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DISSERTATION

Liquid chromatography tandem mass spectrometric methods for the evaluation of selected mycotoxin detoxification strategies

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Abstract

Mycotoxins are toxic secondary metabolites produced by various filamentous fungi that can infect agricultural crops and plants. Due to fungal growth and the production of mycotoxins on the field as well as during transport and storage of harvested commodities, mycotoxin contaminations of food and feed are threats to human and animal health. Consequently, several pre- and post-harvest methods for counteracting mycotoxins have been developed. Pre-harvest strategies are promising approaches focusing on the prevention of mycotoxin production on the field, which can further be achieved by post-harvest prevention methods during transport and storage. Nevertheless, those methods are cost-intensively and cannot fully avoid mycotoxin contamination due to various parameters influencing their efficacy. Consequently, more specific post-harvest techniques ensuring mycotoxin elimination and detoxification (including physical decontamination and *in vivo* methods based on adsorption or biotransformation in the gastrointestinal tract after consumption) subsequently in the food and feed chain are required. The main objective of this thesis was to evaluate the efficacy of different detoxification and thermal processing methods at the post-harvest side. To this end, analytical methods based on liquid chromatography coupled to tandem mass spectrometry for the mycotoxins of interest and their potential metabolites were developed and validated. In detail, certain detoxification techniques for ergot alkaloids, deoxynivalenol (DON), zearalenone (ZEN) and fumonisins were investigated.

The *Rhodococcus erythropolis* strain MTHt3 and its enzymes ErgA and ErgB, which have the ability to degrade ergopeptines, were isolated and identified by BIOMIN Holding GmbH. Within this thesis, the structure elucidation and characterisation of unknown metabolites formed during enzymatic degradation of six different ergot alkaloids by MTHt3, its lysate and the purified enzyme ErgA was conducted. LC-MS/MS measurements of various degradation experiment samples were performed to generate information about molecular weight and fragmentation of the newly formed metabolites. Furthermore, accurate mass measurements as well as ^{1}H-, ^{13}C- and 2D-NMR experiments (performed by the Institute of Applied Synthetic Chemistry, TU Vienna) of isolated metabolites were conducted for structure elucidation. As a result, the formation of two main groups of new metabolites was observed. During the first degradation step, catalysed by the enzyme ErgA, stable and probably non-toxic diketopiperazines as well as ergine hydroxy carboxylic acids (unstable intermediate metabolites that are decomposed to ergine and an oxocarboxylic acid) are formed. In a second degradation step with Erg B, ergine is cleaved to lysergic acid, which has a much lower vasoconstrictive potential. To conclude, structural elucidation of the formed metabolites yielded insight on the mechanism of this enzymatic degradation. Various feed additives claiming *in vivo* detoxification of DON and/or ZEN by a combination of biotransformation and adsorption are commercially available. One aim of this thesis was to evaluate the effectiveness of 20 different products *in vitro* by aerobic and anaerobic degradation experiments. LC-UV-MS/MS measurements

were performed for the quantification of DON, ZEN and related, known compounds as well as for the detection of potential UV-absorbing, unknown metabolites. As a result, under the applied conditions, 4 out of 20 products showed potential biodegrading activity. Only one of those products was able to degrade DON and ZEN completely to less toxic metabolites, while three products were exclusively able to metabolised ZEN (into the more oestrogenic alpha-zearalenol or unknown metabolites). This demonstrates that many products are ineffective in detoxifying mycotoxins and highlights the necessity of *in vitro* experiments for a critical screening. Physical or chemical food processing methods of contaminated goods can achieve mycotoxin reduction, while breakdown products may be formed. For fumonisin B_1 (FB_1) contaminated products, the formation of partially hydrolysed FB_1 ($pHFB_1$), fully hydrolysed FB_1 (HFB_1) and *N*-(1-deoxy-D-fructos-1-yl) FB_1 (NDF) was reported. $pHFB_1$ and HFB_1 are mainly formed during alkaline food treatment as well as during the application of a fumonisin degrading enzyme, while NDF is one major product that is formed during thermal treatment and extrusion of food. The stability of HFB_1 and NDF under human gastrointestinal conditions was evaluated. HFB_1 was partially metabolised during incubation with human faeces, while NDF was partially cleaved during incubation with digestive juices. The possible metabolisation and toxicological relevance in rats, based on the potential alteration of the sphingolipid metabolism, of $pHFB_1$, HFB_1 and NDF was assessed. For this purpose, male Sprague Dawley rats were exposed to diets containing 13.9 µmol/kg of high purity FB_1, $pHFB_1$, HFB_1 and NDF, respectively, for three weeks. By analysing urine and kidney samples of all treatment groups, a significant elevation of a biomarker of effect for fumonisins (sphinganine to sphingosine ratio) was exclusively observed in samples of the group fed with FB_1-diet. In contrast, by measuring the fumonisin derivatives in faeces samples, partial degradation of NDF to FB_1 was demonstrated, thus indicating partial *in vivo* reactivation in the gastrointestinal tract. However, a reduced toxicological relevance of the tested fumonisin derivatives compared to FB_1 and therefore, a potential detoxification by food processing as well as enzymatic degradation was verified.

In conclusion, within this thesis the effectiveness of different post-harvest detoxification strategies for ergot alkaloids, DON, ZEN and fumonisins was investigated. As a result, the current knowledge on promising strategies in regard to their efficacy, formed metabolites and their analysis was extended and the awareness on commercial products was increased.

Kurzzusammenfassung

Mykotoxine sind toxische Sekundärstoffwechselprodukte diverser Schimmelpilze, welche landwirtschaftliche Getreide- und Pflanzensorten befallen können. Durch Pilzwachstum und die Bildung von Mykotoxinen am Feld, sowie beim Transport und bei der Lagerung von geernteten Erzeugnissen, stellen Mykotoxinkontaminationen in Lebens- und Futtermitteln eine Gesundheitsgefährdung für Mensch und Tier dar. Folglich wurden mehrere Methoden vor und nach der Ernte zur Bekämpfung von Mykotoxinen entwickelt. Strategien vor der Ernte sind vielversprechende Ansätze, welche den Schwerpunkt auf die Vermeidung einer Mykotoxinbildung am Feld legen, was zusätzlich nach der Ernte durch Präventionsmaßnahmen beim Transport und bei der Lagerung erzielt werden kann. Diese Methoden sind jedoch kostenintensiv und können aufgrund verschiedener Parameter, welche ihre Wirksamkeit beeinflussen können, Mykotoxinkontaminationen nicht vollständig vermeiden. Folglich sind spezifischere Techniken nach der Ernte später in der Lebens- und Futtermittelkette erforderlich, welche die Beseitigung und Entgiftung von Mykotoxinen gewährleisten (einschließlich physikalischer Dekontamination und *in vivo*-Methoden zur Adsorption oder Biotransformation im Gastrointestinaltrakt nach dem Verzehr). Ziel der Arbeit war die Evaluierung der Wirksamkeit verschiedener Entgiftungs- und thermischer Verarbeitungsmethoden zur Eliminierung von Mykotoxinen. Dazu wurden analytische Methoden zur Messung von Mykotoxinen und ihrer möglichen Metabolite, basierend auf Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie, entwickelt und validiert. Im Detail wurden bestimmte Entgiftungstechniken für Ergotalkaloide, Deoxynivalenol (DON), Zearalenon (ZEN) und Fumonisine untersucht.

Der *Rhodococcus erythropolis*-Stamm MTHt3 und dessen Enzyme ErgA und ErgB, welche dazu befähigt sind, Ergopeptine abzubauen, wurden von BIOMIN Holding GmbH isoliert und identifiziert. Im Rahmen dieser Arbeit wurde die Strukturaufklärung und Charakterisierung von unbekannten Metaboliten durchgeführt, welche während des enzymatischen Abbaus von sechs verschiedenen Ergotalkaloiden durch MTHt3, dessen Lysat und das aufgereinigte Enzym ErgA gebildet wurden. LC-MS/MS-Messungen diverser Abbauversuchsproben wurden verwendet, um Informationen über Molekulargewicht und Fragmentierung neu gebildeter Metabolite zu erhalten. Darüber hinaus wurden präzise Massenmessungen, als auch ^{1}H-, ^{13}C- und 2D-NMR-Messungen (vom Institut für Angewandte Synthesechemie, TU Wien) von isolierten Metaboliten zur Strukturaufklärung durchgeführt. Als Ergebnis wurde die Bildung von zwei Hauptgruppen von neuen Metaboliten beobachtet. Während des ersten Abbauschrittes, welcher durch das Enzym ErgA katalysiert wird, werden stabile und vermutlich nicht-toxische Diketopiperazine, sowie Ergin-Hydroxycarbonsäuren (instabile Zwischenmetabolite, welche in Ergin und einer Oxocarbonsäure zerfallen) gebildet. In einem zweiten Abbauschritt mit ErgB wird Ergin zur Lysergsäure gespalten, welche ein wesentlich geringeres vasokonstriktives Potential aufweist. Zusammenfassend brachte die Strukturaufklärung der geformten Metabolite Erkenntnisse über den Mechanismus dieses enzymatischen Abbaus. Verschiedenste Futtermittelzusätze mit der Behauptung DON und/oder ZEN durch eine Kombination aus

Biotransformation und Adsorption *in vivo* zu entgiften sind im Handel erhältlich. Ein Ziel dieser Arbeit war die Evaluierung der Wirksamkeit von 20 verschiedenen Produkten *in vitro* durch aerobe und anaerobe Abbauversuche. LC-UV-MS/MS-Messungen wurden zur Quantifizierung von DON, ZEN und verwandten, bekannten Verbindungen durchgeführt, sowie zur Detektion möglicher UV-absorbierender, unbekannter Metabolite. Die Ergebnisse zeigten, dass 4 von 20 Produkten unter den verwendeten Bedingungen Potential zum Abbau aufweisen. Nur eines dieser Produkte konnte DON und ZEN vollständig zu weniger toxischen Metaboliten abbauen, während drei Produkte ausschließlich in der Lage waren, ZEN zu metabolisieren (zu östrogenerenm α-Zearalenol oder unbekannten Metaboliten). Dies zeigt, dass viele Produkte Mykotoxine nicht entgiften können und unterstreicht die Notwendigkeit von *in vitro*-Experimenten zur kritischen Untersuchung. Physikalische oder chemische Lebensmittelverarbeitungen von kontaminierten Waren können eine Mykotoxinreduktion bewirken, während Abbauprodukte gebildet werden können. Für Produkte, welche mit Fumonisin B_1 (FB_1) kontaminiert sind, wurde die Bildung von partiell hydrolysiertem FB_1 ($pHFB_1$), vollständig hydrolysiertem FB_1 (HFB_1) und *N*-(1-Deoxy-D-Fructos-1-yl) FB_1 (NDF) berichtet. $pHFB_1$ und HFB_1 werden hauptsächlich während der alkalischen Behandlung von Lebens- und Futtermitteln, als auch während der Anwendung eines Fumonisin-abbauenden Enzyms gebildet, während NDF als Hauptprodukt während der Wärmebehandlung und Extrusion von Lebensmitteln gebildet wird. Die Stabilität von HFB_1 und NDF unter Bedingungen, welche im menschlichen Magen-Darm-Trakt herrschen, wurde bewertet. HFB_1 wurde teilweise im Zuge der Inkubation mit menschlichen Fäkalien metabolisiert, während NDF teilweise bei Inkubation mit Verdauungssäften gespalten wurde. Weiters wurde die mögliche Metabolisierung und toxikologische Relevanz von $pHFB_1$, HFB_1 und NDF in Ratten, basierend auf der möglichen Beeinflussung des Sphingolipid-Metabolismus, evaluiert. Dazu wurden männliche Sprague-Dawley-Ratten für drei Wochen mit Diäten gefüttert, welche jeweils 13.9 µmol/kg hochreines FB_1, $pHFB_1$, HFB_1 und NDF enthielten. Durch die Analyse von Urin- und Nierenproben von allen Fütterungsgruppen wurde eine signifikante Erhöhung eines Fumonisin-spezifischen Biomarkers (Verhältnis von Sphinganin zu Sphingosin) ausschließlich in Proben der Gruppe, welche mit FB_1-kontaminierten Diäten gefüttert wurde, beobachtet. Im Gegensatz dazu konnte mithilfe der Messung der Fumonisin-Derivate in Kotproben ein partieller Abbau von NDF zu FB_1 gezeigt werden, was auf partielle *in vivo*-Reaktivierung im Magen-Darm-Trakt schließen lässt. Dennoch zeigten die getesteten Fumonisin-Derivate eine reduzierte toxikologische Relevanz im Vergleich zu FB_1, was auf eine potentielle Entgiftung während bestimmter Lebensmittelverarbeitungsprozesse oder eines enzymatischen Abbaus hindeutet.

Zusammenfassend wurden im Rahmen dieser Dissertation die Wirksamkeit unterschiedlicher Entgiftungsstrategien nach der Ernte für Ergotalkaloide, DON, ZEN und Fumonisine untersucht. Der gegenwärtige Wissensstand über vielversprechende Strategien konnte v.a. hinsichtlich ihrer Effizient, gebildeter Metabolite und deren Analytik erweitert werden und die Kenntnis über kommerzielle Produkte wurde erhöht.

List of abbreviations

AFB_1	aflatoxin B_1
AFM_1	aflatoxin M_1
AFs	aflatoxins
APCI	atmospheric pressure chemical ionisation
a_W	water activity
CDL	Christian Doppler Laboratory
D3G	deoxynivalenol-3-glucoside
DART	direct analysis in real time
DAS	diacetoxyscirpenol
DHZEN	decarboxylated hydrolysed zearalenone
DKPs	diketopiperazines (cyclic dipeptides)
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol
DONS	DON sulfonates
ECB	European corn borer
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
FBs	B series fumonisins
FHB	Fusarium head blight
GAP	good agricultural practices
GC	gas chromatography
HFB_1	fully hydrolysed FB_1
HPLC	high performance liquid chromatography
HR-MS	high resolution mass spectrometry
HSCAS	hydrated sodium calcium aluminosilicates
HT-2	HT-2 toxin
HZEN	hydrolysed zearalenone
IARC	International Agency for Research on Cancer
IR	infrared
IS	internal standard
IT	ion trap
LC	liquid chromatography
LFD	lateral flow device
LPCP	low pressure cold plasma
m/z	mass-to-charge ratio
MIPs	molecular imprinted polymers
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NDF	*N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1
NIR	near-infrared
NIV	nivalenol
NP	normal-phase
OTA	ochratoxin A
PAT	patulin
$pHFB_1a$, $pHFB_1b$	partially hydrolysed FB_1
Q	quadrupole
QqQ	triple quadrupole
QTL	quantitative trait locus
QTOF	quadrupole time-of-flight
QuEChERS	quick, easy, cheap, effective, rugged and safe
RP	reversed-phase
Sa	sphinganine
So	sphingosine
SPE	solid-phase extraction

SRM	selected reaction monitoring
T-2	T-2 toxin
TCA	tricarballylic acid
TLC	thin layer chromatography
TOF	time-of-flight
UHPLC	ultra-high performance liquid chromatography
ZALs	α- and β-zearalanol
ZEN	zearalenone
α-ZEL	alpha-zearalenol
β-ZEL	beta-zearalenol

Aims and structure of thesis

The presented thesis was conducted within the Christian Doppler Laboratory (CDL) for Mycotoxin Metabolism at the Center for Analytical Chemistry / IFA Tulln (Department for Agrobiotechnology of the University for Natural Resources and Life Sciences Vienna – BOKU). The aims of the CDL are to investigate the metabolism of mycotoxins by plants, microorganisms and animals. The main objectives of this doctoral thesis were related to the metabolism of certain mycotoxins by microorganisms and animals. In detail, in the course of the presented thesis different microbial interactions with mycotoxins for possible detoxification were evaluated as well as an animal experiment was accomplished to verify the effectiveness of detoxification strategies. For this purpose, analytical methods mainly based on liquid chromatography tandem mass spectrometry for the determination of the mycotoxins and their metabolites in different matrices (buffer solutions as well as biological matrices) have been developed, optimised and validated. In particular, four different mycotoxin detoxification strategies have been evaluated: 1) The determination and structure elucidation of unknown metabolites formed during microbial and enzymatic degradation of ergot alkaloids was performed in order to evaluate the potential of the tested enzymes as feed additives that conceivably achieve *in vivo* detoxification (publication #1). 2) The *in vitro* efficacy of different commercially available feed additives claiming detoxification of deoxynivalenol (DON) and zearalenone (ZEN) by metabolisation and additional adsorption was tested separately under aerobic and anaerobic conditions (publication #2). 3) The catabolic fate of fully hydrolysed fumonisin B_1 (HFB_1) (degradation product formed during alkaline treatment of food as well as during microbial degradation) and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF) (product formed by thermal food treatment and extrusion of fumonisin contaminated food or feed in the presence of glucose) compared to FB_1 was investigated and evaluated under human gastrointestinal conditions (publication #3). 4) The toxicological relevance of orally administered FB_1, $pHFB_1$, HFB_1 and NDF in rats was investigated (publication #4).

The presented thesis is written as a cumulative work and consists of three parts. In chapter 1 of the first part (introduction), an overview on mycotoxins (including their occurrence, toxicity and regulations) and their analysis is given. Furthermore, different counteracting strategies, divided into pre- and post-harvest methods, are extensively described and discussed. Chapter 2 focuses on the state-of-the-art in elimination strategies of ergot alkaloids, DON, ZEN and fumonisins and the contribution of the candidate to this topic is summarised. Part two (original works) consists of four publications that have been published in peer-reviewed SCI journals. Three of the publications were written as first-author, while one was published as co-author. For each publication, the contribution and position of the author of this thesis is briefly described. The highlights of the original works are summarised subsequently (highlights). The most relevant achievements of the publications are pointed out in the final part of this thesis (conclusion).

Introduction

1) Mycotoxins

1.1) Definition

Mycotoxins are a large and chemically diverse group of fungal secondary metabolites that have no considerable function for the growth of the fungi. They are produced by a large number of filamentous fungi (e.g. moulds of the genera *Aspergillus*, *Claviceps*, *Fusarium* and *Penicillium*) that parasitize different agricultural crops and plants. About 300 to 400 compounds of low molecular weight are recognized as mycotoxins that show different toxic effects in animals and humans (mycotoxicosis caused for example by acute toxicity, immunotoxic, mutagenic, carcinogenic, teratogenic or oestrogenic effects). The specific toxic effects depend on the age, health and sex of the exposed individual as well as on the mycotoxin type, dose and duration of exposure (Bennett and Klich 2003). Although fungal related epidemics were firstly mentioned around 600 BC (caused by ergot alkaloids) (van Dongen and de Groot 1995), the term mycotoxin as well as the investigation of the linkage between fungal contamination and the presence of toxic compounds in agricultural plants became established in the early 1960s. 100,000 turkeys died in the UK due to the turkey X disease that was traced back to peanut meal contaminated with *Aspergillus flavus* and aflatoxins (AFs) (Rustom 1997). The formation of mycotoxins can occur at different points in the food or feed chain (on the field as well as during harvest and storage) and poor agricultural practices as well as improper post-harvest conditions promote fungal growth and mycotoxin production. Moreover, food processing may chemically modify certain, generally very stable mycotoxins or result in an accumulation. The Food and Agriculture Organization of the United Nations assumed that more than 25% of the world's food and feed are significantly contaminated by mycotoxins (Smith et al. 1994). Recent studies (Streit et al. 2013) showed that this number might be even higher, which can be partly explained by advances in analytical detection capabilities. According to these figures, huge annual losses of several hundred million tons of food and feed as well as corresponding economic losses can be expected (CAST 2003). More than 100 countries worldwide have established specific mycotoxin regulations or detailed guidelines (including general food legislative framework, official controls, maximum levels, prevention and reduction) for many food and feed commodities as well as finished products (van Egmond et al. 2007).

1.2) Groups of mycotoxins

The most relevant groups of mycotoxins, the major compounds, their producing species as well as the affected commodities and toxic effects are summarised in Table 1.

Table 1: Main groups of mycotoxins, major compounds, producing fungi, affected commodities and toxic effects according to Bennett and Klich (2003), Richard (2007), Marin et al. (2013) and Edite Bezerra da Rocha et al. (2014)

Mycotoxin group	Major compounds	Producing species	Affected commodities	Toxicity and symptoms
aflatoxins	aflatoxin B_1, B_2, G_1, G_2	*Aspergillus* spp. (e.g. *A. flavus*, *A. parasiticus*)	maize, peanuts, spices, figs, tree nuts, cottonseeds	carcinogenic, genotoxic, hepatotoxic, immunosuppressive, mutagenic, teratogenic
alternaria toxins	alternariol, alternariol monomethyl ether, altertoxins	*Alternaria* spp. (e.g. *A. alternata*, *A. solani*, *A. tenuissima*)	sunflower seeds, fruits, vegetables	mutagenic, teratogenic
citrinin	citrinin	*Penicillium* spp. (*P. citrinum*, *P. expansum*, *P. viridicatum*, *P. camemberti*), *Aspergillus* spp. (*A. terreus*, *A. niveus*, *A. oryzae*)	wheat, oats, rye, maize, barley	nephrotoxic
ergot alkaloids	ergometrine, ergosine, ergotamine, ergovaline	*Claviceps* spp. (e.g. *C. purpurea*, *C. africana*, *C. fusiformis*), *Neotyphodium* spp.	grasses, rye, millet	gangrenous and convulsive ergotism
fumonisins	fumonisin B_1, B_2, B_3	*Fusarium* spp. (e.g. *F. verticillioides*, *F. proliferatum*), *A. niger*	maize, wheat, rice	possibly carcinogenic, hepatotoxic
ochratoxins	ochratoxin A	*Aspergillus* spp. (e.g. *A. ochraceus*, *A. niger*, *A. carbonarius*), *Penicillium* spp. (e.g. *P. verrucosum*, *P. nordicum*)	rye, barley, coffee, dried fruits, wine, spices, soy products	possibly carcinogenic, immunosuppressive, hepatotoxic, nephrotoxic, genotoxic, teratogenic
patulin	patulin	*Aspergillus* spp. (e.g. *A. clavatus*), *Penicillium* spp. (e.g. *P. expansum*)	apple products	gastrointestinal effects, neurotoxic, immunotoxic, mutagenic
type A trichothecenes	T-2 toxin, HT-2 toxin, diacetoxyscirpenol	*Fusarium* spp. (e.g. *F. sporotrichioides*, *F. langsethiae*, *F. acuminatum*, *F. poae*)	oats, barley, grasses, maize, rice, wheat	immunosuppressive, cytotoxic effects, diarrhoea, haemorrhage
type B trichothecenes	deoxynivalenol, nivalenol, 3- and 15-acetyl-deoxynivalenol	*Fusarium* spp. (e.g. *F. graminearum*, *F. cerealis*, *F. culmorum*,)	maize, barley, oats, wheat	feed refusal, immunosuppressive, vomiting
zearalenone	zearalenone	*Fusarium* spp. (e.g. *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. cerealis*, *F. verticillioides*)	barley, maize, sorghum, wheat	oestrogenic, genotoxic, immunotoxic,

The most important groups of mycotoxins depending on their occurrence as well as on their toxicity are AFs, B series fumonisins (FBs), ergot alkaloids, ochratoxin A (OTA), trichothecenes and zearalenone (ZEN) (CAST 2003). Existing EU legislation related to mycotoxin contamination includes regulations with maximum levels and recommendations with guidance levels for different mycotoxins in food and feed (reviewed by van Egmond et al. 2007 and Cheli et al. 2014). Factors that influence the setting of mycotoxin regulations depend on different scientific, economic and political parameters and are reviewed by van Egmond (2002). Legislation for different food categories, such as Commission Regulation (EC) No. 1881/2006 and amendments, set maximum levels for aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), the sum of AFB_1, AFB_2, AFG_1 and AFG_2, citrinin (in food supplements), deoxynivalenol (DON), the sum of the B series fumonisins FB_1 and FB_2, OTA, patulin (PAT), and ZEN. Indicative values for the type A trichothecenes T-2 toxin (T-2) and HT-2 toxin (HT-2) in cereals and cereal products were provided by Commission Recommendation 2013/165/EU. EC Directive 2002/32 (as amended) set maximum levels for AFB_1 and rye ergot in animal feed, whereas guidance values were defined for DON, the sum of FB_1 and FB_2, OTA, and ZEN (Commission Recommendation 2006/576/EC).

The occurrence of mycotoxins in food and/or feed on regional (Martins et al. 2008; Zinedine and Mañes 2009; Streit et al. 2012) and global level (Placinta et al. 1999; Rodrigues and Naehrer 2012; Schatzmayr and Streit 2013; Streit et al. 2013; Pereira et al. 2014) in different commodities (Scudamore and Livesey 1998) as well as their significance (Didwania and Joshi 2013) have been reported extensively. Nevertheless, the generated occurrence estimation always depends on the analysed samples as well as the mycotoxins covered with the different analytical methods. A good overview of the global occurrence of mycotoxins is given by Schatzmayr and Streit (2013), who summarised the results of an eight-year survey program, monitoring the presence of AFs, DON, FBs, OTA and ZEN in food and feed worldwide (worldwide distribution of the different mycotoxins is shown in Figure 1). Its main drawback is that mycotoxins like alternaria toxins, citrinin, ergot alkaloids, PAT, type A trichothecenes, and emerging mycotoxins were not included in the study.

