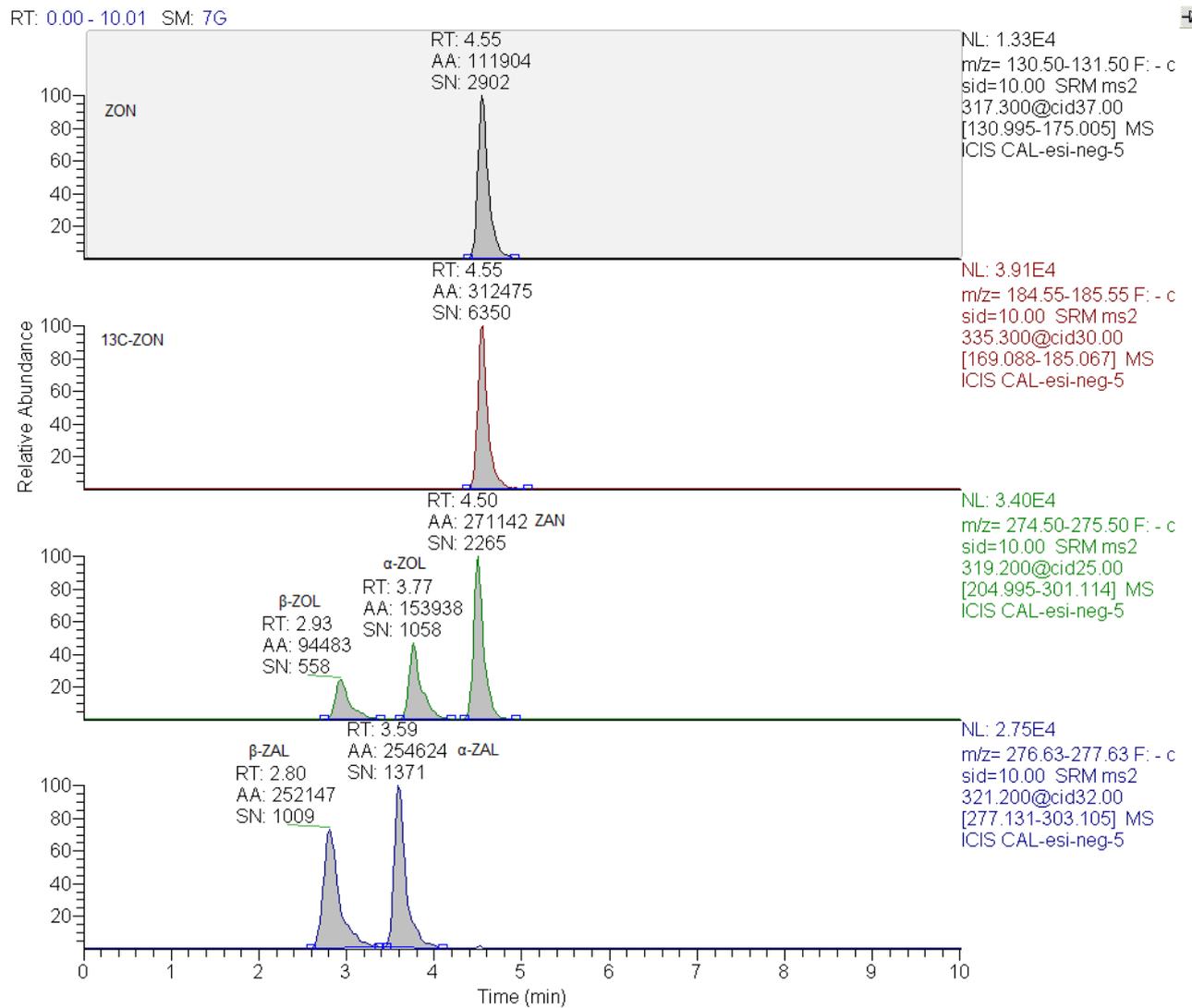
CHAPTER III

Quantitative determination of the *Fusarium* mycotoxins beauvericin, enniatin A, A1, B and B1 in pig plasma using high performance liquid chromatography-tandem mass spectrometry

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Abstract - A sensitive and reliable method was developed for the identification and quantification of beauvericin, enniatin A, A1, B and B1 in pig plasma using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry. Sample clean-up consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in acetonitrile/water (20/80, v/v). The method was in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9980 - 0.9995 and 5.2% - 11.3%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification were 0.1 ng/mL for enniatin A and A1 and 0.2 ng/mL for beauvericin, enniatin B and B1, whereas limits of detection were ≤ 10 pg/mL for all analytes. The method has been applied for the analysis of real plasma samples from one pig that received an oral bolus (0.05 mg/kg BW) of the investigated mycotoxins. At the applied dosage, the results indicated the suitability of the method for use in toxicokinetic studies with enniatines.

1. Introduction

Mycotoxins are secondary metabolites produced by different fungal species contaminating several food and feed commodities. Over 400 mycotoxins have been identified, although only a few of them have been thoroughly investigated because of their distinct toxic effects. The most prevalent mycotoxin producing fungi in moderate climates are *Fusarium* species. Several *Fusarium spp.* are capable of producing well-known mycotoxins like the trichothecenes, fumonisins and/or zearalenone, but also other mycotoxins including beauvericin (BEA) and enniatins (ENNs) such as ENN A, A1, B and B1 (Logrieco et al., 2002).

These *Fusarium* mycotoxins are cyclic hexadepsipeptides consisting of alternating D-α-hydroxyisovaleric acids and N-methyl-L-amino acids (Zhukhlistova et al., 1999) (Figure 1). BEA is a common contaminant of grains and has been detected for example in 6 of 22 Italian maize samples, in concentrations ranging from <1 up to 520 mg/kg, with an average of 102 ± 119 mg/kg. In maize samples from Poland, BEA was detected in 13 of 14 samples, with contamination levels ranging between 5 and 60 mg/kg with an average of 18 ± 11 mg/kg (Logrieco et al., 1993; Ritieni et al., 1997). ENNs have been detected for example in Scandinavian barley (ENN A: 21/22 positive samples, range 2-950 µg/kg and mean contamination level of 67 ± 90 µg/kg; ENN A1: 22/22 contaminated samples, contamination range: 10-2000 µg/kg and mean 282 ±300 µg/kg; ENN B: 22/22 positive samples, range: 4-9760 µg/kg, mean: 1622 ± 1482 µg/kg and ENN B1: 22/22 positive samples, range 11-5720 µg/kg and mean: 1514 ± 1691 µg/kg) (Jestoi, 2008). More recently, ENNs have been detected in rice from Maroc (35/70 samples contaminated with ENNs and for ENN A: maximum level 120 mg/kg, mean 23 ± 15 mg/kg; ENN A1: maximum level 449 mg/kg, mean 258 ± 135 mg/kg; ENN B: maximum level 26 mg/kg, mean 9 ± 1 mg/kg; ENN B1: maximum level 24 mg/kg and mean 9 ± 2 mg/kg) (Sifou et al., 2011).

ENNs and BEA are of interest because they can exert antibacterial, antifungal and phytotoxic effects (Jestoi, 2008; Meca et al., 2010; Meca et al., 2011). On the other hand, they can also be toxic for mammalian cells. Their main mode of action is based on their ionophoric properties (Ivanov et al., 1973). They are capable of transporting cations through the cell membrane, leading to toxic actions by an altered membrane potential (Ivanov et al., 1973; Jestoi et al., 2009). Next to their ionophoric properties, ENNs exhibit other effects, such as inhibiting acyl-coenzyme A:cholesterol acyl transferase (ACAT) and 30,50-cyclo-nucleotide phosphodiesterase enzymes (Tomoda et al., 1992) causing mitochondrial dysfunction

(Tonshin et al., 2010), and the inhibition of multidrug resistance associated protein-1 (ABCG2) and P-glycoprotein (ABCB1) efflux pumps (Dornetshuber et al., 2009). Many *in vitro* studies have reported cytotoxic effects of ENNs and BEA on several cell types, as reviewed by Jestoi (2008). Data on their *in vivo* toxicity are however lacking (Jestoi, 2008). Nevertheless, their possible subclinical effects are of greater importance as these may lead to reduced performance parameters in food producing animals and increased susceptibility to infectious disease. Opinions of the European Food Safety Authority (EFSA) on BEA and ENNs are currently being drafted on request by the European Commission. To evaluate their potential *in vivo* toxic effects, knowledge of their toxicokinetic properties, namely absorption, distribution, metabolisation and excretion (ADME) in livestock is crucial. As an example, no information is available on their oral absorption and bioavailability in any animal species, including pigs. Jestoi et al. (2007, 2009) detected trace level residues of ENNs and BEA in Finnish eggs, poultry meat and liver samples. One other study (Frenich et al., 2011) reported trace levels of BEA in eggs from local retailers in Spain. To date, no other data are available on the tissue distribution and presence of these mycotoxins in other animal tissues or on their toxicokinetic properties in general (Meca et al., 2012). This lack of data could explain why there are no maximum guidance levels set for animal feed yet, in contrast to other mycotoxins (European Commission, 2006).

It is obvious that for assessing animal exposure to BEA and ENNs and to investigate their toxicokinetics, the availability of sensitive and specific validated analytical methods is mandatory. In recent decades, high-performance liquid chromatography (HPLC) has become the most important separation method for the analysis of mycotoxins in food, feed and other matrices (Zollner and Mayer-Helm, 2006). For BEA and ENNs, the most common detection method used to be UV-detection. Nowadays mass spectrometry (MS) is in favour over spectrophotometric techniques because of unambiguous identification, detection and accurate quantification. Furthermore, it allows quantification of multiple residues (Jestoi, 2008; Lattanzio et al., 2011; Songsermsakul and Razzazi-Fazeli, 2008; Uhlig and Ivanova, 2004).

Many high-performance liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods for the analysis of ENNs and BEA in food and feed have been described (Logrieco et al., 1993; Sulyok et al., 2006; Uhlig and Ivanova, 2004; Van Pamel et al., 2011), however, analytical methods for detection of these mycotoxins in biological matrices are scarce

(Frenich et al., 2011; Jestoi et al., 2009; Jestoi et al., 2007). Until now, no method for the analysis of these compounds in plasma has been reported.

The goal of this study was to develop a method which quantifies the *Fusarium* mycotoxins BEA, ENN A, A1, B and B1 in animal plasma, using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry (LC-h-ESI-MS/MS). The final method should be applicable for evaluation of their toxicokinetic properties and oral bioavailability in different animal species. Since a large amount of samples are generated in such experiments, special attention has been paid to the development of a high-throughput sample preparation procedure and LC-MS/MS analysis, but not at the expense of reliability and sensitivity.
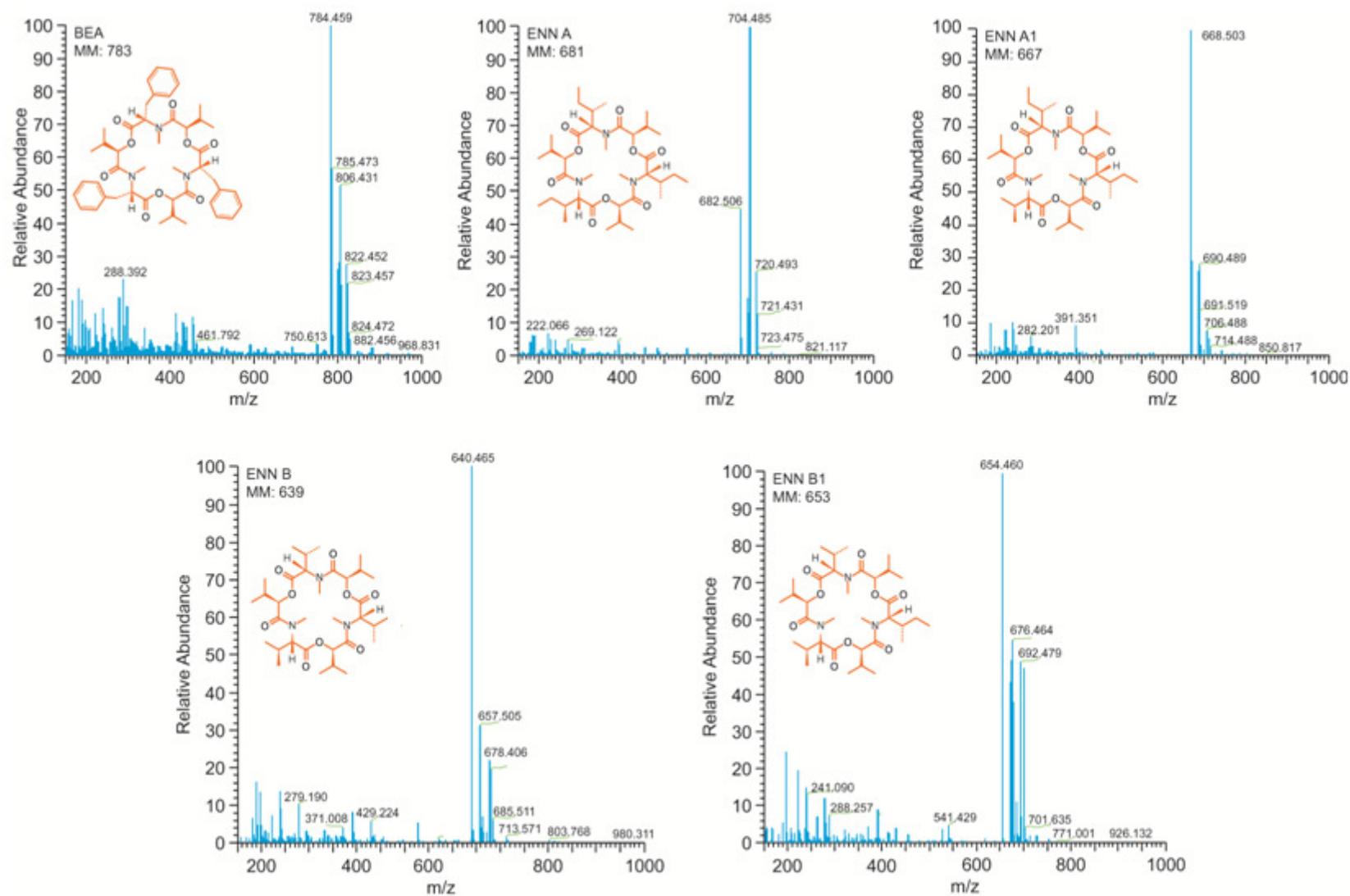
Figure 1. Full mass spectrum (range 150-1000 m/z), chemical structure and molecular mass (MM) of beauvericin (BEA), enniatin (ENN) A, A1, B and B1

2. Materials and Methods

2.1. Chemicals, products and reagents

The analytical standards of ENN A, A1, B and B1 were obtained from Sigma-Aldrich (Bornem, Belgium). Standard of BEA was obtained from Fermentek (Jerusalem, Israel). The internal standard (IS) maduramicin (MAD) was a kind gift from Alpharma (Wilrijk, Belgium). Mycotoxin standards were stored at ≤ -15°C, maduramicin standard at 2-8°C. Water, methanol and acetonitrile (ACN) were of LC-MS grade and obtained from Biosolve (Valkenswaard, The Netherlands). Glacial acetic acid was of analytical grade and obtained from VWR (Leuven, Belgium).

2.2. Preparation of standard solutions

Separate standard stock solutions of all mycotoxins were prepared in ACN (analyte concentration: 1 mg/mL) and were stored at ≤ -15°C (Frenich et al., 2011; Jestoi et al., 2005). A combined working solution of 10 μg/mL of all analytes (except IS) was prepared by transferring 10 μL of each stock solution of 1 mg/mL into an eppendorf tube, followed by further dilution with ACN/water (50/50, v/v) up to a final volume of 1.0 mL. Combined working solutions of 1, 0.1, 0.01 and 0.001 μg/mL were obtained by diluting 100 μL of the 10 times higher concentrated solution with 900 μL ACN/water (50/50, v/v). This method allows leverage to be kept to a minimum. For the IS, a working solution of 1 μg/mL was prepared in ACN/water (50/50, v/v). All working solutions were stored at 2-8°C. The decrease in signal intensity after 4 months storage was for all compounds less than 10% and therefore considered as stable.

2.3. Biological samples

A bolus toxicokinetic study was performed in one piglet (20.2 kg BW). The pig received a single oral intra-gastric bolus of the investigated mycotoxins BEA, ENN A, A1, B and B1 (all 0.05 mg/kg BW). This dose resembled a feed contamination level of 1 mg/kg, since the feed intake of a 20 kg weighing pig is about 1 kg/day. This theoretical feed contamination level was chosen arbitrarily as it lies within the wide range of *Fusarium*-mycotoxins concentration levels that have been detected in feed (~10 μg/kg to > 100 mg/kg) (Jestoi, 2008). The bolus solution was prepared instantly before administration by dissolving the mycotoxin standards

(1 mg of each individual compound) in 1 mL of ACN and further dilution with 50 mL of water. Blood samples were drawn before (0 min) and at 10, 20, 30 and 40 min, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h post-administration in heparinized tubes and centrifuged (2851 x g, 10 min, 4°C). Plasma samples (250 µL) were stored at ≤ -15°C until analysis.

This animal experiment was approved by the Ethical Committee of Ghent University (case number EC 2012_110).

2.4. Sample pretreatment

To 250 µL of plasma were added 12.5 µL of the IS working solution and 750 µL of ACN, followed by a vortex mixing (15 sec) and centrifugation step (8517 x g, 10 min, 4°C). Next, the supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (45 ± 5°C). The dry residue was reconstituted in 200 µL of ACN/water (20/80, v/v). After vortex mixing (15 sec), the sample was transferred into an autosampler vial. The injection volume was 5 µL. One extraction and LC-MS/MS analysis was performed for each sample.

2.5. Liquid chromatography

The chromatographic system consisted of a quaternary, low-pressure mixing pump with vacuum degassing (Surveyor MS pump Plus, ThermoFischer Scientific, Breda, The Netherlands) connected to an autosampler (Autosampler Plus, Thermo Fisher Scientific) with temperature controlled tray and column oven. Chromatographic separation was achieved on a Hypersil Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type (10 mm x 2.1 mm i.d., dp: 3 µm), both from Thermo Fisher Scientific. The temperatures of the column oven and autosampler tray were set a 45°C and 5°C, respectively. Mobile phase A consisted of 0.1% glacial acetic acid in water whereas mobile phase B was ACN. Following gradient elution program was run: 0-0.5 min (70% A, 30% B), 0.5-2.5 min (linear gradient to 20% A), 2.5-8.5 min (20% A, 80% B), 8.5-10.0 min (linear gradient to 70% A), 10.0-12.0 min (70% A, 30% B). Flow rate was set at 300 µL/min.

2.6. Mass spectrometry

The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe operating in the positive ionization mode (all from Thermo Fisher Scientific). Instrument parameters were optimized by syringe infusion of working solutions of 1 µg/mL of each compound (flow rate 10 µL/min) in combination with the mobile phases (50% A, 50% B, flow rate: 200 µL/min).

The following general MS/MS parameters were used: spray voltage: 4000 V, vaporizer temperature: 300 °C, sheath gas pressure: 49 au (arbitrary units), ion sweep gas pressure: 2.0 au, auxilliary gas pressure: 30 au, capillary temperature: 250 °C, source CID collision energy: 10 V, collision pressure: -1.5 mTorr and quad MS/MS bias: 2.9. The resolution for Q1 and Q3 were set at 0.7 full width at half maximum (FWHM).

Acquisition was performed in the selected reaction monitoring (SRM) mode. For each compound, the two most intense product ions of the precursor ion were monitored in the SRM mode for quantification and identification, respectively (see Table 1).

Table 1. SRM transitions and MS/MS parameters for the target analytes

Analyte	Precursor ion (m/z)	Product ions (m/z)		RT (min)	CE (V)	TLO (V)
BEA	784.46 $[M+H]^+$	244.06 261.99	*	5.12	25 24	214
ENN A	704.48 $[M+Na]^+$	350.15 231.99	*	5.45	55 45	180
ENN A1	668.50 $[M+H]^+$	210.05 228.18	*	5.26	29 30	145
ENN B	640.46 $[M+H]^+$	196.15 214.01	*	4.79	28 30	137
ENN B1	654.46 $[M+H]^+$	196.08 214.07	*	5.03	35 35	186
MAD	934.49 $[M+H]^+$	629.23 393.14	*	6.99	24 29	150

m/z: mass to charge ratio, RT: retention time; CE: collision energy; TLO: tube lens offset; the asterix (*) indicates the quantifier ion; BEA: beauvericin; ENN: enniatin; MAD: maduramicin

2.7. In-house method validation

The method was validated according to a validation protocol previously described by De Baere et al. (2011). A set of parameters that were in compliance with the recommendations and guidelines defined by the European Community and with criteria described in the literature, were evaluated (European Commission, 2002; Heitzman, 1994; Knecht and Stork, 1974; VICH GL 46). This includes evaluation of linearity, within- and between-run accuracy, within- and between-run precision (RSD_r and RSD_R, respectively), limit of detection (LOD), limit of quantification (LOQ), specificity, carry-over, extraction recovery (R_E) and signal suppression/enhancement (SSE).

Linearity was evaluated by preparing matrix-matched calibration curves over a concentration range of 0.1 - 200 ng/ml. This concentration range was chosen based on studies with other mycotoxins (De Baere et al., 2011; De Baere et al., 2012; Devreese et al., 2012a). Calibration curve samples (calibration levels (0.1,) 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL) were prepared by applying standard working solutions directly onto the blank plasma samples, followed by a vortex mixing step. After 5 min of equilibration, the calibration curve samples were treated in a similar way as the unknown samples. The correlation coefficients (r) and goodness-of-fit coefficients (g) were calculated and had to be ≥ 0.99 and ≤ 20%, respectively.