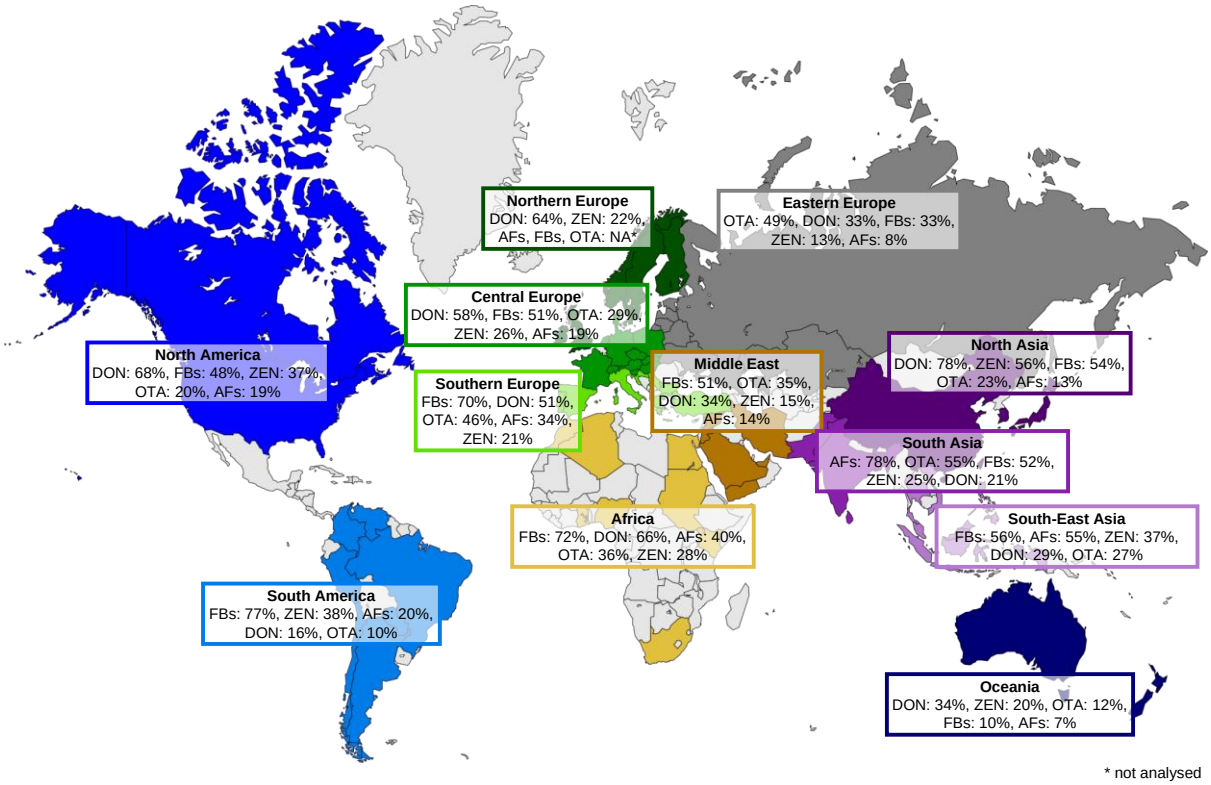


Figure 1: Prevalence of aflatoxins (AFs), deoxynivalenol (DON), B series fumonisins (FBs), ochratoxin A (OTA) and zearalenone (ZEN) in the surveyed regions (prepared after Schatzmayr and Streit 2013)

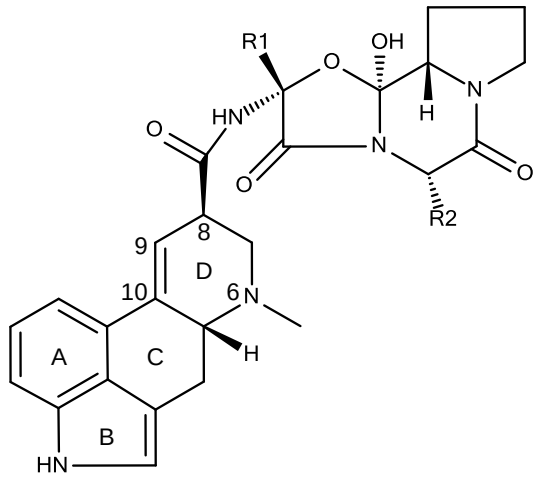
The study showed a higher prevalence of the AFs and fumonisins in the southern countries, whereas DON occurred more frequently in the north, which is in agreement of the growth conditions for the respective fungal species. In general, about 70% of the analysed samples contained detectable amounts of the monitored mycotoxin. In addition, co-occurrence of several mycotoxins was determined in one-third of the measured samples. However, in most of the cases the analysed mycotoxins concentrations were below the European guidance or maximum levels. Nevertheless, due to the risk of co-occurrence of different mycotoxins, synergistic and additive effects gain importance. Toxicological studies have mostly taken into account only one mycotoxin and data on combined toxicity are limited (reviewed by Speijers and Speijers 2004; Grenier and Oswald 2011).

Chapters 1.2.1 - 1.2.4 focus extensively on ergot alkaloids, DON, ZEN and fumonisins due to their importance for the individual publications presented in the part “Original Works”, whereas the other most relevant groups of mycotoxins will be shortly described in chapter 1.2.5.

1.2.1) Ergot alkaloids

Ergot alkaloids are mycotoxins produced by fungi of the genus *Claviceps*, *Neotyphodium*, *Aspergillus* and *Penicillium*. The common chemical structure of ergot alkaloids contains a tetracyclic ergoline ring system that is methylated at the D-ring on N-6 and substituted on the C atom at position 8 (see Figure 2). Depending on the type of substituent on C-8, the group of ergot alkaloids can be divided

into the four subgroups clavine alkaloids, simple lysergic acid derivatives, ergopeptines (with a C9=C10 double bond and a short peptide chain attached on the asymmetric C-8 at the tetracyclic ergoline ring system, Figure 2) and lactam ergot alkaloids (Flieger et al. 1997). Within this thesis (**publication #1**), enzymatic degradation reactions of ergopeptines were investigated.



Compound	R1	R2
ergotamine	CH_3	$CH_2C_6H_5$
ergovaline	CH_3	$CH(CH_3)_2$
ergosine	CH_3	$CH_2CH(CH_3)_2$
ergocristine	$CH(CH_3)_2$	$CH_2C_6H_5$
ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$
ergocryptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$

Figure 2: General structure of ergopeptines with the characteristic substituents R1 and R2 listed in tabular form

Two epimeric forms of ergot alkaloids due to epimerisation of the D-ring C-8 position (C-8-(R) or β and C-8-(S) or α configuration) result from a keto-enol tautomerism after acid attack at the amide oxygen (Lehner et al. 2005). The isomers showing C-8-(R) configuration are biologically active (-*ine* isomers, named ergopeptines like ergotamine, ergovaline and ergosine) and represent lysergic acid amides. The non-bioactive ergopeptinines (-*inine* isomers, e.g. ergotaminine, ergovalinine and ergosinine) showing a C-8-(S) configuration are related to isolysergic acid (Krska and Crews 2008). The epimerisation of ergopeptines to ergopeptinines depends on different parameters (such as pH value, temperature, light, used solvent) and has been reported to occur during storage and extraction of ergot alkaloids from samples (Krska et al. 2008b), during storage of standards in solution (Smith and Shappell 2002; Hafner et al. 2008) as well as during baking (Merkel et al. 2012).

The genus *Claviceps* is a group of approximately 36 different phytopathogenic ascomycete species that parasitizes rye, sorghum, millet, oats, barley, wheat, maize, forage grasses and rice (Schiff 2006). *C. purpurea* infections occur on the seed heads of plants at the time of flowering, which result in a replacement of the developing grain or seed with wintering bodies (named sclerotium, ergot, ergot body or 'mothercorn') (Krska and Crews 2008). Sclerotia are dark, crescent shaped ergot bodies that contain a varying profile of different ergot alkaloids, predominantly ergopeptines (mainly ergosine, ergocornine, ergotamine, α-ergocryptine, ergocristine, and ergovaline), simple lysergic acid derivatives (like ergometrine) and clavine alkaloids (e.g. chanoclavine and agroclavine). Sclerotia from sorghum infected with *C. africana* contained ergopeptines (like dihydroergosine) and clavines (Panaccione 2005). *C. fusiformis* exclusively produce clavine alkaloids (such as agroclavine and

elymoclavine) (Lorenz et al. 2007), while simple lysergic acid derivatives are formed by *C. paspali* (Tudzynski et al. 2001). In addition, several *Aspergillus* and *Penicillium* spp., like *A. fumigatus*, have been reported to produce ergot alkaloids (Flieger et al. 1997; Tudzynski et al. 2001; Panaccione 2005). *Neotyphodium* spp., which live as endophytic symbionts in grasses, were found to form clavines, ergopeptines (mainly ergovaline) and simple lysergic acid derivatives (like ergine) (Panaccione 2005). The ergot alkaloids ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine (plus their corresponding epimers) were defined as the six major ergot alkaloids (EFSA 2005).

Ergotoxicosis, caused by *C. purpurea*, is known in Europe for many centuries. The intoxication, also known as ergotism, manifests with gangrenous and convulsive symptoms. The medieval literature described the harmful effects of ergot-contaminated grains as Saint Anthony's, holy and infernal fire due to the pain and burning sensations in the limbs of people affected by gangrenous ergotism. At that time the ingestion of ergot alkaloid contaminated grain was prevalent due to the fact that rye was grown in larger quantities and many people consumed contaminated rye flour. In 1582, Adam Lonicer firstly described the therapeutic use of sclerotia to stimulate uterine contractions and midwives were already aware of the potential of 'mothercorn' to aid in childbirth or abortion (van Dongen and de Groot 1995). Ergot was subsequently identified as a fungus by von Munchhausen in 1764 (Schiff 2006). More recently, ergot alkaloids have been used therapeutically for example in the treatment of migraine headache, Parkinson's disease and schizophrenia due to their pharmacological properties (Tudzynski et al. 2001).

Nowadays, ergotism has almost been eliminated as a human disease in developed countries due to the development of mechanical grain cleaning and separation techniques (Bennett and Klich 2003; Lauber et al. 2005). However, several recent publications reported higher ergot alkaloid contaminations of rye and rye-based products in Europe as well as rather small harvested ergot bodies (Lauber et al. 2005; Reinhold and Reinhardt 2011; Malysheva et al. 2014). Tendencies of increased *C. purpurea* infections in the recent years (Scott 2009; Koleva et al. 2012) are seemingly associated with the common use of highly ergot susceptible rye hybrid varieties, which represent approximately two-third of the total rye cultivated for example in Germany (Miedaner et al. 2010). Furthermore, ergotoxicosis is an issue particular in animal nutrition for ruminants and other livestock due to contaminated forage grasses (Bennett and Klich 2003; Strickland et al. 2011). Recently, heavy infections of the common cord-grass *Spartina anglica* with *C. purpurea* var. *spartinae* and consequently high concentrations of ergocristine, α-ergocryptine and their epimers along the coastline of the Wadden Sea were reported (Boestfleisch et al. 2015).

The toxic effects of ergot alkaloids are mainly based on their interaction with neurotransmitter receptors (like the D_2 dopamine receptor), which includes binding to the receptor (Larson et al. 1995; Larson et al. 1999) as well as affecting the receptor density (Larson et al. 1994). LD_{50} values after oral administration range between 30 mg/kg body weight (for ergometrine in rabbit) and 3,200 mg/kg body

weight (for ergotamine in mouse), which indicates moderate acute toxicity. Acute exposure to sublethal amounts of ergot alkaloids result in neurotoxic effects, such as tremor, restlessness, muscular weakness, and rigidity (EFSA 2012). Recently, cytotoxic effects and induced apoptosis in different human cell lines have been proven (Mulac and Humpf 2011; Mulac et al. 2013). Two forms of ergotism have been described extensively: the gangrenous and the convulsive type. The gangrenous form causes peripheral vasoconstriction and affects the blood supply of the extremities, whereas the convulsive form results in hallucinations and generates delirium. Those variations in the symptoms may occur due to differences in ergot amounts and compositions in the sclerotia (Schardl et al. 2006). Reported pharmacologic effects on animals include increased body temperature as well as decreased calving and growth rate, body weight gain, reproductive efficiency, milk production, and prolactin secretion (reviewed by Porter and Thompson 1992).

At present, in the European Union ergot alkaloids in food are not regulated so far (EFSA 2012). Furthermore, maximum levels for ergot alkaloid contaminations in animal feed are based exclusively on the percentage of sclerotia in grain (0.1% in rye) (EC Directive 2002/32). Since the differences in size and weight of sclerotia lead to inaccurate physical determination of a contamination rate as well as concentrations show significant variations (EFSA 2005), new regulatory limits based on ergot alkaloid content are required and will probably be established in the next years (van Egmond et al. 2007). However, due to limited toxicological data of individual and combined ergot alkaloid contaminations, guidance values in the EU have only been discussed (EFSA 2005). Limits for ergot alkaloids have been established exclusively for animal feed in Canada (3,000 - 9,000 µg/kg depending on the animal species) and Uruguay (450 µg/kg) (EFSA 2012).

1.2.2) Deoxynivalenol

The phytotoxic mycotoxin DON (Figure 3, A), also known as vomitoxin, belongs to the group of type B trichothecenes, which are generally described in chapter 1.2.5. DON occurs in gains such as maize, wheat, barley, rye, and oats, while other cereals (e.g. sorghum and triticale) are less infected (Döll and Dänicke 2011). The main producers are the *Fusarium* species *F. graminearum* and *F. culmorum*, which occur mainly on the field and are plant pathogens of particular concern in temperate regions, causing Fusarium head blight (FHB) in wheat (reviewed by Creppy 2002). This destructive disease causes necrotic lesions and bleaching of plant tissues (Goswami and Kistler 2004). Further, DON acts as a virulence factor and is responsible for the development of characteristic symptoms after initial infection (reviewed by Audenaert et al. 2014). In general, DON is a prevalent mycotoxin in temperate regions (Döll and Dänicke 2011; Schatzmayr and Streit 2013; Streit et al. 2013) and the most frequently occurring Fusarium mycotoxin in Europe (SCOOP 2003). Chronic exposure might occur in many parts of the world (Turner 2010), while co-occurrences with other trichothecenes and zearalenone are common (Placinta et al. 1999; Bertuzzi et al. 2014).

Numerous comprehensive reviews concerning various toxic effects of DON are available (e.g. Rotter et al. 1996; Pestka and Smolinski 2005; Pestka 2007; Pestka 2010a; Maresca 2013). The toxicity of DON is mainly based on an inhibition of protein synthesis by ribotoxic stress *in vitro* as well as *in vivo*. In addition, on a cellular level, DON causes oxidative stress (Mishra et al. 2014) and disrupts cell signalling, differentiation and proliferation, which induces apoptosis. Acute effects range from emesis, diarrhoea, abdominal distress and malaise to intestinal haemorrhage, necrosis (of bone marrow and lymphoid tissues) and lesions (in kidney and heart). Moreover, mouse LD_{50} values after oral administration range from 46 to 78 mg/kg body weight. Chronic DON exposure leads to growth retardation due to partial feed refusal, immunotoxic effects, anorexia and teratogenic effects, resulting in impaired reproduction and development. The immune system can be either suppressed or stimulated, depending on dose, exposure time as well as frequency and type of assay used for evaluation of these effects. **Publication #2** in this thesis covers the evaluation of one applicable DON degradation technique.

Plants have the ability to metabolise and detoxify DON to its conjugated forms by enzymatic reactions. DON conjugation products of plants refer to the group of biologically modified mycotoxins (Rychlik et al. 2014), which are also well known as masked mycotoxins (reviewed by Berthiller et al. 2007; 2013). Due to changed chemical structures such derivatives mostly remain undetected by conventional analytical methods other than mass spectrometric ones. Reactions of phase II metabolism in plants, which are characterised by a conjugation of polar substances like glucose to the parent mycotoxin, result in the formation of deoxynivalenol-3-glucoside (D3G). The natural occurrence and toxicity of D3G as well as the potential release of the precursor substance (DON) by mammalian digestion have recently been studied. D3G is the main and most prevalent conjugation product of DON in cereal gains (reviewed by Berthiller et al. 2013) and can further be found in cereal based products like beer (Varga et al. 2013), bread and cornflakes (De Boevre et al. 2012). Concerning the thermostability of D3G, a significant decrease in D3G concentrations and corresponding increase in DON contents upon bread baking from contaminated flour was demonstrated (De Angelis et al. 2013). However, factors such as food matrix, processing conditions and type of contamination (naturally or artificially) may influence this circumstance. Furthermore, Kostelanska et al. (2011) reported D3G degradation under heat processing conditions, and five different degradation products with the chemical structures were proposed. Whilst D3G showed reduced *in vitro* inhibition of protein synthesis in wheat (Poppenberger et al. 2003), hydrolytic liberation of DON from D3G by certain gut bacteria was reported (Berthiller et al. 2011). *In vivo* studies on this issue reported lower oral bioavailability and toxicological relevance of D3G compared to DON in rats (Nagl et al. 2012) and pigs (Nagl et al. 2014). Hydrolysis of D3G to DON and further metabolisation during digestion to deepoxy-deoxynivalenol (DOM-1, Figure 3, B) was demonstrated in rats. The epoxide group of DON is mainly responsible for its toxicity and therefore the opening of the epoxide ring results in the formation of the less toxic compound DOM-1 (Sundstøl Eriksen et al. 2004; Dänicke et al. 2010),

which was found to be a degradation product of DON by rumen bacteria (King et al. 1984). Recently, the formation of sulphate conjugates of DON in wheat was reported and their reduced ability to inhibit protein synthesis was demonstrated *in vitro* (Warth et al. 2015).

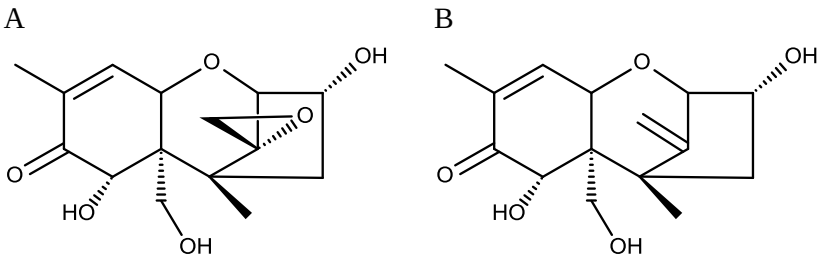


Figure 3: Chemical structure of (A) deoxynivalenol (DON) and (B) deepoxy-deoxynivalenol (DOM-1)

Maximum levels in the European Union for DON in cereals (wheat, barley, rye, oat, maize and products thereof) as food for human consumption range between 200 µg/kg (baby food) and 750 µg/kg, whereas maximum levels for unprocessed food and food not for direct consumption are defined between 1,250 and 1,750 µg/kg (Commission Regulation (EC) No. 1881/2006 and amendments). Guidance values for animal feed (feed and compound feed) are set between 900 µg/kg (complementary and complete feeding stuffs for pigs) and 12,000 µg/kg (maize by-products) (Commission Recommendation 2006/576/EC).

1.2.3) Zearalenone

ZEN (Figure 4, A) is a resorcyclic acid lactone and a common contaminant in cereal crops, which is produced by a variety of *Fusarium* spp. (e.g. *F. graminearum*, *F. culmorum*, *F. cerealis*, and *F. equiseti*). The worldwide occurrence of ZEN is mainly based on the high prevalence of *Fusarium* spp. and the co-occurrence of ZEN with other Fusarium mycotoxins (like trichothecenes and fumonisins) and is an important factor regarding synergistic effects in animals (reviewed by Placinta et al. 1999 and Zinedine et al. 2007). ZEN is very heat stable, whereby a removal and decomposition by thermal treatment can hardly be achieved (reviewed by Gromadzka et al. 2008; Maragos 2010). In addition, ZEN is reported to be stable during storage, milling, processing and cooking of food (Milani and Maleki 2014).

The ZEN related epimeric compounds alpha-zearalenol (α-ZEL) and beta-zearalenol (β-ZEL) (Figure 4, B and C) are formed by fungi (Richardson et al. 1985), by infected plants due to phase I metabolism (Berthiller et al. 2006) as well as during biotransformation in animals and can also be found in naturally contaminated cereals (reviewed by Zinedine et al. 2007).

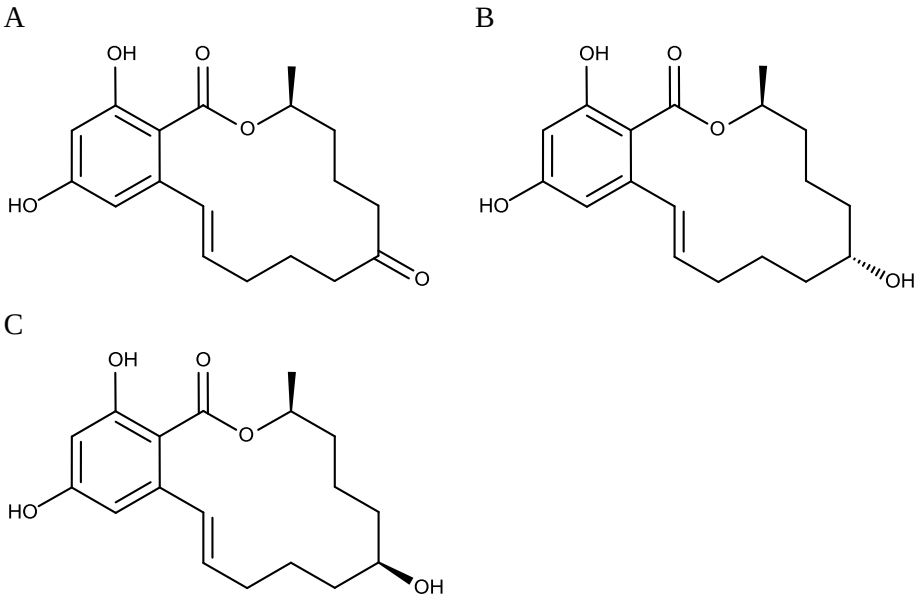


Figure 4: Chemical structure of (A) zearalenone (ZEN), (B) alpha-zearalenol (α-ZEL) and (C) beta-zearalenol (β-ZEL)

ZEN and its derivatives α- and β-ZEL are oestrogenic compounds that act as endocrine disruptors (Metzler et al. 2010) and cause reproductive disorders in farm animals (Bottalico 1998). Their effect is based on the chemical structure similar to naturally occurring oestrogens, resulting in a competitive binding to oestrogen receptors. In general, ZEN is rapidly absorbed after oral administration and metabolisation in intestinal cells to α- and β-ZEL as well as α- and β-zearalanol (ZALs) with a subsequent conjugation to glucuronic acid has been reported (reviewed by Gromadzka et al. 2008). LD_{50} values of ZEN after oral administration in mice range from 2,000 to 20,000 mg/kg body weight, which indicates low acute toxicity. In general, pigs and sheep appear to be more sensitive than rodents. Different chronic effects of ZEN, including liver lesions, haematotoxic, immunotoxic and genotoxic properties, stimulated growth of human breast cancer cells as well as reproductive and development toxicity have been reported (reviewed by D'Mello et al. 1999; Fink-Gremmels and Malekinejad 2007; Zinedine et al. 2007). In this thesis (**publication #2**), the efficacy of one counteracting techniques for ZEN was evaluated.

The natural occurrence of the plant conjugation product zearalenone-14-β-D-glucopyranoside in wheat was proven (Schneweis et al. 2002), whereas 17 different metabolites of ZEN (including most prominently glucosides, malonylglucosides, di-hexose- and hexose-pentose disaccharides of zearalenone, as well as α- and β-ZEL) formed during phase I and phase II metabolism in the model plant *Arabidopsis thaliana* were detected (Berthiller et al. 2006).

Maximum levels in the European Union for ZEN in cereals (wheat, barley, rye, oat, maize and products thereof) and maize oil as food for human consumption range between 20 µg/kg (baby food) and 400 µg/kg, whereas maximum levels for unprocessed food and food not for direct consumption are defined between 100 and 300 µg/kg (Commission Regulation (EC) No. 1881/2006 and

amendments). Guidance values for animal feed (feed and compound feed) range between 100 µg/kg (complementary and complete feeding stuffs for piglets and gilts) and 3,000 µg/kg (maize by-products) (Commission Recommendation 2006/576/EC).

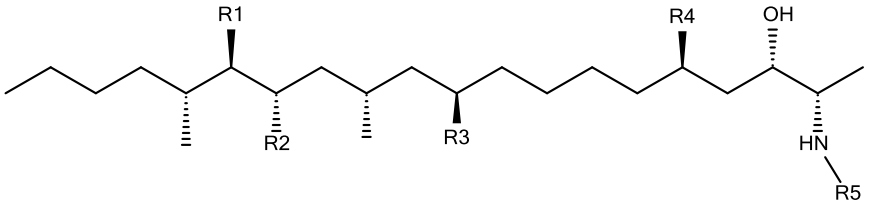
1.2.4) Fumonisins

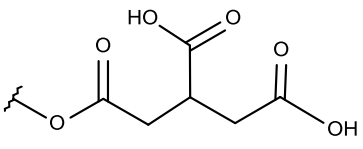
Fumonisins were firstly described in 1988 (Bezuidenhout et al. 1988; Gelderblom et al. 1988) and are polar compounds that are soluble in water as well as in aqueous solutions of acetonitrile and methanol (Waskiewicz et al. 2012). Fumonisins are mostly produced by the *Fusarium* spp. *F. proliferatum*, *F. verticillioides* (formerly *F. moniliforme*) and *F. nygamai*. In addition, the production of fumonisins by *Aspergillus niger* was reported (Frisvad et al. 2007). Fumonisins occur worldwide, predominantly in maize (and maize-based products), which is the most widely cultivated cereal in the world ever since 2001 (FAO 2015). Besides the B series fumonisins (FB_1, FB_2 and FB_3), which are the most prevalent ones, derivatives divided into the A, C and P series have been discovered and isolated so far (Musser and Plattner 1997; Seo and Lee 1999). FB_1 consists of a 20-carbon aminopentol chain with two ester-linked tricarballylic acids (TCAs) (see Figure 5). Various reviews summarise the global occurrence and daily intake of fumonisins (Shephard et al. 1996; Placinta et al. 1999; Soriano and Dragacci 2004a; b). FB_1, FB_2 and FB_3 are reported to occur in naturally contaminated maize grains at a ratio of approximately 12:4:1 (Waskiewicz et al. 2012).