RSD_r (repeatability) and accuracy were determined by analyzing six blank samples spiked at low (1 ng/mL) and high (10 and 100 ng/mL) concentration levels in the same run. The RSD_R (reproducibility) and accuracy were determined by analyzing quality control samples (1, 10 and 100 ng/mL) together with each analytical batch of samples, run on three different days. The acceptance criteria for accuracy were: -50% to +20%, -30% to +10% and -20% to +10% for concentrations <1 ng/mL, between 1 and 10 ng/mL and > 10 ng/mL, respectively. For the precision, the relative standard deviation (RSD, %) had to fall within 2/3 of the values calculated according to the Horwitz equation: $RSD_{max} = 2^{(1-0.5\log Conc)}$ x 2/3 for within-run precision, and within the values calculated according to the Horwitz equation for between-run precision $RSD_{max} = 2^{(1-0.5\log Conc)}$ (European Commission, 2002; Heitzman, 1994). RSD_{max} values for the RSD_r were as follows: 1 ng/mL: 30.2%, 10 ng/mL: 21.3% and 100 ng/mL: 15.1%. For the RSD_R the RSD_{max} values were: 1 ng/mL: 45.3%, 10 ng/mL: 32.0% and 100 ng/mL: 22.6%.

The limit of quantification (LOQ) was the lowest concentration of the analytes for which the method was validated with an accuracy and precision that fell within the recommended ranges (see section accuracy and precision and Table 2). The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing six samples spiked at 0.1 or 0.2 ng/mL, on the same day.

The limit of detection (LOD) was defined as the lowest concentration of the respective analytes that could be recognized by the detector with a signal-to-noise (S/N) ratio of ≥3. The LOD values were calculated using samples spiked at the LOQ level.

The specificity of the method was evaluated with respect to interferences from endogenous compounds. Therefore, one blank sample was analyzed using the above mentioned procedure. The S/N ratio of a possible interfering peak in the blank sample had to be below the S/N ratio of the analyt(es) in the same elution zone at the LOD level.

The carry-over on the LC-MS/MS instrument was evaluated by analyzing a water/ACN sample (50/50, v/v) just after the highest calibrator sample. The eventual analyte concentration in the sample had to be below the LOD.

Recovery experiments were performed according to Matuszewski et al. (2003). In summary, two types of matrix-matched calibration curves were prepared for each analyte, namely by spiking the blank calibrator samples before (spiked) and after extraction (spiked extract). One calibration curve was prepared using standard solutions. The calibration curves were 1/x weighted in order to reduce the risk of leverage. The slopes of the resulting linear, or quadratic for ENN A, 1/x weighted, calibration curves with spiked and spiked extracts samples were compared with the related slopes of the calibration curves with spiked extracts and standard solution, in order to calculate the recovery of the extraction step (R_E) and the signal suppression/enhancement (SSE) due to matrix effects, respectively.

Table 2. Results of the evaluation of linearity (goodness-of-fit coefficient (g), correlation coefficient (r)), extraction recovery (R_E), signal suppression/ enhancement (SSE), limit of quantification (LOQ), limit of detection (LOD) for all analytes

Analyte	Calibration Range (ng/mL)	Spike levels (ng/mL)	g (%)	r	R_E (%)	SSE (%)	LOQ (ng/mL)	LOD (pg/mL)
BEA	0.2-200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	11.30	0.9980	81.94	90.56	0.2	10
ENN A	0.1-200	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	8.48	0.9995	91.03	111.03	0.1	1.1
ENN A1	0.1-200	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	6.19	0.9995	82.28	81.49	0.1	3.3
ENN B	0.2-200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	5.19	0.9992	94.29	91.60	0.2	4.2
ENN B1	0.2-200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	11.31	0.9992	88.31	92.26	0.2	8.1

Note: acceptance criteria: g $\leq$ 20 % (VICH GL 49, 2009), r $\geq$ 0.99 (European Commission, 2002), LOD: S/N > 3 (European Commission, 2002; VICH GL 49, 2009), LOQ: accuracy -50 % to +20 %, RSD_{max} < 30.2 % (European Commission, 2002; Heitzman, 1994), R_E and SSE (Matuszewski et al., 2003)

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation and extraction

The goal of this study was to develop a sensitive LC-MS/MS method for screening and quantitation of BEA and ENNs in pig plasma. These plasma samples can be obtained during assessment experiments to evaluate animal exposure to these mycotoxins (screening) or as a part of toxicokinetic studies (quantitation). Since these surveys or trials generate a large number of samples, the use of a simple and practical sample preparation procedure is advisable in order to reduce the time and cost of analysis. When using a sensitive and specific analytical technique such as LC-MS/MS, clean-up of raw extracts can be kept to a minimum (Van Pamel et al., 2011). However, the most simple procedure, i.e. dilute-and-shoot, was not found suitable for plasma analysis, as this could lead to clogging and serious contamination of the MS instrument. On the other hand, more sophisticated extraction procedures using solid-phase extraction (SPE), for example, are not recommended as they are generally time-consuming and expensive, which is a disadvantage if a large amount of samples have to be analyzed (i.e. for screening or toxicokinetic analysis). Moreover, selecting the right column may sometimes be tricky and when using a silica based SPE column, selectivity is rather low (Songsermsakul and Razzazi-Fazeli, 2008). Such SPE columns have been used as sample preparation technique for BEA and ENNs analysis, although the recoveries are generally lower compared to our sample preparation method (Jestoi et al., 2005). The sample preparation for eggs, meat and liver samples described by Jestoi et al. (2007, 2009) consisted of extraction with ACN followed by SPE (silica). Frenich et al. (2011) used a QuEChERS-based extraction procedure followed by SPE (silica or Oasis HLB) for egg samples. As mentioned for SPE, the disadvantage is mainly the labor when a large amount of samples have to be analyzed. To achieve a good compromise between simplicity of extraction and acceptable sample clean-up, a generic extraction procedure was developed which consisted of a combination of protein precipitation with liquid extraction.

Organic solvents, such as methanol and acetonitrile, are commonly used for deproteinization of plasma samples (Corcuera et al., 2011; De Baere et al., 2011). In our method, 750 µL of ACN was used for combined precipitation of plasma proteins and extraction of mycotoxins,

as it showed high extraction recoveries (R_E, %) for all compounds (ranging from ± 82 to 94 %), as can be seen in Table 2. Acetonitrile gave more clear supernatants after centrifugation compared to methanol, indicating improved deproteinization efficiency. Deproteinization with ACN has also been successfully applied for extracting other mycotoxins from plasma (De Baere et al., 2012; Devreese et al., 2012a). In order to concentrate the samples as much as possible, the supernatants were evaporated under nitrogen and reconstituted in 200 µL acetonitrile/water (20/80, v/v). The addition of organic solvent to dissolve the residue is crucial due to the lipophilicity of the investigated toxins (Jestoi, 2008).

Matrix effects (SSE, %) were evaluated and varied between 90 and 111 % for all compounds (Table 2). These results are excellent taking into account that a generic extraction procedure was used. They correlate well with other matrices such as wheat (Sulyok et al., 2006) and there is less ion suppression compared to egg samples (Frenich et al., 2011; Jestoi et al., 2009). Hence, it can be stated that a generic extraction procedure was developed for the simultaneous extraction of several emerging *Fusarium* mycotoxins from animal plasma. The extraction procedure was straightforward, cheap and combined a high extraction recovery with limited matrix effects on the LC-MS/MS instrument.

3.1.2. Optimization of LC-MS/MS conditions

The Hypersil Gold column was used as good results were obtained with respect to separation of analytes by HPLC within a short analysis time, i.e. <15 min, comparable to other chromatographic methods used for separation of these compounds (De Baere et al., 2012; Frenich et al., 2011; Jestoi et al., 2009).

For the detection of ENNs, the mobile phases reported in the literature consist generally of a combination of water and an organic solvent (methanol or acetonitrile) (Jestoi, 2008). To facilitate the ionization process in mass spectrometry, often volatile acids are added (formic acid, acetic acid) (Jestoi, 2008). In our experiments the best sensitivity and separation for all investigated compounds was achieved using 0.1 % acetic acid in water (A) and ACN (B) as mobile phase (Supplementary Material Figure 2S). For other mycotoxins, including DON, ZON, T-2, OTA, FB1 and AFB1, methanol seems to be a more suitable organic solvent for the separation of those compounds (Jestoi et al., 2005).

The MS instrument was operated in the positive ion electrospray mode (ESI +) as this was the most sensitive mode, confirmed by previous studies (Jestoi et al., 2007; Logrieco et al., 1993; Meca et al., 2012). Using ESI, adducts are often formed, i.e. with NH_4^+, Na^+, K^+ and Ca^{2+} (Jestoi et al., 2005). This is however dependent on type of mobile phases and MS parameters. Uhlig and Ivanova (2004) showed that a higher capillary temperature favors protonation ($[M+H]^+$) instead of $[M+NH_4]^+$ adduct formation for ENNs and BEA. Despite adduct formation, the majority of the molecules are protonated and consequently used for detection and quantification by several other authors (Jestoi et al., 2007; Jestoi et al., 2009; Uhlig and Ivanova, 2004). In our experiments sodium adduct formation was also present, but $[M+H]^+$ ions gave the most intense signal except for ENN A where the $[M+Na]^+$ ion was monitored, as did Frenich et al. (2011). The full scan MS spectra of precursor and product ions are shown in Figure 1 and 2, respectively.

Acquisition was performed in the SRM mode, which means that for each analyte the two most intense precursor ion > product ion transitions were monitored. The two most intense product ions were generated both with an automated procedure and manually by comparing the signal intensity with applying increased collision energy. The same product ions were retrieved by both methods and comparable collision energies were found. The product ion with the highest intensity was selected as quantifier, while the other ion was used as qualifier. In Table 1 the MS/MS conditions for all target analytes are shown.
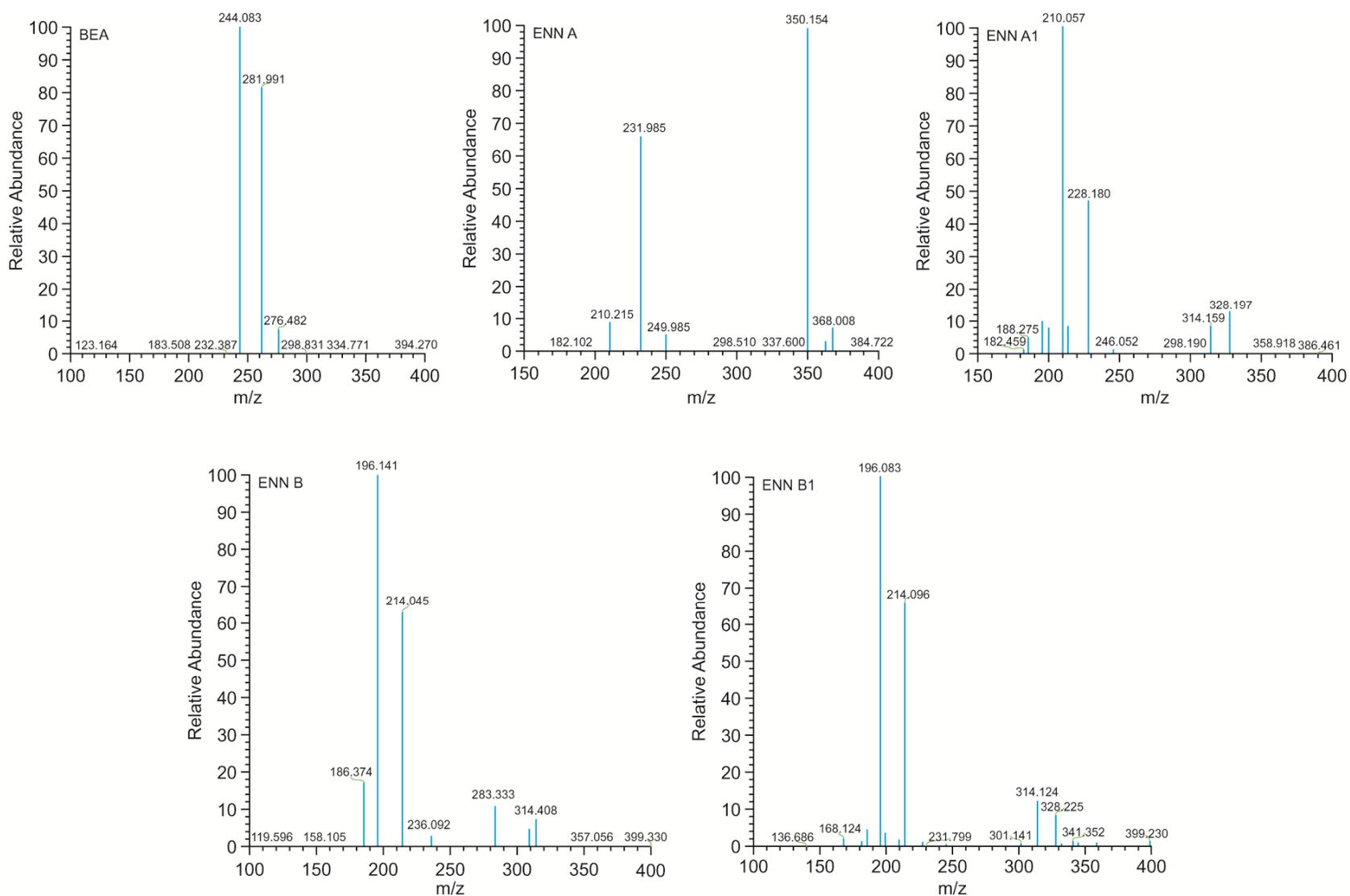
Figure 2. Full mass spectrum (100-400 m/z) of the product ions of beauvericin (BEA), enniatin (ENN) A, A1, B and B1

3.1.3. Internal standard

Other authors (Frenich et al., 2011; Jestoi et al., 2009; Jestoi et al., 2007) describing methods for quantification of ENNs and BEA in biological matrices did not use an internal standard (IS). However, it is preferable to use an IS, because this leads to maximal compensation for losses during extraction, signal suppression/enhancement effects during LC-MS/MS analysis, and general analysis errors (Songsermsakul and Razzazi-Fazeli, 2008). Isotopically labeled IS are seen as the ideal IS, because of their similar physico-chemical properties as the target compound (Spanjer et al., 2008). However, these IS are very costly or not commercially available for ENNs nor BEA, in contrast to other mycotoxins (Devreese et al., 2012a). Then compounds having similar physicochemical properties are often used as IS. Ionophoric coccidiostats are closely related to ENNs and BEA, especially based on their chemical structure and properties (Jestoi et al., 2007), therefore they can be used as an IS (Van Pamel et al., 2011). In our study maduramicin was used since it showed improved fragmentation patterns compared to others such as valinomycin.

However, it has to be considered that coccidiostats are frequently used as feed additives in a practical situation for animal production. Therefore, it must be verified before the start of the analysis that MAD is not present in the animal feed, e.g. by analyzing the feed or an animal plasma sample without the addition of the IS (MAD). Otherwise contamination of plasma samples with MAD can occur, which should be avoided when performing toxicokinetical studies or assessing exposure.

3.2. Method validation

The following parameters were evaluated for each mycotoxin: linearity, within- and between-run precision and accuracy, LOQ, LOD and specificity. The results are shown in Table 3.

Table 3. Results of the within-run and between-run precision and accuracy evaluation for the analysis of emerging *Fusarium* mycotoxins in pig plasma determined by a multi-mycotoxin LC-MS/MS method

Analyt	Theoretical concentration (ng/mL)	Mean concentration ± SD (ng/mL)	Precision, RSD (%)	Accuracy (%)
BEA	0.2 [a]	0.2 ± 0.01	6.9	1.1
	1 [a]	1.0 ± 0.13	12.1	4.4
	1 [b]	1.0 ± 0.11	11.0	0.6
	10 [a]	10.5 ± 0.29	2.8	4.7
	10 [b]	10.0 ± 1.38	13.8	0.1
	100 [a]	100.4 ± 5.03	5.0	0.4
	100 [b]	105.5 ± 11.45	10.9	5.5
ENN A	0.1 [a]	0.1 ± 0.01	10.8	7.6
	1 [a]	1.0 ± 0.02	1.9	2.3
	1 [b]	0.9 ± 0.11	12.0	-6.1
	10 [a]	9.8 ± 0.20	2.1	9.3
	10 [b]	9.0 ± 2.80	30.9	-9.5
	100 [a]	103.3 ± 9.66	9.3	3.3
	100 [b]	105.3 ± 14.4	13.7	5.3
ENN A1	0.1 [a]	0.1 ± 0.01	10.0	13.1
	1 [a]	1.0 ± 0.15	14.5	0.5
	1 [b]	1.1 ± 0.10	9.4	8.9
	10 [a]	10.8 ± 0.73	6.7	8.3
	10 [b]	10.3 ± 0.44	4.2	2.7
	100 [a]	109.5 ± 7.62	7.0	9.5
	100 [b]	100.0 ± 7.49	7.5	0.0
ENN B	0.2 [a]	0.2 ± 0.02	10.8	4.4
	1 [a]	1.1 ± 0.07	6.7	6.9
	1 [b]	1.0 ± 0.09	9.0	1.1
	10 [a]	10.6 ± 0.74	7.0	5.7
	10 [b]	10.5 ± 0.55	5.2	4.7
	100 [a]	101.4 ± 1.69	1.7	1.4
	100 [b]	102.9 ± 6.96	6.8	2.9
ENN B1	0.2 [a]	0.2 ± 0.03	13.6	5.8
	1 [a]	1.0 ± 0.04	3.4	4.7
	1 [b]	1.1 ± 0.08	7.0	6.3
	10 [a]	10.4 ± 0.59	5.7	3.5
	10 [b]	10.2 ± 0.71	7.0	1.9
	100 [a]	97.9 ± 4.12	4.2	-2.1
	100 [b]	105.9 ± 7.83	7.4	5.9

[a] Within-run accuracy and precision (n=6); [b] Between-run accuracy and precision (n=12); SD: standard deviation; RSD: relative standard deviation; Acceptance criteria: accuracy: < 1 ng/mL: -50% to +20%, 1-10 ng/mL: -30% to +10%, > 10 ng/mL: -20% to +10%, within-run precision (RSD_{max}): 1 ng/mL: 30.2%, 10 ng/mL: 21.3% and 100 ng/mL: 15.1%, between-run precision: 1 ng/mL: 45.3%, 10 ng/mL: 32.0% and 100 ng/mL: 22.6%.

Matrix-matched calibration graphs were linear over the working concentration range for all tested mycotoxins, with r values between 0.9980 and 0.9995 and g values between 5.2 and 11.3 %. For all compounds except ENN A, the best fitting graphs were linear and 1/x weighted. For ENN A, the best fitting was obtained by applying quadratic, 1/x weighted regression since a small deviation from linearity was observed for concentrations above 50

ng/ml. As can be seen from Table 3, values for r (0.9995) and g (8.48 %) fell within the accepted ranges, indicating the reliability of the quadratic calibration model for quantitation of ENN A in real samples up to a concentration level of 200 ng/ml .

The within- and between-run accuracy and precision were tested at three different concentration levels and fell within the acceptability ranges. The limits of quantification (LOQ) were 0.1 ng/mL for ENN A and A1 and 0.2 ng/mL for ENN B, B1 and BEA. These limits are comparable (Jestoi et al., 2009; Jestoi et al., 2007) or ten-fold lower (Frenich et al., 2011) than other LC-MS/MS methods quantifying ENNs and BEA in other biological matrices.

The limits of detection (LOD) were calculated taking into account a S/N ratio of 3 and were 10 pg/mL, for BEA, or lower, for all other analytes. Several studies reported that plasma concentrations of mycotoxins in general fall within the lower ppb (ng/mL) range. After bolus administration of DON, ZON, OTA and AFB1 to pigs (all 0.05 mg/kg BW), mean plasma concentrations ranged between 0 and respectively 30.1, 2.1, 132.0 and 2.1 ng/mL (Devreese et al., 2012a). After feeding a DON contaminated diet (7.5 mg/kg feed) to broiler chickens for 21 consecutive days, mean plasma levels were 3.87 ± 0.86 ng/mL at day 7, 2.66 ±2.02 ng/mL at day 14 and <LOQ (1.25 ng/mL) after 21 days (De Baere et al., 2011; Osselaere et al., 2012). In human plasma samples, OTA was detected at very low concentrations, 0.25 ± 0.03 ng/mL (Munoz et al., 2009). When developing a method for assessing mycotoxin exposure in animals by analyzing plasma, it is therefore a prerequisite to reach these low LOQ and LOD levels. Moreover, for toxicokinetic studies, dosing high mycotoxin concentrations to animals is no longer ethically acceptable as it compromises animal welfare. From the results shown in Table 3, it can be concluded that the developed method succeeded in quantifying the investigated ENNs in spiked matrix-matched samples at concentration levels which can be expected in real pig plasma samples after administration of one oral bolus of 0.05 mg/kg BW (range: 0.1/0.2 ng/ml – 200 ng/ml). For BEA, the results for accuracy and precision fell within the specified ranges for the analysis of matrix-matched samples that were spiked in the same concentration range (0.2 ng/ml to 200 ng/ml). However, taken into account the low oral bioavailability of BEA (see section 3.3), it is clear that a higher oral dosing (> 0.05 mg/kg BW) is needed to reach quantifiable plasma levels.

An analyzed blank sample did not demonstrate a possible interfering peak with a S/N ratio above the S/N ratio of the analyt(es) in the same elution zone at the LOD level, testifying the good specificity of the method.

No carry-over was present, as there were no peaks detected in the same retention time zone of the compounds of interest, as can be seen in Figure 1S (Supplementary Material).