In general, fumonisins are classified in Group 2B (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (IARC 1993). FB_1 is the most thoroughly studied fumonisin and its consumption is associated with animal disease such as equine leukoencephalomalacia (effects on heart, central nervous system, liver and brain in horses) and porcine pulmonary edema (respiratory distress and cyanosis in pigs followed by death due to acute pulmonary edema and hydrothorax). Although poorly absorbed from the gastrointestinal tract, FB_1 is toxic to the cardiovascular system in pigs and horses, nephrotoxic to a wide range of animal species and hepatotoxic to all tested animal species (reviewed by Voss et al. 2007). The toxicity of fumonisins is mainly based on the inhibition of the enzyme ceramide synthase (Norred et al. 1992; Merrill et al. 1993; Riley et al. 1996), which is essential for the biosynthesis of ceramides and catalyses the acylation of the sphingoid base sphinganine (Sa) as well as the re-acylation of sphingosine (So) (Shephard et al. 2007). Ceramides are bioactive lipids that play a key role in the modulation of membrane physical properties and have been identified as essential signal molecules mediating cellular pathways including regulation of cell differentiation, proliferation, adhesion and apoptosis (reviewed by Zheng et al. 2006 and Morales et al. 2007). The inhibition of the ceramide synthase due to the structural similarity of fumonisins to sphinganine and sphingosine leads to a disruption of the sphingolipid metabolism resulting in an elevation in intracellular Sa levels and in the ratio of Sa to So (Desai et al. 2002). This promoting Sa accumulation has repeatedly been reported to correlate with the onset of apoptosis (reviewed by Voss et al. 2007). The elevation of the free Sa/So ratio in biological

matrices (e.g. tissues, urine, and blood) due to the consumption of fumonisin contaminated feed was suggested as a useful biomarker in animal studies (reviewed by Shephard et al. 2007).

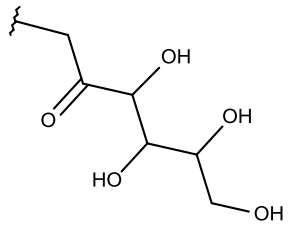
In general, food processing can chemically alter fumonisins resulting in derivatisation or cleavage of those precursors (reviewed by Humpf and Voss 2004; Bullerman and Bianchini 2007; Shephard et al. 2013b). For example, alkaline treatment, like nixtamalisation of maize, which is used for the production of tortillas, tortilla chips, tamales, hominy and others, leads to a hydrolysis of FB_1 to partially and fully hydrolysed FB_1 by the gradual cleavage of the tricarballylic acid moieties at the C-14 and C-15 position ($pHFB_1a$ or $pHFB_1b$ and HFB_1, see Figure 5) (Hendrich et al. 1993; Voss et al. 1996). Moreover, thermal food treatment and extrusion of fumonisin contaminated food or feed may result in the formation of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF) (see Figure 5) in the presence of reducing sugars (Poling et al. 2002; Jackson et al. 2012). Within this thesis, the gastrointestinal stability of HFB_1 and NDF (**publication #3**) as well as the effects of orally administered FB_1, $pHFB_1$, HFB_1 and NDF in rats (**publication #4**) was investigated.





tricarballylic acid (TCA)

Compound	R1	R2	R3	R4	R5
FB_1	TCA	TCA	OH	OH	H
FB_2	TCA	TCA	H	OH	H
FB_3	TCA	TCA	OH	H	H
$pHFB_1a$	TCA	OH	OH	OH	H
$pHFB_1b$	OH	TCA	OH	OH	H
HFB_1	OH	OH	OH	OH	H
NDF	TCA	TCA	OH	OH	deoxy-D-fructos-1-yl



deoxy-D-fructos-1-yl

Figure 5: Chemical structure of fumonisin B_1 (FB_1), FB_2 and FB_3 as well as partially hydrolysed FB_1 ($pHFB_1a$, $pHFB_1b$), hydrolysed FB_1 (HFB_1) and *N*-(1-deoxy-D-fructos-1-yl) FB_1 (NDF) with the characteristic substituents R1 - R5 listed in tabular form

Besides the free forms of fumonisins and derivatives, masked fumonisins like covalently (bound fumonisins) and non-covalently bound forms (hidden fumonisins) can occur and may be generated during extrusion of contaminated maize products (reviewed by Berthiller et al. 2013). Alkaline

hydrolysis is required for analysis and hidden fumonisins seem to be responsible for low comparability of quantifications results determined by different laboratories (Dall'Asta et al. 2009). Whilst the occurrence of bound fumonisins has been attributed to the covalent bond formation between fumonisins and matrix compounds such as carbohydrates and proteins during food processing (Seefelder et al. 2003), a high prevalence of hidden fumonisins in maize (Oliveira et al. 2015; Szabó-Fodor et al. 2015), maize silage (Latorre et al. 2015), maize-based products (Falavigna et al. 2012) and corn flakes (Kim et al. 2003) was reported.

In vivo, minor conversion of FB_1 to $pHFB_1$ and HFB_1 by hydrolytic action most likely realised by gut microbiota during gastro-intestinal metabolisation has been reported (Shephard et al. 1994a; b; Shephard et al. 1995; Fodor et al. 2007; Fodor et al. 2008), although no major metabolisation reactions of FB_1 were detected. In addition, partial *in vivo* acetylation of FB_1 (Harrer et al. 2013; Harrer et al. 2015) and HFB_1 (Seiferlein et al. 2007) resulting in the formation of cytotoxic *N*-acyl-metabolites as well as the conjugation to sulphated HFB_1 (Hopmans et al. 1997) was demonstrated.

Commission Regulation (EC) No. 1881/2006 and amendments set maximum levels in the European Union for the sum of fumonisins FB_1 and FB_2 in maize as food for human consumption between 200 µg/kg (baby food) and 1,000 µg/kg, whereas maximum levels for unprocessed food and food not for direct consumption are defined between 1,400 and 4,000 µg/kg. Guidance values for the sum of FB_1 and FB_2 were defined between 5,000 µg/kg (for pigs, horses, rabbits and pet animals) and 50,000 µg/kg (for adult ruminants and minks) in animal feed (feed and compound feed) (Commission Recommendation 2006/576/EC).

1.2.5) Other groups of mycotoxins

Aflatoxins, including AFB_1, AFB_2, AFG_1 and AFG_2, are difuranocoumarins produced by *Aspergillus* species that are mainly found in hot and humid climates. AFB_1 is one of the most potent natural carcinogens and AFs are classified in Group 1 (carcinogenic to humans) by the IARC (IARC 1993). Besides frequent chronic effects caused by extended exposure to small AFs amounts, acute poisoning due to highly contaminated food or feed results for example in abdominal pain, vomiting, pulmonary or cerebral oedema, necrosis, and death (Marin et al. 2013). The toxicity of AFs is based on its conversion by cytochrome P450 enzymes to a reactive 8,9-epoxide form, which is capable of binding to both DNA and proteins (Bennett and Klich 2003). AFM_1, a hydroxylated metabolisation product of AFB_1, is classified in Group 2B (possibly carcinogenic to humans) by the IARC (IARC 1993) and can be found in mammals tissues and fluids (milk and urine) due to AFB_1-contaminated food or feed and further metabolisation in the liver (Richard 2007). AFs are quite stable and therefore might be a problem in processed foods, like in roasted nuts (Marin et al. 2013).

More than 30 potentially toxic compounds from different *Alternaria* species have been isolated and the most important Alternaria toxins belong to the three structural classes dibenzopyrone (alternariol,

alternariol monomethyl ether, altenuene), perylene (altertoxins I, II, III) and tetramic acid derivatives (tenuazonic acid). AAL-toxins produced by *A. alternata* f.sp. *lycopersici* are structurally and toxicologically related to fumonisins (reviewed by Abbas et al. 1996), another major group of mycotoxins, which were described in chapter 1.2.4. The acute toxicity of Alternaria toxins are low, whereas teratogenic, foetotoxic, mutagenic and carcinogenic effects as well as liver and kidney damages have been described (reviewed by Ostry 2008).

A variety of *Penicillium* and *Aspergillus* spp., including species which are used in cheese (*P. camemberti*) and Asiatic food production (*A. oryzae*), as well as the fungi *Monoascus ruber* and *Monoascus purpureus* (used industrially in red pigment production), have the ability to produce citrinin. Citrinin was firstly isolated from *P. citrinum* and was associated with a yellow rice disease in Japan in 1971 (reviewed by Edite Bezerra da Rocha et al. 2014). Citrinin is nephrotoxic and has the ability to depress RNA synthesis in murine kidneys due to synergistic effects with ochratoxin A, whereas its acute toxicity depends on the animal species (Bennett and Klich 2003).

OTA is the most widespread ochratoxin showing nephrotoxic, immunosuppressive and genotoxic as well as hepatotoxic effects. Its chemical structure contains an L-phenylalanine group bound to one isocoumarin substitute (Edite Bezerra da Rocha et al. 2014). OTA inhibits various phenylalanine-binding enzymes, is rated in Group 2B (possibly carcinogenic to humans) by the IARC (IARC 1993) and represent a chemically very stable compound (Marin et al. 2013). It is slowly eliminated from the body, which results in common OTA levels detectable in human serum (reviewed by Reddy and Bhoola 2010).

PAT is a toxic, water-soluble lactone that inhibits a variety of enzymes due to its strong affinity to sulfhydryl moieties (Marin et al. 2013). Acute effects range from epithelial cell degeneration, intestinal haemorrhage to pulmonary congestion, whereas the chronic toxicity is based on carcinogenic, immunotoxic, neurotoxic and genotoxic effects. Cellular level effects include inhibition of protein and DNA synthesis as well as disruption of plasma membrane, transcription and translation (reviewed by Moake et al. 2005).

In general, trichothecenes are a large group of mycotoxins produced by different fungi of the genus *Fusarium, Trichoderma, Stachybotrys, Trichothecium*, and others. Their toxicity is based on the ability to inhibit eukaryotic protein synthesis by different interferences like binding to the 60S ribosomal subunit (Marin et al. 2013) in the initiation, elongation and/or termination steps (Edite Bezerra da Rocha et al. 2014). They are common food and feed contaminants and their consumption results in vomiting and alimentary hemorrage, whereas direct contact causes dermatitis. The name trichothecene is based on trichothecin, which was one of the firstly isolated compounds of this group of mycotoxins (Bennett and Klich 2003). The group of trichothecenes contains more than 180 different compounds with a common tetracyclic sesquiterpenoid ring system and can be divided into four types (type A - D,

with A and B being the most prevalent) depending on the differences in the functional groups. Type A trichothecenes are mainly represented by T-2, HT-2 as well as diacetoxyscirpenol (DAS), while DON, nivalenol (NIV) and their acetylated precursors 3-, 15-acetyl-deoxynivalenol and fusarenon-X belong to type B trichothecenes (Pestka 2010b; Marin et al. 2013). In general, it has been shown, that type A trichothecenes are more toxic to mammals than those of type B based on their acute toxicity (Placinta et al. 1999). T-2, DAS as well as DON are the most extensively studied trichothecenes. The toxicity of T-2 and DAS is based on their cytotoxic activity as well as their immunosuppressive effects. Furthermore, T-2 and DAS appear to be responsible for a human disease called alimentary toxic aleukia with symptoms such as vomiting, hematopoietic tissues damages as well as skin inflammations (Bennett and Klich 2003). Moreover, T-2 inhibits DNA and RNA synthesis and can be metabolised to HT-2 *in vivo*, while the latter represent approximately two-third of the sum of T-2 and HT-2 in naturally contaminated commodities. Due to this *in vivo* metabolisation, the toxic effects of T-2 and HT-2 cannot be differentiated. In general, trichothecenes are quite stable during food processing and thermal treatment (Marin et al. 2013).

Fusaproliferin, beauvericin, enniatins, and moniliformin are Fusarium toxins that are considered as emerging mycotoxins with limited available occurrence data (reviewed by Jestoi 2008 and Marin et al. 2013). Due to a possible co-occurrence with other mycotoxins, synergistic effects may be expected. Fusaproliferin, a bicyclic sesterterpene with pathogenic and teratogenic effects is produced by *F. proliferatum*, *F. subglutinans*, *F. circinatum*, *F. antophilum*, and others. Its highest occurrence was repeatedly reported from the Mediterranean region (Santini et al. 2012). Beauvericin and enniatins (enniatine A, A1, B and B1) are cytotoxic compounds that are mainly produced by *F. avenaceum*, *F. tricinctum*, *F. sporotrichioides*, and *F. poae* (Marin et al. 2013). Moniliformin, which occurs as sodium or potassium salt of the semisquaric acid (Jestoi 2008) and inhibits several thiamine pyrophosphate dependent enzymes, can be produced by *F. proliferatum*, *F. verticillioides*, *F. tricinctum*, *F. avenaceum*, and *F. subglutinans* (Marin et al. 2013).

1.3) Determination of mycotoxins and biomarker methods

1.3.1) Determination of mycotoxins

Due to the global occurrence of mycotoxins and their potential threat to human and animal health, suitable analytical tools for qualitative and quantitative analysis of mycotoxins and their metabolites in different matrices (like food, feed and biological samples) are required for regulatory and scientific purposes. Mycotoxins are a structurally and chemically diverse group of compounds with a varying concentration range in various agricultural commodities. Hence, many different analytical methods have been developed and the method of choice has to be selected and evaluated carefully. In general, methods for the quantification of mycotoxins in cereals are comprised of a representative sampling, the optimised sample preparation and clean-up as well as the analytical technique including mainly separation and detection.

Usually, in agricultural crops mycotoxin contaminations are heterogeneously distributed and may be concentrated in 'hot-spots'. Therefore, sampling represents an important and crucial step and a representative sample is essential for the precision and accurate determination of mycotoxin levels. The issue of correct sampling, the design of sampling protocols and plans as well as suitable sampling equipment has been reported extensively (Campbell et al. 1986; Coker et al. 1995; Whitaker 2006; Maestroni and Cannavan 2011). Methods of sampling and analysis for the official control of the level of mycotoxins in foodstuff in the European Union are laid down in Commission Regulation (EC) No. 401/2006.

With the majority of analytical techniques a direct detection of mycotoxins in milled cereal samples is not achievable and therefore sample preparation procedures are required (Krska et al. 2008a). Sample preparation includes for example solvent extraction to transfer the target analytes from the solid matrix into a liquid phase, enrichment of the compound of interest, and clean-up strategies to reduce interfering matrix components. The choice of the adequate sample preparation depends on the analyte, the matrix as well as on the final separation and detection technique (Pereira et al. 2014). During conventional solid-liquid extraction procedures the mycotoxins are extracted from ground cereal samples by mechanical shaking with different mixtures of solvents, sometimes in addition with acidic or alkaline modifiers. Additionally, quicker and automated alternatives such as pressurized liquid or microwave-assisted extraction as well as improved extraction procedures (e.g. supercritical fluid extraction with CO_2) have been developed (reviewed by Krska et al. 2008a; Razzazi-Fazeli and Reiter 2011; Pereira et al. 2014).

Solid-phase extraction (SPE) methods with various SPE cartridges have been established for clean-up of raw extracts and simultaneous enrichment of the analyte. In general, SPE protocols consist of the application of the sample to the packed column with the retaining of the analyte on the column and the removal of matrix components during the sample loading and washing step. Subsequently, the analyte is eluted from the column using different solvents. The most common sorbent materials for SPE columns in the field of mycotoxin analysis range from reversed-phase materials (e.g. C_8 and C_{18}) to cation or anion exchangers and mixed-mode phases (reviewed by Turner et al. 2009). Fully automated online SPE methods have been applied recently (Campone et al. 2015; Drzymala et al. 2015). Moreover, clean-up protocols based on SPE suitable for multi-mycotoxin analysis in different matrices have been published (Njumbe Ediage et al. 2015). In addition, packing materials based on immunoaffinity and hydrophilic interaction chromatography can be employed (Krska et al. 2008a). Alternatively, mycotoxin specific molecular imprinted polymers (MIPs) and aptamers are also considered as potential and practicable SPE packing materials. The compound of interest is specifically bound by selected, polymerised monomers in case of MIPs (Pereira et al. 2014) and by short DNA and RNA strains in case of aptamers (Razzazi-Fazeli and Reiter 2011; Rouah-Martin et al. 2014). MycoSep® columns with a mixture of ion exchange resins, celite, charcoal and other materials

can be used for clean-up of mycotoxin containing samples (AFs, trichothecenes, ochratoxins, ZEN, moniliformin or PAT) due to the removal of matrix components by retaining on the column (Krska et al. 2008a). A fast and easy multiresidue extraction and clean-up method, called QuEChERS (abbreviation for Quick, Easy, Cheap, Effective, Rugged and Safe), have been repeatedly applied to the analysis of mycotoxins (reviewed by Pereira et al. 2014). QuEChERS methods consist of an extraction with aqueous acetonitrile (sometimes containing acids), a salting out step with sodium chloride and magnesium sulphate followed by the addition of a small amount of SPE sorbent material to the extract (named disperse solid-phase extraction) and was initially developed for pesticide analysis (Anastassiades et al. 2003). Recently, the dilute-and-shoot approach without any clean-up has often been used in combination with mass spectrometry (MS) for multi-mycotoxin analysis in food matrices (Malachova et al. 2014; Hickert et al. 2015) as well as for the analysis of multiple mycotoxin biomarkers in biological samples (Warth et al. 2012; Shephard et al. 2013a; Gerding et al. 2014).

The analytical techniques for qualitative and quantitative analysis of mycotoxins usually consist of a separation and detection system. Conventionally, separation strategies are based on chromatographic methods. The term chromatography was defined by the International Union on Pure and Applied Chemistry as "a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other moves in a definite direction (mobile phase)" with a gaseous (gas chromatography, GC) or a liquid (liquid chromatography, LC) mobile phase. Various interactions between the compounds of interest and the stationary phase result in different retention times of the analytes. Depending on the stationary phase, column chromatography is an option in which the stationary bed is within a tube (a solid or coated particles packed column as well as columns where the stationary phases are along the inside wall), whereas during planar chromatography the stationary phase is on a plate. Separation based on GC is usually achieved on a column, whereas LC can be performed on a column as well as planar (Ettre 1993).

Separation methods based on GC in combination with electron capture or MS detection were reported to be used for the analysis of mycotoxins, but less frequently compared to other techniques (reviewed for example by Krska et al. 2008a, Turner et al. 2009, Shephard 2011 and Pereira et al. 2014). Additional derivatisation reactions like silylation or polyfluoroacylation prior to GC separation and analysis are required due to the low volatility of most mycotoxins. The thermal instability of some mycotoxins and the subsequent degradation during heated injections represents another disadvantage of GC analysis (Turner et al. 2009).

Thin layer chromatography (TLC), a traditional and popular method based on planar LC, was widely used for the analysis of mycotoxins for many years and is still used in developing countries (reviewed by Turner et al. 2009 and Shephard 2011). The currently used LC techniques are based on high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography

(UHPLC), that have superseded techniques like TLC and are characterised by small particles as stationary phase in an analytical column (most commonly 5 µm for HPLC, less than 2.5 µm for UHPLC resulting in improved speed, resolution and sensitivity) and high back pressures of several hundred bar (Snyder et al. 2010; Shephard 2011; Pereira et al. 2014). Normal- and reversed-phase (NP and RP) columns, depending on the polarity of the toxins, are used for HPLC separation (Turner et al. 2009). While the stationary phase is more polar than the mobile phase during NP chromatography, RP chromatography is characterised by a more polar mobile phase compared to the stationary phase (Ettre 1993). Nowadays, mainly RP in combination with gradient elution is used, in which the percentage of organic solvent of the mobile phase is increased during the elution process. When target analytes in the samples are ionisable compounds (containing one or more acidic or basic functional groups) and have to be separated on a RP column, the pH value of the mobile phase is one of the most important parameters for retention control. Therefore, pH modifiers for the mobile phases are commonly used to ensure the presence of non-ionised compounds that are less polar and more strongly retained in RP. While acids are more retained at low pH values, bases are more retained at higher pH values. LC detectors for the continuous monitoring and detection of the analytes leaving the column are based on measurements of UV/Vis-absorbance (e.g. for DON, AFs, citrinin, PAT and moniliformin), refractive index, fluorescence (e.g. for OTA, ZEN, citrinin, PAT, ergot alkaloids and AFs), electrochemical parameters, light scattering properties (e.g. for fumonisins) and mass spectrometric information (reviewed by Krska et al. 2007 and Turner et al. 2009). An extensive review summarising conventional and advanced HPLC detectors was published by Kaushal et al. (2011).

HPLC separation coupled to MS detection has become a powerful and the state-of-the-art technique in the qualitative and quantitative analysis of mycotoxins due to crucial advantages over the last decade (reviewed extensively by Zöllner and Mayer-Helm 2006; Spanjer 2011; Pereira et al. 2014). These include the high sensitivity and selectivity, the application to multi-mycotoxin analysis and the additional information about mass-to-charge ratios (*m/z*) and fragment ions of the analytes). For a feasible combination of LC with MS, appropriate interfaces achieve the evaporation of the effluent delivered by a capillary from the HPLC system from the liquid to the gaseous phase and generate ionised molecules. Different types of ion sources have been developed, with the atmospheric pressure ionisation techniques, in particular the atmospheric pressure chemical ionisation (APCI) and the electrospray ionisation (ESI), as the most relevant ones for the analysis of mycotoxins (Krska et al. 2008a). While APCI is suitable for compounds with medium or low polarity, ESI can be used for polar or even ionised compounds. In addition, direct analysis in real time (DART) ionisation, another atmospheric pressure technique, was successfully applied for the analysis of different mycotoxins (Vaclavik et al. 2010; Busman et al. 2015). The used ionisation process in DART is based on excited-state helium atoms that produce reactive species for analyte ionisation. This technique facilitates the analysis under ambient conditions without a prior separation step (Maragos and Busman 2010). Recently, imaging techniques based on laser-ablation electrospray ionisation or silica plate imprinting

laser desorption/ionisation mass spectrometry have been applied for the simultaneous detection of various contaminants, including mycotoxins, on different food matrices (de Oliveira et al. 2014; Nielen and van Beek 2014). In general, the ionisation for subsequent MS detection can be achieved in the positive as well as negative ionisation mode, generating mainly protonated and deprotonated molecular ions ($[M+H]^+$, $[M–H]^–$) of the analysed mycotoxins. Type B trichothecenes are mainly analysed as acetate or formate adducts in ESI(–) by adding acetic or formic acid and their ammonium salts to the mobile phase due to low ionisation efficiencies of the protonated and deprotonated molecular ions (Pereira et al. 2014). Matrix components that are co-eluting in the LC system with the compounds of interest may influence the ionisation efficiencies of the analytes (named matrix effects), resulting in suppressed or enhanced signals. By using an adequate clean-up of the analysed sample or calibration methods (such as matrix matched calibration, standard addition or stable isotope labelled analogues of the target analytes as internal standards, IS), possible matrix effects can be omitted or compensated (Krska et al. 2008a). After ionisation, the compounds are separated in the mass analyser under high vacuum according to their *m/z* ratio. Finally, the separated ions are counted by a detector. Different designs of mass analysers, like quadrupole (Q), ion trap (IT), time-of-flight (TOF) and orbitrap, have been developed. Nowadays, the most commonly used mass spectrometers are combinations, which enable tandem MS (MS/MS) and consist of two or more mass analysers in a row (like a triple quadrupole (QqQ) mass spectrometer). Those designs allow serial mass spectrometric measurements and fragmentation of the analytes in a collision cell for the generation of fragment ions that are characteristic for the precursor ion. This results in increased signal-to-noise ratios. In the field of quantitative mycotoxin analysis triple quadrupole (QqQ) mass spectrometers or a hybrid of quadrupoles with a linear IT (QTrap instruments) are often used (with MS/MS in the selected reaction monitoring (SRM) mode) (Krska et al. 2007; Pereira et al. 2014), while high resolution MS (HR-MS) techniques with a high mass accuracy, such as time-of-flight (TOF, QTOF) and orbitrap are mainly used in the area of screening for unknown compounds (metabolomics and metabolite identification) (Ates et al. 2014; Klitgaard et al. 2014). In this thesis, two generations of QTrap mass spectrometers (QTrap and 4000 QTrap, AB Sciex) (**publication #1, #2, #3** and **#4**) and a QTOF instrument (6550 iFunnel QTOF, Agilent Technologies) (**publication #1**) were used in different measurement modes.

Capillary electrophoresis, another chromatographic separation technique based on the charge and mass dependent migration in an electric field, has been used as well for several mycotoxins (Wilkes and Sutherland 1998; Pena et al. 2002; Shephard 2011), but its application is rather rare.

Screening methods based on rapid test systems commonly provide qualitative results (presence or absence for single mycotoxins or whole mycotoxin groups), while screening assays for semi-quantitative and quantitative analysis have recently been developed. Screening techniques that can mainly be applied without any clean-up include immunochemical assays (e.g. enzyme-linked immunosorbent assays (ELISA), lateral flow devices (LFD), dip-stick tests and immunosensors),

biosensors, micro-arrays and non-destructive, optical measurements (mainly infrared, IR). Immunochemical methods are tests using the interaction between the target analyte (antigen) and a selected, specific antibody. Usually, the non-covalent antibody-antigen binding is detected with a colour reaction. Concerning the design of ELISA tests, direct and indirect formats can be distinguished. A direct ELISA refers to the use of an enzyme-labelled primary antibody that is binding to the epitope of the compound of interest. During an indirect ELISA the analyte is bound by a primary antibody, which is detected by an enzyme-labelled secondary antibody. A substrate of this enzyme is added subsequently to achieve a measurable colour reaction. The terms "direct ELISA" and "indirect ELISA" are used in case of the adherence of the antigen, which is the compound interest, on the plastic of the microtiter plates. Sandwich ELISA tests, also feasible in direct and indirect formats, use a capture antibody is immobilised on the surface of the microtiter plates. A typical sandwich ELISA is more precisely a non-competitive ELISA test, where the signal is direct proportional to the analyte concentration. In contrast, during a competitive ELISA test, that has been developed for small analytes with only one epitope, a competition between a marked tracer and the analyte results in signals that are indirect proportional to the analyte concentration. At present, commercial ELISA test kits are available for most of the major mycotoxins with 96-well microtiter plates and are the most commonly used immunochemical assays (reviewed by Goryacheva et al. 2007; Krska et al. 2007; Goryacheva and De Saeger 2011; Pereira et al. 2014). Incubation times between 0.5 and 2 h are required and the intensities of the colour reaction are measured subsequently with a photometer. The advantages are the easy, fast and cheap use as well as the portable and simple equipment, while the main disadvantages are the single use and the possible cross-reactivity of the antibody with compounds that are structurally related to the target analyte. Turner et al. (2009) summarised common ELISA protocols for the determination of mycotoxins. Alternatively, LFDs and dip-tick tests are membrane-based immunoassay methods that use antibodies immobilised on a test strips. The technique of lateral-flow immunoassays for the detection of mycotoxins was reviewed by Anfossi et al. (2013). Immunosensors for the determination of mycotoxins (reviewed by Goryacheva et al. 2007 and Pereira et al. 2014) consist of an antibody specific for the target analyte, an electrochemical, optical or piezoelectric transducer and a processing system (Krska et al. 2007). The development and application of micro-arrays, biosensors and other emerging techniques (e.g. MIPs) have been promoted in the last 10 years and are reviewed extensively (Maragos 2004; Logrieco et al. 2005; Tothill 2011). One promising, newly developed method for the detection of mycotoxins is based on the use of aptamers (for example in biosensors or dipstick assays). Especially for the detection of AFB_1 (Shim et al. 2014; Castillo et al. 2015), OTA (Rhouati et al. 2013; Zhu et al. 2015) and fumonisins (Yue et al. 2014), several reports regarding the use and development of aptamers have recently been published. In an excellent review, the current state of electrochemical biosensors for the determination of mycotoxins is summarised (Vidal et al. 2013). Non-destructive, optical methods, mainly based on IR spectroscopic techniques, like Fourier Transform mid-infrared spectroscopy with

attenuated total reflection or near-infrared (NIR) transmittance spectroscopy, represent promising and fast screening methods that can be applied for the analysis of mycotoxins (Krska et al. 2008; Pereira et al. 2014; Wang et al. 2014). Data interpretation and extraction of relevant spectral information has to be achieved with chemometric analysis, such as a principal component analysis or the development of calibration models like neural networks.

Independent of the chosen separation and detection system, the validation of the used method for the target analytes in the measured matrices is a crucial and essential step. This confirmation is required to provide reliable and comparable data (Krska et al. 2008a; Eurachem 2014). The issue of quality assurance includes sampling, validation of the analytical methods, proficiency testing as well as certified reference materials and has been reviewed extensively (van Egmond 1995; Brera and Miraglia 1996; FAO/WHO/UNEP 1999a; Krska et al. 2005). A method validation typically covers the determination of apparent recoveries, matrix effects, linearity, repeatability as well as limits of detection and quantification.

1.3.2) Biomarker methods

In the field of mycotoxin research, biomarkers are biochemical or molecular indicators that are measured in biological samples (easily-accessed biological fluids, tissues or faeces) and result from the exposure or biological response to a mycotoxin. Biomarkers of exposure are most commonly the parent mycotoxins or metabolisation products, while biomarkers of effect are mechanism-based biomarkers (Groopman and Kensler 1999; Baldwin et al. 2011). In general, the determination of mycotoxin biomarkers is useful for exposure assessment and related fields as well as in animal studies for a better understanding of mycotoxin-related diseases and the responsible biological effects. Exposure and risk assessment as well as exposure analysis in the field of mycotoxin research are efforts that investigate the source and effects of contaminants in biological systems, evaluate the risk and estimate or measure the dose, frequency and duration of a possible exposure. In addition, the efficacy of decontamination approaches to reduce toxicity *in vivo* can be evaluated by applying the analysis of toxin-specific biomarkers. The application of biomarker analysis in mycotoxin research has been reviewed extensively. In particular, the status quo of developed, validated and applied biomarker methods in humans and animals were reviewed by Riley et al. (2011) as well as Routledge and Gong (2011). In addition, exposure assessments for mycotoxins based on biomarker analysis and the differences in the supplied information compared to traditional food analysis (Miraglia et al. 1996; Turner et al. 2012) as well as analytical challenges regarding biomarker analysis in urine (Warth et al. 2013) were discussed.

An excellent summary on the status of biomarker development as well as on available biomarkers in animals and humans was published by Baldwin et al. (2011). AFB_1 biomarkers are based on the toxic effects of the parent mycotoxin and are the most extensively studied and validated biomarkers in the

field of mycotoxin research. Table 2 displays some relevant biomarkers of exposure and effect in animals for DON, FB_1 and ZEN, which represent mycotoxins primarily important for this thesis.

Table 2: Biomarkers of exposure and effect in animals for DON, FB_1 and ZEN (modified after Baldwin et al. 2011)

Mycotoxin	Biomarker	Type	Biological matrix
DON	DON, DON-glucuronide	biomarker of exposure	urine, tissue, faeces
	proinflammatory cytokines	biomarker of effect	blood, tissue
FB_1	levels of FB_1	biomarker of exposure	faeces, urine
	elevation of intracellular Sa levels and the Sa to So ratio	biomarker of effect	blood, tissue
ZEN	levels of ZEN, ZELs and ZALs	biomarker of exposure	urine, faeces
	glucuronic acid-conjugates	biomarker of exposure	urine, faeces
	endocrine disruption	biomarker of effect	tissue

Within this thesis (**publication #4**), the analysis of fumonisin biomarkers of exposure and effect were applied to samples collected in the course of a rat feeding experiment in order to evaluate sphingolipid metabolism related toxicity and possible gastro-intestinal metabolisation of different fumonisin analogues. Interested readers are referred to excellent reviews on fumonisin biomarkers, e.g. Turner et al. (1999), Shephard et al. (2007) and van der Westhuizen et al. (2013).

1.4) Counteracting mycotoxins

Fungal growth as well as mycotoxin production can occur on the living plants in the field (pre-harvest; field fungi) as well as on the agricultural crops during storage (post-harvest; storage fungi). *Aspergillus* species parasitize commodities both pre- and postharvest, whereas *Fusarium* spp. occur more likely pre-harvest and *Penicillium* spp. are frequent post-harvest contaminants (Bryden 2009). Consequently, methods for controlling and counteracting mycotoxins focus on these aspects and can be divided into pre- and post-harvest techniques. While pre-harvest approaches most commonly focus on the prevention of mycotoxin production, post-harvest methods are mainly related to elimination and detoxification strategies. Detoxification of mycotoxins can only be achieved by chemical reactions transforming the mycotoxins into less toxic compounds. An overview of different methods for counteracting mycotoxins is given in Figure 6.

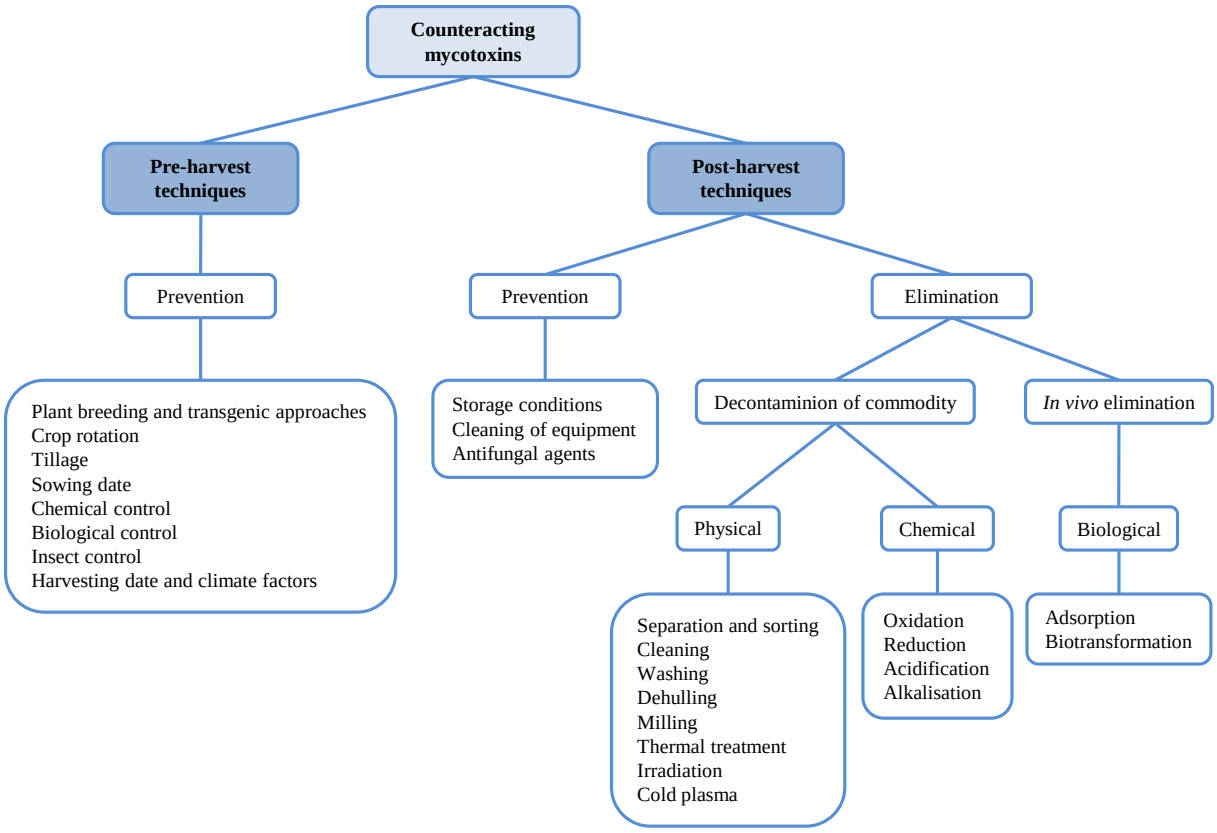


Figure 6: Different pre- and post-harvest methods for counteracting mycotoxins

Chapters 1.4.1 and 1.4.2 focus on pre- and post-harvest techniques in general, whereas chapter 2 ("Elimination strategies of selected mycotoxins") (2.1 - 2.4) extensively reviews post-harvest elimination strategies for ergot alkaloids, DON, ZEN and fumonisins.

1.4.1) Pre-harvest techniques

Pre-harvest techniques that are the most effective ones are those addressing prevention of mycotoxins on the field. Therefore, good agricultural practices (GAP) carried out prior to fungal infection and mycotoxin production on the field have been developed. Most of the published pre-harvest techniques were developed for counteracting Fusarium infection and contamination because *Fusarium* spp. mainly represent field fungi. Prevention of fungal and mycotoxin contamination in the crop requires integrated approaches, where different agricultural practices play various roles (reviewed by Edwards 2004). The methods include plant breeding, crop rotation, tillage, chemical control and others.

1.4.1.1) Plant breeding and transgenic approaches

Plant breeding can be used for susceptible crops to produce lines that provide good resistance to different fungal species. For instance, resistance to FHB caused by *Fusarium* spp. was developed and studied extensively (reviewed by Prat et al. 2014). Resistance to Fusarium disease is highly prioritised in almost all wheat growing regions. Hence, different research programs have been established to study FHB disease as well as the host plant responses in order to select and breed for Fusarium resistance. Willyerd et al. (2012) reported that planting resistant cultivars together with appropriate

fungicide application result in most stable FHB and DON control. The determination of the genetic characters of interest and the development of a selection tool that allows the breeder to distinguish between desired and non-desired genotypes, are essential conditions for plant breeding. Hence, quantitative train loci (QTLs) that are stretches of DNA containing or linked to genes that underlie a quantitative trait have been identified for Fusarium resistance in wheat (Buerstmayr et al. 2003; Snijders 2004). However, some of those QTLs are coincident with genes encoding morphological plant characteristics and an improvement may lead to adverse effects in agronomic properties. In addition, increased conversion of DON to D3G in resistant wheat lines probably due to the enhanced expression of a DON-glucosyltransferase was demonstrated (Lemmens et al. 2005). Nevertheless, it was shown that the FHB resistance of some cultivars significantly influenced DON production (Mesterházy 2002).

Promising transgenic approaches (reviewed by Cary et al. 2009) include anti-Fusarium proteins (Dahleen et al. 2001) as well as antibody-mediated Fusarium prevention (Hu et al. 2008). Furthermore, specific maize varieties showing resistance to aflatoxin production were studied (Windham and Williams 1998; Tubajika and Damann 2001). Moreover, Panaccione et al. (2001) demonstrated the absence of ergovaline production in *Neotyphodium* knockout strains generated by genetic modification of the grass-endophyte.

Although plant breeding and transgenic methods are very promising approaches, it is obvious, that such techniques are costly and require years until registering and marketing of new cultivars.

1.4.1.2) Crop rotation

Cropping systems in which maize is repeatedly cultivated (Reddy et al. 2007) or rotated with wheat (reviewed by Munkvold 2003a) seems to increase disease epidemics of *Aspergillus flavus* and *Fusarium graminearum*. Orlando et al. (2010) reported that the highest concentrations of T-2 and HT-2 after Fusarium infection occurred in spring barley grown after another spring barley or small-grain cereals as a previous crop. In addition, crop rotation with non-cereals was found to reduce mycotoxin concentration as well as various Fusarium infestations (Bernhoft et al. 2012). Therefore, avoiding adverse and preferring beneficial cropping systems and rotations might have a positive impact, although there is little direct evidence for the success of this approach.

1.4.1.3) Tillage

Tillage, the agricultural preparation of soil, includes several techniques, such as ploughing, rototilling and harrowing. Ploughing achieves that the top 10 - 30 cm of soil are inverted, whereas minimum tillage mixes the crop debris with the top 10 - 20 cm of soil. It was shown that no use of tillage after wheat or maize increased DON levels due to FHB in the following wheat crops compared to ploughing (Dill-Macky and Jones 2000) and that deeper tillage lowers the number of isolated *Fusarium* spp. (Steinkellner and Langer 2004). In contrast, Orlando et al. (2010) showed that tillage

practices did not decrease T-2 and HT-2 toxin levels after Fusarium infection. Consequently, the elimination of crop residues often containing fungal spores is likely a crucial and essential step in especially DON prevention of the next crop.

1.4.1.4) Sowing date

Fungal contamination of plants is promoted when the crop flowering stage occurs at the time of fungal spore release and change in sowing date can significantly influence fungal infection and mycotoxin contamination (Jouany 2007). Earlier sowing dates or the use of winter varieties that develop earlier than spring varieties can reduce Fusarium contamination level (Orlando et al. 2010). Significant AFB_1 reductions associated with early planting after infection by *Aspergillus flavus* were reported by (Jones et al. 1981). However, annual weather changes challenge this method.

1.4.1.5) Chemical control

Fungicides are widely used to control fungal contamination and a broad range of substances has been tested against mycotoxin producing fungi in greenhouse trials as well as under field condition. In general, commercially available fungicides contain substances related to the groups of triazoles, imidazoles and synthetic strobilurins. Triazole and imidazole fungicides inhibit the fungal biosynthesis of ergosterol, which is an essential component of fungal cell membranes (Ankley et al. 2007). Synthetic strobilurins affect the mitochondrial electron transport chain, which results in an inhibition of fungal spore development.

In literature, results concerning the ability of fungicides to reduce fungal and mycotoxin contamination under field conditions are contradictory (reviewed for *Fusarium* and *Aspergillus* contamination by D'Mello et al. 1998). While the commercially available triazole fungicide cyproconazole in combination with the imidazole prochloraz as well as the triazole tebuconazole plus the synthetic strobilurin azoxystrobin significantly increased yield and reduced FHB disease severity and DON content in wheat compared with inoculated non-treated control, tetraconazole showed reduced efficacy (Haidukowski et al. 2005). Another study supporting these findings demonstrated that treatment with prochloraz and tebuconazole reduced FHB incidence and DON concentration in winter wheat grain (Blandino et al. 2006). Edwards et al. (2001) reported that the triazole metconazole and tebuconazole markedly reduced the *Fusarium* amount in harvested wheat grains, whereas azoxystrobin did not show any effects against *Fusarium* spp. compared with the untreated control. Besides the contradictory resulting evidence concerning the effectiveness of fungicides, increased or stimulated mycotoxin production due to sub-lethal exposure to fungicides has also been demonstrated (D'Mello et al. 1998). Different aromatic plant essential oils without characterisation of the responsible substances have been positively tested for their antifungal properties (Rana et al. 1997; Velluti et al. 2003; 2004).

1.4.1.6) Biological control

Different biological control agents such as bacteria, fungi and yeasts have been shown to be feasible to complement chemical control in perspective of an integrated management to reduce the impact of mycotoxin producing fungi (reviewed by Tsitsigiannis et al. 2012 and Chulze et al. 2015). Microbial antagonists or competitors sprayed on plants at different stages of plant flowering (Khan et al. 2001; Dal Bello et al. 2002; Diamond and Cooke 2003; Xue et al. 2009) or at the fungi growth phase (Bacon et al. 2001) reduced FHB severity. More research including the investigation of further potentials and risks as well as of the applicability of such techniques is needed for subsequent registration of possible commercially available products.

1.4.1.7) Insect control

Physical injuries of grains caused by mechanical stress and/or insect activity often provide initial infection sites for field fungal species. The European corn borer (ECB) (*Ostrinia nubilalis*) has been associated with Fusarium infection of maize kernels and stalks due to crop damage as well as spore transfer from the larvae. CryIA(b), an endotoxin produced by some strains of the bacterium *Bacillus thuringiensis*, is pathogenic for certain insects, like ECB (reviewed by Aronson et al. 1986 ; Hoefte and Whiteley 1989). Genes encoding for CryIA(b) have been cloned and inserted into the genome of several maize genotypes. For Bt maize hybrids lower incidence of infection by *Fusarium* spp. and reduced severity of disease symptoms (Munkvold et al. 1997) as well as decreased fumonisin concentration was proven (Munkvold et al. 1999). In contrast, others have found that the effect of Bt hybrids on reducing DON contamination only occurred in fields with high intensities of ECB infestation (Schaafsma et al. 2002) or to a lesser extent compared to the decrease of fumonisin concentrations (Bakan et al. 2002). This is likely due to the fact that insects play a key role in the dispersal of *Fusarium* ear rot pathogens like *F. verticillioides*, but less for *Gibberella* ear rot pathogens like *F. graminearum* (reviewed by Munkvold 2003b).

1.4.1.8) Harvesting date and climate factors

The physiological stage of the plants can have consequences for the final fungal and mycotoxin contamination, thus choosing a suitable harvesting date is essential. Jones et al. (1981) reported significant AFB_1 reductions associated with early harvest and a direct correlation between FB_1 production by *F. moniliforme* in maize and kernel development was demonstrated (Warfield and Gilchrist 1999). Furthermore, Lauren et al. (2007) reported a continuous increased NIV and DON contamination due to Fusarium infection in maize when a crop is left standing for a prolonged period after the kernel moisture has reached a harvestable content. Concerning climate factors, increased DON concentrations due to low temperature as well as increased HT-2 concentrations due to high air humidity before harvest was demonstrated (Bernhoft et al. 2012).

1.4.2) Post-harvest techniques

In case of poor pre- and post-harvest practices that promote fungal growth (during agricultural and harvesting practices, drying, handling, storage, transport and packaging) as well as due to the fact that even the best pre-harvest management cannot totally prevent mycotoxin contamination post-harvest techniques are required. Consequently, various strategies for counteracting mycotoxin after harvesting have been developed and can be divided into prevention as well as elimination techniques.

Post-harvest prevention methods cover correct cleaning of farming equipment, proper storage conditions as well as the application of antifungal agents during storage (FAO/WHO/UNEP 1999b). Drying should be performed as soon as possible after harvest in order to decrease water activity (a_W) to approximately 0.7, which has to be monitored and controlled during storage. Additionally, grain damage caused by mechanical stress or insect activity has to be avoided. Antifungal agents such as organic acids, antioxidants, essential oils (Abdollahi et al. 2011; Lima de Sousa et al. 2013) and fungicides can be applied for preventing fungal spoilage (reviewed by Varga et al. 2010). However, due to the high costs this approach is hardly used (Chulze 2010). Furthermore, grains should not be mixed and long-time storage should be avoided, while a moisture content < 15% as well as reduced temperature should be applied (reviewed by Jard et al. 2011). In addition, high carbon dioxide levels (> 75%) in combination with minor presence of oxygen were reported to reduce fungal growth during storage (reviewed by Magan and Aldred 2007).

Post-harvest elimination strategies include decontamination methods of the harvested commodities (physical and chemical methods) as well as *in vivo* elimination techniques (biological methods based on adsorption or biotransformation in the gastrointestinal tract after consumption) (see Figure 6) and are further described in chapters 1.4.2.1 - 1.4.2.3.

1.4.2.1) Physical methods

Physical methods for the elimination and decontamination of mycotoxins in harvest commodities include the use of techniques based on sorting, cleaning, washing, dehulling, milling, thermal treatment, irradiation and cold plasma. While the efficacy of sorting, cleaning, washing, dehulling and milling is mainly based on the removal of infected plant materials, thermal treatments may reduce the contamination by chemical transformation of the mycotoxins. Irradiation and cold plasma methods are promising sterilisation techniques for the reduction of fungal material, while also toxin decomposition seems possible.

Selective separation as well as sorting out approaches based on differences in density, colour or size achieves the removal of infected and rotten goods after harvest (reviewed by Varga et al. 2010 and Jard et al. 2011). For instance, flotation methods for the separation of aflatoxin-contaminated grains, kernels, seeds and nuts have been developed (He and Zhou 2010). However, flotation was reported to be less effective compared to hand sorting (Matumba et al. 2015). Modern separation techniques are

automated single kernel applications with spectroscopic detection (visible or NIR) (Pearson et al. 2004; McMullin et al. 2015). Recently, commercially available high-speed sorters have been manufactured. However, robust and affordable high throughput sorters still have to be developed (Fox and Manley 2014). Moreover, separation and sorting methods do not detoxify mycotoxins and just highly infected material is removed.

Subsequent cleaning results in removed husks, hair, dust and shallow particles (Varga et al. 2010), while effects of sorting and cleaning show a high variability (Scudamore 2008). Effects of sorting and cleaning on mycotoxin content in wheat are reviewed by Cheli et al. (2013).

During washing procedures with water or sodium carbonate solutions additional decontamination of mycotoxins in grains can be performed (Varga et al. 2010). Concerning DON and ZEN, pressure washing of wheat grains with water in combination with oven, infrared or microwave drying achieved decreased mycotoxin content of up to 89%, while washing with sodium carbonate, chlorinated water or sodium hydroxide solutions resulted in reduced DON and ZEN levels, with a reduction of 38-91% and 32-84%, respectively (Yener and Köksel 2013). Unfortunately, no degradation products or reaction mechanisms were discussed.

Subsequent dehulling can be applied to further eliminate mycotoxin contaminations (Varga et al. 2010). For instance, by applying dehulling procedures a decrease of *Fusarium* spp. and DON content in naturally infected durum wheat samples (Rios et al. 2009), reduced DON and ZEN levels in naturally infected barley, maize, and rye (Trenholm et al. 1991) as well as a decrease in fumonisin levels in maize (Fandohan et al. 2006) up to 70% was demonstrated. However, the efficacy of dehulling techniques was reported to be extremely variable (summarised by Cheli et al. 2013).

In general, mycotoxins are concentrated in extremely contaminated portions of harvested goods and are heterogeneously distributed in a lot. By applying milling procedures mycotoxins may be redistributed and concentrated in different mill fractions, while a destruction of the mycotoxins cannot be achieved. In the dry milling process, although mycotoxins were present in all fractions (Scudamore 2008), they were mainly concentrated in the germ and bran fraction (reviewed by Bullerman and Bianchini 2007). For instance, higher levels in the bran fraction (usually used as animal feed) as well as lower levels in the flour (particle size usually < 200 µm) after milling of wheat was demonstrated for DON (Lancova et al. 2008), ZEN (Trigo-Stockli et al. 1996), T-2 and HT-2 (Pascale et al. 2011). In contrast, higher DON and NIV concentrations in the flour fractions as well as lower concentrations in the bran fractions due to high rainfall before harvest was reported (Edwards et al. 2011). During wet milling of maize, mycotoxins may be distributed in gluten, fibre and germ fractions or are dissolved in the steep water (reviewed by Bullerman and Bianchini 2007). After wet milling, the more polar mycotoxins NIV and DON, were found at high concentrations in steep water, while ZEN was pronounced in the solid fractions (gluten, fibre and germ) (Lauren and Ringrose 1997). The most

recent findings concerning redistribution of mycotoxins in wheat milling fractions was reviewed by Cheli et al. (2013). Similar trends of different studies with wheat indicate that levels of mycotoxins are lower in fractions used for human consumption and higher in fractions mainly intended for animal feed. However, a high variability of the reported distributions was shown.

Mycotoxins are very stable compounds and removal or decomposition by thermal treatment can hardly be achieved. For example, mycotoxin contaminations cannot be or can only slightly be reduced by normal cooking conditions, like boiling and frying. In particular, heat treatment of ZEN does not lead to a significant elimination. While torrefaction of aflatoxin and pressure cooking of aflatoxin and OTA contaminated commodities results in a reduction up to 80%, temperatures up to 220 °C are needed for a complete removal of fumonisins and a partial instability of DON. In contrast, extrusion processes have the ability to reduce AFs, DON, ZEN and FB_1 levels (reviewed by Bullerman and Bianchini 2007; Jard et al. 2011). However, the efficacy of thermal treatment techniques is extremely variable and depends on many different parameters such as mycotoxin content, type of mycotoxin, pH and a_W value as well as duration of heat treatment (reviewed by Jard et al. 2011). In addition, thermal treatment as well as extrusion may result in chemically modified mycotoxin derivatives with unknown toxicological relevance.

In general, ionising radiation (α-, β-, γ- and X-rays as well as short-wavelength UV radiation) has antimicrobial properties from which β-, γ-, X-rays and UV radiation are used in the food industry for pasteurisation. Due to high costs and consumer concerns irradiation techniques are not as common as other conventional methods (Calado et al. 2014), although an overall dose of 10 kGy has been considered as safe and effective (FAO/IAEA/WHO 1981). Irradiation can be applied to eliminate or reduce the present moulds and mould spores in food and feed. Nevertheless, mycotoxin contaminations may remain on or in the irradiated goods, although fungal material was destroyed. Consequently, the efficacy of ionising radiation to eliminate mycotoxins has been investigated extensively (reviewed by Calado et al. 2014). The efficacy of UV irradiation to reduce levels of DON and ZEN on dried filter paper strips was reported (Murata et al. 2008). Moreover, it was demonstrated that γ-irradiation achieved a decomposition of OTA in liquid media (Varga et al. 2010) and AFB_1 is sensitive to X- and γ-rays, UV and microwaves (Jard et al. 2011; Markov et al. 2015). However, insufficient or incomplete destruction of OTA and AFs by γ-irradiation in tested almond, feed and pepper samples were reported by Jalili et al. (2012) and Di Stefano et al. (2014a; b). The efficacy of irradiation methods is influenced by different fungi and commodity depending parameters, such as mycotoxin content, fungal load, fungal species, spore age, absorbed dose and moisture content. Hence, it was concluded that irradiation should only be applied in combination with pre- and post-harvest techniques addressing the prevention of fungal infections and that irradiation strategies are insufficient in detoxifying contaminated commodities (Calado et al. 2014). Alternatively, ultrasonic treatment,

which is not related to irradiation techniques, was reported to reduce decontaminations of trichothecenes, ZEN, OTA and AFs in aqueous media (He and Zhou 2010).