3.3. Biological samples

To demonstrate the applicability of the developed mycotoxin method, one piglet was administered an intra-gastric bolus of BEA, ENN A, A1, B and B1 (dose 0.05 mg/kg BW). Blood samples were drawn before and at different time points post-administration. One blood sample was drawn per time-point and analyzed once using the described method. In Figure 3, a LC-MS/MS chromatogram is demonstrated showing the SRM traces of the target analytes for the analysis of a pig plasma sample that was taken at 40 min after the oral administration of 0.05 mg/kg BW of the target analytes. Following analysis, a plasma concentration-time profile for ENN A, A1, B and B1 was set-up and shown in Figure 4. Interestingly, there is a big difference in oral absorption between the different ENNs although they have a similar chemical structure. ENN B seems to have the highest oral absorption, followed by ENN B1, A1, A and finally BEA. The maximal plasma concentrations (C_{max}) for ENN B, B1, A1 and A were respectively 73.4, 35.2, 11.6 and 6.8 ng/mL. The time to maximal plasma concentration (T_{max}) was 20 min after bolus administration for ENN B1, B and A1, whereas the T_{max} for ENN A was 30 min post-administration. For BEA, no plasma concentration-time profile could be designed as the plasma concentration was above the LOQ level at only 2 time points, i.e. 0.51 ng/mL at 30 min and 0.82 ng/mL at 40 min post-administration. The elimination rate of all ENNs is fast and comparable to DON (Devreese et al., 2012b; Knecht and Stork, 1974). With the dose tested, the applicability of the developed method for toxicokinetical analysis of ENNs has been demonstrated. As BEA has now demonstrated to have a lower oral bioavailability, higher dosing is needed to reach quantifiable plasma levels. These results should be taken into account when a comprehensive animal experiment would be conducted to determine the toxicokinetic parameters of these mycotoxins.
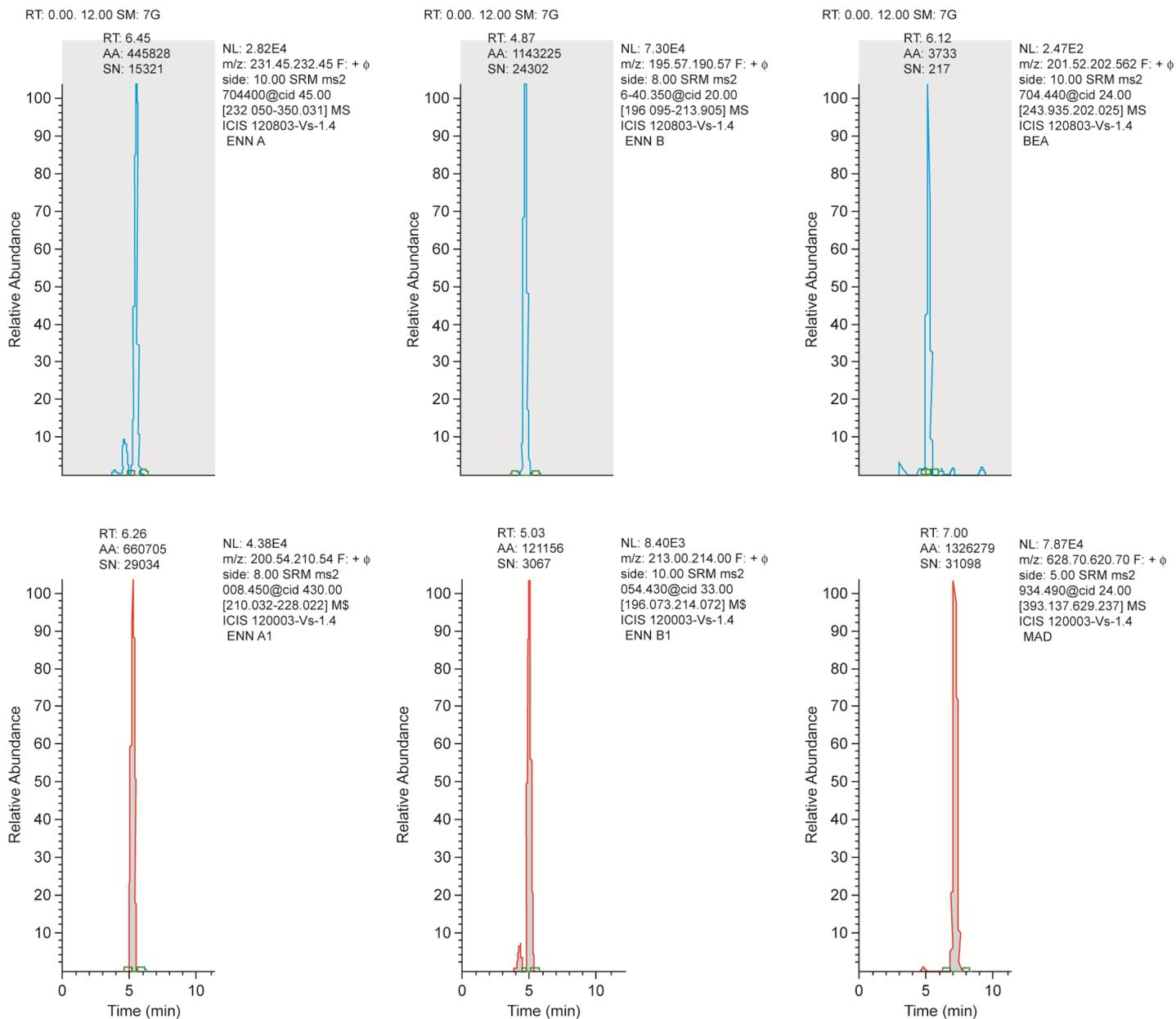
Figure 3. LC-MS/MS chromatogram showing the SRM traces of the target analytes for the analysis of a pig plasma sample that was taken at 40 min after the oral administration of 0.05 mg/kg BW of the target analytes
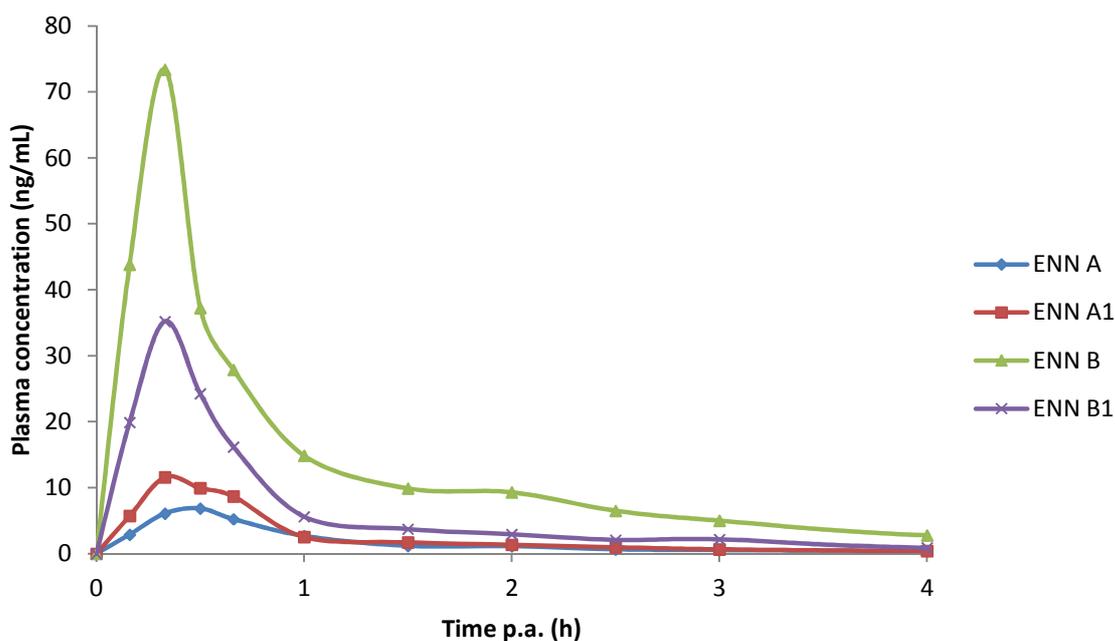
Figure 4. Plasma concentration time profile of ENN A, A1, B and B1 after single oral bolus administration of the target analytes (dose 0.05 mg/kg BW) to a pig

4. Conclusions

This paper describes a sensitive and specific LC-MS/MS method for the analysis of several important *Fusarium* mycotoxins in pig plasma. All analytes were isolated from plasma by performing a generic, fast and low-cost sample preparation procedure. The extracted samples were analyzed using a multi-mycotoxin method which can be applied in the field of animal exposure assessment and toxicokinetic studies. The method was successfully validated and the applicability for toxicokinetical analysis of ENNs was shown by the analysis of incurred plasma samples that were taken from a pig which received an oral bolus of the investigated mycotoxins.

In conclusion, this is - to our knowledge - the first paper describing the simultaneous detection and quantification of these emerging *Fusarium* mycotoxins in animal plasma.

5. Acknowledgments

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7. Supplementary Material
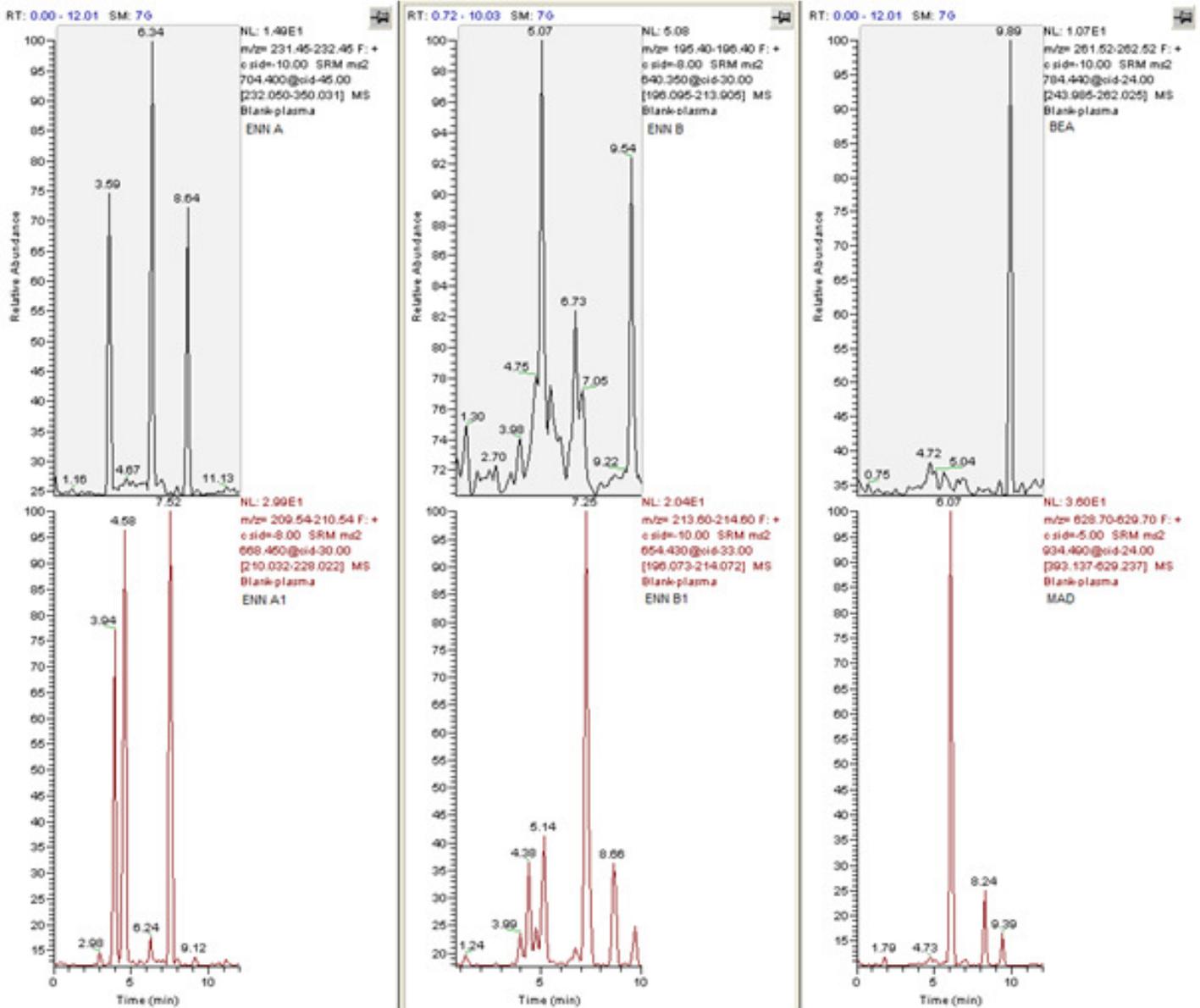
Figure 1. LC-MS/MS chromatogram showing the SRM traces of the target analytes for the analysis of a blank pig plasma sample
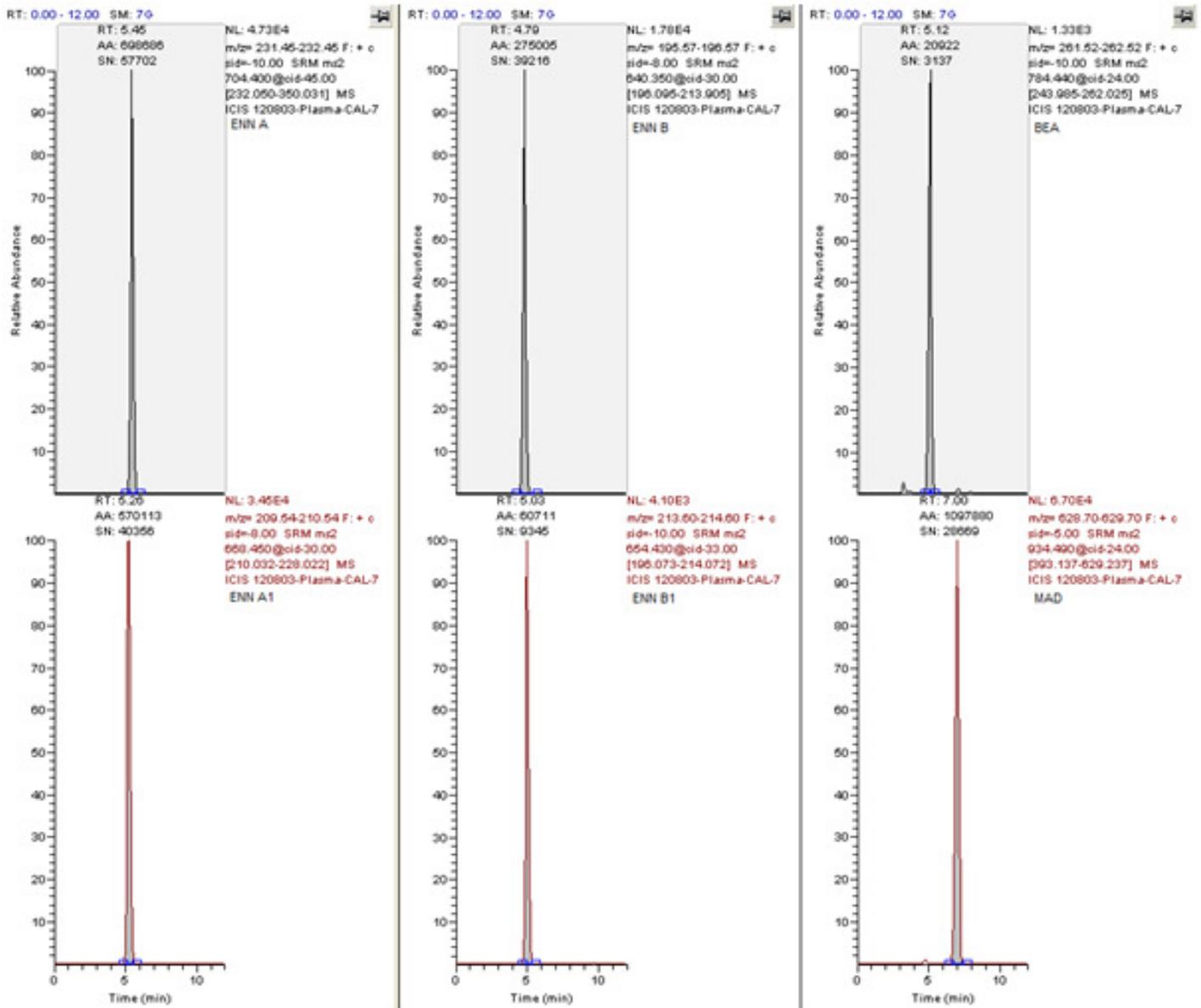
Figure 2. LC-MS/MS chromatogram showing the SRM traces of the target analytes for the analysis of (a pig plasma spiked with the analytes beauvericin (BEA), enniatin (ENN) A, A1, B, B1 and the internal standard maduramicin (MAD) at 10 ng/mL

CHAPTER IV

New bolus models for *in vivo* efficacy testing of mycotoxin detoxifying agents in relation to EFSA guidelines, assessed using deoxynivalenol in broiler chickens

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Abstract - In this study, three new models were developed for efficacy testing of mycotoxin detoxifiers in relation to recent European guidelines. In the first model, deoxynivalenol was given to broiler chickens as an intra-crop bolus together with a mycotoxin detoxifier in order to study the plasma concentration-time profile of deoxynivalenol. In the second model the same oral bolus was given, preceded by an oral bolus of mycotoxin detoxifier, to make sure the detoxifier was present in the whole intestinal tract when the mycotoxin was administered. In the third model, the mycotoxin detoxifier was mixed in the feed of broiler chickens, and after one week feeding, deoxynivalenol was given as an oral bolus. In order to evaluate the efficacy of these agents, plasma concentration-time profiles were set up and the main toxicokinetic parameters were compared. Two commercially available mycotoxin detoxifiers were tested, but they were not able to lower the oral availability of deoxynivalenol. As a positive control, activated carbon was used. We showed that activated carbon significantly reduced the absorption and oral availability of deoxynivalenol in all three models. Therefore, it can be concluded that these models are able to demonstrate the efficacy of mycotoxin detoxifiers in relation to EFSA guidelines.

Keywords – mycotoxins – deoxynivalenol - efficacy testing - mycotoxin detoxifier – modeling - legal assessment

1. Introduction

The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production (Wu, 2007). Consequently, a variety of methods for the decontamination of feed have been developed, but mycotoxin detoxifying agents (mycotoxin detoxifiers) seem to be the most promising and are therefore most commonly used (Jard, et al., 2011, Kolosova and Stroka, 2011). These detoxifiers can be divided into two different classes, namely mycotoxin binders and mycotoxin modifiers. These two classes have different modes of action; mycotoxin binders adsorb the toxin in the gut, resulting in the excretion of toxin-binder complexes in the faeces, whereas mycotoxin modifiers transform the toxin into non-toxic metabolites (EFSA, 2009). The extensive use of these additives has led, in 2009, to the establishment of a new group of feed additives: 'substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action' (European Commission, 2009). Evidently, the efficacy of these products for their adsorbing or degrading ability should be tested. Many *in vitro* methods have been developed ranging from single-concentration studies to classical isotherm studies (binder concentration fixed, toxin concentration increasing) and beyond, to more complex set-ups such as gastro-intestinal tract models (EFSA, 2009). Nevertheless, in recent guidelines the European Food Safety Authority (EFSA) has stated that *in vitro* tests do not fully prove the efficacy of mycotoxin detoxifiers (EFSA, 2010) and that *in vivo* trials should be performed. Although these *in vivo* trials can report non-specific parameters such as organ weight, performance parameters (e.g. growth rate, feed conversion rate) and blood serum parameters (e.g. total protein, albumin, key enzymes), they are not sufficient as proof of efficacy of mycotoxin detoxifiers. In addition, specific parameters should be measured based on toxicokinetic studies including the bioavailability and absorption/excretion of the toxin. For each mycotoxin the EFSA has proposed specific end-points. For deoxynivalenol (DON), the most relevant end-point is measuring DON and its major metabolite (deepoxy-deoxynivalenol or DOM-1) in plasma.

In their guidelines, the EFSA proposes feeding trials in which the mycotoxin and detoxifier are mixed in the feed (steady-state design). In these experimental set-ups the pre-sampling period should not be shorter than seven days, and the blood samples should be collected

over a five-day period during feeding (EFSA, 2010). However, these trials are labor intensive and quite complicated to perform. Moreover, in a three week feeding trial with broiler chickens where the maximum allowed level of 5 mg DON/kg feed (European Commission, 2006) was added to the feed, no plasma concentrations of DON and DOM-1 could be measured when sampled on a weekly basis (Osselaere, et al., 2012). This indicates that a model where DON and detoxifier are mixed in the feed, is not an appropriate way to prove the efficacy of mycotoxin detoxifiers for DON in broiler chickens using EFSA endpoints.

Therefore, we propose oral bolus models which are easy to perform, have a straightforward design and can easily be adopted by the feed additive producing industry. All three proposed models in our study are in relation to the EFSA guidelines, stating that specific parameters should be evaluated, based on *in vivo* toxicokinetic or ADME studies (absorption, distribution, metabolisation and excretion) (EFSA, 2010).

To the author's knowledge, no studies have been published yet according to these recent EFSA guidelines. Broiler chickens were chosen as they are convenient to handle and blood collections can be easily performed. Moreover, poultry meat represents one-third of all meat produced globally, indicating the major importance of the broiler chicken industry (Scanes, 2007). As mycotoxin, deoxynivalenol was used as it is the most common mycotoxin found in European feed commodities. In a recent study it was found that 78% of European feed samples were contaminated with DON (Monbaliu, et al., 2010). Deoxynivalenol is produced by several fungi of the *Fusarium* genus and it impairs the protein synthesis by binding to the 60S ribosomal unit and therefore interferes with the activity of peptidyltransferase. Trichothecenes can also cause the 'ribotoxic stress syndrome' by activating mitogen-activated protein kinases (MAPKs) (Pestka, 2007). Poultry seem to be relatively resistant to DON compared to other species, especially pigs. Nevertheless, low to moderate levels of this toxin can cause several effects, which influence immunological and performance parameters (Awad, et al., 2006).