Another recently investigated sterilisation strategy is the promising application of cold plasma to reduce microbial contaminations, spore germination, mycotoxin production as well as mycotoxin content at low temperatures. In general, this technique, divided into thermal and non-thermal plasmas, uses a gas containing free electrons, ions and neutral particles. Non-thermal plasmas with a mean kinetic energy of more than 1 eV and an ambient gas temperature can cause chemical reactions and trigger excitation stated by electron and radical impact as well as emitted UV radiation. Advantages of this method in the field of medical engineering are the high efficacy at low temperatures, the precise and just in time generation of the plasma as well as low impact on the product matrix (Schlüter et al. 2013). The sterilisation effects of low pressure cold plasma (LPCP) using air gases and sulphur hexafluoride (SF_6) on nuts artificially contaminated with *Aspergillus parasiticus* as well as a 50% reduction in total AFs was demonstrated (Basaran et al. 2008). In addition, a significant reduction of *Aspergillus* spp. and *Penicillium* spp. by LPCP on artificially contaminated seeds was reported, while food quality as measured by the germination of wheat and beans seeds was only marginally effected (Selcuk et al. 2008). Moreover, Ouf et al. (2015) showed that the application of a double atmospheric pressure argon cold plasma jet system caused a reduction of the viability of *A. niger* spores on artificially contaminated date palm fruits and a decrease of FB_2 and OTA contents. However, data on antimicrobial activity are still too scarce, especially in regard to the possible application range and process parameters are often described incompletely. Potential physical, chemical, biochemical and microbiological changes in the treated goods induced by plasma treatment have rarely been investigated so far. In addition, possible impacts resulting in allergic compounds in the treated products are conceivable (Schlüter et al. 2013).

1.4.2.2) Chemical methods

Chemical detoxification methods using oxidising, reducing, acidic and alkaline chemicals have been developed. Decontamination strategies based on chemical transformation can cause changes in the molecular structure and biological activity of the degraded mycotoxins.

It was shown that oxidising agents, such as ozone and hydrogen peroxide, can attack the double bonds of trichothecenes, although moisture content is a critical parameter influencing the efficacy (reviewed by He and Zhou 2010). Ozone, which can be applied for microbial inactivation, mycotoxin degradation and insect killing, is a fumigant with minor effects on grain quality (reviewed by Tiwari et al. 2010). In detail, the efficacy of a continuous ozone treatment to reduce *Aspergillus flavus* counts on the surface of maize kernels was reported (McDonough et al. 2011), while insufficient aflatoxin reduction was observed. In contrast, ozone was reported to reduce aflatoxin and DON concentration in maize as well as OTA in nuts, grains and vegetables (reviewed by He and Zhou 2010; He et al. 2010;

Varga et al. 2010). Degradation of Fusarium toxins in maize by hydrogen peroxide was demonstrated and decreased toxicity was proven after ZEN degradation *in vivo* (reviewed by He and Zhou 2010).

Reduction by for example ascorbic acid, sodium bisulfite ($NaHSO_3$) and sodium metabisulfite ($Na_2S_2O_5$) results in a decrease of mycotoxin levels, particular for AFB_1 and DON (reviewed by He et al. 2010 and Jard et al. 2011). The reaction of sulphur reagents, such as $Na_2S_2O_5$ and sodium sulphite, with DON causes the formation of three different DON sulfonates (DONS) named DON sulfonate 1, 2 and 3 (Schwartz et al. 2013). The toxicity of DONS-1, -2 and -3, investigated with three different toxicity tests (yeast bioassay, effects on ribosomes and the viability of IPEC-J2 cells), was reduced compared to DON by factors of at least 330, 29 and 33, respectively (Schwartz-Zimmermann et al. 2014).

Treatments with acidic chemicals, such as propionic acid or sulphur dioxide, were also reported to reduce mycotoxin contamination by controlling mycotoxin producing fungi (He and Zhou 2010), while acidification with HCl has been shown to induce a 20% reduction of AFB_1 levels within 24 h (reviewed by Jard et al. 2011). Acidic inactivation of AFB_1 with citric acid and the formation of a less mutagenic derivative AFD_1 in maize grain has been demonstrated (Mendez-Albores et al. 2005). Alternatively, other carboxylic acids, such as formic, propionic or sorbic acids, as well as sodium hypochlorite are useful for OTA degradation (Varga et al. 2010).

Alkaline treatment with ammonia and sodium hydroxide were reported to deactivate aflatoxins in peanuts as well as meal of peanut, oilseed and jojoba (reviewed by He and Zhou 2010), while the formation of AFD_1 during ammonia application on maize was demonstrated (reviewed by Jard et al. 2011). In addition, almost complete degradation of OTA in wheat, maize, cocoa and barley can be achieved (reviewed by Varga et al. 2010). Chemical transformation of DON and NIV resulting in for example an opening of the 12,13-epoxy group by alkaline treatment was demonstrated. Moreover, a strategy using acidic and alkaline chemicals for two different fractions of the raw material and mixing the fractions after treatment was reported to reduce aflatoxin contaminations in products like cottonseed, peanuts, maize, wheat, coconuts (He and Zhou 2010). However, alkaline treatment is a cost-intensive strategy, which may decrease food quality by high levels of reagents interacting with the commodities (Jard et al. 2011).

In summary, chemical transformation based on the reaction with different oxidising, reducing, acidic and alkaline agents results in decreased mycotoxin levels. However, chemically modified mycotoxin derivatives with unknown toxicological relevance may be formed. In addition, within the European Union regulation for human food products chemical treatments for detoxification of mycotoxins are not allowed (Commission Regulation (EC) No. 466/2001).

1.4.2.3) Biological methods

Biological elimination strategies aiming to detoxify mycotoxins *in vivo* after the consumption of contaminated commodities are based on the adsorption or biotransformation of the ingested toxins.

In case of feed contamination, mycotoxin adsorption can be achieved by feed additives that have the ability to bind mycotoxins in the gastrointestinal tract of animals. This results in reduced bioavailability and subsequent elimination of the toxin-binder complex via faeces. Adsorbing materials consist of silica-based inorganic compounds (aluminosilicates), carbon-based organic polymers or yeast cell-wall derived binders (EFSA Report 2009). One important requirement for mycotoxin-adsorbing agents is the stability of the toxin-binder complex throughout the entire gastrointestinal tract, which has to be evaluated carefully. The efficacy of binding agents depends on the physical properties and chemical structure of both the adsorbing material and the target compound (Daković et al. 2005). Silicate binders, representing one group of extensively studied adsorbing agents, include bentonites, montmorillonites, hydrated sodium calcium aluminosilicates (HSCAS) and zeolites. Other relevant and commercially available mycotoxin-binding feed additives mainly contain activated charcoal, yeast (living yeast or yeast cell wall components), bacteria (mainly lactic acid bacteria), fibres or synthetic polymers (e.g. cholestyramine and polyvinylpyrrolidone). In general, the effectiveness of binders can be tested *in vitro* and *in vivo*. During *in vitro* testing, the binding efficacy and maximum binding capacity at different pH values is evaluated by single adsorption and adsorption isotherms studies (EFSA Report 2009). Many published *in vitro* studies have the disadvantage of testing single formulations used for research work, which do not refer to the complex product mixtures available on the market. In addition, the comparability of *in vitro* studies can hardly be ensured due to the impact of different experimental conditions (e.g. % of binder, incubation parameters, toxin concentration, pH and type of medium) on the amount of toxin bound per kg of used binder (discussed by Fruhauf et al. 2012). Consequently, percentage of binder, toxin concentration and pH value have recently been defined for *in vitro* studies testing the AFB_1 binding efficacy of bentonites by an EURL-evaluated method (Commission Implementing Regulation (EU) No. 1060/2013). Most of the *in vivo* studies assess the efficacy of adsorption by recording performance parameters (e.g. body weight gain, feed intake, mortality) or biological markers (concentration of toxin and/or metabolisation products in biological matrices) (Huwig et al. 2001; EFSA Report 2009). The *in vitro* and *in vivo* efficacy of different binding agents has been reviewed repeatedly (Huwig et al. 2001; EFSA 2009; Jard et al. 2011; Di Gregorio et al. 2014). In general, high adsorption rates of AFB_1 and alleviated effects by binding agents were reported frequently *in vitro* as well as *in vivo*, whereas the efficacy against ZEN, FB_1 and OTA was limited or variable and practically zero against trichothecenes. In detail, for HSCAS, a sufficient efficacy against AFB_1 was demonstrated *in vitro* as well as *in vivo* (reviewed by Ramos and Hernandez 1997). In addition, the European Panel on Additives and Products or Substances used in Animal Feed stated that bentonite has the potential of sufficient aflatoxin binding in feed of ruminants (EFSA 2011). In contrast, limited or no *in vitro* and *in vivo* effectiveness of

silicate binders to adsorb other mycotoxins was reported. Exceptions were a calcium montmorillonite and chemically modified montmorillonites that showed high *in vitro* binding capacities for ergotamine and ZEN, respectively. *In vitro*, activated charcoal has the ability to adsorb most of the mycotoxins in varying extent, whereas reduced or no efficacy was demonstrated *in vivo* due to an additional, unspecific binding of nutrients. While *in vitro* adsorption of AFB_1, ZEN, OTA, T-2 and HT-2 by yeast derived products was occasionally reported, *in vivo* effects were alleviated for AFB_1, slightly reduced for DON and almost not effected for ZEN, FB_1, OTA and T-2. For lactic acid bacteria, *in vitro* adsorption was shown for AFB_1, NIV, ZEN and OTA, while insufficient *in vitro* adsorption for DON and FB_1 was reported. Synthetic polymers were occasionally reported to bind AFB_1, ZEN, FB_1 and OTA *in vitro*, whereas controversial and insufficient results for an *in vivo* efficacy are reported (reviewed by Jard et al. 2011). In conclusion, the application of one single binding agent cannot achieve the adsorption of most types of mycotoxins and therefore, mixtures as well as modified adsorbents have to be used (reviewed by Huwig et al. 2001). An excellent and critical review on mycotoxin binders provided by Kolosova and Stroka (2011) discusses the disadvantages and restrictions, such as the limited efficacy against mycotoxins apart from AFB_1, further research that is required, as well as the low comparability of *in vitro* and *in vivo* studies.

Alternatively, another biological approach represents the *in vivo* biotransformation and degradation of mycotoxins into less toxic compounds by microorganisms or isolated enzymes. A wide range of microorganisms capable of degrading certain mycotoxins have been isolated from different sources, such as soil, infected plants or seeds, rumen fluids and gut microbiota. For example, detoxification of AFB_1 in contaminated commodities can be achieved by certain bacterial strains (e.g. *Flavobacterium aurantiacum*, *Stenotrophomonas maltophilia* and *Bacillus subtilis*), as well as by macroscopic fungi, such as *Pleurotus ostreatus* and *Armillariella tabescens*. Moreover, *Aspergillus*, *Rhyzopus* and *Penicillium* spp. as well as the yeast *Trichosporon mycotoxinivorans* have been reported to degrade OTA (reviewed by Jard et al. 2011). Most of the isolated microorganisms do not fulfil the generally recognised as safe (GRAS) status. For more details, interested readers are referred to extensive reviews by Halasz et al. (2009), He et al. (2010), Kolosova and Stroka (2011) and McCormick (2013), while chapters 2.1, 2.2, 2.3 and 2.4 deal with biological degradation strategies for ergot alkaloids, DON, ZEN and fumonisins, respectively. The long-standing investigation and development of mycotoxin degrading agents include the screening and selection of promising microorganisms, the optimisation and improvement of culture conditions, the evaluation of the degradation reaction as well as the optimisation of product formulations (spray- or freeze-dried and mixed with carrier material). Disadvantages of microorganisms directly used for mycotoxin degradation are on the one hand based on various parameters influencing the growth and function of the applied strains (e.g. pH value, temperature, availability of O_2, media, and duration). On the other hand, living microorganisms adding to food or feed have to overcome toxicological and regulatory concerns (GRAS status required). Consequently, the identification of genes encoding for the responsible enzymes and the subsequent

production and application of the enzymes catalysing the degradation reactions seem to be the most promising workflow (reviewed by He and Zhou 2010; Jard et al. 2011).

2) Elimination strategies of selected mycotoxins

Within this chapter, post-harvest strategies for the elimination and detoxification of ergot alkaloids, DON, ZEN and fumonisins are summarised and discussed. In addition, the scientific contributions of the author of this thesis as well as the main finding of the included publications related to detoxification strategies are reported.

2.1) Detoxification of ergot alkaloids

Modern grain cleaning and separation methods after harvest in milling facilities are the most effective techniques concerning post-harvest elimination of *C. purpurea* contamination (Bennett and Klich 2003). Normally, sclerotia can be sorted out from grain kernels after harvested together due to their different shape and size. However, the occurrence of rather small sclerotia as a result of hot and dry summers was reported (Lauber et al. 2005). Alternatively, physical methods suitable for the elimination and detoxification of ergot alkaloids in grains are mainly based on thermal treatment occasionally in combination with high pressure and/or steam. Thermal treatment (200 °C) of sclerotia was reported to result in an ergot alkaloid reduction of approximately 93% within 4 h, while epimerisation of the *-ine* isomers to the *-inine* forms was detected (Young et al. 1983). In addition, the authors demonstrated reduced ergot alkaloid concentrations in sclerotia by autoclaving (25% reduction) and showed reduced toxic effects in growing chicken. Although ergot alkaloids are photosensitive, in that study UV irradiation had no significant influence on total ergot alkaloid content or individual composition in sclerotia. A promoted epimerisation of the β configuration (*-ine*) to the α configuration (*-inine*) in addition to a decreased total ergot alkaloid content of approximately 10% in sclerotia during hydrothermal treatment was confirmed by Mainka et al. (2005).

Publications on the chemical treatment and detoxification of ergot alkaloids are limited. In principle, instability of ergot alkaloids during alkaline treatment and the subsequent formation of ergine and lysergic acid was reported (Jacobs and Craig 1934; Basmadjian and Floss 1971). While chlorine treatment on sclerotia reduced greatly total ergot alkaloid content (approximately 90%) within 6 h, hydrogen chloride and sulphur dioxide showed only moderate effects (less than 20%) (Young et al. 1983). In contrast, ammonia and ozone treatment tested in this study did not alter total ergot alkaloid levels in sclerotia.

Various adsorbent materials have been tested for their ability to bind different ergot alkaloids *in vitro* as well as for their efficacy to reduce adverse effects *in vivo*. For example, high *in vitro* binding affinity of clay-based mycotoxin binders (adsorption up to 97%) (Huebner et al. 1999; Malysheva et

al. 2013) and surfactant-modified zeolite (Tomasevic-Canovic et al. 2003) towards ergopeptines was demonstrated. Further, Merrill et al. (2007) reported the ability of yeast-derived cell wall preparations to prevent mycotoxicoses caused by feeding high-alkaloid tall fescue *in vivo*. In contrast, HSCAS did not reduce adverse effects of *Neotyphodium* contaminated seed and resulted in an impaired absorption of Mg, Mn and Zn *in vivo* (rats and sheep), although *in vitro* adsorption of ergotamine at a pH of 7.8 or lower was proven (Chestnut et al. 1992).

A limited number of publications exist on the biological degradation of ergot alkaloids. For instance, a decrease in ergovaline content caused by microbial activity in earthworm digestive tracts was demonstrated (Rattray et al. 2010), while the overall microbiome present in worm-gut cultures was reported. Unfortunately, no information about possible formed metabolites was presented.

Publication #1 in this thesis describes the determination and structural elucidation of unknown metabolites formed during microbial and enzymatic degradation of ergot alkaloids. In detail, the background of this work was the isolation of an ergopeptine degrading strain from soil and its identification as a *Rhodococcus erythropolis* strain MTHt3 (DSM 25948) (Thamhesl et al. 2015). In addition, the characterisation of the enzymes ErgA and ErgB catalysing the degradation reaction was achieved. Within publication #1, the efficacy of this biodegradation was evaluated. Therefore, the unknown metabolites formed during degradation of six different ergopeptines by the isolated strain MTHt3, its lysate and the purified enzyme ErgA as well as the mechanism of the enzymatic degradation were identified and characterised. For this purpose, LC-MS/MS and LC-HR-MS methods were developed and applied. Additionally, two main metabolites were isolated by preparative HPLC and NMR measurements were performed. Besides ergine and lysergic acid, two main types of metabolites, namely unstable ergine hydroxy carboxylic acids (reacting further to ergine) and stable, probably non-toxic diketopiperazines (cyclic dipeptides, DKPs) were detected. As a result, the isolated *R. erythropolis* MTHt3 single strain and its enzymes are the first extensively characterised microbial based agents capable of degrading ergopeptines. By the characterisation of the degradation products and the enzymatic reaction, the potential of the tested enzymes as feed additives to achieve safe *in vivo* detoxification of ergot alkaloids was evaluated.

2.2) Detoxification of deoxynivalenol

Concerning the physical, chemical and biological elimination of DON, publications investigating different strategies (including washing, dehulling, milling, thermal treatment, irradiation, treatment with oxidising, reducing and alkaline chemicals, as well as adsorption) are discussed in chapter 1.4.2. In addition, by applying optical one-pass sorting, average DON levels in wheat were reduced by 49% (Delwiche et al. 2005), while cleaning of wheat resulted in an average DON reduction of 23% prior to food processing (Visconti et al. 2004). For thermal treatment and food processing, in general variable efficacies in DON reduction have been reported. Reduced DON levels during bread baking and pasta boiling have been observed (reviewed by Kushiro 2008). In contrast, an increase in DON content

during fermentation for dough preparation and bread-baking and little effects of extrusion cooking has been demonstrated (reviewed by Milani and Maleki 2014). On the one hand, the reported variability in DON reduction could be caused by not considering the moisture content of the analysed food as assumed by Scudamore et al. (2009). On the other hand, the most efficient step in food processing may hardly be identified. Possible DON degradation products formed during food processing and available toxicological studies are summarised by Kushiro (2008). During baking a conversion of DON into an isomer with unknown toxicological relevance, named isoDON, may occur. Degradation of DON into products connected to the less cytotoxic norDON series (norDON A, B, and C) due to thermal and alkaline treatment has been observed.

Several adsorbent materials and feed additives have been tested for their DON binding efficacy *in vitro* as well as *in vivo*. For instance, insufficient *in vitro* binding ability against DON was reported for clay minerals (Thimm et al. 2001) as well as for HSCAS, humic substances, activated charcoal and yeast cell-wall derived products (Döll et al. 2004; Avantaggiato et al. 2005; Sabater-Vilar et al. 2007; Kong et al. 2014), while minor or no alleviation of harmful effects of DON was observed *in vivo* for polymeric glucomannan adsorbents (Swamy et al. 2003; Dänicke et al. 2007), yeast-derived products and bentonite (Shehata et al. 2004; Weaver et al. 2014). In contrast, a moderate *in vitro* binding of DON by activated carbon was demonstrated (Avantaggiato et al. 2004) and prevention of some harmful effects of DON was observed *in vivo* for clay-based binders (Qiang et al. 2011; Sief et al. 2012) and activated carbon (Devreese et al. 2012). The high variability of *in vitro* and *in vivo* results is based on the significant influence of different experimental conditions on the achieved binding efficacy. In general, most of the mineral clays and commercial adsorbing materials bind DON only weakly *in vitro* and are mainly ineffective *in vivo*.

Different microbes with the ability to enzymatically convert DON to less toxic products have been identified. Various studies used mixed cultures of microorganisms and only few single strains capable in degrading DON have been identified. In general, complete mineralisation of DON by microorganisms can hardly be achieved and two possibilities of DON detoxification by bacteria seem promising (reviewed by Zhou et al. 2008, Awad et al. 2010, Karlovsky 2011 and McCormick 2013). On the one hand, an oxidation at the hydroxyl group on C-3 to 3-keto DON (for example by a soil bacterium of the *Agrobacterium-Rhizobium* group) can be achieved (Shima et al. 1997), in some cases followed by the reduction of 3-keto DON to 3-epi DON (see Figure 7 for the chemical structures of both DON metabolites) by aerobic bacteria (*Nocardioides* and *Devosia* spp.) (Ikunaga et al. 2011; Sato et al. 2012). On the other hand, a reductive de-epoxidation by anaerobic bacteria isolated from animal digestive system (e.g. DSM 11798 strain BBSH 797 of the *Coriobacteriaceae* family) to DOM-1 (Figure 3) was reported (King et al. 1984; Binder et al. 1997; Fuchs et al. 2002). The de-epoxidation activity exclusively under anaerobic conditions limits the practical application of these strains. Consequently, strain BBSH 797, which was developed into a commercial product, has to be stabilised

by freeze-drying and embedding into protective substances (such as organic polymers) (reviewed by Kolosova and Stroka 2011).

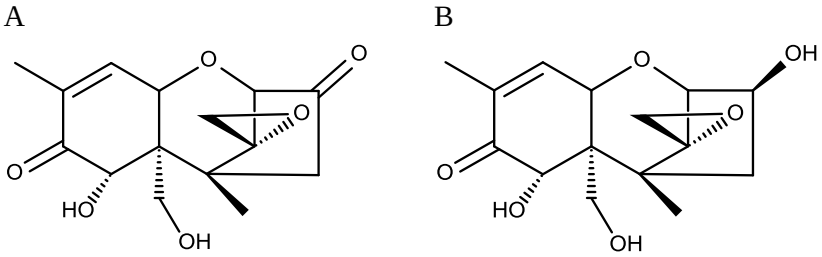


Figure 7: Chemical structure of (A) 3-keto DON and (B) 3-epi DON

Fungi, such as *Aspergillus tubingensis* and *Alternaria alternata*, as well as *Bacillus* spp. (*B. licheniformis* and *B. subtilis*) were reported to transform DON, while the chemical structure of the formed products were not investigated (reviewed by Karlovsky 2011). In general, various publications testing the possible biological transformation of DON are available. Additionally, several commercially available feed additives claiming to detoxify DON *in vivo* are marketed, frequently without a scientific proof of their efficacy. However, the potential and effective application of microorganisms to detoxify contaminated feed *in vivo* is limited. For example, failing in counteracting DON *in vitro* by enzymatic and microbial products was demonstrated (Döll et al. 2004; Kong et al. 2014). Moreover, no commercial feed additive based on a pure enzyme capable in degrading DON has been successfully developed so far, although a promising enzyme has been investigated (Ito et al. 2013). *In vivo*, controversial results on the efficacy of DON degrading feed additives are reported. While prevention of some harmful effects caused by DON was achieved in dairy cows (Kiyothong et al. 2012) and broilers (Sarandan et al. 2012) due to the application of a microbial product, no alleviation of DON induced symptoms was observed in pigs (Dänicke et al. 2004) and broilers (Dänicke et al. 2003) using the same product. Furthermore, Dänicke and Döll (2010) demonstrated, that a probiotic feed additive containing spores of *Bacillus subtilis* and *B. licheniformis* was not able to alleviate performance depressing effects of DON in piglets.

The aim of **publication #2** of this thesis was to evaluate the *in vitro* efficacy of different feed additives claiming detoxification of DON and ZEN and is discussed in more detail at the end of the following chapter.

2.3) Detoxification of zearalenone

Regarding physical, chemical and biological elimination strategies for ZEN, various studies evaluating the efficacy of different methods (including washing, dehulling, milling, thermal treatment, irradiation, treatment with oxidising chemicals, and adsorption) are summarised in chapter 1.4.2. An excellent review on the toxicity, occurrence, metabolism, regulations and detoxification was published by Zinedine et al. (2007). Furthermore, insufficient degradation of ZEN during the baking process

(fermentation with *S. cerevisiae* followed by baking at 200 °C) was reported (Cano-Sancho et al. 2013).

For the potential adsorption of ZEN many different binding agents have been tested *in vitro* as well as *in vivo* and controversial results are reported. For example, *in vitro* adsorption of ZEN was demonstrated for mineral clays and humic substances (Sabater-Vilar et al. 2007; Santos et al. 2011), a surfactant-modified zeolite (Tomasevic-Canovic et al. 2003), activated carbon (Avantaggiato et al. 2003; Bueno et al. 2005), a modified yeast glucan (Freimund et al. 2003), *Saccharomyces cerevisiae* strains (Armando et al. 2012), cholestyramine (Ramos et al. 1996; Döll et al. 2004) and *Aspergillus japonicus* conidia (Jard et al. 2009). In contrast, *in vitro* insufficient ZEN reduction was reported for bentonite (Döll et al. 2004), a carbon/aluminosilicate-based product (Avantaggiato et al. 2007) and yeast cell-wall derived products (Fruhauf et al. 2012). *In vivo*, alleviation of symptoms caused by ZEN was shown for a montmorillonite clay (Wang et al. 2012), a natural clay (Jiang et al. 2010), activated carbon (Afriyie-Gyawu et al. 2005), while minor or no prevention of harmful effects of ZEN was observed for organophilic montmorillonite clays (Lemke et al. 2001a), modified clinoptiolite (Nesic et al. 2005) and esterified glucomannan derived from yeast cell-wall (Bursian et al. 2004).

Concerning biodegradation of ZEN, different microorganisms have been reported, capable of degrading ZEN into preferably non-toxic metabolites. While various microorganisms, such as *S. cerevisiae*, *Rhizopus* spp. and *Alternaria alternata*, reduced ZEN to the still oestrogenic metabolites α- and β-ZEL, non-oestrogenic metabolisation products like 2,4-dimethoxyl zearalenone, zearalenone-14-sulfate and zearalenone-14-β-D-glucoside were formed by *Cunninghamella bainieri*, *Rhizopus arrhizus* and *Thamidium elegans*, respectively (reviewed by McCormick 2013). In addition, ring cleavage and ZEN degradation by the yeast *Trichosporon mycotoxinivorans* MTV into a non-oestrogenic metabolite termed ZOM-1 (Vekiru et al. 2010) as well as by the mycoparasitic fungus *Gliocadium roseum* into the non-oestrogenic hydrolysed zearalenone (HZEN) and decarboxylated hydrolysed zearalenone (DHZEN) (El-Sharkawy and Abul-Hajj 1988) was demonstrated. From the latter fungus, the enzyme lactonohydrolase catalysing this detoxification have been isolated and the responsible genes have been identified (Takahashi-Ando et al. 2002). The protein sequence of an esterase (from *Rhodococcus* or *Nocardia* spp.) catalysing the conversion of ZEN into DHZEN has been patented for the insertion into transgenic plants (reviewed by Jard et al. 2011). *In vitro* and *in vivo* studies on commercial microbial and enzymatic based feed additives are limited. For instance, minor *in vitro* reduction of ZEN due to adsorption and enzymatic degradation by two commercially available products was demonstrated (Döll et al. 2004). *In vivo*, no prevention of ZEN induced symptoms could be achieved in broilers (Dänicke et al. 2001), while alleviation of some harmful effects caused by ZEN was observed in pigs (Cheng et al. 2006; Brydl et al. 2014).

The aim of **publication #2** of this thesis was to evaluate the *in vitro* efficacy of different feed additives claiming detoxification of DON and ZEN by metabolisation and additional adsorption. In detail, 20

commercially available products were tested separately under aerobic and anaerobic conditions at pH 7.0 for possible DON and ZEN degradation. Aerobic as well as anaerobic incubation conditions were applied for up to 72 h to enable mycotoxin reduction by microorganisms as well as by enzymes or binding materials stated to be present in the tested products. Sample aliquots were taken during incubation and measured by LC-UV-MS/MS. Mass transitions of DON, ZEN and related metabolites were monitored and evaluated. Additionally, UV signals at 220 nm and 270 nm were recorded for the detection of possibly formed toxins-related UV absorbing compounds. Our *in vitro* results revealed that only a minority of the tested products are able to detoxify the mycotoxins. Those important findings demonstrate that many of the currently commercialized products in fact endanger the health of farm animals rather than protecting it and emphasise the need of proper scientific evidence to prove the claims of those products.

2.4) Detoxification of fumonisins

Concerning physical, chemical and biological elimination strategies for fumonisins, literature discussing the efficacy of different strategies (including dehulling, thermal treatment, cold plasma and adsorption) is given in chapter 1.4.2. Moreover, a fumonisin reduction by 71% due to optimised maize sorting (separating visibly infected maize kernels from good maize kernels) and an additional reduction by 13% due to washing with water for 10 min at 25 °C were observed (van der Westhuizen et al. 2010). After commercial dry milling, fumonisin content was usually lower in grits and higher in bran and germ fractions. By applying a wet milling process, fumonisins were found in steep water as well as in the gluten, fibre and germ fractions, while no fumonisins were detected in the starch fractions. Fumonisins are very heat stable and reduction by thermal treatment can only be achieved by frying, extrusion and roasting processes at temperatures above 150 °C. In addition, it has been shown that the use of glucose increases fumonisin reduction during those food processes (reviewed by Milani and Maleki 2014). One possible explanation for a higher reduction of fumonisins in the presence of reducing sugars is the conversion of FB_1 into NDF (see Figure 5) (Poling et al. 2002; Jackson et al. 2012). This reaction is regarded as detoxification due to the blockage of the primary amino group of FB_1 (Howard et al. 2002), which is relevant for the toxic effects of fumonisins (Gelderblom et al. 1993). Reduced toxicity of extruded maize grits contaminated with FB_1 in the presence of glucose was demonstrated *in vivo* (Voss et al. 2008; Jackson et al. 2012).

Nixtamalisation of maize represents an alkaline treatment with lime that is extensively performed, e.g. in Mexico, for the production of tortillas and tortilla chips. If fumonisin contaminated maize is used, alkaline treatment will result in the hydrolysis of FB_1 to $pHFB_1$ and HFB_1 (Hendrich et al. 1993). A reduction of total fumonisins (fumonisins and their hydrolysed derivatives) in tortillas can be achieved by nixtamalisation due to the remaining of those compounds in the residual lime and washing water (Palencia et al. 2003). In addition, reduced elevation of the Sa to So ratio in cells treated with extracts of tortillas was demonstrated. Moreover, cinnamon oil was reported to reduce levels of FB_1 (Xing et

al. 2014), while degradation products were not investigated. Furthermore, a deamination of FB_1 in aqueous solution with $NaNO_2$ at pH 1.0 and 5 °C was reported to reduce toxicity in a *Hydra attenuata* bioassay (Lemke et al. 2001b).

Several binding agents have been tested for their ability to bind fumonisins *in vitro* as well as for their efficacy to reduce adverse effects *in vivo*. For instance, *in vitro* binding of FB_1 by montmorillonite and HSCAS (Aly et al. 2004), activated carbons (Galvano et al. 1997), and cholestyramine (Solfrizzo et al. 2001b; Avantaggiato et al. 2005) was demonstrated. Prevention of some harmful effects of FB_1 was reported *in vivo* for a yeast-cell wall derived product (Siloto et al. 2013). By analysing fumonisin biomarkers, no elevation of the Sa to So ratios, indicating sufficient binding, was observed after feeding a commercial silica-based binder (Denli et al. 2015), and cholestyramine (Solfrizzo et al. 2001b; Avantaggiato et al. 2005). In contrast, HSCAS and bentonite were not effective in binding FB_1 *in vitro* (Galvano et al. 1997; Solfrizzo et al. 2001b). Furthermore, an elevation of the Sa to So ratios that indicates insufficient FB_1 binding was observed *in vivo* for activated carbon (Solfrizzo et al. 2001a) and esterified glucomannan derived from yeast cell-wall (Bursian et al. 2004).

In general, microbial degradation of FB_1 is based on the removal of the two TCA moieties by hydrolysis to generate $pHFB_1$ and finally HFB_1, followed by a deamination to produce 2-keto HFB_1 (reviewed by Hartinger and Moll 2011). A fumonisin-degrading bacterium *Sphingopyxis* sp. has been isolated from soil and the two enzymes catalysing this two-step degradation (a carboxylesterase and an aminotransferase) have been characterised (Heinl et al. 2010; 2011; Hartinger et al. 2011). After identification of the responsible genes and optimisation of the enzyme production, the *in vivo* efficacy of the carboxylesterase has been proven (Grenier et al. 2013) and the enzyme has been commercialized as an enzyme-based feed additive (named FUMzyme®) by BIOMIN Holding GmbH. From the black yeast *Exophiala spinifera*, isolated from field-grown maize kernels, a carboxylesterase and an amine oxidase have been identified as fumonisin-degrading enzymes (reviewed by McCormick 2013). Another FB_1 degrading bacterial strain, related to the *Delftia/Comamonas* group, has been isolated from soil and four degradation products have been tentatively identified as heptadecanone ($C_{17}H_{34}O$), isononadecene ($C_{19}H_{38}$), octadecenal ($C_{18}H_{34}O$) and eicosane ($C_{20}H_{42}$) (Benedetti et al. 2006).

Within **publication #3** of this thesis the stability and catabolic fate of HFB_1 and NDF compared to FB_1 was investigated and evaluated under human gastrointestinal conditions. As already mentioned, HFB_1 represents a degradation product formed during alkaline treatment of food as well as during microbial degradation catalysed by a carboxylesterase. For NDF, one major product formed by thermal food treatment and extrusion of fumonisin contaminated food or feed in the presence of glucose, liberation of the parent forms during mammalian digestion may occur. In detail, NDF was chemically synthesised and purified using preparative HPLC due to the lack of available analytical standards for further experiments. An *in vitro* digestion assay was applied by incubating the target compounds with

the proper amounts of salts, enzymes and digestive juices (gastric and duodenal juice, as well as bile) at 37 °C. For the *in vitro* human colonic fermentation assay, freshly collected faeces in buffer solution with growth medium were incubated at 37 °C under anaerobic conditions with the toxin derivatives. Samples were taken during both assays, inactivated by adding acetonitrile and directly measured by LC-MS/MS. The results of this study indicate a partial cleavage of NDF during simulated digestion, while its degradation products have not been fully identified. Besides that, the stability of NDF towards human gut microbiota was demonstrated. In contrast, HFB_1 was partially metabolised into unknown compounds after 24 h of incubation by gut microbiota. The applied *in vitro* assay provided easily and quickly first indications of the stability and potential metabolisation of HFB_1 and NDF under human gastrointestinal conditions. In conclusion, the partial cleavage of NDF during simulated digestion and the observed metabolisation of HFB_1 by the gut microbiota emphasise the need of animal experiments to investigate the toxicological relevance of those compounds *in vivo*.

Publication #4 in this thesis describes the toxicological relevance of orally administered FB_1, $pHFB_1$, HFB_1 and NDF in rats. In detail, the sphingolipid metabolic effect and possible metabolisation of those fumonisin derivatives was investigated and compared. For this purpose, production of the toxins and further purification, stability tests and inclusion of toxins (via spiked water) into a mixture of commercial rat feed and supplemented maize was required for the preparation of the experimental diets. 20 male Sprague Dawley rats were exposed to diets containing 13.9 µmol/kg of high purity FB_1, $pHFB_1$, HFB_1 and NDF, respectively (n = 4), for three weeks. Biomarkers of effect (elevation of the Sa to So ratio in urine and kidney samples) as well as biomarkers of exposure (excreted fumonisin derivatives in urine and faeces) were analysed by developed and validated LC-MS/MS based methods. As a result, Sa/So ratios in urine and kidney samples were significantly elevated in samples of the positive control group fed with FB_1 containing diet. In contrast, orally administered $pHFB_1$, HFB_1 and NDF did not affect sphingolipid metabolism in rats. Nevertheless, partial decomposition of NDF to FB_1 was observed in faeces samples, suggesting partial liberation during digestion *in vivo*. By producing the compounds of interest in high purity and feeding those fumonisin derivatives to rats, the current knowledge of their toxicological relevance was extended. In conclusion, all tested compounds were of lower toxicological relevance compared to FB_1.

Original Works - Overview

Ergot alkaloids

Publication #1

Hahn, I., Thamhesl, M., Apfelthaler, E., Klingenbrunner, V., Hametner, C., Krska, R., Schatzmayr, G., Moll, W.D., Berthiller, F. and Schwartz-Zimmermann, H.E. (2015) Characterisation and determination of metabolites formed by microbial and enzymatic degradation of ergot alkaloids. World Mycotoxin J, DOI: 10.3920/WMJ2014.1807.

DON and ZEN

Publication #2

Hahn, I., Kunz-Vekiru, E., Twaruzek, M., Grajewski, J., Krska, R. and Berthiller, F. (2015) Aerobic and anaerobic *in vitro* testing of feed additives claiming to detoxify deoxynivalenol and zearalenone. Food Addit Contam Part A, DOI: 10.1080/19440049.2015.1023741.

Fumonisins

Publication #3

Cirlini, M., **Hahn, I.**, Varga, E., Dall'Asta, M., Falavigna, C., Calani, L., Berthiller, F., Del Rio, D. and Dall'Asta, C. (2015) Hydrolysed fumonisin B_1 and *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1: stability and catabolic fate under simulated human gastrointestinal conditions. Int J Food Sci Nutr 66, 98-103.

Publication #4

Hahn, I., Nagl, V., Schwartz-Zimmermann, H.E., Varga, E., Schwarz, C., Slavik, V., Reisinger, N., Malachová, A., Cirlini, M., Generotti, S., Dall'Asta, C., Krska, R., Moll, W.D. and Berthiller, F. (2015) Effects of orally administered fumonisin B_1 (FB_1), partially hydrolysed FB_1, hydrolysed FB_1 and *N*-(1-deoxy-D-fructos-1-yl) FB_1 on the sphingolipid metabolism in rats. Food Chem Toxicol 76, 11-18.

Publication #1

Hahn, I., Thamhesl, M., Apfelthaler, E., Klingenbrunner, V., Hametner, C., Krska, R., Schatzmayr, G., Moll, W.D., Berthiller, F. and Schwartz-Zimmermann, H.E. (2015) Characterisation and determination of metabolites formed by microbial and enzymatic degradation of ergot alkaloids. World Mycotoxin J, DOI: 10.3920/WMJ2014.1807.

The author of this thesis was responsible for the design of the degradation experiments and the further development and optimisation of an LC-MS/MS method for the determination of ergot alkaloids and their metabolites. In addition, the candidate performed the metabolite isolation and purification, as well as the measurement of the optical rotation. Furthermore, the LC-HR-MS analysis for metabolite characterisation was conducted by the author of this thesis. Moreover, the candidate analysed and interpreted all generated data, elucidated the strucutre of the unknown metabolites, evaluated the degradation reactions and characterised the metabolites. The manuscript was written by the author of this thesis and then extended and revised by all co-authors.

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Characterisation and determination of metabolites formed by microbial and enzymatic degradation of ergot alkaloids

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RESEARCH ARTICLE

Abstract

Ergot alkaloids are frequent contaminants of cereal crops. Strategies for their inactivation include the use of microorganisms or enzymes as feed additives capable of degrading ergot alkaloids. Recently, an ergopeptine-degrading *Rhodococcus erythropolis* strain MTHt3 (DSM 25948) has been isolated from soil and the involved enzymes ErgA and ErgB have been identified. The aim of the current study was to characterise the metabolites formed by degradation of various ergopeptines with the MTHt3 strain, its lysate and the purified enzyme ErgA. Using preparative HPLC, ^{1}H-, ^{13}C- and 2D-NMR as well as HR-MS measurements, two main groups of metabolites formed during microbial and enzymatic degradation of ergopeptines were identified: diketopiperazines (cyclic dipeptides, DKPs) and unstable ergine hydroxy carboxylic acids. However, degradation by strain, lysate and enzyme yielded different end-products. Whereas DKPs were transient and lysergic acid the only final product upon incubation of ergopeptines with the *R. erythropolis* strain, incubation with the lysate resulted in formation of lysergic acid and two isomeric DKPs at different ratios. Enzymatic degradation by ErgA yielded only one DKP isomer and ended at the stage of the ergine hydroxy carboxylic acids which then spontaneously degraded to ergine. In conclusion, we succeeded in identification of metabolites formed by microbial and enzymatic degradation of ergot alkaloids which is a crucial step in the future development of feed additives for gastro intestinal detoxification of ergopeptines in farm animals.

Keywords: detoxification, mycotoxins, mass spectrometry, NMR, *Rhodococcus*

1. Introduction

Ergot alkaloids are mycotoxins produced by the fungus *Claviceps purpurea*, other species of the grass parasitic genus *Claviceps* and grass endophytes, like *Neotyphodium* species. Different *Aspergillus* and *Penicillium* fungi were also reported to produce certain ergot alkaloids (Flieger *et al.*, 1997). *C. purpurea*, a ubiquitous pathogen of cereals, parasitises the seed heads of plants like forage grasses, maize, wheat, barley, oats, millet, sorghum, rice, and rye at the time of flowering (Krska and Crews, 2008). The formed sclerotia mainly contain ergometrine, ergotamine (Figure 1), ergosine, ergocristine, ergocryptine and ergocornine (EFSA, 2012).

Ergotism, which is also known as ergotoxicosis or Saint Anthony's Fire, is caused by the consumption of ergot alkaloid containing food or feed. This issue is a problem in animal nutrition (particularly in chicken, cattle, horses, sheep and pigs) (Bennett and Klich, 2003). The gangrenous form of ergotism influences the blood supply of the limbs and causes peripheral vasoconstriction, whereas the convulsive form generates delirium as well as hallucinations due to the interaction of ergot alkaloids with neurotransmitter receptors (Schiff, 2006). This is caused by the structural homology of ergot alkaloids to neurotransmitters like dopamine (Larson *et al.*, 1999). Nowadays, the most widespread cases of ergotism in livestock are caused by the intake of tall fescue (*Festuca*

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arundinacea), a cool-season perennial grass infected by the ergot alkaloid producing endophytic fungus *Neotyphodium coenophialum* (Cheeke, 1995; Porter and Thompson, 1992). Besides vasoconstrictive activity (Merhoff and Porter, 1974), different pharmacologic effects on animals have been reported. These include decrease of prolactin concentration in serum, immune response, body weight gain, dry matter intake, reproductive efficiency and milk production, as well as increase of body temperature (Porter and Thompson, 1992; Rhodes *et al.*, 1991; Schuenemann *et al.*, 2005).

The tetracyclic ergoline ring, which is methylated on the N-6 nitrogen atom and substituted on the C-8 position, is a common structural feature of ergot alkaloids. Depending on the C-8 substituent, ergot alkaloids can be divided into clavine alkaloids, simple lysergic acid derivatives, ergopeptines and lactam ergot alkaloids. In the group of ergopeptines a short peptide chain is attached at the C-8 position of the ergoline ring structure (Flieger *et al.*, 1997). In general, the stereocenter at C-8 gives rise to two epimeric forms of ergot alkaloids (C-8-(R) and C-8-(S) configuration). The biologically active ergopeptines (e.g. ergotamine and ergovaline) are lysergic acid amides, show a C-8-(R) configuration and represent the left-hand rotation isomers. In contrast, the less toxic ergopeptinines are the right-hand rotation isomers, exhibit a C-8-(S) configuration and represent isolysergic acid derivatives (e.g. ergotaminine and ergovalinine). Epimerisation of ergopeptines into ergopeptinines occurs during storage or extraction of ergot alkaloids from raw materials and has been reported to take place during storage of ergot alkaloid standards in solution (Smith and Shappell, 2002).

Published approaches for the reduction of ergot alkaloids in feed include the application of clays (Huebner *et al.*, 1999) or other adsorbent materials based on yeast cell walls (Merrill *et al.*, 2007). However, *in vitro* results cannot be applied to animals directly and *in vivo* efficacy of binders has not yet been proven. Another elimination strategy represents the use of microorganisms or enzymes capable of degrading ergot alkaloids (Rattray *et al.*, 2010). Isolation and characterisation of an ergopeptine degrading strain (*Rhodococcus erythropolis* MTHt3, DSM 25948) from soil (M. Thamhesl, unpublished results), as well as the characterisation of the responsible enzymes ErgA and ErgB has recently been achieved. ErgA, an alpha/beta-hydrolase, catalyses the degradation of different ergopeptines to ergine and in a second step, the amidase ErgB is responsible for further metabolisation of ergine to lysergic acid.

The aims of this work were the characterisation and identification of metabolites formed during degradation of different ergopeptines by the strain MTHt3, its lysate and the purified enzyme ErgA. A further objective was to provide insights into the mechanism of the enzymatic degradation. As the isolated *R. erythropolis* MTHt3 as well as its enzymes ErgA and ErgB are the first extensively characterised biocatalysts capable of degrading ergopeptines, the results of this study will provide the basis for future *in vivo* applications of the enzymes as feed additives for gastro intestinal detoxification of ergot alkaloids.

2. Materials and methods

Reagents and standards

All chemicals used for enzyme purification and preparation of cultivation media, buffer solutions, as well as dimethyl sulfoxide (DMSO), deuterated chloroform ($CDCl_3$, 99.8%) and formic acid (all analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), chloroform, dichloromethane, ethanol (EtOH), methanol (MeOH), trifluoroacetic acid (all LC grade), glacial acetic acid (p.a.) and ammonia solution (25%, p.a.) were purchased from VWR International GmbH (Vienna, Austria). Water was purified with a Purelab Ultra system (ELGA LabWater, Celle, Germany). Ammonium formate solution was purchased from Agilent Technologies (Waldbronn, Germany). Ergot alkaloids were obtained as crystalline substances. Ergine, erginine, ergotaminine, lysergic acid and isolysergic acid were provided by Alfarma s.r.o. (Cernošice, Czech Republic), ergocornine, ergocristine, α-ergocryptine and ergosine by Prof. M. Flieger (Czech Academy of Sciences, Prague, Czech Republic), ergovaline by Prof. F. Smith (Auburn University, Auburn, AL, USA) and ergotamine (as ergotamine tartrate) by Sigma-Aldrich. Standard stock solutions of the known compounds were prepared in a concentration range between 5 and 200 mg/l by dissolving aliquots of solid compounds in ACN and diluting in ACN:water (20:80, v/v) which were stored at -20 °C. Calibration solutions of the identified metabolites 244-1 and 244-2 were prepared after isolation on a preparative HPLC system and assessment of the UV purity between 12 and 3,000 ng/ml for quantitative analysis.

Development of an LC-MS/MS method for the determination of ergot alkaloids and their metabolites

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on a 1290 Infinity ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies) coupled to a 4000 QTrap mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turbo V electrospray ionisation (ESI) source. Chromatographic separation was achieved at 25 °C on a RP Gemini C18 column (150×4.6 mm i.d., 5 µm particle size; Phenomenex, Aschaffenburg, Germany) equipped with a 4×3 mm C18 security guard cartridge. The injection volume was 5 µl and the flow rate was 0.8 ml/min. Mobile phase A was composed of MeOH:water:acetic acid (10:89.9:0.1, v/v/v) and mobile phase B of MeOH:water:acetic acid

(80:19.9:0.1, v/v/v), both containing 0.5% of aqueous ammonia solution (12.5%, v/v). The following gradient was used: 0-1 min hold at 10% B; 1-9 min 10-63% B; 9-12 min 63-100% B; 12-17.9 min hold at 100% B; 17.9-18 min 100-10% B and 18-20 min hold at 10% B. Mass spectrometric detection included electrospray ionisation in positive mode and analysis in the enhanced full scan (EMS), enhanced product ion scan (EPI) and selected reaction monitoring (SRM) mode. For all analyses, the ion spray voltage was 4,000 V and the source temperature 500 °C. In the EMS mode, ions were scanned between *m/z* 100 and 600, the collision energy (CE) was set to 25 eV and the declustering potential (DP) to 70 V. In the EPI mode, a DP of 70 V was used and selected precursor ions were fragmented at collision energies between 10 and 40 eV depending on the analyte. Fragment ions were scanned between *m/z* 50 and 390. SRM parameters of the commercially available (ergine, ergocornine, ergocristine, α-ergocryptine, ergosine, ergotamine, ergovaline and lysergic acid) and later on isolated compounds (244-1 and 244-2 metabolites) were determined by syringe pump infusion of single analyte solutions in MeOH:water (50:50, v/v) and software controlled optimisation. The SRM parameters for the 355-1, 355-2, 383-1 and 383-2 metabolites were optimised by selecting the mass transitions on the base of EPI spectra and by systematic testing of DP and CE values. Mass transitions and parameters used in the SRM mode are listed in Table 1 which gives an overview of the investigated ergopeptines and their metabolites. For data analysis the software Analyst version 1.5.2 (AB Sciex) was used. Quantitative analysis was carried out on the basis of linear calibration curves (peak area versus concentration) established in a concentration range between 1 and 500 ng/ml for the individual analytes.

Table 1. Mass transitions and selected reaction monitoring parameters on a 4000 QTrap of investigated ergot alkaloids and monitored metabolites.

Analyte	Retention time (min)	Precursor ion (*m/z*) $[M + H]^+$	DP (V)	Product ion (*m/z*) (CE in eV)	
				Quantifier ion	Qualifier ion
Ergot alkaloids					
Ergocristine and ergocristinine	13.9 16.1	610.7	66	223.3 (40)	305.0 (40)
Ergotamine and ergotaminine	15.3 16.4	582.6	66	223.3 (47)	208.1 (59)
Ergocryptine and ergocryptinine	16.0 17.8	576.7	66	223.3 (40)	305.0 (40)
Ergocornine and ergocorninine	15.1 16.7	562.6	66	223.3 (40)	277.1 (40)
Ergosine and ergosinine	14.5 15.8	548.6	76	223.3 (40)	208.1 (40)
Ergovaline and ergovalinine	14.4 15.1	534.6	76	223.3 (45)	208.1 (63)
Lysergic acid and isolysergic acid	7.6 8.1	269.3	66	44.0 (43)	154.0 (63)
Ergine and erginine	10.6 12.6	268.3	56	223.3 (29)	208.1 (35)
Ergine hydroxy carboxylic acids					
383-1 and 383-2	9.7 12.4	384.2	76	268.1 (30)	223.3 (30)
355-1 and 355-2	7.8 10.3	356.2	76	223.3 (30)	268.1 (30)
Diketopiperazines					
244-1 and 244-2	10.0 10.3	245.1	61	70.1 (65)	154.2 (33)
210-1 and 210-2	9.0 9.4	211.1	61	70.1 (65)	138.2 (40)
196-1 and 196-2	6.8 7.1	197.1	61	70.1 (65)	154.2 (33)

Cultivation of strains, preparation of lysates and purification of the enzyme ErgA

Strain *R. erythropolis* MTHt3 (M. Thamhesl, unpublished results) was grown overnight in tryptone soy broth (Oxoid Limited, Basingstoke, UK) at 25 °C and 200 rpm. Biomass (10 ml) was harvested by centrifugation (17,696×*g*, 3 min, 4 °C), washed and suspended in the same volume of 50 mM sodium phosphate buffer (pH 7.0). An optical density (OD_{600}) of 2.0 was adjusted and suspensions were used for degradation experiments. For preparation of lysates from MTHt3, harvested pellets of 180 ml biomass (OD_{600} 4.6) were washed with 50 mM sodium phosphate buffer (pH 7.0), suspended in 9 ml distilled water and lysed by two passes through a French Press (Thermo Electron Corporation, Waltham, MA, USA) at 40,000 psi cell pressure. The lysates were clarified (17,696×*g*, 15 min, 4 °C) and filtered with a 0.2 µm filter (Filtopur, Sarstedt, Nümbrecht, Germany). *Escherichia coli* ArcticExpress(DE3) strains harbouring plasmid pET-28a containing gene *ergA*, encoding *R. erythropolis* MTHt3 hydrolase ErgA were grown overnight in Luria-Bertani medium (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl) with 20 µg/ml gentamycin and 50 µg/ml kanamycin at 37 °C and 150 rpm. Cultures were diluted 50-fold in modified M9ZB medium (Studier *et al.*, 1990) containing the M9 salts, 10 g/l tryptone instead of NZ-amine, 5 g/l yeast extract, 1 mM $MgSO_4$ and 0.4% glucose and incubated for 3 h at 30 °C and 200 rpm. After transfer to 11 °C (Ecotron incubator, Infors HT, Bottmingen, Switzerland) and incubation for 10 min, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM. Induced cultures were incubated at 11 °C and 200 rpm for 24 h. The pellet of 500 ml culture was suspended in 10 ml binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) containing 60 mM imidazole and lysed by two passes through a French Press at 1,280 psi cell pressure. Lysate was clarified by centrifugation (4,500×*g*, 30 min, 4 °C) and filtration (0.2 µm). Enzyme purification was performed with an ÄKTA™prime system (GE Healthcare Life Science, Uppsala, Sweden). The supernatant (8 ml) was loaded on a 1 ml HisTrap™ HP column (GE Healthcare), pre-equilibrated with 10 ml binding buffer containing 60 mM imidazole, with a flow rate of 0.3 ml/min. Column was washed with 20 ml binding buffer containing 60 mM imidazole. Hydrolase ErgA was eluted by increasing imidazole concentration to 500 mM in a linear gradient over 30 ml. During elution fractions of 1 ml were collected. Pre-equilibration, washing and elution were performed with a flow rate of 1 ml/min. After SDS-PAGE analysis fractions containing ErgA were pooled. Imidazole was removed by dialysis (Spectra/Por® Dialysis Membrane MWCO 6-8.000; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) with binding buffer for 18 h at 4 °C with stirring and one buffer exchange after 1 h. Protein concentration was determined by using the Pierce™ BSA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions. Glycerin with a final concentration of 25% was added to the pooled fractions and the solution was stored at -20 °C.