In our models, we tested two commercially available mycotoxin detoxifiers on their ability to lower the oral bioavailability of DON. The first product is a mycotoxin binder, composed of esterified glucomannan (GMA) derived from the cell wall of *Saccharomyces cerevisiae* yeasts. Unspecific *in vivo* trials have shown the ability of the GMA product to counteract the

negative effects of DON on performance parameters and blood biochemical parameters in broiler chickens and pigs (Aravind, et al., 2003, Faixova, et al., 2006, Swamy, et al., 2004). The second product is a combination of mycotoxin binder and modifier. The bentonite fraction (binder) has a high affinity towards aflatoxins, but not towards DON (Avantaggiato, et al., 2005) as aflatoxins are hydrophilic planar structures with a high affinity for planar surfaces. In contrast, DON is a non-ionisable molecule with a more polar structure and a bulky epoxy group and therefore not easily bound by mycotoxin binders (EFSA, 2009). Nevertheless, this product also contains a yeast, claimed to be able to open the C-12,13 epoxide ring, converting DON into a non-toxic metabolite DOM-1 (Awad, et al., 2010, Diaz, et al., 2005). This mycotoxin detoxifier showed potential in diminishing the deleterious effects of DON on growth performance and other non-specific parameters in pigs (Plank, et al., 2009). However, Dänicke et al. (2003) could not show benefits of this detoxifier on performance and blood chemical parameters in poultry. As positive control, activated carbon was used as it proved to adsorb various compounds, including mycotoxins such as DON (Avantaggiato, et al., 2004, Cavret, et al., 2010).

2. Materials and methods

2.1. Animals and housing conditions

For each bolus model, thirty-two twenty-one-day-old healthy broiler chickens (Ross 308, Poeke, Belgium) were randomly allotted in 4 groups of eight chickens, males and females equally divided. The animals were housed in pens of 4 m^2/pen (8 animals/pen), one week before the start of the experiment to adapt to the environment. Blank feed was given *ad libitum* during the trial. The light schedule was 20 h light, 4 h darkness. The temperature was kept between 18 and 25°C. The relative humidity was between 40 and 80%. The bedding of the pens consisted of wood shavings, allowing the animals to perform their natural dust bathing and foraging behaviour.

This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, case number EC 2011_14).

2.2. Feed

Commercially available broiler feed (Bromix Plus®) was obtained from Versele-Laga (Deinze, Belgium). This feed was analyzed for the presence of mycotoxins by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Fytolab, Zwijnaarde, Belgium). The analyzed mycotoxins were aflatoxin B1, B2, G1 and G2, cytochalasin E, deoxynivalenol, 3-acetyl-deoxynivalenol, nivalenol, fumonisin B1 and B2, T-2 and HT-2 toxin, ochratoxin A, zearalenone, α- and β-zearalenol. The concentrations of the mycotoxins were all below the limit of detection (LOD), which was 100 µg/kg for DON, 3-acetyl-DON and nivalenol and between 0.5 and 50 µg/kg for the other mycotoxins. The animals received this blank feed during the complete trial.

2.3. Mycotoxins and detoxifiers

Deoxynivalenol used for the animal experiments was purchased as a powder from Fermentek (Jerusalem, Israel). The administered dose of 0.750 mg DON/kg BW was calculated based on the maximally allowed concentration in poultry feed, i.e. 5 mg/kg (European Commission, 2006), and the daily feed intake, i.e. 150 g/kg BW. The mycotoxin was dissolved in ethanol *pro analysis* and water of HPLC quality (1:8, v/v), in order to obtain a stock solution of 1 mg/mL, which was used for dosing the broiler chickens.

The standards of DON and DOM-1, used for the analytical experiments, were purchased from Sigma-Aldrich (Bornem, Belgium) and were dissolved in acetonitrile (ACN) to obtain stock solutions of 1 mg/mL. Working solutions were used to prepare matrix-matched calibrators and quality control samples in plasma. These working solutions were prepared by mixing appropriate volumes of the stock solution with ACN and water (1:1, v/v), both of HPLC quality. The internal standard ($^{13}C_{15}$-DON, 25 µg/mL ACN) was obtained from Biopure (Tulln, Austria).

Two commercially available mycotoxin detoxifiers were used. The first product was a mycotoxin binder, composed of glucomannan derived from the cell wall of *Saccharomyces cerevisiae* yeasts. The second product was a combination of a mycotoxin binder (i.e. bentonite) and a modifier (a yeast). The mycotoxin detoxifiers were administered at a dose of 1 g/kg BW for the intra-crop bolus. The negative control group was given blank feed (1

g/kg BW) instead of a detoxifier. Both detoxifier and blank feed were suspended in 5 mL of water in a syringe immediately before administration into the crop, and flushed afterwards with 1 mL of water. This administration was performed using the tubing of a catheter (14G, 2", Vasofix® Braunüle®) (Braun, Melsungen, Germany). The positive control group received activated carbon (AC) (1 g/kg BW) (NORIT Carbomix®, KELA Pharma, Sint-Niklaas, Belgium) suspended in water, also by means of an intra-crop bolus.

2.4. Study design

2.4.1. Bolus model 1

The animals were divided into four groups of eight animals. Each group received a different treatment. The animals in the Detoxifier 1, Detoxifier 2, Negative Control and Positive Control group received a bolus of DON and mycotoxin detoxifier 1, DON and mycotoxin detoxifier 2, DON and blank feed and DON and AC, respectively. Feed was withheld for 12 h before the bolus administration, until 4 h post-administration.

Following the administration, blood samples were taken from the leg vein at different time points, at 0 (just before bolus administration), 15, 30, 45 min, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h post-administration. The samples were centrifugated (2851 x *g*, 10 min, 4°C), and plasma was stored at ≤ -15°C until further analysis.

2.4.2. Bolus model 2

The experiment was similar to experiment 1, except that the mycotoxin detoxifier was now not only given at the same time of the DON bolus, but also 1 and 2 hours before the DON administration as an intra-crop bolus ('preload' of the animals with the detoxifier).

2.4.3. Bolus model 3

In this experiment the mycotoxin detoxifiers were mixed in the blank feed at a dose of 2 kg/ton feed, as recommended by the manufacturers. This feed was given from the start of the experiment onwards, i.e. one week before the bolus administration, until the last blood sampling point (8 h post-bolus administration). In this experiment there was no special feed

deprivation period.

Table 1. Schematic representation of the different bolus model designs

Bolus model 1	Acclimatization period Diet: blank feed	Feed deprivation		Oral administration detoxifier/blank feed + DON	Blood sampling
Bolus model 2	Acclimatization period Diet: blank feed	Feed deprivation	Oral administration detoxifier/blank feed	Oral administration detoxifier/blank feed + DON	Blood sampling
Bolus model 3	Acclimatization period Diet: blank feed + detoxifier	No deprivation		Oral administration detoxifier/blank feed + DON	Blood sampling
Time line	-8 d to -1 d	-12 h	-2 h	0 h	0 h to +8 h

2.5. Plasma analysis

The plasma concentrations of DON and DOM-1 were determined by LC-MS/MS, based on a validated method with modifications in the sample preparation and chromatography set-up (De Baere, et al., 2011). Briefly, 250 µl of plasma was spiked with 12.5 µL working solution (1 µg/mL) of internal standard (IS) ($^{13}C_{15}$-DON). This was followed by adding 750 µL of ACN. Next, the samples were vortexed (15 sec) and centrifugated (8517 x *g*, 10 min, 4°C). The supernatant was then evaporated using a gentle nitrogen stream (40 ± 5°C). The dry residue was reconstituted in 200 µL of a 95/5 (v/v) mixture of mobile phase A/B. The mobile phase A consisted of 0.1 % glacial acetic acid in water of UHPLC quality. Mobile phase B consisted of methanol of UHPLC quality. After vortex mixing and filtering through a Millex® filter (0.22 µm), the sample was transferred to an autosampler vial, and an aliquot (10 µL) was injected onto the LC-MS/MS instrument.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MS pump Plus and an autosampler, type Autosampler Plus, from Thermo Fisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type, both from Thermo Fisher Scientific (Louvain-la-Neuve, Belgium). A gradient elution was performed: 0-1 min (95% A/5% B), 4 min (linear gradient to 80% B), 4-5.1 min (20% A/80% B), 5.6 min (linear gradient to 95% A), 5.6-8 min (95% A/5% B). The flow rate was 300 µL/min. The LC column effluent was interfaced to a TSQ® Quantum Ultra triple

quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe (Thermo Fisher Scientific), operating in the negative ionization mode. Following selected reaction monitoring (SRM) transitions were monitored used for identification and quantification: for DON m/z 355.1 > 265.1* and 355.1 >295.1, for DOM-1 m/z 339.1 > 59.1 and 339.1 > 249.1* and for $^{13}C_{15}$-DON m/z 370.1 > 279.1* and 370.1 > 310.1. The asterix (*) indicates the ion used for quantification. The limit of quantification (LOQ) of DON and DOM-1 was 1 ng/mL and the limit of detection (LOD) of DON 0.1 ng/mL and 0.19 ng/mL of DOM-1.

2.6. Toxicokinetic and statistical analysis

The following toxicokinetic parameters were calculated (WinNonlin 6.2.0, Phoenix, Pharsight corp., USA) using non-compartmental analysis: area under the plasma concentration-time curve from time 0 to infinite (AUC_{0-inf}), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), elimination half-life ($T_{1/2el}$), elimination rate constant (k_{el}) and relative oral bioavailability (relative OBB). This relative OBB was calculated according to the formula: relative OBB $= \frac{AUC0-inf\,(DON+Detoxifier)}{AUC0-inf\,(DON)}$ x 100. Using non-compartmental analysis, a better estimate of C_{max} could be made, which is of great importance for the interpretation of the data. The absorption rate constant (k_a) and the absorption half-life ($T_{1/2a}$) were calculated using one-compartmental analysis. The statistical analysis was performed with SPSS via one-way ANOVA (SPSS 19.0, IBM, USA). The significance level was set at 0.05.

3. Results

The plasma concentration-time profiles of DON after bolus administration with or without detoxifier (model 1), after bolus administration preceded by preload with blank feed or a detoxifier (model 2) and after a bolus administration of DON preceded by one week feeding of blank feed with or without mycotoxin detoxifier added, are shown in Figure 1. The main toxicokinetic parameters are summarized in Table 2. Plasma concentrations of the main metabolite of DON, DOM-1, were not detected. Moreover, the concentration of DON in all samples of the positive control group (DON+AC) were below LOQ and therefore, no toxicokinetic parameters could be calculated for this group.

Table 2. Main toxicokinetic parameters of DON after administration of DON and blank feed (negative control) or DON and a detoxifier (detoxifier 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are given as mean values ± SD

Toxicokinetic parameter	Model 1			Model 2			Model 3		
	Negative control	Detoxifier 1	Detoxifier 2	Negative control	Detoxifier 1	Detoxifier 2	Negative control	Detoxifier 1	Detoxifier 2
$AUC_{0\text{-}inf}$ (ng.h/mL)	10.22 ± 3.15	24.71 ± 10.75*	15.60 ± 5.91	14.86 ± 2.66	20.23 ± 5.20	19.42 ± 3.50	12.13 ± 4.99	10.58 ± 1.68	12.02 ± 5.77
C_{max} (ng/mL)	8.22 ± 2.69	23.74 ± 12.00*	15.21 ± 6.11	14.13 ± 2.25	19.26 ± 7.25	22.56 ± 10.43	12.22 ± 7.42	9.83 ± 4.06	9.68 ± 6.28
T_{max} (h)	0.66 ± 0.16	0.59 ± 0.28	0.47 ± 0.22	0.50 ± 0.25	0.50 ± 0.19	0.38 ± 0.16	0.57 ± 0.22	0.53 ± 0.35	0.69 ± 0.27
k_a (h^{-1})	1.76 ± 0.29	10.48 ± 10.40*	3.90 ± 2.27*	17.20 ± 19.50	11.14 ± 10.76	24.66 ± 22.37	13.19 ± 5.98	8.72 ± 4.05	10.04 ± 3.52
$T_{1/2a}$ (h)	0.41 ± 0.07	0.22 ± 0.18*	0.26 ± 0.13*	0.20 ± 0.16	0.22 ± 0.15	0.18 ± 0.16	0.29 ± 0.19	0.27 ± 0.21	0.32 ± 0.15
k_{el} (h^{-1})	1.29 ± 0.48	1.70 ± 0.45	1.47 ± 0.45	0.95 ± 0.48	1.04 ± 0.30	0.95 ± 0.13	1.24 ± 0.25	1.02 ± 0.28	1.01 ± 0.29
$T_{1/2el}$ (h)	0.70 ± 0.27	0.44 ± 0.11	0.57 ± 0.21	0.73 ± 0.37	0.80 ± 0.29	0.75 ± 0.11	0.59 ± 0.13	0.82 ± 0.31	0.86 ± 0.38
Relative OBB (%)		256 ± 109*	153 ± 58		136 ± 35	131 ± 24		87 ± 14	99 ± 47

$AUC_{0\text{-}inf}$ = area under the plasma concentration-time curve from time 0 to infinite; C_{max} = maximal plasma concentration; T_{max} = time to maximal plasma concentration; k_a = absorption rate constant; $T_{1/2a}$ = absorption half-life; k_{el} = elimination rate constant; $T_{1/2el}$ = elimination half-life; OBB = oral bioavailability; the asterix (*) indicates a significant difference (p<0.05) compared to the negative control group

Figure 1. Plasma concentration-time profile of DON after administration of DON and blank feed (negative control) or DON and a detoxifier (detoxifying agent 1 or 2) in broiler chickens (n=8), using model 1, 2 and 3. Results are presented as mean values + SD.
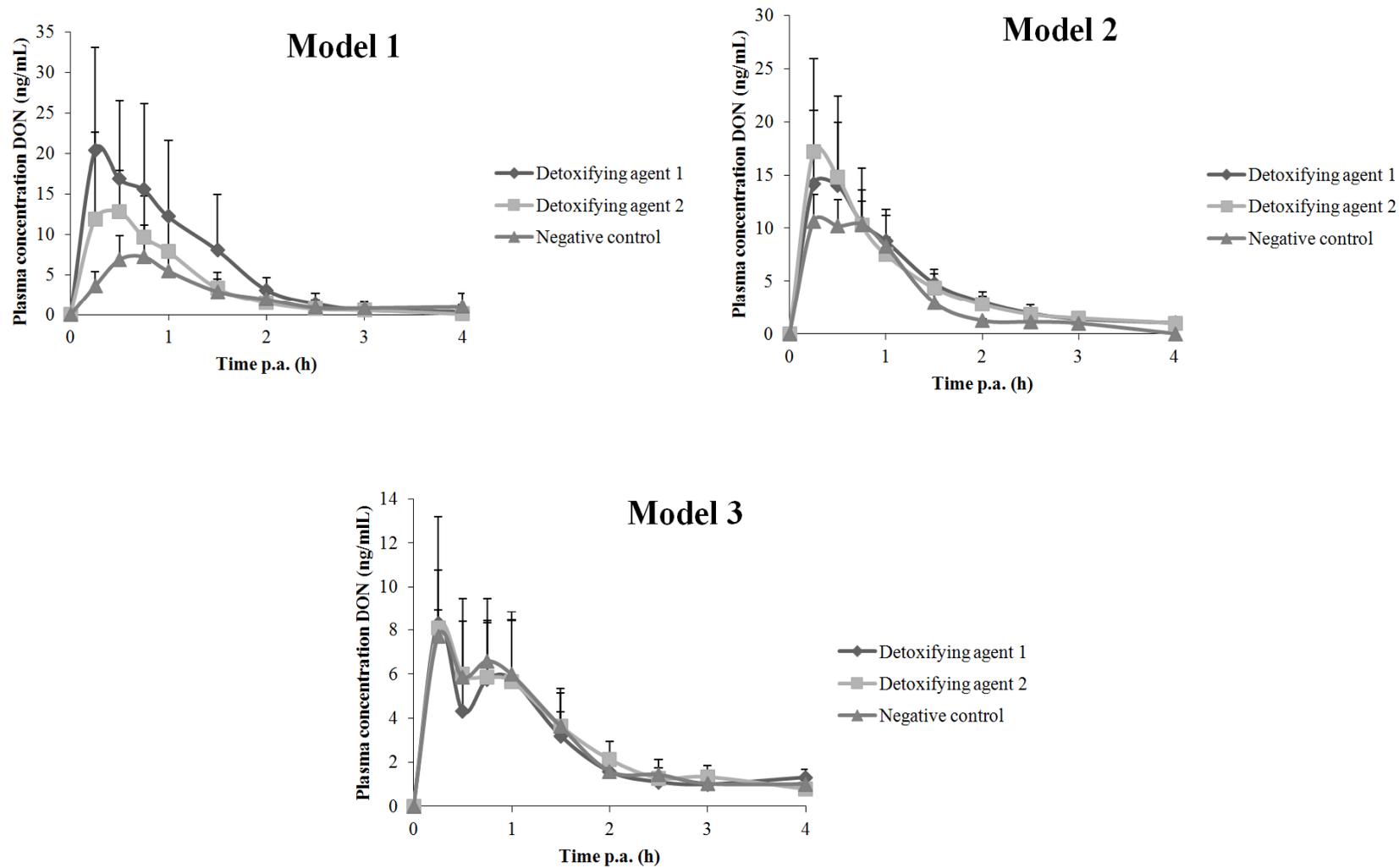
4. Discussion

Up till now, no straightforward models for *in vivo* efficacy testing of mycotoxin detoxifiers, in relation to the recent EFSA guidelines, have been reported. No studies have been published on the ability of mycotoxin detoxifiers to lower the oral bioavailability of mycotoxins in poultry. Dänicke et al. (2001) studied the excretion kinetics of zearalenone (ZON) in broiler chickens and the efficacy of a mycotoxin detoxifier to alter the excretion of ZON. No difference in toxicokinetic parameters were found after bolus administration of ZON with or without the mycotoxin detoxifier. The enterohepatic recirculation of ZON and the rapid passage of the detoxifier through the intestinal tract was put forward as a possible explanation.

Previous studies (Döll, et al., 2004, Sabater-Vilar, et al., 2007) have evaluated the *in vitro* binding or biotransforming ability of different mycotoxin detoxifiers, including those used in this study. In those screening studies, none of the tested products were able to effectively bind DON, except for activated carbon. These *in vitro* findings correlate with our findings, where no significant differences in toxicokinetic parameters were found between the detoxifier groups and the negative control group, except in the first bolus model. Surprisingly, a significant higher AUC_{0-inf}, relative oral bioavailability, C_{max} and k_a and a shorter $T_{1/2a}$ were found in the detoxifying group 1 compared with the negative control group. However, this relates with the study by Goossens et al. (2012) in which the interaction between a yeast derived mycotoxin detoxifier and the antibiotic doxycycline was investigated in pigs. It was found that the detoxifier, in combination with T-2 toxin, enhanced the oral absorption of the drug. A recent study showed a significant influence of a mycotoxin detoxifier on the oral absorption of oxytetracycline in broiler chickens (Osselaere, et al., 2012). Again, an increased oral bioavailability in the detoxifier group was observed. The mechanisms of this interaction still have to be elucidated and are currently being investigated. Most probably, these effects are not related to a direct interaction between drug and detoxifier. Possible indirect effects such as promotion of intestinal health, altered intestinal immunological parameters, influence on intestinal mucus production, etc. can be put forward.

In the present study, activated carbon was used as a positive control. This product is a basic

universal antidote which adsorbs various compounds, including mycotoxins such as DON (Avantaggiato, et al., 2004, Cavret, et al., 2010). However, the commercial use of AC in practice should be avoided in order to minimize the risk of a diminished nutrient absorption as well as the impairment of nutritional value (Avantaggiato, et al., 2004, Ramos, et al., 1996). In all of the three bolus models, the plasma concentration of DON was below LOQ, indicating the efficient adsorption of DON by AC in the intestinal tract. Therefore, we can conclude that the 3 models developed in this study are able to demonstrate the (in)efficacy of mycotoxin detoxifiers. Further research should be performed on testing these models with other mycotoxins and detoxifiers.

5. Conclusions

It can be stated that three suitable *in vivo* models for efficacy testing of mycotoxin detoxifiers were developed. The reliability of the models was demonstrated using activated carbon. The two mycotoxin detoxifiers used in this study were not able to lower the oral bioavailability of DON.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

7. Acknowledgements

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CHAPTER V

Interaction between tylosin and bentonite clay from a pharmacokinetic perspective

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Abstract - In this study, the interaction between bentonite and tylosin was investigated in broiler chickens, based on pharmacokinetic characteristics obtained *in vivo*. Also a gluco-mannan binder was evaluated for possible interactions with tylosin absorption. Simultaneous oral administration of bentonite and tylosin significantly lowered plasma levels of tylosin and reduced the area under plasma concentration-time curve (AUC_{0-inf}), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}) and relative oral bioavailability (OBB). These results prove unambiguously the binding of tylosin by bentonite. Simultaneous administration of tylosin, in the drinking water or feed, and bentonite, mixed in the feed as a mycotoxin binder, should therefore be avoided. No interaction with gluco-mannan was demonstrated, therefore tylosin and this binder can be safely co-administered in animal feed.

Mycotoxin binders are commonly used in the feed additive industry to prevent mycotoxicosis in farm animals (Kolosova and Stroka, 2011). These products are deemed to adsorb mycotoxins in the gut and consequently prevent mycotoxin absorption. Recently, the European Food Safety Authority (EFSA, 2010) stated that too little is known about the interaction between mycotoxin binders and veterinary drugs. Therefore more studies are needed to investigate possible interactions at the level of intestinal absorption.

Some suggestions have been made about a possible interaction between macrolide antibiotics and bentonite clay (EFSA, 2011), however no specific proof has been put forward yet. The Canadian Bureau of Veterinary Drugs (1992) reported a case of lack of efficiency of tylosin when fed concurrently with bentonite to bovines. Shryock et al. (1994) reported that tilmicosin added to the diet in broiler chickens prevented development of airsacculitis after inoculation with *Mycoplasma gallisepticum*, whereas a combined use of tilmicosin and bentonite did not prevent air-sac lesions or clinical symptoms, indicating a possible negative interaction between both. Therefore, the goal of this study was to investigate the capacity of bentonite to bind tylosin, based on specific pharmacokinetic characteristics instead of clinical symptoms which can be biased. A commercially available glucomannan (GMA) mycotoxin binder was also included in the study.

Bentonite clays are inorganic mycotoxin binders, generally impure clays consisting mostly of montmorillonite, a layered silicate ($Al_2O_3 \bullet 4SiO_2 \bullet H_2O$). They exhibit a high affinity towards aflatoxins but not towards other mycotoxins (Phillips et al., 1988). Besides their aflatoxin binding potency, the inclusion of 2% bentonite in broiler feed improves weight gain, feed efficiency and feed intake (Ali et al., 1996; Katouli et al., 2010). Tylosin (Figure 1) is a macrolide antibiotic produced by fermentation of *Streptomyces* strains. It consists of a substituted 16-membered lactone, an amino sugar (mycaminose) and two neutral sugars, mycinose and mycarose. Tylosin is used extensively as a therapeutic substance in the treatment of mycoplasmosis in poultry and livestock (Ashenafi et al., 2011).

Figure 1. Chemical structure of tylosin

Twenty-four 3-week-old broiler chickens (Ross 308) were randomly allocated in two groups. After a one-week acclimatization period, the animals were famished for 12 h. This was followed by an oral intra-crop bolus. One group received a bolus of tylosin (24 mg/kg BW, Tylan Soluble, Elanco Animal Health). The other two groups received a bolus of tylosin (24 mg/kg BW) and bentonite (Toxisorb Classic, Süd-Chemie) or GMA (Alltech Inc., Lexington, KY, USA) (both 100 mg/kg BW). The dose of tylosin administered was according to the leaflet. The dose of the mycotoxin binders was based on the daily feed intake of the animals (100 g/kg BW) and the dose recommended by the manufacturer (1 kg/ton). Blood samples were taken from the leg vein just before (0 h) and at different time points 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after bolus administration. The samples were centrifugated (2851 x g, 10 min, 4°C), and plasma was stored at ≤ -15°C until further analysis. The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (case number EC 2011_014). The sample preparation for tylosin analysis in plasma was performed as follows: samples (250 µL) were spiked with 25 µL of a working solution of the internal standard (gamithromycin, 1 µg/mL in water/acetonitrile, 50/50, v/v) (Merial). Next, 250 µL of acetonitrile (ACN) were added, samples were vortexed (15 s) and centrifugated (8517 x g, 10 min, 4°C). The supernatant was passed through a Millex GV-Nylon filter (0.20 µm) and transferred into an autosampler vial. An aliquot (5 µL) was injected onto the LC-MS/MS instrument. The LC system consisted of Surveyor MS pump Plus and an autosampler, type Autosampler Plus, both from Thermo Fisher Scientific. Chromatographic separation was achieved on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a

guard column of the same type, both from Thermo Fisher Scientific. The mobile phase A consisted of 10 mM ammonium acetate in water of UHPLC quality, acidified to pH 3.5 with glacial acetic acid. Mobile phase B was ACN of UHPLC quality. A gradient elution was performed at a flow rate of 300 µL/min: 0-0.5 min (80% A/20% B), 0.5-1 min (linear gradient to 90% B), 1-4.5 min (10% A/90% B), 4.5-5 min (linear gradient to 80% A), 5-8 min (80% A/20% B). The LC column effluent was interfaced to a TSQ Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe (Thermo Fisher Scientific), operating in the positive ionization mode. Detection was performed in the selected reaction monitoring (SRM) mode and following transitions were monitored and used for identification and quantification (*): m/z 777.5 > 157.8 and 777.5 > 619.3* for respectively gamithromycin and m/z 916.5 > 173.8* and 916.5 > 772.4 for tylosin. The limit of quantification (LOQ) was 10 ng/mL and the limit of detection (LOD) 0.09 ng/mL.

After LC-MS/MS analysis, plasma concentration-time profiles of tylosin were compared (Figure 2). It was shown that bentonite significantly lowered plasma concentrations of tylosin. This was confirmed with pharmacokinetic analysis, using non-compartmental modeling (WinNonlin 6.2.0, Phoenix, Pharsight) (Table 1). No significant interaction between tylosin and GMA could be demonstrated. Statistical analysis was performed via one-way ANOVA (SPSS 19.0, IBM). Following pharmacokinetic characteristics reflecting drug absorption were significantly altered by addition of bentonite to the oral bolus: area under the plasma concentration-time curve from time 0 to infinite ($AUC_{0\text{-}inf}$), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}) and relative oral bioavailability (relative OBB). This relative OBB was calculated according to the formula: relative OBB = $AUC_{0\text{-}inf}$ (Tylosin+Bentonite) / $AUC_{0\text{-}inf}$ (Tylosin) x 100. No significant effect on elimination parameters were found, including elimination rate constant (K_{el}) and elimination half-life ($T_{1/2el}$).
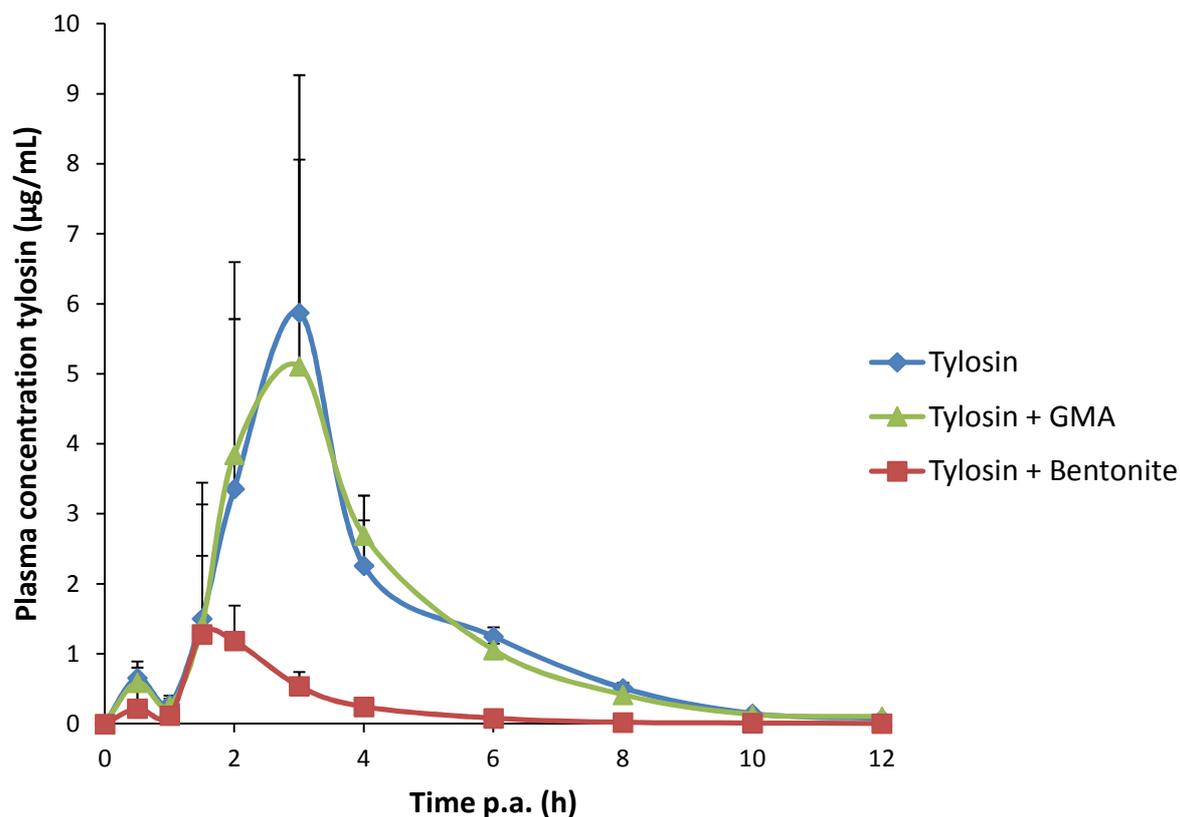
Figure 2. Plasma concentration-time profile of tylosin after a single oral bolus administration of tylosin (24 mg/kg BW) with or without bentonite or glucomannan (GMA) (100 mg/kg BW) to broiler chickens. Values are presented as mean + SD (n=8)

Table 1. Main pharmacokinetic parameters of tylosin after a single oral bolus administration of tylosin (24 mg/kg BW) with or without bentonite or glucomannan (GMA) (100 mg/kg BW) to broiler chickens. Values are presented as mean ± SD (n=8)

	Tylosin	Tylosin + Bentonite	Tylosin + GMA
AUC_{0-inf} (µg.h/mL)	17.06 ± 5.31	2.89 ± 1.14 **	16.37 ± 4.14
C_{max} (µg/mL)	7.08 ± 3.64	1.66 ± 0.77 **	5.84 ± 1.67
T_{max} (h)	3.00 ± 0.33	1.92 ± 0.42 *	2.92 ± 0.38
k_{el} (h^{-1})	0.51 ± 0.08	0.59 ± 0.23	0.52 ± 0.14
$T_{1/2el}$ (h)	1.42 ± 0.21	1.67 ± 0.99	1.49 ± 0.51
relative OBB (%)	100.00	23.30 ± 7.22 ***	98.04

AUC_{0-inf} : area under plasma concentration-time profile from time 0 to infinite; C_{max}: maximal plasma concentration; T_{max}: time to maximal plasma concentration; K_{el}: elimination rate constant; $T_{1/2el}$: elimination half-life; relative OBB: relative oral bioavailability; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

These results confirm the suggestion made by other authors (Canadian Bureau of Veterinary Drugs, 1992; EFSA, 2011; Shryock et al., 1994) that bentonite is able to bind macrolide antibiotics. The mechanism behind is the replacement of mineral cations by organic cations such as tylosin. The combined use of tylosin and bentonite in animal husbandry should therefore be avoided, as this interaction can have serious consequences. Lowered plasma concentrations of antibiotics may implicate therapy failure and enhanced microbial resistance development. In contrast, tylosin can be safely co-administered with GMA in poultry feed as no interaction was observed using the developed *in vivo* model.

Future research should be performed on the interaction with other veterinary drugs and different mycotoxin binders. This research should be based on specific pharmacokinetic parameters instead of unspecific clinical symptoms, as only pharmacokinetic parameters can really prove a decreased oral bioavailability of the drug, due to adsorption.

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CHAPTER VI

Efficacy testing of a glucomannan mycotoxin binder towards the effects of feed-borne *Fusarium* mycotoxins in turkey poults based on specific and unspecific parameters

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Abstract - An experiment was conducted to investigate the effects of feeding grains naturally contaminated with *Fusarium* mycotoxins and a yeast derived glucomannan mycotoxin adsorbent (GMA) on selected unspecific and specific parameters in turkey poults. Two hundred and forty 1-day-old male turkey poults were fed the experimental diets for twelve weeks. Experimental diets were formulated with control grains, control grains + 0.2% GMA, contaminated grains, or contaminated grains + 0.2% GMA. Deoxynivalenol (DON) was the major contaminant of the contaminated grains and its concentrations varied from 4.0 to 6.5 mg/kg in the contaminated diets. Unspecific parameters measured included: performance parameters, plasma biochemistry profiles, morphometry and CD8^{+} T-lymphocyte counts in the duodenum. Plasma concentrations of DON and de-epoxydeoxynivalenol (DOM-1) were used as specific parameter. Performance parameters and plasma biochemistry were altered by DON and GMA but this was not consistent throughout the trial. Deoxynivalenol reduced the villus height and apparent villus surface area in the duodenum. This effect was prevented by GMA supplementation to the diets. Deoxynivalenol elevated the total CD8^{+} T-lymphocyte count in the duodenum but this effect was not prevented by GMA. No significant differences in plasma concentrations of DON and DOM-1 between contaminated and contaminated + GMA groups were observed, suggesting that GMA did not prevent DON absorption.

1. Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungal species. They are of great interest because of their deleterious effects on animal health and performance, which consequently leads to economic losses in animal production (Wu, 2007). A variety of methods to prevent the adverse effects of mycotoxins have been developed. Utilizing mycotoxin detoxifying agents (mycotoxin detoxifiers) is the most commonly used preventative method (Jard et al., 2011; Kolosova and Stroka, 2011). These detoxifiers can be divided into two different classes, namely, mycotoxin binders and mycotoxin modifiers. Mycotoxin binders adsorb the toxin in the gut, resulting in the excretion of toxin-binder complex in faeces, whereas mycotoxin modifiers transform the toxin into non-toxic metabolites (Kolosova and Stroka, 2011).

Mycotoxin detoxifiers should be tested for their mycotoxin binding or degrading ability *in vitro* as well as *in vivo*. *In vitro* models are a powerful tool to screen and select a large number of compounds. However, only *in vivo* trials can fully proof the efficacy of mycotoxin detoxifiers as mycotoxin adsorption or biotransformation *in vivo* is influenced by physiological variables and the composition of feed (Lemke et al., 2001). Up to day, mycotoxin detoxifiers are commonly evaluated *in vivo* by so-called unspecific parameters. Those include animal performance (e.g. growth rate, feed intake and feed conversion rate), plasma biochemistry (e.g. concentration of proteins, enzymes and minerals), effect on immune function and histological changes in different tissues. As the criteria are unspecific, differences obtained between treated and untreated animals cannot be solely attributed to the efficacy of the detoxifier. There may be some confounding effects such as immuno-modulating activity of β-glucans and antioxidant action of other feed components. Due to the lack of specificity of these parameters, the European Food Safety Authority (EFSA) recently proposed other end-points based on specific toxicokinetic parameters (EFSA, 2010). As mycotoxin binders are deemed to adsorb mycotoxins in the gut, a lowered oral absorption is expected. According to the EFSA, the most relevant parameter to evaluate the efficacy of these products against mycotoxins is the plasma concentration of these toxins or their main metabolites (Devreese et al., 2012).

The goal of the present study was: (1) to determine the effects of the *Fusarium* mycotoxins, mainly deoxynivalenol (DON), on specific and unspecific parameters in turkey poults, and (2)

to evaluate the efficacy of a yeast derived glucomannan mycotoxin binder (GMA). The selection of unspecific parameters for this trial was based on previous research (Girish and Smith, 2008; Girish et al., 2008; Girish et al., 2010; Yunus et al., 2012a; Yunus et al., 2012b). These included performance parameters, plasma biochemistry profile, morphometry and CD8$^+$ cell population in the duodenum. Specific parameters, plasma concentrations of DON and its main metabolite de-epoxydeoxynivalenol (DOM-1), were selected as advised by the EFSA (EFSA, 2010).

2. Materials and methods

2.1. Experimental birds and diets

Two hundred and forty one-day-old male Hybrid turkey poults (Hybrid Turkeys, Kitchener, ON, Canada) were individually weighed and randomly distributed in 12 pens at the Arkell Poultry Research Station of the University of Guelph (Guelph, ON, Canada). Three pens were randomly assigned to each of the four different diets. The temperature and lighting programs were followed according to standard recommendations of the supplier. Birds were managed as has been prescribed by the Canadian Council on Animal Care with the Animal Utilization Protocols approved by the Animal Care Committee of the University of Guelph (CCAC, 2009).

Four different corn-, wheat- and fish meal-based diets were formulated for each rearing phase including starter (0-3 w), grower (4-6 w), developer (7-9 w) and finisher (10-12 w). The diets met the standard nutritional specifications for turkey poults. The mycotoxin contaminated diets were formulated to the nutrient specifications of the control diets by replacing control corn with corn naturally contaminated with *Fusarium* mycotoxins. GMA-supplemented diets contained 0.2% GMA (Mycosorb®, Alltech Inc., Lexington, KY, USA).

2.2. Analysis of dietary mycotoxin concentrations

Dietary concentrations of DON, 3-acetyl-DON (3-aDON), 15-acetyl-DON (15-aDON), nivalenol (NIV), fusarenan-X (FUS-X), neosolaniol (NEO), diacetoxyscirpenol (DAS), T-2 toxin (T-2) and HT-2 toxin (HT-2) were analyzed using gas chromatography-mass spectrometry (GC-MS) according to Raymond et al. (2003). The limits of detection (LODs) were 0.06, 0.05, 0.05, 0.12, 0.11, 0.07, 0.06, 0.04 and 0.06 µg/g, respectively. Ochratoxin A (OTA) and zearalenone

(ZON) concentrations were analyzed by high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Wang et al., 2008). The LODs were respectively 0.2 and 20 ng/g. The sum of aflatoxin (AF) B1, B2, G1 and G2 concentrations and the sum of fumonisin B1 (FB1) and B2 (FB2) concentrations were analyzed by enzyme-linked immunosorbent assay (ELISA) and the LODs were 1.0 and 25.0 ng/g, respectively.

2.3. Body weight, feed intake and feed conversion ratio

Poults were individually weighed at placement and at the end of each growth phase. Feed consumption was measured for each pen at the end of each growth phase. Weight gain, feed intake, and feed conversion ratio (FCR) were calculated. Feed intake and FCR were adjusted for mortalities when necessary.

2.4. Plasma and tissue collection

At week 1 and the end of starter phase (week 3), 4 birds per pen (12 birds/diet) were euthanized by cervical dislocation. Blood was sampled from the jugular vein prior to euthanasia. Blood was centrifugated (2851 x g, 4°C, 10 min) and plasma was collected and frozen (≤ -15°C) until analysis. Proximal and intermediary sections of the *duodenum descendens* were collected and flushed with 0.9% saline for immunohistochemistry and histology, respectively. For immunohistochemistry, tissue samples were embedded in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and snap frozen in liquid nitrogen. Frozen tissue samples were sectioned at 5 μm thickness, placed on Superfrost® Excell® glass slides (Fisher Scientific, Ottawa, ON, Canada) and stored at -20°C until further staining. For histology, tissues were fixed in 10% neutral-buffered formalin for 48 hr, then trimmed and processed for hematoxylin and eosin staining (Girish and Smith, 2008).

At the end of the grower, developer and finisher phase, blood was collected from the wing vein (12 animals/diet) and plasma was separated as described above.

2.5. Plasma biochemistry

Plasma concentrations of Ca, P, total protein, albumin, globulin, albumin:globulin ratio, glucose, cholesterol, total bilirubin, γ-glutamyltransferase (GGT), aspartate aminotransferase (AST), creatine kinase (CK), amylase, lipase, uric acid, lactate dehydrogenase (LDH), bile acid

and glutamate dehydrogenase (GLDH) were determined by a Hitachi 911 Chemistry Analyzer (Roche Diagnostics, Montreal, QC, Canada).

2.6. Quantification of DON, DOM-1 in plasma

Detection and quantification of DON and DOM-1 in plasma was performed as described by Devreese et al. (2012). Briefly, 250 µL of plasma was spiked with 12.5 µL of a working solution (1 µg/mL) of internal standard (IS) ($^{13}C_{15}$-DON). This was followed by adding 750 µL of acetonitrile (ACN). Samples were vortexed (15 sec) and centrifuged (10 min, 2851 x *g*, 4°C). The supernatant was then evaporated using a gentle nitrogen stream (40 ± 5°C). The dry residue was reconstituted in 200 µL of a 95/5 (v/v) mixture of mobile phase A/B. The mobile phase A consisted of 0.1% glacial acetic acid in water of ultra performance liquid chromatography (UHPLC) quality. Mobile phase B consisted of methanol of UHPLC quality. After vortex mixing and filtering through a Millex® PVDF-filter (0.22 µm), the sample was transferred to an autosampler vial, and an aliquot (10 µL) was injected onto the LC-MS/MS instrument. The LC system consisted of a quaternary, low pressure mixing pump with vacuum degassing (Surveyor MS pump Plus, Thermo Fisher Scientific, Breda, the Netherlands), and an autosampler (Autosampler Plus, Thermo Fisher Scientific). Chromatographic separation was achieved on a Hypersil-Gold column (50 mm x 2.1 mm i.d., dp: 1.9 mm) in combination with a guard column of the same type (Thermo Fisher Scientific, Louvain-la-Neuve, Belgium). A gradient elution was performed: 0–1 min (95% A/5% B), 4 min (linear gradient to 80% B), 4–5.1 min (20% A/80% B), 5.6 min (linear gradient to 95% A), 5.6–8 min (95% A/5% B). The flow rate was 300 µL/min. The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionisation (h-ESI) probe (Thermo Fisher Scientific), operating in the negative ionisation mode. Following selected reaction monitoring (SRM) transitions were monitored and used for quantification: for DON m/z 355.1 > 265.1* and 355.1 >295.1, for DOM-1 m/z 339.1 > 59.1 and 339.1 > 249.1* and for $^{13}C_{15}$-DON m/z 370.1 > 279.1* and 370.1 > 310.1. The asterisk (*) indicates the ion used for quantification. The limit of quantification (LOQ) for DON and DOM-1 was 0.5 ng/mL and LODs for DON and DOM-1 were 0.09 ng/mL and 0.11 ng/mL, respectively.