Degradation experiments of different ergopeptines

Degradation experiments of the six different ergopeptines ergocornine, ergocristine, α-ergocryptine, ergosine, ergotamine, ergovaline and the epimer ergotaminine were performed using different biocatalysts and conditions. In addition, negative controls only containing the toxins or the biocatalysts were incubated. Degradation experiments with biomass of MTHt3 were performed with strain suspensions previously adjusted to an OD_{600} of 2. The solutions were diluted 1:50 (v:v) in 50 mM sodium phosphate buffer pH 7.0 containing 10 mg/l ergotamine or another single ergopeptine in equimolar concentration and were incubated under shaking at 200 rpm in Erlenmeyer flasks. The lysate of strain MTHt3, prepared as described above, was diluted 1:20 (v:v) with water containing 10 mg/l ergotamine or another single ergopeptine in equimolar concentration. Degradation experiments with the purified enzyme ErgA were performed by adding 1.5 µg/ml ErgA to water:ACN (96:4, v/v) containing the tested toxins. All degradation experiments were carried out at 25 °C in the dark. Sample aliquots were taken at the beginning and after 0.5, 1, 2, 3, 6, 8, 24 and 48 h of incubation and adjusted to 20% ACN for inactivation of the enzyme. Finally, the samples were clarified by centrifugation (2,655×*g*, 20 °C, 10 min), the supernatants were transferred into brown glass vials and stored at -20 °C until LC-MS/MS analysis.

Metabolite isolation and purification

Two separate large-scale degradation experiments of ergotamine were performed, one using the lysate of MTHt3, one the purified enzyme ErgA. To this end, 25 ml of the clarified and filtered lysate was added to 475 ml of a 46 mg/l ergotamine solution (water:DMSO, 95:5, v/v). The mixture was incubated at 25 °C for 48 h. The degradation of ergotamine by ErgA was performed as follows: 12.12 ml of a 0.28 mg/ml ErgA solution was mixed with 2,108 ml of a 10 mg/l ergotamine solution (water:ACN, 96:4, v/v) and incubated at 25 °C for 2 h. After incubation the solutions were frozen at -20 °C overnight and freeze-dried. The residues were re-dissolved in ACN:water (20:80, v/v), clarified by centrifugation (2,655×*g*, 20 °C, 10 min) and the isolation of the formed metabolites was performed on a preparative 1100 series HPLC system (Agilent Technologies) coupled to a multi-wavelength detector. Chromatographic separations were carried out at a flow rate of 16 ml/min and a temperature of 25 °C on a RP Gemini NX C18 column (110 Å, 150×21.2 mm i.d., 5 µm particle size; Phenomenex) protected by a C18 security guard column (10×10 mm). Different mobile phase compositions and gradients were used for isolation of the metabolites. (1) One metabolite

termed 244-1 was isolated from the reaction batch obtained by degradation of ergotamine by the lysate of MTHt3. Mobile phases were composed of MeOH:water:acetic acid (phase A: 10:89.9:0.1, phase B: 80:19.9:0.1, v/v/v) and the same gradient as described for LC-MS/MS analysis was used. Fractions containing the 244-1 metabolite were collected between 9 and 11 min using a wavelength of 210 nm for detection. (2) Two metabolites termed 355-1 and 244-2 were isolated in two separate chromatographic runs from the residue obtained upon degradation of ergotamine by the purified enzyme ErgA. In a first step, the same alkaline eluents and the gradient as used for LC-MS/MS analysis were employed for the separation of the 355-1 metabolite. Fractions containing the 355-1 metabolite were collected between 6 and 8 min after UV detection at 230 nm, acidified by addition of acetic acid, frozen at -20 °C and lyophilized, for stability reasons. Fractions containing the coeluting pair of the 244-2 metabolite and ergine were collected, evaporated to dryness on a rotary evaporator and the residues were re-dissolved in 10 ml of ACN:water (20:80, v/v). In a second step, the 244-2 metabolite was isolated under the same conditions as described above for isolation of the 244-1 metabolite (acidic mobile phase). Fractions of the 244-2 metabolite were collected between 9 and 11 min after peak detection at 210 nm. All fractions containing pure 244-1 or 244-2 metabolite were taken to dryness on a rotary evaporator.

Nuclear magnetic resonance spectroscopy of the 244-1 and 244-2 metabolites

For structure elucidation of the 244-1 and 244-2 metabolites, ^{1}H, ^{13}C, $^{1}H^{1}H$ correlation spectroscopy, $^{1}H^{13}C$ heteronuclear single quantum correlation, and $^{1}H^{13}C$ heteronuclear multiple bond correlation spectra were recorded. NMR spectra were obtained from $CDCl_3$ solutions on a Bruker Avance DRX-400 FT-NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at ambient temperature, and chemical shifts were established on the basis of residual solvent resonances (7.26 ppm for ^{1}H NMR, 77.0 ppm for ^{13}C NMR). All pulse programs were taken from the Bruker software library. The NMR data were evaluated using TopSpin 1.3 (Bruker BioSpin GmbH).

Optical rotation of the 244-1 and 244-2 metabolite

For determination of the absolute stereochemistry of the isolated 244-1 and 244-2 metabolites, the optical rotation was measured at 20 °C and 589 nm on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Waltham, MA, USA). To this end, the 244-1 metabolite was dissolved in MeOH and CH_2Cl_2 at a concentration of 0.076 g/100 ml and the 244-2 metabolite in EtOH at a concentration of 0.086 g/100 ml.

Metabolite characterisation by LC-HR-MS analysis

High-resolution mass spectrometry measurements were used for further characterisation of the metabolites of the investigated ergopeptines. UHPLC-MS/MS analysis was carried out on a 1290 Infinity LC system coupled to a 6550 iFunnel QTOF mass spectrometer (both Agilent Technologies) equipped with a dual Jet Stream electrospray ion source of the same supplier. Chromatographic separation was accomplished at 30 °C on an Agilent Zorbax SB-C18 column (150×2.1 mm, 1.8 µm) at a flow rate of 0.25 ml/min. Mobile phase A was composed of water:formic acid (99.9:0.1, v/v) and mobile phase B of MeOH:formic acid (99.9:0.1, v/v). Both mobile phases contained 5 mM ammonium formate. The injection volume was 1 µl and the gradient was set as follows: 0-0.5 min hold at 10% B; 0.5-20 min linear increase to 100% B; 20-22 min hold at 100% B; 22-22.1 min return to 10% B; 22.1-25 min 10% B for re-equilibration. In addition, flow injections of one non-retained metabolite (pyruvic acid in negative mode) were carried out at 50% B (mobile phase A: water:formic acid 99.9:0.1, v/v; mobile phase B: MeOH:formic acid 99.9:0.1, v/v) using a restriction capillary instead of a column. MS analysis in the positive as well as the negative ionisation mode and MS/MS analysis in the positive ionisation mode was performed at a gas temperature of 130 °C, drying gas flow of 12 l/min, nebulizer pressure of 50 psi, sheath gas temperature of 350 °C and sheath gas flow of 10 l/min. A capillary voltage of 4,000 V, nozzle voltage 500 V and fragmentor 175 V were used and data acquisition was performed in the 2 GHz extended dynamic range mode. In MS experiments, ions were scanned from *m/z* 100-1,000 (3 spectra/s acquisition rate and 2,714 transients/spectrum) in the positive mode and from *m/z* 50-1,000 (acquisition rate 3 spectra/s and 2,704 transients/spectrum) in the negative ionisation mode. In MS/MS experiments, ions were scanned from *m/z* 50-500 (5 spectra/s acquisition rate and 1,579 transients/spectrum) at collision energies of 10 and 30 eV in the targeted-MS/MS mode with a precursor isolation width of 1.3 *m/z*. Continuous mass calibration was performed on the basis of the ions *m/z* 121.0508 and 922.0097 in the positive mode and the ions *m/z* 119.0363 and 966.0007 in the negative mode (Agilent ES TOF reference mass solution). The Agilent Technologies software MassHunter (Data Acquisition version B.05.01, Qualitative Analysis version B.06.00 and Molecular Structure Correlator version B.05.00) was used for instrument control, data evaluation and structure assignment of the major fragment ions.

3. Results

Development of an LC-MS/MS method for the determination of ergot alkaloids and their metabolites

An LC-MS/MS based method for the simultaneous analysis of six ergopeptines, their corresponding epimers (ergoinines), and their metabolites was gradually developed on a AB Sciex 4000 QTrap system. First, SRM parameters of available compounds were determined by software controlled optimisation. Then, measurements of selected degradation experiment samples in the EMS and in the EPI mode provided *m/z* values of protonated molecular ions and of fragment ions of newly formed metabolites. The overall degradation reaction of ergotamine by the lysate of the stain *R. erythropolis* MTHt3 and by the enzymes ErgA and ErgB is shown in Figure 1. The limits of detection (LODs) and limits of quantitation (LOQs) in neat solvent solution were established at signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively (Table 2).

Degradation experiments with different ergopeptines

Six different ergopeptines (ergocornine, ergocristine, α-ergocryptine, ergosine, ergotamine and ergovaline) and one ergopeptinine (ergotaminine) were incubated separately with the strain MTHt3, its lysate and the purified enzyme ErgA. Samples taken at different time points were analysed by LC-MS/MS in the SRM, EMS and EPI mode. Compared to negative controls, incubation of ergopeptines with strain, lysate or enzyme resulted in formation of several metabolites which were, depending on the biocatalyst, stable end products, unstable intermediates and/or substrates for further metabolisation. Degradation of all ergopeptines by the strain MTHt3 or by its lysate followed a two-step metabolisation with ergine as intermediate and lysergic acid as final product. Due to epimerization of ergopeptines in solution, erginine and isolysergic acid were also detected in these samples. Incubation of ergopeptines with the purified enzyme ErgA ended at the stage of ergine. Further metabolites formed upon incubation of ergopeptines with MTHt3, its lysate or ErgA were a transient pair of epimers termed 355-1, 355-2 (degradation products of ergotamine, ergovaline and ergosine) and 383-1, 383-2 (degradation of ergocornine, ergocristine, ergocryptine), respectively. Three

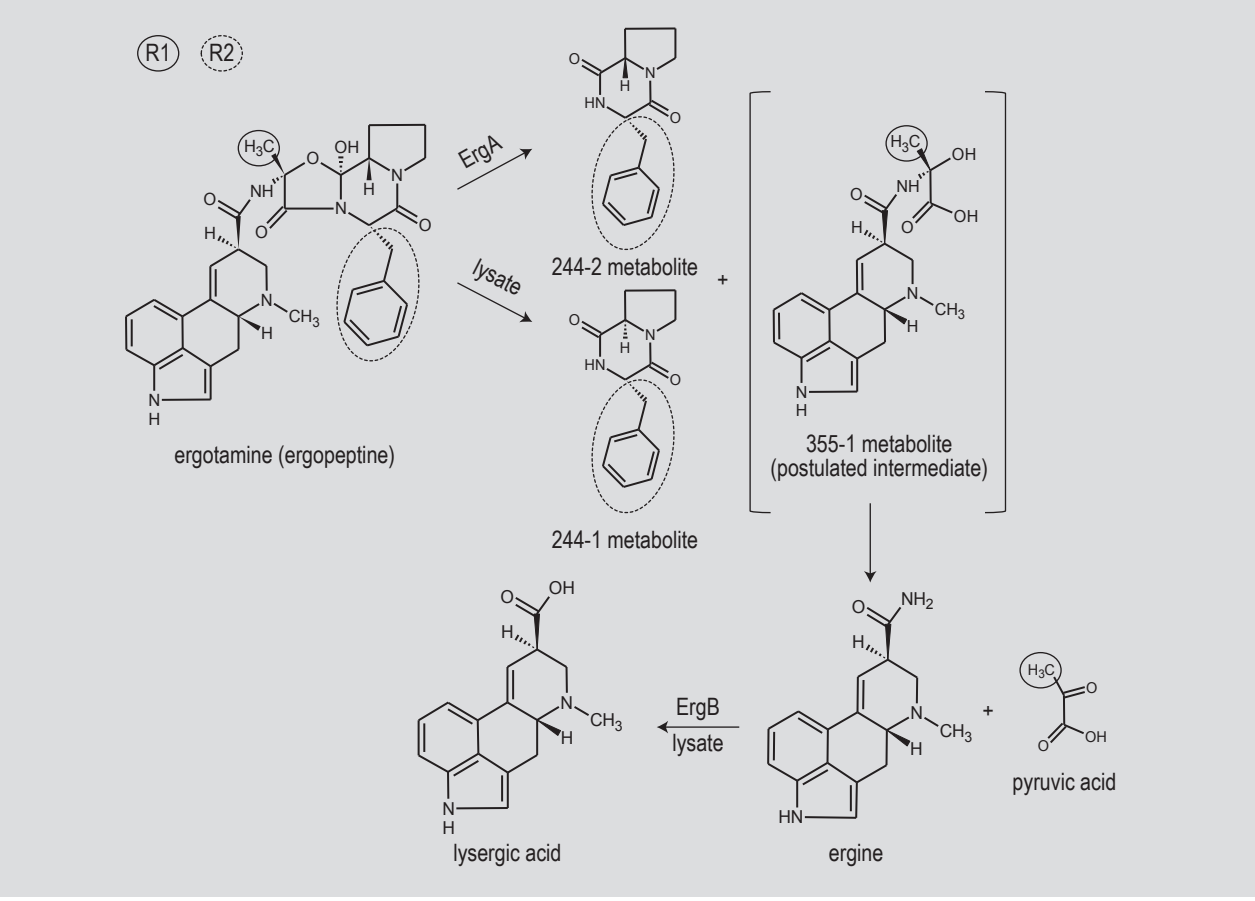

Figure 1. Postulated degradation scheme of ergotamine by ErgA or by the lysate with further metabolisation of ergine to lysergic acid, catalysed by ErgB (ergopeptine characteristic substituents R1 and R2 are circled).

Table 2. Limits of detection (LODs), limits of quantification (LOQs) and upper end of linear range in aqueous standards.

Compound	LOD (ng/ml)	LOQ (ng/ml)	Upper linear range (ng/ml)
Ergotamine	1.5	3.2	1000
Ergotaminine	1.4	3.5	1000
Lysergic acid	0.21	0.93	300
Isolysergic acid	0.11	0.35	300
Ergine	0.04	0.14	300
Erginine	0.20	1.1	300
244-2 metabolite	2.6	12.7	3,000

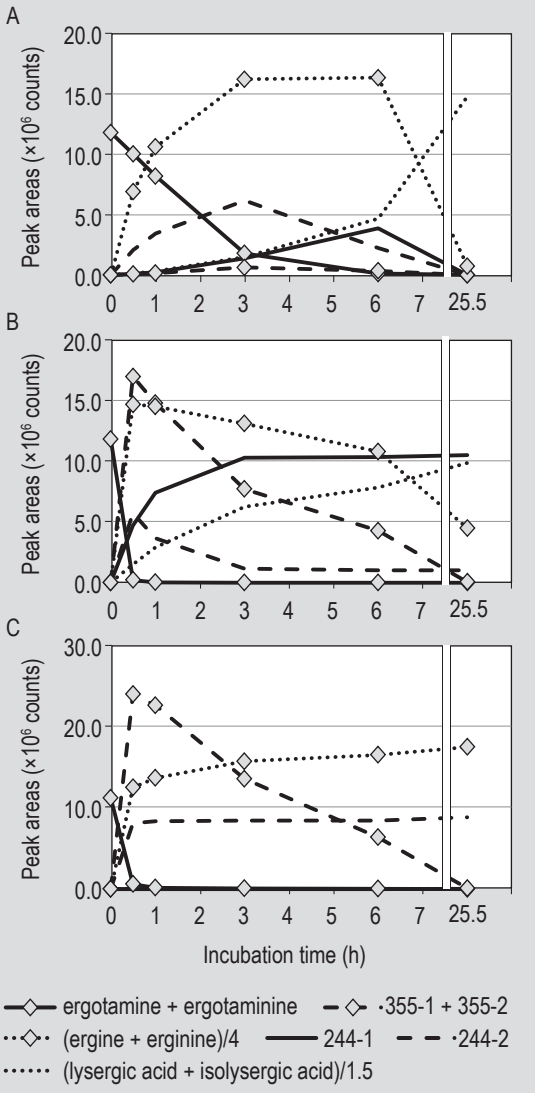

Figure 2. Time course of metabolites upon degradation of ergotamine by (A) the MTHt3 strain, (B) the MTHt3 lysate and (C) the purified enzyme ErgA.

further epimeric pairs named 196-1, 196-2 (metabolites of ergovaline, ergocornine), 210-1, 210-2 (degradation products of ergocryptine, ergosine) and 244-1, 244-2 (degradation of ergotamine, ergocristine) were either intermediate (during degradation by the strain) or stable end products (during metabolisation by the lysate or ErgA). As described below, the epimers 355-1 and 355-2 as well as 383-1 and 383-2 were later on found to spontaneously degrade to ergine and erginine, whereas the metabolites 196-1, 196-2, 210-1, 210-2, 244-1 and 244-2 could be identified as epimeric cyclic dipeptides (diketopiperazines; DKPs).

In Figure 2A, the time course of metabolite formation and disappearance upon degradation of ergotamine by MTHt3 is illustrated. Degradation of ergotamine was almost complete (1% remaining) after 6 h of incubation. Formation of ergine reached a maximum between 3 and 6 h, but was followed by conversion of the formed ergine into lysergic acid between 6 and 25.5 h. After 3 h of incubation, formation of the 244-2 metabolite and of the 355 metabolites reached a maximum, whereas formation of the 244-1 metabolite was greatest after 6 h. After 25.5 h, the only detectable reaction products were the end product lysergic acid at its maximum concentration and ergine (5% of its maximum concentration).

The time course of degradation of ergotamine by the lysate of MTHt3 is depicted in Figure 2B. Degradation by the lysate was faster compared to degradation by the strain and complete after 0.5 h. At the same time, the 355 metabolites and 244-2 reached a clear maximum. Equally, formation of ergine was greatest between 0.5 and 1 h. After their respective maximum, these three metabolites were gradually converted into the stable end-products of lysergic acid (ergine and 355 metabolite via ergine) and 244-1 metabolite. After 6 h incubation of ergotamine with the lysate of MTHt3 a 244-1/244-2 ratio of 10:1 was reached.

As illustrated in Figure 2C, degradation of ergotamine by the purified enzyme ErgA was equally rapid as by the lysate. Likewise, formation of the 355 metabolites showed a maximum after 0.5 h and was followed by degradation. However, upon degradation by the enzyme, ergine and the 244-2 metabolite turned out to be the end products. A chromatogram showing the metabolites formed upon degradation of ergotamine for 0.5 h by ErgA is given in Figure 3.

The intensities of ergotamine and ergotaminine, ergine and erginine, lysergic acid and isolysergic acid and of the two individual epimeric compounds 355-1 and 355-2 as well as 383-1 and 383-2 changed in the same way over the incubation period independent of the biocatalyst, which is explained by formation of the *-inine* form from the *-ine* form upon epimerization in solution. Therefore, the summed

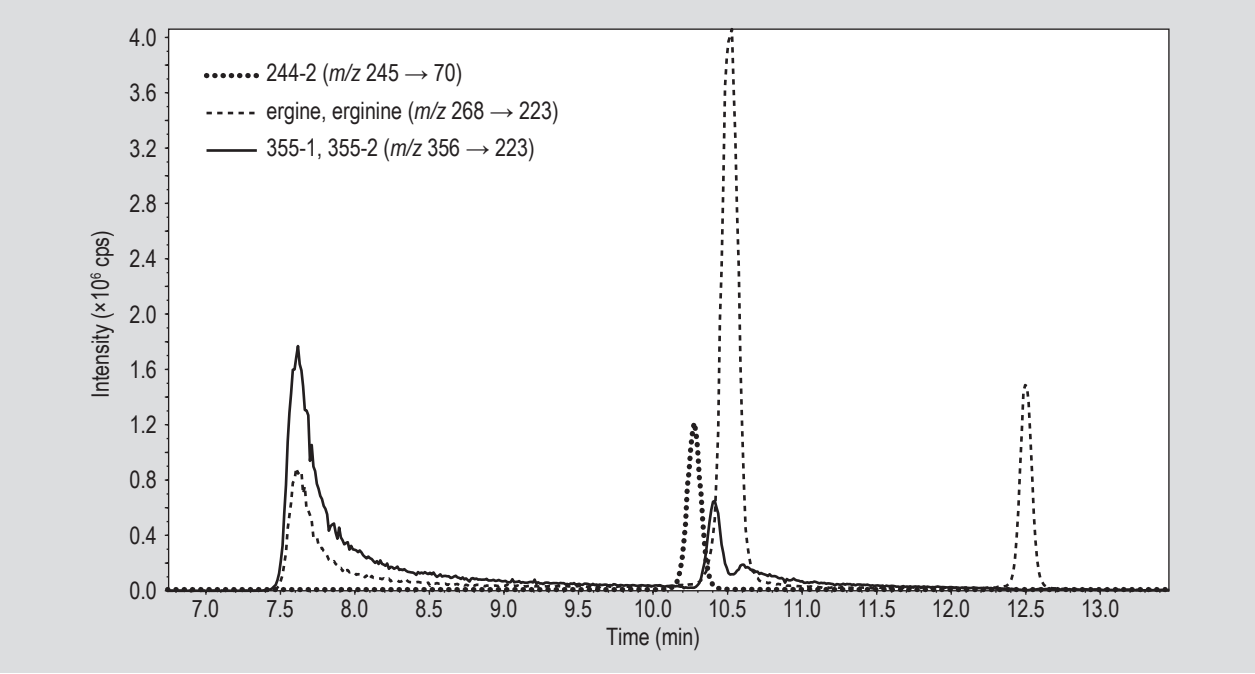

Figure 3. ESI(+) LC-MS/MS SRM chromatogram of a 10 mg/l ergotamine solution degraded for 0.5 h by enzyme ErgA.

peak areas of the epimeric compounds are given in Figure 2. However, the two diketopiperazine compounds were characterised by changing ratio of the epimers over the incubation time. In addition, the selection of biocatalyst greatly influenced the formation pattern of the individual diketopiperazine metabolites.

Characterisation of the diketopiperazines

Predominant formation of the 244-1, 210-1 and 196-1 metabolites by the lysate and exclusive formation of 244-2, 210-2 and 196-2 by ErgA facilitated separate production and isolation of the dipeptide metabolites. For economic reasons, ergotamine was chosen as model compound and degraded in two separate large scale degradation experiments. The 244-1 and 244-2 metabolites were isolated for subsequent structure elucidation by NMR. The UV purity of the isolated compounds assessed by LC-UV at 210 nm was 99.5 (244-1) and 86.1% (244-2), respectively.

By 1D- and 2D-NMR measurements both 244 metabolites were identified as cyclic Pro-Phe dipeptides (3-(phenylmethyl)-hexahydropyrrolo[1,2-*a*]pyrazine-1,4-diones). Assignment of the relative configuration of the two isomers was carried out by comparison of their ^{1}H and ^{13}C NMR spectra with literature data (Fdhila *et al.*, 2003; Hendea *et al.*, 2006).

However, from NMR data, the absolute configuration of the two DKPs could not be determined. Therefore, the optical rotation was measured and compared with literature data. It was reported that the $[\alpha]_D$ of these proline-containing

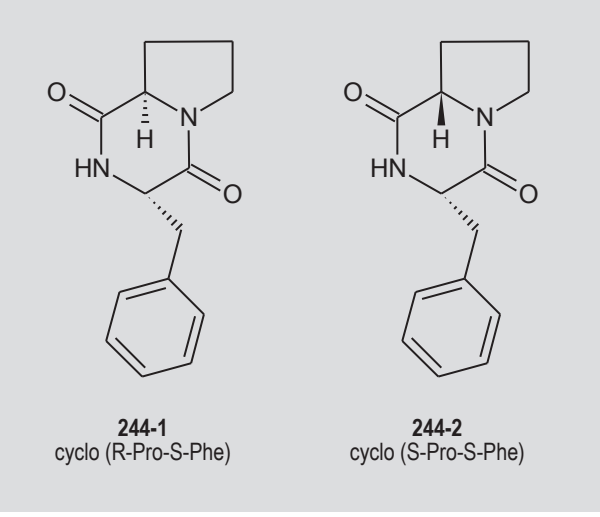

Figure 4. Chemical structure and absolute configuration of the 244 metabolites: 244-1 (left, predominantly formed during ergotamine degradation by the lysate of the strain MTHt3) and 244-2 metabolite (right, formed during ergotamine degradation by the enzyme ErgA).

DKPs was either negative or positive depending only on the absolute configuration of the proline part (S or R) (Adamczeski *et al.*, 1995). The 244-1 metabolite had an $[\alpha]_D$ of +59° in MeOH and an $[\alpha]_D$ of +14° in CH_2Cl_2 at 20 °C. The $[\alpha]^{20}_D$ values of 244-1 was positive in the two different solvents which indicated an R configuration for proline and matched very well with literature data (+67° in MeOH) (Wang *et al.*, 2010). In the case of the 244-2 metabolite, an $[\alpha]_D$ value of -91° in EtOH was measured at

20 °C which suggested an S proline configuration and the measured value was in line with published data (-93.9° in EtOH) (Jayatilake *et al.*, 1996). The chemical structure and absolute configuration of the 244-1 and 244-2 metabolite are given in Figure 4.