2.7. Immunohistochemistry analysis (CD8$^+$ T-lymphocytes)

Immunohistochemistry analysis was performed according to Girgis et al. (2010a) with some minor modifications. Tissue sections were fixed with ice-cold acetone for 10 min. Endogenous peroxidase activity was inhibited by incubating the tissues for 10 min with 3% H_2O_2 prepared in phosphate buffered saline (PBS) containing 0.3% normal goat serum. Blocking of nonspecific sites was done by incubating the tissues for 30 min with 5% normal goat serum in PBS. Sections were then incubated with anti-chicken CD8a (clone CT-8) primary antibodies (Southern Biotech, Birmingham, AL, USA) for 30 min, diluted 1:25 in blocking buffer. Biotinylated goat anti-mouse IgG (H + L) (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody at a dilution of 1:250 in blocking buffer. Sections were incubated with the secondary antibody for 30 min. Avidin–biotin–peroxidase system (Vectastain® ABC kit, Vector Laboratories) was used for immunoperoxidase staining according to the manufacturer's protocol. Enzyme-linked antibodies were then visualized by reaction with 3,3-diaminobenzidine–H_2O_2 solution (DAB substrate kit for peroxidase, Vector Laboratories). Tissue sections were counterstained with hematoxylin (Fisher Scientific), and mounted with Cytoseal-60 (Richard-Allan-Scientific, Kalamazoo, MI, USA).

Within each tissue section, 5 random fields were captured using a 5x objective lens of a computer-aided light microscope imager (Openlab®, Perkin Elmer, Waltham, MA, USA). Images were scanned using ImageScope® software (Aperio Technologies, Vista, CA, USA). The percentage of CD8$^+$-stained area in mucosa and submucosa for each section was determined and the mean value was calculated for each bird.

2.8. Morphometry analysis

Morphometry of duodenal sections was done as described by Girgis et al. (2010b). Briefly, formalinized duodenal sections were trimmed, dehydrated, cleared, and paraffin embedded. Sections, 5 µm thick, were placed on glass slides and processed by hematoxylin and eosin stain. Morphometric measurements were performed on 20 axially sectioned well oriented villi selected from each segment, using a computer-aided light microscope image with Openlab® software (Perkin Elmer). Morphometric measurements included villus height (VH) from the tip of the villus to the crypt, crypt depth from the base of the villi to the submucosa, villus width (VW; average of VW at one-third and two-third of the villus) and

muscularis thickness from the submucosa to the serosa. Apparent villus surface area (AVSA) was calculated by the formula: (VW at one third + VW at two thirds of the height of the villus)/2 × villus height.

2.9. Statistical analysis

For performance, plasma biochemistry and DON/DOM-1 analysis, means were taken of each replicate (4 birds/replicate). These means (3 replicates/diet) were compared between groups using one-way ANOVA after determination of normality and homogeneity of variances (SPSS 19.0, IBM, Chicago, IL, USA). Data are expressed as mean of the replicate means ± standard error of the mean (SEM).

Morphometry and immunohistochemistry results were analyzed in a similar way, but with 20 and 5 measurements in each slide, respectively.

3. Results

3.1. Dietary mycotoxin concentrations

DON was the major mycotoxin contaminant detected in experimental diets. Diets also included lesser amounts of 15-aDON, OTA, ZON, aflatoxins and fumonisins (Table 1).

Table 1. Dietary mycotoxin concentrations of experimental diets

	Control	Control + GMA	Contaminated	Contaminated + GMA
Starter Diet				
DON (µg/g)	0.06	0.12	5.20	6.50
15-aDON (µg/g)	ND	ND	0.49	0.57
OTA (ng/g)	0.66	ND	0.61	1.10
ZON (µg/g)	ND	ND	0.37	0.67
Aflatoxins (ng/g)	ND	ND	1.40	1.10
FB1+FB2 (ng/g)	ND	ND	29	25
Grower Diet				
DON (µg/g)	0.39	0.38	6.50	6.10
15-aDON (µg/g)	0.10	0.11	0.55	0.51
OTA (ng/g)	ND	ND	0.77	0.77
ZON (µg/g)	ND	0.02	0.59	0.66
FB1+FB2 (ng/g)	0.42	0.76	1.20	0.33
Developer Diet				
DON (µg/g)	0.31	0.33	4.00	4.20
15-aDON (µg/g)	ND	ND	0.45	0.55
OTA (ng/g)	0.31	ND	0.29	0.28
ZON (µg/g)	0.03	0.03	0.67	0.79
FB1+FB2 (ng/g)	ND	0.25	0.26	0.30
Finisher Diet				
DON (µg/g)	0.10	0.16	4.50	5.50
15-aDON (µg/g)	ND	ND	0.45	0.57
OTA (ng/g)	ND	ND	0.35	0.28
ZON (µg/g)	ND	ND	0.42	0.44
FB1+FB2 (ng/g)	ND	0.40	0.28	0.29

ND = not detected (<LOD)
LOD: DON = 0.06 µg/g, 15-aDON = 0.05 µg/g, OTA = 0.2 ng/g, ZON = 0.02 µg/g, Aflatoxins = 1.0 ng/g, FB1-FB2 = 25.0 ng/g
Other tested mycotoxins were 3a-DON, NIV, FUS-X, NEO, DAS, T-2, HT-2. Dietary concentrations fell below the respective LOD of 0.05, 0.12, 0.11, 0.07, 0.06, 0.04 and 0.06 µg/g

3.2. Performance parameters

Except for the starter phase, no significant differences in body weight, weight gain, feed intake or FCR were observed (Table 2). Birds receiving the contaminated diet showed a significantly higher body weight and body weight gain in the starter phase.

Table 2. Effect of different diets on performance parameters of turkey poults

	Control	Control + GMA	Contaminated	Contaminated + GMA
Starter Diet				
Body Weight (kg/bird)	0.56 ± 0.024	0.56 ± 0.064	0.59 ± 0.028*	0.55 ± 0.029
Weight Gain (kg/bird)	0.50 ± 0.003	0.50 ± 0.006	0.52 ± 0.003*	0.49 ± 0.003
Feed Intake (kg/bird)	0.89 ± 0.005	0.89 ± 0.020	0.94 ± 0.018	1.04 ± 0.082
FCR	1.79 ± 0.007	1.80 ± 0.034	1.79 ± 0.029	2.12 ± 0.155
Grower Diet				
Body Weight (kg/bird)	2.18 ± 0.055	2.26 ± 0.120	2.28 ± 0.0181	2.22 ± 0.009
Weight Gain (kg/bird)	1.63 ± 0.031	1.70 ± 0.011	1.69 ± 0.015	1.67 ± 0.009
Feed Intake (kg/bird)	2.69 ± 0.077	2.76 ± 0.031	2.75 ± 0.093	2.68 ± 0.022
FCR	1.65 ± 0.035	1.62 ± 0.008	1.62 ± 0.041	1.60 ± 0.017
Developer Diet				
Body Weight (kg/bird)	5.26 ± 0.055	5.26 ± 0.034	5.32 ± 0.046	5.38 ± 0.012
Weight Gain (kg/bird)	3.06 ± 0.017	2.99 ± 0.026	3.04 ± 0.028	3.17 ± 0.017
Feed Intake (kg/bird)	7.12 ± 0.157	7.15 ± 0.084	6.86 ± 0.189	6.77 ± 0.055
FCR	2.33 ± 0.038	2.39 ± 0.046	2.25 ± 0.049	2.14 ± 0.029
Finisher Diet				
Body Weight (kg/bird)	9.35 ± 0.133	9.33 ± 0.035	9.11 ± 0.087	9.17 ± 0.091
Weight Gain (kg/bird)	4.09 ± 0.078	4.07 ± 0.070	3.78 ± 0.047	3.79 ± 0.088
Feed Intake (kg/bird)	11.49 ± 0.262	10.83 ± 0.153	10.40 ± 0.453	10.40 ± 0.181
FCR	2.81 ± 0.011	2.67 ± 0.074	2.75 ± 0.106	2.75 ± 0.046

* significantly different compared to the control ($P<0.05$)
Values represent the overall mean of the replicate means (n=3) ± SEM

3.3. Plasma biochemistry

Differences in plasma biochemistry profiles were observed amongst the different dietary groups. However, no parameter was consistently altered in any rearing phase. Also no consistent trend between control and contaminated or contaminated + GMA diets could be observed. The results of the parameters showing a significant difference between groups is shown in Table 3.

Table 3. Altered plasma chemistry parameters in turkey poults fed the experimental diets

	Control	Control + GMA	Contaminated	Contaminated + GMA
Starter Diet				
CK (U/L)	2584.00 ± 160.407[a]	3681.17 ± 232.031[b]	4229.08 ± 337.814[b]	4136.33 ± 62.322[b]
Amylase (U/L)	654.33 ± 33.422[a]	663.75 ± 5.774[a,b]	757.17 ± 52.218[b]	705.67 ± 50.069[b]
Grower Diet				
P (mmol/L)	2.53 ± 0.053[a]	2.60 ± 0.063[a,b]	2.43 ± 0.061[a]	2.93 ± 0.055[b]
Developer Diet				
LDH (U/L)	488.33 ± 21.586[a]	597.33 ± 16.807[b]	576.33 ± 5.132[a,b]	510.33 ± 7.441[a,b]
Finisher Diet				
Cholesterol (mmol/L)	3.52 ± 0.073[a,b]	3.23 ± 0.032[a]	3.28 ± 0.058[a]	3.74 ± 0.042[b]

A different superscript letter indicates a significant difference ($P < 0.05$)
Values represent the overall mean of the replicate means (n=3) ± SEM

3.4. Morphometry of the duodenum

Table 4 shows the results of the morphometrical analysis of duodenal sections examined at the end of the starter phase. Birds fed the contaminated diet showed a significantly lower VH and AVSA. This negative effect was prevented by addition of GMA into the contaminated diet.

3.5. Immunohistochemistry

A significant higher population of CD8^{+} T-lymphocytes was observed in the duodenum of turkeys fed either the contaminated or the contaminated + GMA diet after three weeks of feeding (Table 4).

Table 4. Morphometrical and immunohistochemical analysis of the duodenum at the end of the starter phase (3 weeks)

	Control	Control + GMA	Contaminated	Contaminated + GMA
Morphometry				
Villus height (µm)	2205.12 ± 35.481	2222.45 ± 30.209	1952.25 ± 23.974***	2253.29 ± 21.949
Crypt depth (µm)	239.59 ± 17.750	250.96 ± 6.346	243.60 ± 9.846	252.37 ± 7.486
Submucosa thickness (µm)	29.61 ± 0.336	28.41 ± 0.659	27.58 ± 0.501	27.68 ± 0.385
Villus width down (µm)	134.87 ± 2.452	137.75 ± 1.704	140.35 ± 2.148	132.60 ± 1.175
Villus width up (µm)	134.79 ± 1.208	139.47 ± 1.456	136.63 ± 1.221	133.79 ± 1.012
Mean villus width (µm)	134.83 ± 1.568	138.61 ± 1.446	138.49 ± 1.499	133.19 ± 1.015
Apparent villus surface area (mm²)	2977.03 ± 68.976	3077.88 ± 51.697	2701.71 ± 51.642*	3004.66 ± 45.027
CD8^{+} T-lymphocytes				

Positivity (%)	2.53 ± 0.293	2.77 ± 0.384	4.89 ± 0.391 *	6.04 ± 0.512 ***

* and *** indicate a significant difference compared to the control at 0.05 < P < 0.01 and P <0.001 respectively
Values represent the overall mean of the replicate means (n=12) ± SEM

3.6. DON, DOM-1 concentrations in plasma

No DON or DOM-1 was detected in any plasma samples of the birds fed the control or control + GMA diet. DON and DOM-1 plasma concentrations were detectable in all analyzed samples of birds fed the contaminated or the contaminated + GMA diet. No significant differences, however, were observed. The results are presented in Table 5.

Table 5. Plasma concentrations of deoxynivalenol (DON) and de-epoxydeoxynivalenol (DOM-1) after feeding different experimental diets

	Control	Control + GMA	Contaminated	Contaminated + GMA
Week 1				
DON (ng/mL)	ND	ND	1.14 ± 0.334	1.27 ± 0.337
DOM-1 (ng/mL)	ND	ND	2.45 ± 0.198	2.82 ± 0.178
Starter Diet				
DON (ng/mL)	ND	ND	2.09 ± 0.490	2.28 ± 0.415
DOM-1 (ng/mL)	ND	ND	2.28 ± 0.043	2.40 ± 0.048
Grower Diet				
DON (ng/mL)	ND	ND	3.21 ± 0.337	3.06 ± 0.409
DOM-1 (ng/mL)	ND	ND	9.51 ± 0.638	9.12 ± 1.230
Developer Diet				
DON (ng/mL)	ND	ND	1.71 ± 0.212	1.38 ± 0.267
DOM-1 (ng/mL)	ND	ND	4.00 ± 0.210	3.73 ± 0.419
Finisher Diet				
DON (ng/mL)	ND	ND	1.00 ± 0.036	1.11 ± 0.133
DOM-1 (ng/mL)	ND	ND	2.11 ± 0.217	2.20 ± 0.352

ND = Not Detected (<LOD)
Values represent the overall mean of the replicate means (n=3) ± SEM

4. Discussion

The goal of this study was to evaluate the effect of a yeast derived mycotoxin binder on selected unspecific parameters and specific toxicokinetic markers after feeding a naturally contaminated diet (± 5 mg DON/kg feed) to turkey poults.

Trichothecenes, including DON, target the 60S ribosomal unit, where they stop the elongation-termination step during protein synthesis (Ueno, 1984). Trichothecenes also inhibit DNA and RNA synthesis, which is a secondary effect due to protein synthesis inhibition (Ueno, 1985). Moreover, they activate mitogen-activated protein kinases (MAPKs) and induce apoptosis in a process called 'ribotoxic stress response' (Pestka, 2007). As a consequence of MAPK activation, DON increases the expression and stability of cyclooxygenase-2 (COX-2) mRNA confirming its role in the inflammatory process (Moon and Pestka, 2002). The most sensitive cell types are therefore those with a high turn-over rate such as intestinal cells and leucocytes.

Enterocytes are particularly exposed to the effects of DON as this mycotoxin is almost completely absorbed in the duodenum (Dänicke et al., 2010; Eriksen et al., 2003). The effects of DON on intestinal morphology, morphometry and immunology of poultry have been described before (Girish and Smith, 2008; Girgis et al., 2010a,b; Girish et al., 2010; Yunus et al., 2012a). Changes primarily occur in the duodenum, the absorption site of DON.

In the current study, birds fed the contaminated diet showed significant reduction of the VH and AVSA at the end of the starter phase in the duodenum. This effect is most probably attributed to DON, the major contaminant of the diets. Moreover, adding GMA to the contaminated diet counteracted this effect. These findings were previously also described by Girish and Smith (2008). The reproducibility of the results confirms the pronounced beneficial effects of GMA on this unspecific parameter.

CD8$^+$ T-lymphocytes were selected as immunological marker in the intestine as the effects of feeding a DON contaminated diet already showed to alter this cell type count in cecal tonsils (Girish et al., 2010). The present results demonstrate a significantly higher influx of CD8$^+$ T-lymphocytes in the duodenum of turkeys fed the contaminated diet compared to the controls. GMA was not able to counteract this effect as no significant difference was observed between the contaminated and contaminated + GMA group. The dose of DON is

an important variable as with a lower dose (3 mg DON/kg feed), no significant differences between control and contaminated feeding groups were observed in a previous feeding trial with turkeys (Girish et al., 2010). We opted to use 5 mg/kg as it is the maximally allowed contamination level for poultry feed in Europe (European Commission, 2006) and Canada (FAO, 1997). Also, when feeding naturally contaminated diets, the entire 'mycotoxin profile' could play a role on the outcome. Next to the variation in contamination level of the major contaminant (DON), co-contaminants could have an additive or synergistic effect.

Since poultry are relatively resistant to the effects of DON in terms of performance parameters, high concentrations are needed to cause detrimental effects (Awad et al., 2012). It should be noted that diets containing purified DON may be less toxic than naturally contaminated diets due to the presence of masked mycotoxins, such as DON-3-glucoside, which add to the toxicity of the diet but are not detected by routine feed analyses (Sasanya et al., 2008). Awad et al. (2008) described that broiler chickens can tolerate up to 15 mg DON/kg without a decrease in BW gain. Moreover, diets contaminated with up to 10 mg DON/kg did not affect FCR of turkey poults (Xu et al., 2011). Also other studies did not observe altered poultry performance when fed DON contaminated diets (Dänicke et al., 2001; Awad et al., 2006; Yegani et al., 2006). In contrast, Swamy et al. (2002) found significant linear declines in growth rate and feed consumption of broiler chickens fed increasing levels of grains naturally contaminated with *Fusarium* mycotoxins. In the present study, BW gain was remarkably increased in the DON contaminated group (starter phase). A growth promotion by moderate DON levels has already been described previously in broiler chickens (Kubena et al., 1985; Kubena et al., 1989) and turkeys (Xu et al., 2011). This could be attributed to an effect of hormesis. Hormesis represents the stimulatory effects caused by low levels of potentially toxic agents (Stebbing et al., 1982; Calabrese, 2008). In this concept, living organisms overreact on the presence of a toxin or poison at low concentrations. This could lead, as in the current study for example, to a transient increase of growth rate.

In general, there is no simple dose-response relationship between dietary DON concentrations and growth performance or feed consumption in turkey poults (Dänicke et al., 2001; Xu et al., 2011). Therefore, animal performance parameters, including growth rate, feed intake and FCR, are not suitable to test the efficacy of mycotoxin detoxifiers in turkey poults.

Alterations in plasma biochemistry profile is often used as a parameter to demonstrate the deleterious effects of mycotoxins. Feeding diets naturally contaminated with 5 mg DON/kg lowered albumin concentrations and GGT activity in broiler chickens (Swamy et al., 2002). Another broiler study conducted with 3 mg DON/kg feed concluded that DON lowered total protein, albumin and P plasma concentrations (Klapacova et al., 2011). Ghareeb et al. (2012) did not only observe reduced protein concentrations after feeding 10 mg DON/kg feed, but also reduced uric acid concentrations. In turkey poults, feeding 3 mg DON/kg did not alter total protein, albumin, minerals or enzyme activities, but elevated uric acid concentrations in the plasma (Girish et al., 2008). The reduced protein concentrations observed in several studies on broiler chickens is attributed to the inhibitory effect of DON on protein synthesis. In the current study, no alterations in total protein, albumin, globulin or albumin:globulin ratio were observed in turkeys fed the contaminated diet, which agrees with the findings reported by Girish et al. (2008). For all other enzymes and minerals monitored, no trend was found in literature nor in this study. It can be concluded that, although significant differences can be observed, plasma biochemistry is not consistent enough to be considered a reliable parameter for efficacy testing of mycotoxin detoxifiers.

A direct approach to evaluate the efficacy of a mycotoxin binder against DON absorption is measuring DON and DOM-1 levels in plasma (EFSA, 2010). After a single bolus administration of 0.75 mg DON/kg BW to broiler chickens, equally to 5 mg DON/kg feed, plasma concentrations of DON were low (maximal plasma concentration, C_{max}, 26.1 ng/mL and absolute oral bioavailability of 19.3%), time to maximal plasma concentration (T_{max}) was short, 35 min, and DON was rapidly excreted (elimination half-life, $T_{1/2el}$, 38 min) (Osselaere et al., 2013). This implicates the need for very sensitive methods which quantify DON/DOM-1 at lower ng/mL levels. This can be achieved via LC-MS/MS and such methods have been developed at our department (De Baere et al., 2011; Devreese et al., 2012). To our knowledge, the present study is the first to detect DON and DOM-1 in turkey plasma. After chronic feeding of DON contaminated diets to broiler chickens, plasma concentrations of DON may tend to be lower due to an adaptation mechanism (Yunus et al., 2012a). When broilers were fed 7.5 mg DON/kg feed, average plasma concentrations were 3.87 and 2.66 ng/mL in week 1 and 2 respectively, but were <LOQ of 1.25 ng/mL after three weeks (De Baere et al., 2011; Osselaere et al., 2012). In the present study, plasma concentrations of DON and DOM-1 increased with a maximum at the end of the grower phase. This could be

attributed to intestinal damage, as demonstrated with the morphometry indices, leading to increased DON absorption. Adaptation to DON as feed contaminant, as described by Osselaere et al. (2012) and Yunus et al. (2012a), was observed at a later stage of the trial. In the developer and finisher phase, both DON and DOM-1 plasma concentrations tend to decrease again. When plasma concentrations are compared amongst groups, no significant differences between contaminated and contaminated + GMA group could be demonstrated at any of the sampling points. This suggests that supplementation of the contaminated diets with GMA did not alter DON absorption in turkey poults.

5. Conclusions

Feeding naturally DON contaminated diets to turkey poults altered some unspecific parameters such as growth rate, plasma biochemistry profile, duodenal villus height and apparent villus surface area and CD8^{+} T-lymphocyte count in the duodenum. A yeast derived mycotoxin binder, GMA, was partially effective in preventing those effects. Performance parameters and plasma biochemistry profiles were not found suitable to evaluate the efficacy of mycotoxin binders on DON absorption in turkey poults as they were not consistent. GMA was able to counteract the negative effects of DON on duodenal morphometry but did not alter the increased influx of CD8^{+} T-lymphocytes. Plasma concentrations of DON and DOM-1 were not altered by the addition of GMA to the diet, suggesting that GMA was ineffective in decreasing DON absorption. These data suggest that the beneficial effects of GMA are due to another mechanism than DON adsorption in the gut.

6. Acknowledgments

7. References

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GENERAL DISCUSSION

Mycotoxin detoxifying agents (mycotoxin detoxifiers) are commonly used as feed additives to prevent the deleterious effects of mycotoxins on animal health (Jard et al., 2011). These products should be evaluated for their *in vitro* and *in vivo* efficacy as well as possible interactions with the oral absorption of veterinary drugs. A lot of different variables have to be taken into account when developing models for *in vitro* and *in vivo* tests. A more general discussion on mycotoxin detoxifiers is presented, linked to the results obtained in this thesis.

Mycotoxin concentration levels

Mycotoxin binding is determined by the concentration of both the mycotoxin and the detoxifier. This can be demonstrated by *in vitro* adsorption isotherms, where a known amount of mycotoxin is added to a known amount of test product in an aqueous solution. The results are usually reported as '%ads', the fraction of toxin bound to the adsorbing agent. At low mycotoxin concentrations, the binding of mycotoxin to the binder will depend on the affinity between both (affinity constant or K_a). With increasing concentration of mycotoxin, the amount adsorbed will increase more slowly and finally reach a point at which the binder is saturated, i.e. the capacity (n) of the mycotoxin binder (EFSA, 2009). When, at very high mycotoxin concentrations, the binder is saturated, the only way to increase the %ads is by increasing the amount of binder.

In *in vivo* trials, high feed contamination levels are used to evoke negative effects on animal performance (Kolosova and Stroka, 2011). Mycotoxin levels in feedstuffs which lower zootechnical performance depend on both the animal species in question and on the type of mycotoxin. This implies that each mycotoxin at a given level of contamination can cause a specific sensitivity depending on the animal species: for example, pigs are more sensitive to fumonisin B1 (FB1), zearalenone (ZON) and deoxynivalenol (DON) than poultry. The EFSA states (EFSA, 2010) that maximum levels set by the EC should be used, for example 0.9 or 5 mg DON/kg feed for pigs or poultry, respectively (**Chapter IV and VI**). However, these maximum levels are 'safe' values, established at a level that should not impair animal performance. For example, the no observed adverse effect level (NOAEL) on zootechnical performance of pigs is 0.04 mg/kg BW/day (Bergsjo et al., 1992). This resembles a dietary feed intake of 0.9 mg DON/kg feed. These maximum levels limit the use of performance parameters to evaluate the effect of a mycotoxin detoxifier. Other parameters should be

used, and as proposed by the EFSA, toxicokinetic parameters are promising in this context. If a mycotoxin binder adsorbs the mycotoxin in the gut, lowered concentrations will be detected in organ tissues and body fluids such as plasma. A prerequisite however is the availability of sensitive methods capable to detect and quantify mycotoxins in those matrices at the low ng/mL level. A specific goal of our research was developing such methods as they were unavailable at the time being (**Chapter II and III**) (De Baere et al., 2011; Devreese et al., 2012 and 2013). The methods were successful in quantifying several mycotoxins such as DON, de-epoxydeoxynivalenol (DOM-1) and ochratoxin A (OTA) in animal plasma administered at the levels proposed by the EFSA. The methods were unsuccessful for others such as aflatoxin B1 (AFB1) and T-2 toxin (T-2) because of the very low maximum levels, e.g. 0.02 mg/kg for AFB1, or a fast elimination ($T_{1/2el}$ of T-2 = 3.9 min in broiler chickens). Another issue is that for beauvericin (BEA) and enniatins (ENNs) no maximum levels in feed have been established by the EC. The developed method for quantifying BEA and ENNs in pig plasma was successful for enniatin A, A1, B and B1 at the administered dose (0.05 mg/kg) and may now be used for future efficacy studies. However, this dose was set arbitrarily. Efficacy and safety studies with such arbitrarily set contamination levels due to the lack of guidance levels, might not be accepted by the EFSA anymore once guidance levels are established.

Furthermore, the developed methods were used to evaluate the efficacy of mycotoxin detoxifiers towards DON absorption in poultry (**Chapter IV and VI**). The maximally allowed guidance level of DON contamination was used (5 mg DON/kg feed). Very low DON plasma levels were measured, ranging between 1 and 20 ng/mL. These levels can only be quantified using highly sensitive LC-MS/MS equipment. As a result, this may urge the need to use higher contamination levels than those proposed by the EFSA when it comes to efficacy testing of mycotoxin detoxifiers.

Mycotoxin standards or feed naturally contaminated with mycotoxins

As a source of mycotoxins, both spiked feed (used in **Chapter IV**) or naturally contaminated feed (used in **Chapter VI**) can be used in *in vivo* trials. The advantage of mycotoxin standards is that the amount of toxin administered to the animal is exactly known. It is also easier to reproduce results using standards, when the exact same amount can be chosen in

consecutive experiments. Next, no co-contamination is present, which could exert synergistic or additive effects. Besides the high purchase costs, the main drawback of using mycotoxin standards is that it does not really represent the natural situation.

The use of naturally contaminated feed materials is preferred by the EFSA and other research groups (Dänicke et al., 2003; EFSA, 2010; Girish et al., 2010). It resembles the situation in animal husbandry far more than using standards. Co-contaminants are often present which makes it harder to interpret the obtained results as a different 'mycotoxin profile' of the feed can lead to a different outcome. Also a larger variation of contamination level will be present as mycotoxin contamination is not homogenous throughout the entire feed batchv(Jard et al., 2011). Another key factor are masked mycotoxins which lead to an underestimation of the total mycotoxin contamination. Masked mycotoxins are usually plant derivatives of mycotoxins which are not detected by conventional analytical techniques (Berthiller et al., 2013). The plant itself modifies the mycotoxin to a less harmful compound, in order to reduce plant toxicity. Plant derived masked mycotoxins can be divided in two classes, the conjugated, such as DON-3-glucoside, and bound varieties such as protein bound FB1 (Park et al., 2004; Sasanya et al., 2008). Next to the plant derivatives, also food or feed processing can alter mycotoxins, forming masked mycotoxins. However, most of these compounds formed during food processing are less toxic than their precursors (Berthiller et al., 2013). The question remains whether these conjugated forms are hydrolyzed *in vivo*, resulting in increased exposure to the native toxin.

Does glucomannan binds deoxynivalenol?

Glucomannan (GMA) is an organic mycotoxin binder derived from cell wall components of *Saccharomyces cerevisiae* yeasts. Based on *in vitro* assays, GMA has shown to effectively adsorb DON, T-2, ZON, OTA and AFB1 (Bejaoui et al., 2004; Freimund et al., 2003; Yiannikouris et al., 2004; Yiannikouris et al., 2006). The β-D-glucan fraction of yeast cell walls is directly involved in the binding process with ZON, and the structural organization of β-D-glucans modulates the binding strength. Hydrogen and van-der-Waals bonds have been evidenced in the glucan-mycotoxin complexes (Jouany, 2007; Shetty and Jespersen, 2006; Yiannikouris et al., 2004; Yiannikouris et al., 2006). These *in vitro* results correlate well with the results obtained in our *in vitro* model (**Chapter I**). In our cell culture experiments using

IPEC-J2 cells grown on Transwell® inserts, GMA lowered the passage of DON through the epithelial monolayer (37-57%). GMA was also partially effective in reducing the negative effects of cytotoxic DON concentrations on cellular viability and integrity (57 and 65%, respectively).

The next question is whether the *in vitro* results could be confirmed *in vivo*. Several studies have demonstrated beneficial effects of GMA against the detrimental consequences of DON in both pigs and poultry.

In pigs, feeding DON reduced brain neurochemistry hormones (dopamine, dihydroxyphenylacetic acid and norepinephrine) (Swamy et al., 2002). It also increased serum IgA and IgM concentrations and altered performance parameters (reduced feed intake and weight gain). In a study by Diaz-Llano and Smith (2007), GMA addition was able to counteract the alterations of serum biochemical parameters induced by DON in sows. However, no positive effect on feed intake and body weight gain was seen. Also Dänicke et al. (2007) did not observe improved productive parameters in pigs following GMA addition in a DON contaminated feed.

Furthermore, GMA has shown to counteract most of the alterations in plasma biochemical parameters caused by a DON contaminated diet in chickens (Faixova et al., 2010). In a study by Swamy et al. (2004), GMA could also prevent the decrease in B-cell count induced by DON. GMA also promoted the growth of villi and intestinal recovery after coccidial challenge of broiler chickens (Girgis et al., 2010). It has preventive effects on alterations in brain neurochemistry of turkeys (Girish et al., 2008a). Also in turkeys, GMA counteracted the effects of DON on intestinal morphology (Girish and Smith, 2008), animal performance, some alterations in plasma biochemistry and the effects of CD8$^+$ T cell-mediated delayed-type hypersensitivity response (Girish et al., 2008b).

In conclusion, GMA is able to counteract many effects induced by DON on animal performance, blood biochemistry, histology, immunology and other parameters in several animal species. May these effects be attributed to the binding of DON by GMA in the intestinal tract or are other mechanisms involved? To elucidate this, GMA was included as mycotoxin detoxifier in our *in vivo* model based on the toxicokinetics of DON. In **Chapter IV**, oral bolus models were developed in which DON with or without GMA was given as an intra-

crop bolus to broiler chickens. In the first model, a significant higher AUC_{0-inf}, C_{max}, k_a and relative oral bioavailability (OBB) and a shorter $T_{1/2a}$ were found in the DON + GMA group compared to the group only administered DON. No significant differences in toxicokinetic parameters between both groups in bolus model 2 and 3 were observed. Another trial was performed with DON and GMA in turkey poults (**Chapter VI**). Birds were reared for twelve weeks and fed different diets: control (minimally contaminated), control + GMA, DON contaminated (5 mg/kg) and contaminated + GMA. Several unspecific parameters were monitored, as well as DON and DOM-1 plasma concentrations. GMA was able to counteract some effects of DON on unspecific parameters, however the feed additive was not able to lower plasma concentrations of DON or DOM-1 in any of the rearing phases.

As a general conclusion, it was shown in this work that GMA is partly effective in adsorbing DON *in vitro*, but the *in vivo* situation is complicated by many other variables. Although GMA exerts beneficial effects, they cannot be attributed to binding of DON by GMA *in vivo*. What other mechanism is involved, is not clear up to day. GMA is composed of mannan oligosaccharide (MOS) and β-glucan. MOS has a distinct effect on the intestinal microflora. Spring et al. (2000) demonstrated a reduction of prevalence and concentration of *Salmonella* strains in broiler chickens ceca when fed MOS. Besides, it is often used as a prebiotic to increase animal performance. Addition of MOS to the diet of broiler chickens and turkeys increased their growth rate and decreased their feed conversion (Hooge, 2004a, 2004b; Rosen, 2007). MOS would also have an advantageous effect on intestinal morphology and function. It enhances the nutrient digestibility, increase villus surface area and stimulates mucus production (Yang et al., 2008; Baurhoo et al., 2009). The second compound of GMA, namely β-glucan, can promote the immune system by activating T-lymphocytes (Rop et al., 2009) (**Chapter VI**). It also stimulates intestinal recovery after coccidial challenge in poultry (Girgis et al., 2010), increases the intestinal cell rate proliferation leading to changes in mucosal structure (Iji, 1999), sequesters bile acids in the gut (Chen and Huang, 2009), and even exerts anti-tumoral activity (Yalin et al., 2005). All these, and other factors, may account for the beneficial effects of GMA, indirectly counteracting the effects of DON.

Specific biomarkers to evaluate the efficacy of mycotoxin detoxifiers

To evaluate the efficacy of mycotoxin detoxifiers, measuring specific biomarkers in tissues or body fluids is the method of choice according to the EFSA (EFSA, 2010). For DON, the parent compound in plasma has demonstrated to be a sensitive biomarker for exposure and for efficacy testing in broiler chickens (**Chapter II and IV**). Its main metabolite DOM-1 was not suited as a biomarker in broiler chickens, in contrast to turkeys, where plasma levels of DOM-1 were higher and less variable compared to DON (**Chapter VI**). This demonstrates that biomarkers should be assessed for each species individually and cannot be generalized. Also phase II metabolites might be suited as biomarker for DON. At least in humans, DON-glucuronides were validated as biomarker (Warth et al., 2012). The hepatic glucuronidation pattern of DON is species dependent, therefore the suitability of DON-glucuronide as biomarker will be species dependent as well. Humans and pigs extensively glucuronidate DON, in contrast to poultry species (Maul et al., 2012).

Suitable biomarkers for assessing the efficacy of mycotoxin detoxifiers towards other mycotoxins should be investigated as well. For T-2 for example, 70% of the metabolite residue is present in its glucuronide form (Corley et al., 1985). This metabolite might therefore be more suited as biomarker compared to T-2 itself or HT-2. This is acknowledged by our results since no plasma levels of the T-2 nor HT-2 could be detected after oral administration of a relatively high dose of T-2 to pigs (0.05 mg/kg BW) (**Chapter II**).

Next to the parent compound or its metabolites, biomarkers can also include compounds related to the mode of action of the mycotoxin. This is the case for fumonisin B1 (FB1). FB1 specifically acts as an inhibitor of the ceramide synthesis, leading to altered levels of sphinganine (Sa) and sphingosine (So) in tissues and body fluids. The Sa:So ratio was suited as biomarker for fumonisin exposure in mammals and avian species (Haschek et al., 2001).

Efficacy and safety testing of mycotoxin detoxifiers: involvement of different stakeholders

Since *in vitro* and *in vivo* models are now available to evaluate the efficacy of mycotoxin binders and modifiers and to investigate possible interactions with oral absorption of veterinary drugs, the next question arises: who should evaluate the efficacy and safety of both types of products?

Several partners are involved: governmental instances, academia and manufacturers of those feed additives.

The outline on how new detoxifiers should be evaluated has to be provided by governmental instances. However, they should take the available know-how and equipment into account. When the EFSA proposed their guidelines for *in vivo* efficacy and safety testing of mycotoxin detoxifiers in 2010 (EFSA, 2010), no models were available at that time. Such guidelines should be drafted in agreement with the involved manufacturers. To our knowledge, since the launch of their guidelines one registration dossier was submitted, demonstrating the ability of bentonite clay to bind AFB1, to be used in all animal species. The efficacy on ruminants could be demonstrated by an altered excretion profile, lower aflatoxin M1 (AFM1) excretion in milk due to lowered AFB1 absorption from the intestinal tract. For the other animal species, the manufacturer provided numerous studies to demonstrate the efficacy. However, they were based on unspecific parameters including zootechnical parameters, blood proteins, organ weights and histopathology. The EFSA concluded (EFSA, 2011) that the data provided were insufficient to currently prove the efficacy of bentonite clays as no evidence based on specific toxicokinetic parameters was given.

It was up to the academia to fill the gap between what legislators want and the know-how available to manufacturers. The models described in this thesis are in close relation to the EFSA guidelines and provide an added value for the involved manufacturers which can adopt them. It does require some efforts as manufacturers are used to evaluate their products using adsorption studies and/or feeding trials evaluating performance parameters. For new registration dossiers, manufacturers should also demonstrate that their products can be safely used in combination with medicated feed or drinking water. Another option is to prohibit the use of the detoxifier in combination with additives from the group of antibiotics, chemotherapeutics, coccidiostats and other frequently used medical substances in poultry and pig industry.

On the other hand, a huge variety of mycotoxin detoxifiers are commercially available today, without fully evaluating the safety of those products. In our opinion, it is up to the academia to draw attention on these interactions and consequences for animal health, microbial resistance and even public health. Evaluating all available drugs and all available commercial detoxifiers is, however, out of the scope of the academia.

Future perspectives

A first future perspective could be developing an *ex vivo* model using the Ussing chamber technology. An Ussing chamber consists of two halves clamped together with an insert containing a full-thickness intestinal sample. Similar parameters can be evaluated as in the developed *in vitro* model, namely tissue integrity based on transepithelial electrical resistance (TEER) and passage through the intestinal tissue explant. This *ex vivo* model can be used for efficacy and safety testing of mycotoxin binders. It leads to a higher throughput compared to *in vivo* trials, but it is more labor intensive than the developed *in vitro* model.

Mycotoxin modifiers are supposed to biotransform mycotoxins into less toxic metabolites. The Simulator of Human Intestinal Microbial Ecosystem (SHIME) is a dynamic *ex vivo* model simulating the gastro-intestinal tract by consecutive reactors each containing stable microbial communities. The SHIME could be used to evaluate the efficacy (biotransformation capability) and safety (identification and characterization of the formed metabolites) of these products in the presence of the intestinal microbiota.

The *in vitro* and *in vivo* efficacy models described in this thesis were validated with DON. It would definitely be interesting to use the developed models with other mycotoxins. The most appropriate biomarkers should be selected first. These biomarkers can be the mycotoxin itself, metabolites or compounds related to the mode of action (as stated above).

The *in vitro* and *in vivo* safety models developed were based on a direct interaction between mycotoxin detoxifier and veterinary drugs. However, as stated above, a more complex interaction between detoxifier, veterinary drug and mycotoxin was described by means of the *in vivo* model (Goossens et al., 2012; Osselaere et al., 2013). It would be of interest to investigate if the results of this more complex interaction could be reproduced with the *in vitro* model.

Future research should not only focus on adsorption of veterinary drugs by mycotoxin detoxifiers but also enhanced oral bioavailability, as demonstrated with doxycycline in pigs (Goossens et al., 2012) and oxytetracycline in broilers (Osselaere et al., 2012). Enhanced oral bioavailability, leading to increased tissue concentrations might impair the withdrawal time of veterinary drug formulations and possibly lead to the occurrence of undesirable residues

(above the Maximum Residue Limit, MRL) of the antibiotic in edible tissues. On the other hand, if the detoxifier leads to increased plasma concentrations, this could, from a theoretical point of view, be compensated by reducing the dosage of the antibiotic.

In **Chapter III**, a sensitive LC-MS/MS method for simultaneous determination and quantification of BEA and ENNs in pig plasma was described. At the time being, little is known about the *in vivo* toxicokinetics and toxicodynamics of these mycotoxins. The presented method can now be applied to conduct a comprehensive animal experiment to elucidate the toxicokinetics of these mycotoxins in swine. Eventually, this would provide a better understanding on their *in vivo* effects.

Recently, there are indications that mycotoxins and mycotoxin detoxifiers could also interfere with the pathogenesis of infectious disease. More specifically, DON and T-2 potentiate *Salmonella* Typhimurium in pigs (Vandenbroucke et al., 2011; Verbrugghe et al., 2012). Moreover, the addition of GMA to T-2 contaminated diet (87 µg T-2/kg feed) did not only alleviate the reduced body weight gain of the pigs, it also reduced the intestinal colonization of *S.* Typhimurium, although not significantly. Nevertheless, *in vitro* findings suggests that GMA binds *Salmonella* bacteria (Verbrugghe et al., 2012). These results open new research perspectives regarding the interaction between mycotoxins, mycotoxin detoxifiers and infectious agents.

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SUMMARY

Mycotoxins are considered a major issue worldwide because of their harmful effects on animals. This leads to great economic losses, especially in pig and poultry industry. The most commonly used method to counteract the negative impact of mycotoxins on animals is adding mycotoxin detoxifying agents (mycotoxin detoxifiers) to feed. These feed additives, so-called mycotoxin binders or mycotoxin modifiers, either adsorb or biotransform mycotoxins in the gastrointestinal tract, respectively.

These products should be tested on their ability to bind or modify mycotoxins *in vitro* as well as *in vivo* (efficacy). At the time being, no reliable *in vitro* models were available which were both high-throughput and mimic the gastrointestinal tract nor were there *in vivo* models available which evaluate the efficacy of mycotoxin detoxifiers based on specific toxicokinetic parameters.

Next to the efficacy of these products, questions have been raised on possible interactions with the oral absorption of veterinary drugs. Furthermore, no *in vitro* nor *in vivo* models were available to evaluate the safety, regarding drug absorption, of these products.

The **general introduction** of this thesis starts with an overview of the most toxic and prevalent mycotoxins. Next, pre- and postharvest methods to reduce the impact of mycotoxins is provided including mycotoxin detoxifiers. Finally, regulations of the European Commission (EC) and the European Food Safety Authority (EFSA) are summarized, focusing on maximum levels of mycotoxin contamination in feed and guidelines to evaluate the efficacy and safety of mycotoxin detoxifiers, respectively.

The general **aim** of this doctoral research was to develop *in vitro* and *in vivo* models for testing the efficacy of mycotoxin detoxifiers and their possible interactions with the oral absorption of veterinary drugs.

In **Chapter I** an *in vitro* model was developed to evaluate the efficacy of mycotoxin binders and possible interactions with veterinary drug adsorption. Intestinal porcine epithelial cells derived from the jejunum (IPEC-J2) were cultivated on Transwell® cell culture inserts. In the first experiments, deoxynivalenol (DON) was added at 0.5 µg/mL or 1 µg/mL, with or without activated carbon (AC) or a commercially available glucomannan binder (GMA). A concentration of 1 µg/mL decreased cellular viability (based on a neutral red cytotoxicity

assay) and cellular integrity (based on measurements of transepithelial electrical resistance, TEER). On the other hand, a concentration of 0.5 µg/mL DON did not exert any cytotoxic effect nor did it damage the cellular integrity. AC was used as a positive control binder and completely alleviated the negative effects of 1 µg/mL DON. Furthermore, it almost completely (>99%) reduced the passage through the epithelial monolayer, mimicking intestinal absorption, at both 1 and 0.5 µg/mL DON. These results prove that our model is effective in demonstrating the ability of mycotoxin binders to bind DON. The commercial binder, GMA, was partially effective in preventing the cytotoxic effects and cellular integrity damage of DON. Moreover, it reduced the transepithelial passage of DON with 37% to 57% compared to AC, at both cytotoxic and non-cytotoxic concentrations of DON.

In the second experiments, a drug interaction model was developed. The interaction between tylosin (20 µg/mL) and mycotoxin binders was investigated as some authors suggest binding of macrolide antibiotics to bentonite clays. Indeed, a bentonite clay, used as positive control, decreased the passage of tylosin through the epithelial monolayer with 82%, indicating binding of tylosin by bentonite. This indicates that the combined use of bentonite and tylosin in the feed could lead to therapy failure. GMA did not alter the passage of tylosin, indicating safe combined use.

The next step after introducing the *in vitro* model was to develop *in vivo* models based on specific pharmaco- or toxicokinetic parameters. In order to do so, sensitive methods were needed to detect and quantify mycotoxins in animal plasma.

In **Chapter II** a sensitive and reliable multi-mycotoxin method was developed for the identification and quantification of several toxicological important mycotoxins such as DON, de-epoxydeoxynivalenol (DOM-1), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZON), zearalanone (ZAN), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1) in pig plasma using liquid chromatography combined with heated electrospray ionization triple quadrupole tandem mass spectrometry (LC-h-ESI-MS/MS). Sample cleanup consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in water/methanol (85/15, v/v). Each plasma sample was analyzed twice, i.e. once in the ESI+ and ESI- mode, respectively. For the performance of toxicokinetic studies with individual mycotoxins, even more sensitive analyte-specific LC–

MS/MS methods were developed. The multi-mycotoxin and analyte-specific methods were in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9974–0.9999 and 2.4–15.5%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification for the multi-mycotoxin and analyte-specific methods ranged from 2 to 10 ng/mL and 0.5 to 5 ng/mL, respectively, whereas limits of detection fell between 0.01–0.52 ng/mL and < 0.01–0.15 ng/mL, respectively.

Several *Fusarium spp*. are capable of producing well-known mycotoxins like trichothecenes, fumonisins and/or ZON, but also other mycotoxins including beauvericin (BEA) and enniatins (ENNs) such as enniatin (ENN) A, A1, B and B1. Several studies have reported cytotoxic effects of ENNs and BEA on several cell types. Data on their *in vivo* toxicity are however lacking. To investigate their toxicokinetic properties and to evaluate the possible effects of mycotoxin binders on adsorption of BEA and ENNs, methods to detect and quantify these compounds in plasma are again mandatory. Therefore, in **Chapter III** a sensitive and reliable method was developed for the identification and quantification of BEA, ENN A, A1, B and B1 in pig plasma using LC-h-ESI-MS/MS. The same sample preparations was used as for the previously described mycotoxins except for the resuspension solvent (acetonitrile/water, 80/20, v/v). The method was in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9980 and 0.9995 and between 5.2% and 11.3%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification were 0.1 ng/mL for enniatin A and A1 and 0.2 ng/mL for BEA, ENN B and B1, whereas limits of detection were ≤ 10 pg/mL for all analytes. The method has been applied for the analysis of incurred plasma samples from one pig that received an oral bolus (0.05 mg/kg BW) of the investigated mycotoxins. At the applied dosage, the results indicated the suitability of the method for use in toxicokinetic studies with ENNs.

Another specific aim was to develop *in vivo* models based on toxicokinetic parameters using the described analytical methods. In **Chapter IV**, three bolus models were developed, assessed using DON in broiler chickens. In the first model, DON was given to broiler chickens

as an intra-crop bolus together with a mycotoxin detoxifier in order to study the plasma concentration–time profile of DON. In the second model, the same oral bolus was given, preceded by an oral bolus of mycotoxin detoxifier, to make sure the detoxifier was present in the whole intestinal tract when the mycotoxin was administered. In the third model, the mycotoxin detoxifier was mixed in the feed of broiler chickens, and after 1 week feeding, DON was given as an oral bolus. In order to evaluate the efficacy of these detoxifiers, plasma concentration–time profiles were set up and the main toxicokinetic parameters were compared. Two commercially available mycotoxin detoxifiers were tested, but they were not able to lower the oral availability of DON. As a positive control, AC was used. We showed that AC significantly reduced the absorption and oral availability of DON in all three models. It was concluded that these models are able to demonstrate the efficacy of mycotoxin detoxifiers in relation to EFSA guidelines.

After the *in vivo* efficacy model, a similar bolus model for testing the interaction of mycotoxin detoxifiers with oral absorption of veterinary drugs was developed in **Chapter V**. As an interaction between tylosin and bentonite clay was observed *in vitro*, these compounds were used to develop the *in vivo* model. The interaction between bentonite and tylosin was investigated in broiler chickens, based on pharmacokinetic characteristics. Simultaneous oral administration of bentonite and tylosin significantly lowered plasma levels of tylosin and reduced the area under the plasma concentration–time curve (AUC_{0-inf}), maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}) and relative oral bioavailability. The results proved unambiguously the binding of tylosin by bentonite. Simultaneous administration of tylosin (in the drinking water or feed) and bentonite (mixed in the feed as a mycotoxin binder) should therefore be avoided. A commercially available GMA binder was also evaluated in this study. It did not alter the oral absorption of tylosin and can therefore be safely co-administered with tylosin in animal feed.

The final goal for *in vivo* modeling was to evaluate the correlation between unspecific and specific parameters for efficacy testing of mycotoxin detoxifiers. In **Chapter VI**, an experiment was conducted to investigate the effects of grains naturally contaminated with *Fusarium* mycotoxins and a yeast derived mycotoxin binder (GMA) on selected unspecific

and specific parameters in turkey poults. Two hundred-forty 1-day-old male turkey poults were fed diets up to twelve weeks, formulated with minimally contaminated grains (control), control + 0.2% GMA, contaminated grains, or contaminated grains + 0.2% GMA. DON was the major contaminant of the contaminated grains and concentrations varied around 5 mg/kg feed. Following unspecific parameters were selected: performance parameters, plasma biochemistry profile, morphometry and CD8$^+$ T-lymphocyte count of the duodenum. Plasma concentrations of DON and DOM-1 were used as specific parameter and were correlated to the unspecific parameters.

Zootechnical and plasma biochemistry parameters were altered by DON and/or GMA but were not consistent along the trial and cannot be used as parameters for efficacy testing of the mycotoxin detoxifier in turkey poults. DON reduced the villus height and apparent villus surface area in the duodenum. This effect was prevented by GMA addition to the diet. DON elevated the total CD8$^+$ T-lymphocyte count in the duodenum but this effect was not prevented by GMA. No significant differences in plasma concentrations of DON and DOM-1 between contaminated and contaminated + GMA groups were observed, concluding that GMA did not prevent DON absorption. The effect of GMA on morphometry parameters can therefore not be attributed to DON adsorption in the gut. As the CD8$^+$ T-lymphocyte count in the duodenum correlates well with DON and DOM-1 plasma concentrations it can be concluded that this unspecific parameter is suitable to evaluate the efficacy of the mycotoxin binder on adsorption of DON in turkey poults.

SAMENVATTING

Mycotoxines worden wereldwijd beschouwd als een belangrijk probleem omwille van hun schadelijke effecten op mens en dier. Dit leidt tot beduidende economische verliezen, voornamelijk in de varkens- en pluimveehouderij. De meest toegepaste methode om de schadelijke effecten van mycotoxines tegen te gaan, is het inmengen van mycotoxine detoxificerende producten in veevoeder. Deze voederadditieven kunnen onderverdeeld worden in mycotoxine binders, die de mycotoxines in het gastro-intestinaal stelsel adsorberen, en mycotoxine modifiërs, die mycotoxines in het gastro-intestinaal stelsel transformeren en gedeeltelijk of totaal onschadelijk maken.

Deze voederadditieven dienen getest te worden op hun mogelijkheid om mycotoxines te detoxificeren zowel *in vitro* als *in vivo*. Bij de aanvang van het onderzoek waren er geen betrouwbare *in vitro* modellen beschikbaar, die zowel een grote verwerkingscapaciteit hebben als de omstandigheden van het gastro-intestinaal stelsel nabootsen. Bovendien waren er geen *in vivo* modellen voorhanden die de efficaciteit van deze producten toetsten gebaseerd op specifieke toxicokinetische parameters. Naast het testen van de efficaciteit van deze producten zijn er ook bedenkingen bij hun veiligheid, vooral wat betreft mogelijke interacties met de orale absorptie van veterinaire geneesmiddelen. Er waren eveneens noch *in vitro* noch *in vivo* modellen beschikbaar die de veiligheid van deze producten konden evalueren.

De **algemene inleiding** van dit proefschrift vangt aan met een overzicht van de meest toxische en relevante mycotoxines. Vervolgens worden methoden beschreven die de impact van mycotoxines kunnen tegengaan, zowel vóór als na het oogsten. Mycotoxine detoxificerende producten vallen hier ook onder. Uiteindelijk wordt de belangrijkste Europese regelgeving, uitgaande van de Europese Commissie (EC) en de Europese Autoriteit voor de Voedselveiligheid (EFSA), beschreven. Daarbij werd uitgegaan van maximaal toegelaten gehaltes van mycotoxines in veevoeder en richtlijnen om de efficaciteit en veiligheid van mycotoxine detoxificerende producten te evalueren.

De algemene **doelstelling** van dit doctoraatsonderzoek was om *in vitro* en *in vivo* modellen te ontwikkelen die de efficaciteit van mycotoxine detoxifiërs kunnen bepalen, evenals hun mogelijke interacties met de orale absorptie van veterinaire geneesmiddelen.

In **Hoofdstuk I** werd een *in vitro* model ontwikkeld om de efficaciteit van mycotoxine binders na te gaan, evenals de mogelijke interacties ervan met veterinaire geneesmiddelen. Darmepitheelcellen van het jejunum van varkens (IPEC-J2) werden gekweekt op Transwell® celcultuur inserts. Tijdens de eerste experimenten werd 0,5 of 1 μg/mL deoxynivalenol (DON) aan de inserts toegevoegd met of zonder actieve kool (AK) of een commercieel beschikbare glucomannaan binder (GMA). Een concentratie van 1 μg/mL gaf aanleiding tot een significante daling van de cellulaire leefbaarheid (vastgesteld aan de hand van de neutraal rood cytotoxiciteitstest) en cellulaire integriteit (vastgesteld aan de hand van transepitheliale elektrische weerstandsmetingen, TEER). Een concentratie van 0,5 μg/mL daarentegen, had geen invloed op de cellulaire leefbaarheid of integriteit. AK werd gebruikt als een positieve controle en was inderdaad in staat om de schadelijke effecten van 1 μg/mL DON volledig teniet te doen. Meer zelfs, AK verminderde de passage van DON doorheen de epitheliale monolaag bijna volledig (>99%), zowel bij 0,5 als 1 μg/mL. Deze resultaten tonen aan dat het model in staat is om de efficaciteit van mycotoxine detoxificerende agentia *in vitro* aan te tonen. GMA was in staat om de negatieve effecten van DON op de cellulaire viabiliteit en integriteit deels te verhinderen. Het additief verminderde de transepitheliale passage van DON met 37% tot 57% in vergelijking met AK en dit zowel bij cytotoxische als niet-cytotoxische concentraties, respectievelijk.

In een tweede reeks experimenten werd een veiligheidsmodel ontwikkeld. De interactie tussen het macrolide antibioticum tylosine (20 μg/mL) en mycotoxine binders werd geëvalueerd aangezien sommige auteurs voorafgaandelijk mogelijke interacties tussen macrolide antibiotica en bentoniet klei rapporteerden. Bentoniet werd gebruikt als positieve controle en dit additief reduceerde de passage van tylosine doorheen de epitheliale monolaag met 82%, ten gevolge van tylosine-bentoniet binding. Het gecombineerd gebruik van tylosine en bentoniet klei in veevoeder kan bijgevolg tot therapiefalen van tylosine leiden. De GMA binder had geen effect op de transepitheliale tylosine passage, zodat beiden samen veilig gebruikt zouden kunnen worden in veevoeder.

Een volgende specifieke doelstelling na de ontwikkeling van het *in vitro* model bestond er in *in vivo* modellen te ontwikkelen gebaseerd op farmaco- of toxicokinetische parameters. Daarvoor werden eerst gevoelige analysemethoden op punt gesteld om mycotoxines te kunnen detecteren en kwantificeren in dierlijk plasma.

In **Hoofdstuk II** werd een multi-mycotoxine analyse methode ontwikkeld voor identificatie en kwantificatie van verschillende toxicologisch belangrijke mycotoxines zoals DON, de-epoxydeoxynivalenol (DOM-1), T-2 toxine (T-2), HT-2 toxine (HT-2), zearalenone (ZON), zearalanone (ZAN), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), ochratoxine A (OTA), fumonisine B1 (FB1) en aflatoxine B1 (AFB1) in varkensplasma gebruik makende van vloeistofchromatografie gecombineerd met elektrospray ionisatie triple quadrupool tandem massaspectrometrie (LC-h-ESI-MS/MS).

De staalvoorbereiding bestond uit een deproteïnizatie stap met acetonitrile, gevolgd door evaporatie van het supernatans en heroplossen van het droog residu in water/methanol (85/15, v/v). Elk plasmastaal werd tweemaal geanalyseerd, in ESI+ en ESI- modus. Voor de uitvoering van specifieke toxicokinetische studies met individuele mycotoxines, werden meer gevoelige analiet-specifieke LC-MS/MS methoden ontwikkeld. De multi-mycotoxine en analiet-specifieke methoden werden gevalideerd: matrix-afgestemde calibratiecurven werden opgesteld voor elke analiet en correlatiecoëfficiënten en goodness-of-fit coëfficiënten varieerden, respectievelijk, tussen 0,9974-0,9999 en 2,4-15,5%. De precisie en accuraatheid binnen één analyse en tussen verschillende analysen viel binnen de aanvaardbare grenzen. De kwantificatielimieten voor de multi-methode en analiet-specifieke methoden varieerden, respectievelijk, tussen 2 en 10 ng/mL en 0,5 en 5 ng/mL. De detectielimieten varieerden, respectievelijk, tussen 0,01–0,52 ng/mL en < 0,01–0,15 ng/mL.

Verschillende *Fusarium spp.* kunnen welgekende mycotoxines zoals de trichothecenen, fumonisinen en/of ZON produceren, maar ook minder gekende toxines zoals beauvericine (BEA) en enniatines (ENNs), waaronder enniatine (ENN) A, A1, B en B1. Verschillende studies hebben de cytotoxische effecten van ENNs en BEA reeds aangetoond op verschillende celtypes. Gegevens in verband met hun *in vivo* toxiciteit daarentegen ontbreken. Om de toxicokinetische eigenschappen te onderzoeken en het effect van mycotoxine detoxificerende voederadditieven op de absorptie van ENNs of BEA te evalueren, zijn methoden noodzakelijk om deze componenten te identificeren en kwantificeren in plasma. Bijgevolg werd er een methode ontwikkeld voor identificatie en kwantificatie van BEA, ENN A, A1, B en B1 in varkensplasma gebruik makende van LC-h-ESI-MS/MS, zoals beschreven in **Hoofdstuk III**. De staalvoorbereiding was identiek aan deze vermeld in het voorgaand hoofdstuk, met uitzondering voor wat het heroplossingsolvens betreft (acetonitrile/water,

80/20, v/v). De methode werd opnieuw gevalideerd: matrix-afgestemde calibratiecurven werden opgesteld voor elke analiet en correlatiecoëfficiënten en goodness-of-fit coëfficiënten varieerden, respectievelijk, tussen 0,9980-0,9995 en 5,2-11,3%, respectievelijk. De precisie en accuraatheid binnen één analyse en tussen verschillende analysen viel binnen de aanvaardbare grenzen. De kwantificatielimiet was 0,1 ng/mL voor ENN A en A1 en 0,2 ng/mL voor BEA, ENN B en B1. De detectielimiet was ≤ 10 pg/mL voor alle componenten. De methode werd succesvol toegepast op biologische stalen afkomstig van een varken dat een orale bolus (0,05 mg/kg LG) van de onderzochte mycotoxines toegediend kreeg. De resultaten bevestigden de toepasbaarheid van de methode voor toxicokinetische studies met ENNs, althans wanneer deze worden toegediend aan de geteste dosering.

In de volgende fase was het de bedoeling om, met behulp van de op punt gestelde analysemethoden, *in vivo* modellen te ontwikkelen gebaseerd op toxicokinetische parameters. Daarom werden drie orale bolus modellen ontwikkeld, getoetst aan de hand van DON bij mestkippen (**Hoofdstuk IV**). In het eerste model werd DON oraal in de krop toegediend met of zonder een mycotoxine detoxifiër om het plasmaconcentratie-tijdsprofiel van DON op te stellen. In het tweede model werd dezelfde orale bolus toegediend, echter voorafgegaan door een orale bolus van de mycotoxine detoxifiër zodat het additief reeds in het gastro-intestinaal stelsel aanwezig was vóór toediening van DON. In het derde model werd het mycotoxine detoxificerende additief ingemengd in het voeder en werd, na 1 week voederen, DON als bolus toegediend. Om de efficaciteit van de additieven na te gaan werden plasmaconcentratie-tijdsprofielen van DON opgesteld en de belangrijkste toxicokinetische parameters onderling vergeleken. Twee commercieel beschikbare mycotoxine detoxifiërs werden onderzocht, maar deze waren niet in staat om de orale absorptie van DON te verminderen. Als positieve controle werd opnieuw AK ingesloten in de proefopzet. AK was effectief in het verlagen van de orale absorptie en biologische beschikbaarheid van DON in alle modellen. Aldus kan men concluderen dat de ontwikkelde modellen in staat zijn de efficaciteit van mycotoxine detoxificerende producten aan te aantonen, in relatie met de richtlijnen van de Europese Autoriteit voor de Voedselveiligheid.

Een volgende doelstelling, na de ontwikkeling van *in vivo* efficaciteitsmodellen, bestond erin een gelijkaardig bolus model te ontwikkelen om de interactie tussen mycotoxine detoxifiërs

en de orale absorptie van veterinaire geneesmiddelen na te gaan (**Hoofdstuk V**). De interactie tussen tylosine en bentoniet werd reeds *in vitro* aangetoond en werd vervolgens getoetst *in vivo*. Mestkippen kregen een orale bolus van tylosine toegediend, al dan niet gecombineerd met bentoniet klei. Gelijktijdige toediening van bentoniet en tylosine verlaagde de plasmaconcentraties van tylosine significant. Dit bleek uit de verminderde oppervlakte onder het plasmaconcentratie-tijdsprofiel (AUC_{0-inf}), maximale plasmaconcentratie (C_{max}), tijd tot maximale plasmaconcentratie (T_{max}) en relatieve orale biologische beschikbaarheid. Deze resultaten toonden ondubbelzinnig aan dat tylosine interageert met door bentoniet. Simultane toediening van beide producten in het voeder of drinkwater is daarom niet aangewezen. GMA werd ook ingesloten in de proefopzet, maar dit additief veranderde de farmacokinetische parameters van tylosine niet. Bijgevolg kunnen tylosine en GMA veilig samen in voeder ingemengd worden.

De finale doelstelling van *in vivo* modellering bestond er in om de correlatie na te gaan tussen niet-specifieke en specifieke parameters voor efficaciteitsstudies van mycotoxine detoxifiërs. Daarom werd een experiment uitgevoerd om het effect na te gaan van granen natuurlijk gecontamineerd met *Fusarium* mycotoxines en een mycotoxine binder (GMA), op zowel specifieke als niet-specifieke parameters bij kalkoenen. Dit werd beschreven in **Hoofdstuk VI**. Tweehonderd veertig eendagskuikens kregen verschillende diëten, namelijk controle (minimaal gecontamineerd), controle + 0.2% GMA, gecontamineerd of gecontamineerd + 0.2% GMA. DON was de voornaamste contaminant met een gehalte van ongeveer 5 mg/kg. De volgende niet-specifieke data werden geëvalueerd: zoötechnische prestaties, biochemisch plasmaprofiel, duodenale morfometrie en CD8^{+} T-lymfocytenaantal in het duodenum. De gemeten plasmaconcentraties van DON en DOM-1 werden gebruikt als specifieke parameter en gecorreleerd aan de niet-specifieke data.

De zoötechnische gegevens en het biochemisch plasmaprofiel werden gewijzigd ten gevolge van DON en/of GMA, maar dit bleek niet consistent te zijn gedurende de ganse fokperiode zodat deze parameters niet kunnen gebruikt worden om de efficaciteit van mycotoxine detoxifiërs bij kalkoenen na te gaan. DON zorgde voor kortere villi en verminderde schijnbaar villus oppervlakte in het duodenum. Dit effect werd teniet gedaan door GMA toevoeging in het voeder. DON zorgde tevens voor een toegenomen influx van CD8^{+} T-lymfocyten in het duodenum maar dit effect bleef gehandhaafd in aanwezigheid van GMA.

Er werden geen significante verschillen in DON of DOM-1 plasmaconcentraties aangetoond, waaruit kan geconcludeerd worden dat GMA niet in staat is om de absorptie van DON te verlagen. Het positieve effect van GMA op villuslengte en schijnbaar villus absorptieoppervlak kon daarom niet te wijten zijn aan adsorptie van DON aan GMA. Het aantal $CD8^+$ T-lymfocyten in het duodenum correleerde met de plasmaconcentraties van DON en DOM-1, zodoende kan deze niet-specifieke parameter als kandidaat merker beschouwd worden om de efficaciteit van de mycotoxine binder tegen DON in kalkoenen te evalueren.

CURRICULUM VITAE

Mathias Devreese werd geboren op 9 oktober 1986 te Roeselare. Na het beëindigen van het hoger secundair onderwijs aan het Instituut Onze-Lieve-Vrouw van Vreugde te Roeselare, richting Wetenschappen-Wiskunde, startte hij in 2004 met de studies diergeneeskunde aan de Universiteit Gent. Hij behaalde in 2010 het diploma van Master in de diergeneeskunde (optie herkauwers) met grote onderscheiding. Onmiddellijk na het afstuderen trad hij in dienst bij de vakgroep Farmacologie, Toxicologie en Biochemie. In januari 2011 vatte hij zijn doctoraatsonderzoek aan met als doel *in vitro* en *in vivo* modellen te ontwikkelen om de efficaciteit van mycotoxine binders en modifiers na te gaan en hun interactie met de orale absorptie van veterinaire geneesmiddelen te bestuderen. Dit onderzoek werd gefinancierd door een IWT-strategische onderzoeksbeurs. Vanaf september 2012 verbleef hij 4 maanden aan het Department of Animal & Poultry Science (University of Guelph, Canada) als wetenschappelijk medewerker. Ook vervolledigde hij in 2013 het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent. Mathias Devreese is auteur en mede-auteur van meerdere wetenschappelijke publicaties, gaf presentaties op verschillende internationale congressen en is reviewer voor diverse tijdschriften in zijn vakgebied.

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