Furthermore, the DKP metabolites of other investigated ergopeptines were additionally analysed by high-resolution mass spectrometry in positive ionisation mode for further characterisation. The accurate mass obtained by QTOF full scan measurements of both epimeric forms of the 244 metabolites (244-1 and 244-2) with retention times of 10.32 and 10.60 min was 244.1212 which corresponds to a molecular formula of $C_{14}H_{16}N_2O_2$ and is in line with structures determined by NMR measurements. The accurate mass of the epimeric DKP metabolites formed from ergocornine and ergovaline (196-1 and 196-2) with retention times of 7.14 and 7.34 min was 196.1212 and the sum formula $C_{10}H_{16}N_2O_2$. DKP metabolites derived from ergocryptine and ergosine (210-1 and 210-2) with retention times of 9.36 and 9.68 min had an accurate mass of 210.1368 and a sum formula of $C_{11}H_{18}N_2O_2$. Assuming analogy between DKP metabolites of different ergopeptines and considering results of NMR and optical rotation measurements of the 244 metabolites, the 196-1 metabolite was tentatively identified as cyclo(R-Pro-S-Val) and the 196-2 metabolite as cyclo(S-Pro-S-Val). In the same way, the 210-1 and 210-2 metabolites were tentatively assigned the structures cyclo(R-Pro-S-Leu), and cyclo(S-Pro-S-Leu). In all measurements, the deviation of the measured from the exact mass was ≤2 ppm.

Characterisation of the 355 and 383 metabolites

The second novel type of metabolites were the transient compounds 355-1, 355-2 (degradation of ergotamine, ergovaline, ergosine) and 383-1, 383-2 (degradation products of ergocornine, ergocryptine and ergocristine). As gradual decomposition of the 355 metabolites was observed after preparative HPLC isolation, stability tests in solution were carried out in order to determine the most beneficial conditions for purification and in order to assess the suitability of the 355 metabolites for structure elucidation by NMR. Hence, different conditions and methods for up-concentration were compared. As a result, lower temperature, slightly acidic pH value (pH 3), shorter storage time as well as faster handling and dark conditions resulted in increased stability. Therefore, after isolation of the 355-1 metabolite by preparative HPLC, fractions were immediately acidified and frozen until freeze-drying. Despite those beneficial conditions, significant degradation to ergine (approximately 84%) was observed. Equally, NMR analysis was not possible because of gradual degradation of the 355 metabolites during measurement. Consequently, tentative structure elucidation was carried out by accurate mass measurements in positive ionisation mode of the up-concentrated preparative HPLC fractions and the degradation samples. HR-MS measurements in the full scan mode of the 355 metabolites with retention times of 5.33 and 5.65 min yielded an accurate mass of 355.1532 and the proposed sum formula was $C_{19}H_{21}N_3O_4$. The accurate mass obtained by QTOF full scan measurements of both epimeric forms of the 383 metabolites with retention times of 7.94 and 8.82 min was 383.1845 which correspond to a molecular formula of $C_{21}H_{25}N_3O_4$. In all full scan measurements, the deviation of the measured from the exact mass was <2 ppm, in all MS/MS measurements <5 ppm. The major fragments of the 355 metabolites, the 383 metabolites and ergine observed in HR-MS/MS measurements are summarised in Table 3.

Table 3. Fragmentation of the precursors *m/z* 384.1918, *m/z* 356.1605 and *m/z* 268.1444 (CE = 30 eV). Relative intensities are defined as intensity of the fragment ion in percent of the basepeak fragment ion.

Product ion, exact (*m/z*)	Relative intensity (%) Precursor ion (*m/z*)			Proposed formula of the product ion
	384.1918	356.1605	268.1444	
268.14499	27	40	5	$[C_{16}H_{18}N_3O]^+$
225.10279	20	20	8	$[C_{14}H_{13}N_2O]^+$
223.12352	100	100	100	$[C_{15}H_{15}N_2]^+$
221.10787	8	20	11	$[C_{15}H_{13}N_2]^+$
208.07624	27	50	46	$[C_{14}H_{10}NO]^+$
197.10787	13	10	28	$[C_{13}H_{13}N_2]^+$
192.08132	8	20	18	$[C_{14}H_{10}N]^+$
180.08132	13	20	41	$[C_{13}H_{10}N]^+$

According to the stereochemistry of the ergopeptines compared to the ergopeptinines, the 355-1 and the 383-1 metabolites represented the left-hand rotation isomers (C-8-(R) configuration), whereas the 355-2 and 383-2 metabolites belonged to the right-hand rotation isomers (C-8-(S) configuration). The left-hand rotation isomers were the main compounds formed upon degradation of ergopeptines (see Figure 3 for ergotamine). In a degradation experiment of ergotaminine by ErgA performed to confirm that the stereochemistry of ergoline ring containing metabolites is not changed during microbial or enzymatic degradation, the 355-2 metabolite was the main reaction product (3% 355-1 peak area compared to 355-2).

Decomposition of the 355 and 383 metabolites to ergine was assumed to result in a second cleavage product. Isopropylglyoxylic acid (dimethyl pyruvic acid) was expected to occur as decomposition product of the 383 metabolites. A compound with an accurate mass of 116.0473 corresponding to the sum formula $C_5H_8O_3$ was measured

(error <2 ppm) in standard solutions of isopropylglyoxylic acid and in degradation samples of ergocryptine by MTHt3 lysate by HR-MS in the negative ionisation mode.

Pyruvic acid was expected to occur as decomposition product of the 355 metabolites. However, UHPLC-HR-MS measurements of concentrated preparative HPLC fractions of the 355-1 metabolite in which ergine could be detected did not reveal any additional metabolite. One possible reason might be low retention of small ionic compounds under the chosen HPLC conditions. Therefore, the same samples and standard solutions of pyruvic acid were measured by direct infusion HR-MS in the negative ionisation mode. Under these conditions, a compound with an accurate mass of 88.0160 corresponding to a sum formula of $C_3H_4O_3$ was detected. Considering decomposition of the 355-1 metabolite to ergine and pyruvic acid and adding results from HR-MS measurements, a structure of the 355-1 metabolite was proposed (Figure 1).

Summary of the degradation reactions

Figure 1 shows the overall degradation reaction of ergotamine by the enzyme ErgA and by the lysate of the strain MTHt3. The strain MTHt3 as well as the lysate also contain the enzyme ErgB, which is responsible for further metabolisation of ergine to lysergic acid. A common feature of all investigated ergopeptines is that they differ only in the residues at C2' (R1 at ring E of the peptide part) and at C5' (R2 at ring F of the peptide part), respectively. In Figure 1, the two substituents R1 and R2 are circled. These differences are also reflected in the formed metabolites, which are summarised in Table 4.

In order to exclude side reactions and to confirm quantitative formation of DKP metabolites (the common end products upon degradation of ergopeptines by lysate and enzyme), the concentration of both 244 metabolites was determined in a solution of ergotamine degraded by the lysate of MTHt3. Within 30 min a nearly complete degradation of ergotamine and ergotaminine was observed and simultaneously, 244-2 and afterwards 244-1 were formed. From 3-24 h, the molar concentrations of 244-1 and 244-2 were 96% and 10%, respectively, of the initial molar concentration of ergotamine. The 244-1 metabolite represents a final product of this reaction and reached its maximum molar concentration after 3 h.

4. Discussion and conclusion

The six different ergopeptines (ergocornine, ergocristine, α-ergocryptine, ergosine, ergotamine and ergovaline) and one ergopeptinine (ergotaminine) were incubated with different biocatalysts. To this end, an LC-MS/MS method for the determination of the precursors, intermediates and products of the reaction had to be developed. Different eluents and columns were used to optimise the chromatographic conditions. Strain *R. erythropolis* MTHt3 (isolated from a soil sample) as well as the lysate degraded the investigated ergopeptines in a two-step metabolisation. The first step catalysed by ErgA cleaved ergopeptines into two products: DKPs (named 196, 210 and 244 metabolites) and intermediate metabolites termed 355 and 383 metabolites. The latter decomposed spontaneously to ergine and an oxocarboxylic acid (pyruvic and isopropylglyoxylic acid, respectively) due to instability. In the second degradation step, which is catalysed by ErgB, ergine was cleaved to lysergic acid. Increased *in vitro* transport across ruminant gastric tissue was stated for lysergic acid (Hill *et al.*, 2001). Nevertheless, lysergic acid showed low toxicity based on LD_{50} values after intravenous injection (EFSA, 2012) and no bioaccumulation of lysergic acid in contrast to ergovaline was proven (Klotz *et al.*, 2009). Furthermore, the vasoconstrictive effects are 1000 times less pronounced with lysergic acid compared to ergovaline (Klotz *et al.*, 2008). DKPs corresponding to cyclic dipeptides were reported to occur frequently in nature (Yang *et al.*, 2009) and have been suggested to represent a new class of quorum-sensing molecules for cell-density dependent signalling mechanisms used by bacteria (Campbell *et al.*, 2009). Furthermore, the antimicrobial activity of various DKPs was observed (210-1, 210-2, 244-1 and 244-2 metabolites) (Kumar *et al.*, 2013) and it was demonstrated that cyclo(S-Pro-S-Leu) (210-2 metabolite) inhibits aflatoxin production from the fungus *Aspergillus parasiticus* (Yan *et al.*, 2004). DKPs are generally believed to be non-toxic. However, mild cytotoxicity at

Table 4. Investigated ergot alkaloids, their characteristic substituents (R1 and R2) and the resulting metabolites.

Ergopeptine	R1	R2	Ergine hydroxy carboxylic acids	Diketopiperazines	Oxo carboxylic acid
Ergotamine	CH_3	$CH_2C_6H_5$	355 metabolite $C_{19}H_{21}N_3O_4$	244 metabolite $C_{14}H_{16}N_2O_2$	Pyruvic acid $C_3H_4O_3$
Ergovaline	CH_3	$CH(CH_3)_2$	355 metabolite $C_{19}H_{21}N_3O_4$	196 metabolite $C_{10}H_{16}N_2O_2$	Pyruvic acid $C_3H_4O_3$
Ergosine	CH_3	$CH_2CH(CH_3)_2$	355 metabolite $C_{19}H_{21}N_3O_4$	210 metabolite $C_{11}H_{18}N_2O_2$	Pyruvic acid $C_3H_4O_3$
Ergocristine	$CH(CH_3)_2$	$CH_2C_6H_5$	383 metabolite $C_{21}H_{25}N_3O_4$	244 metabolite $C_{14}H_{16}N_2O_2$	Isopropylglyoxylic acid $C_5H_8O_3$
Ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$	383 metabolite $C_{21}H_{25}N_3O_4$	196 metabolite $C_{10}H_{16}N_2O_2$	Isopropylglyoxylic acid $C_5H_8O_3$
Ergocryptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	383 metabolite $C_{21}H_{25}N_3O_4$	210 metabolite $C_{11}H_{18}N_2O_2$	Isopropylglyoxylic acid $C_5H_8O_3$

100 µM for cyclo(S-Pro-S-Val) (196-2 metabolite) was observed (Kumar *et al.*, 2014) and one report of possible toxicity was published for two cyclic dipeptides (the 196 metabolite cyclo(-Pro-Val-) and cyclo(-Pro-Tyr-)) which were reported to be toxic *in vitro* to Japanese black pine suspension cells and seedlings of *Pinus thunbergii* (Guo *et al.*, 2007).

The 196-1, 210-1 and 244-1 epimers were formed predominantly by the lysate after minor initial formation of 196-2, 210-2 and 244-2. In contrast, the 196-2, 210-2 and 244-2 epimers were formed mainly by ErgA, which did not cause formation of the 196-1, 210-1 and 244-1 epimers. On the basis of these data, it was concluded that in a first step, 196-2, 210-2 and 244-2 are formed during degradation of ergopeptines by ErgA. Another unknown enzyme expressed by the strain MTHt3 and also present in the lysate is assumed to be responsible for the subsequent conversion into the 196-1, 210-1 and 244-1 epimers.

NMR analysis of the isolated 355-1 metabolite was not possible because of gradual degradation to ergine. Consequently, tentative structure elucidation was carried out by accurate mass measurements. The eight most abundant fragments of the 355 and the 383 metabolites are consistent with the most abundant fragments of ergine which indicates a similar structure of these three compounds. The 355 metabolites, the 383 metabolites as well as ergine that still contain the ergoline ring showed common fragment ions characteristic for the tetracyclic ergoline ring system (e.g. *m/z* 268, 223, 208, 180), which were also described repeatedly in the literature (Lehner *et al.*, 2004). Recently, a holistic approach for the identification of less studied or novel ergot alkaloids based on HR-MS and ion trap MS technology to study the fragmentation pattern was published (Arroyo-Manzanares *et al.*, 2014). In this study, the product ion *m/z* 223 was common for all ergopeptines and proved to be the most abundant fragment. The proposed structures of the fragment ions *m/z* 268.14499, *m/z* 223.12352, *m/z* 208.07624 and *m/z* 180.08132 are in line with the structure assignment performed by the MassHunter MSC software based on targeted-MS/MS measurements of the precursor *m/z* 384.1918, *m/z* 356.1605 and *m/z* 268.1444 (see Table 3 for the proposed sum formulas and the relative intensities of the observed product ions).

Both oxocarboxylic acids (isopropylglyoxylic and pyruvic acid) have already been described in the literature as degradation products after alkaline and acidic hydrolysis of the ergopeptines investigated in the current study (Stoll, 1945).

In summary, two main groups of metabolites were formed during microbial and enzymatic degradation of various ergopeptines. On the one hand, unstable ergine hydroxy carboxylic acids which spontaneously degrade to ergine were detected. On the other hand, formation of DKPs, which are probably non-toxic cyclic dipeptides, was observed. An analytical method for the determination of the ergopeptines and their degradation products has successfully been developed. Structure elucidation was essential to explore the mechanism of the enzymatic degradation reaction. The DKP metabolites will be needed for future experiments studying enzyme kinetics. After development and establishment of a large-scale production process with suitable expression systems, the utilisation of the enzymes as a feed additive for ergopeptine and ergine degradation seems practicable. Whether or not the usage of the enzymes as potential feed additive is safe as well as efficient is the scope of further experiments, including animal trials.

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Publication #2

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The candidate conducted all incubation experiments and was responsible for the LC-UV-MS/MS analysis. Furthermore, the author of this thesis interpreted all generated data and evaluated the potential of the tested feed additives to reduce DON and ZEN. The candidate wrote the manuscript, which was then revised by all co-authors.

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Aerobic and anaerobic *in vitro* testing of feed additives claiming to detoxify deoxynivalenol and zearalenone

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Deoxynivalenol (DON) and zearalenone (ZEN) are mycotoxins produced by fungi of the genus *Fusarium* which frequently contaminate maize and grain cereals. Mycotoxin-contaminated feed endangers animal health and leads to economic losses in animal production. Several mycotoxin elimination strategies, including the use of commercially available DON and ZEN detoxifying agents, have been developed. However, frequently there is no scientific proof of the efficacy of such adsorbents and degrading products. We therefore tested 20 commercially available products claiming to detoxify DON and/or ZEN either by biodegradation (4 products) or a combination of degradation and adsorption (16 products) under aerobic and anaerobic conditions at approx. pH 7. Under the applied conditions, a complete reduction of DON and consequent formation of the known non-toxic metabolite DOM-1 was exclusively observed in samples taken from the anaerobic degradation experiment of one product. For all other products, incubated under aerobic and anaerobic conditions, a maximum DON reduction of 17% after 72 h of incubation was detected. Aerobic and anaerobic incubation of only one tested product resulted in complete ZEN reduction as well as in the formation of the less-toxic metabolites DHZEN and HZEN. With this product, 68–97% of the toxin was metabolised within 3 h. After 24 h, a ZEN reduction ≥ 60% was obtained with four additional products during aerobic incubation only. Six of the 20 investigated products produced α- and/or β-ZEL, which are metabolites showing similar oestrogenic activity compared to ZEN. Aerobic and anaerobic degradation to unknown metabolites with unidentified toxicity was obtained with 10 and 3 products, respectively. The results of our study demonstrate the importance of *in vitro* experiments to critically screen agents claiming mycotoxin detoxification.

Keywords: degradation; trichothecenes; *Fusarium*; mass spectrometry; mycotoxins

Introduction

Both deoxynivalenol (DON), a mycotoxin belonging to the group of type B trichothecenes, as well as zearalenone (ZEN) are produced by fungi of the genus *Fusarium* (e.g. *F. graminearum*). As frequent contaminants of maize and small grain cereals in Europe (Bottalico & Perrone 2002; EFSA 2013; Marin et al. 2013) they are often co-occurring. The toxicity of DON is based on the inhibition of eukaryotic protein synthesis and is associated with acute adverse gastrointestinal effects such as vomiting both in animals and humans. The main long-term effects on animals exposed to DON are anorexia, altered nutritional efficiency and reduced weight gain (Pestka 2007). In addition to ZEN, the related epimeric metabolites alpha-zearalenol (α-ZEL) and beta-zearalenol (β-ZEL) are formed naturally by fungi (Richardson et al. 1985). Although relatively low acute toxicity is observed, ZEN as well as α- and β-ZEL show oestrogenic properties (Minervini et al. 2005). The oestrogenic activity of those compounds is based on structural similarities to the female hormone 17β-estradiol and subsequent competitive binding to oestrogen receptors. In animals, especially in pigs, which are one of the most susceptible animal species, dietary ZEN exposure results in reproductive disorders, carcinogenicity, genotoxicity, endocrine effects as well as immunotoxicity, reduced embryo survival, vulvar dilatation, retention or absence of milk and rectal prolapse (Zinedine et al. 2007).

Mycotoxin-contaminated feed for livestock may severely endanger health and productivity (D'Mello et al. 1999). Consequently, many different strategies for counteracting mycotoxins, including prevention as well as elimination techniques, have been developed. Elimination strategies based on the application of feed additives range from adsorption in the gastrointestinal tract of animals by silica-based inorganic compounds, carbon-based organic polymers and yeast cell-wall derived binders (EFSA Report 2009) to microbial or enzymatic approaches aiming to biotransform and degrade mycotoxins to non-toxic metabolites *in vivo*. DON and ZEN are well known to be biotransformed by different microorganisms (reviewed by McCormick 2013). For example, degradation of DON to de-epoxy-deoxynivalenol (DOM-1) based on de-epoxidation of trichothecenes into a diene by rumen

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microorganisms was reported (King et al. 1984) and has been studied intensively (Binder et al. 1997; Fuchs et al. 2002). Furthermore, Ikunaga et al. (2011) isolated a DON-degrading *Nocardioides* strain and identified the novel metabolite 3-*epi*-DON. Concerning ZEN degradation, Vekiru et al. (2010) investigated biotransformation into a non-oestrogenic metabolite termed ZOM-1 by the yeast *Trichosporon mycotoxinivorans*. In addition, formation of the non-oestrogenic metabolites hydrolysed zearalenone (HZEN; Fruhauf et al. manuscript in preparation) and decarboxylated hydrolysed zearalenone (DHZEN) by a *Gliocadium roseum* strain (El-Sharkawy & Abul-Hajj 1988) and the responsible isolated enzyme lactonohydrolase was demonstrated (Takahashi-Ando et al. 2002).

Many different feed additives which claim to reduce certain mycotoxins after consumption of contaminated feed in the gastrointestinal tract of animals are available commercially. The practical application of such products to reduce DON contamination has often been questioned. Insufficient effectiveness of several commercially available adsorbents and other materials merchandised as DON-detoxifying agents for contaminated feed has been shown (Döll et al. 2004; Avantaggiato et al. 2007; Awad et al. 2010; Dänicke & Döll 2010). For ZEN, controversial results are reported. While almost complete reduction by mineral clays or humic substances was reported (Sabater-Vilar et al. 2007), insufficient adsorption of ZEN by commercial binder materials based on yeast cell-wall was demonstrated (Fruhauf et al. 2012). Only a few *in vitro* studies with enzymatic and/or microbial-based commercial products testing possible ZEN detoxification were published scientifically. For example, Döll et al. (2004) demonstrated ZEN reduction by 5% and 17% for two commercially available products with a mode of action based on adsorption and enzymatic degradation.

Studies evaluating the performance of feed additives for DON and/or ZEN reduction are limited. Therefore, the aim of the current study was to test and evaluate the efficacy of 20 commercially available products that claim *in vivo* detoxification of DON and ZEN by biodegradation or a combination of degradation and adsorption. Consequently, aerobic and anaerobic degradation experiments were performed for 72 h to establish the conditions enabling mycotoxin degradation by microorganisms as well as by enzymes and/or mycotoxin adsorption by binding materials present in the tested feed additives. This study raises the awareness on numerous ineffective products currently on the market and emphasises the need for proper scientific evidence to prove the claims of those products.

Materials and methods

Reagents and standards

Acetonitrile (ACN) and methanol (MeOH) (both LC grade) were purchased from VWR International GmbH (Vienna, Austria). Water was purified with a Purelab Ultra system (ELGA LabWater, Celle, Germany). All chemicals used for preparation of cultivation media and buffer solutions as well as ammonium acetate (MS grade) were purchased from Sigma-Aldrich (Vienna, Austria). The ZEN metabolites HZEN, DHZEN and ZOM-1 were prepared after isolation on a preparative HPLC system as described by Kunz-Vekiru (2009), whereas stock solutions of DON, DOM-1, ZEN, α-ZEL and β-ZEL were obtained from Romer Labs GmbH (Tulln, Austria).

Feed additives and positive controls

We have chosen 20 commercially available products claiming DON and/or ZEN detoxification by microbial or enzymatic processes. Additional binding is claimed for some of these products. The materials were tested *in vitro* under aerobic and anaerobic conditions. In the case of three products (products no. 3, 4 and 5), two different batches were analysed to investigate product variations. Product details including ingredients and mode of action (as stated on the product labels and/or manufacturers' webpages) are listed in Table 1. The claimed composition of the products was as follows: product 1, potentiated bentonite and enzyme complex (xylanase, beta-glucanase and pectinase); product 2, various enzymes; product 3, Na-Ca-aluminosilicate, yeast wall extract (mannan-oligosaccharides (MOS) and beta-glucans) and *Saccharomyces telluris* strains (CH 7, CH 12, CH 23 and CH 27); product 4, clinoptilolite, *Saccharomyces cerevisiae* (*S. cer.*), enzymes (esterase and epoxidase) and vitamin E; product 5, enzymes (epoxidase and esterase), hydrated sodium calcium aluminosilicate (HSCAS), chitosan, vitamin C and natural extracts; product 6, HSCAS, enzymatic complex (amylase and protease), esterified glucomannans, ammonium acetate, propionic and acetic acids; product 7, enzymes, vitamins, prebiotics and liver protectors; product 8 (liquid product), *S. cer.* extract, bentonite, citric, phosphoric, lactic and formic acids, propylenglycol, probiotics (*Lactobacilli* sp., *Enterococcus faecium, Bifidobacteria* and *Streptococcus* sp.), amino acids, vitamins and minerals; product 9 (liquid product), enzymes, bacteria dried extract, fructooligosaccharides, natural plant extracts, organic acids (e.g. lactic acid), *S. cer.*, vitamins and minerals; product 10, epoxide reductase, peptidase and esterase; product 11, HSCAS, inactivated *S. cer., S. cer.* extract, calcium propionate, antioxidants mixture and botanicals; product 12, bentonite, diatomaceous earth, inactivated yeast, phycophytic substance, plant extracts and BBSH 797; product 13, HSCAS, *S. cer.*, MOS, betaglucan, enzymes, fructooligosaccharides, probiotics (*L. acidophilus, Bifidobacterium longhum* and *thermophilum* and *Streptococcus faecium*) and prebiotics; product 14, anti-caking bentonite,

Table 1. Tested products, claimed composition and toxin related mode of action (stated on the product labels and/or manufacturers' webpages).

	Claimed ingredients						Claimed mode of action		
Product no.	Enzymes	Bacteria	Yeast and yeast components	Clay minerals	Natural extracts	Vitamins	Adsorption	Enzymatic degradation	Others
1	x			x			x		Enzymatic conversion by addition of polar groups
2	x							x	
3			x	x			x		Biotransformation
4	x		x	x		x	x	x	
5	x			x	x	x	x	x	Transformation
6	x			x			x		Biotransformation by enzymatic activity
7	x					x	x		Decomposition
8		x	x	x		x	x	x	"Bio-inactivation"
9	x	x	x	x	x	x	x		Decomposition
10	x							x	
11			x	x			x	x	"Bio-inactivation"
12		x	x	x	x			x	
13	x	x	x	x			x	x	
14			x	x	x		x		Chemical biotransformation
15			x					x	
16				x	x		x		"Bioneutralisation"
17				x	x		x		"Bioneutralisation"
18				x			x	x	
19			x	x			x		Biotransformation
20			x	x	x	x	x		Biotransformation

diatomaceous earth, silicon dioxide, gelling agent lecithin, dehydrated yeast, flavouring *Tagetes erecta* oily extract and calcium carbonate; product 15, *S. cer.*; product 16, herbal extracts, propionic and acetic acids, clinoptilolite; product 17, herbal extracts, clinoptilolite; product 18, calciumcarbonate, magnesium oxide, bentonite, montmorillonite, E321, propyl gallate and ethoxyquin; product 19, a combination of aluminosilicates, yeast cell-wall extract and antioxidants; product 20, sodium aluminosilicate, calcium aluminosilicate, dried yeast culture extract, dried molasses, vitamin A, vitamin E, manganese sulphate and vegetable oil.

Products no. 1, 3 (batch 1), 4 (batch 1), 5 (batch 1), 7, 8, 9, 14, 16, 17, 18 and 19 were purchased commercially, whereas products no. 2, 3 (batch 2), 4 (batch 2), 5 (batch 2), 6, 10, 11, 12, 13, 15 and 20 were received from a cooperation partner.

All used positive controls were obtained from the Biomin Research Center (Tulln, Austria) for quality control purposes during the experimental work. An aerobic DON-degrading bacterium was used as an aerobic DON positive control, whereas during anaerobic incubation a lyophilisate of the anaerobic DON-biotransforming strain BBSH 797 (Fuchs et al. 2002; Commission Implementing Regulation (EU) No 1016/2013) was served as a positive control. In addition, a ZEN-degrading lactonohydrolase, catalysing the degradation of ZEN to HZEN and DHZEN (Kunz-Vekiru 2009), was chosen as aerobic and anaerobic ZEN positive control.

LC-UV-MS/MS analysis

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) for the determination of DON, DOM-1, ZEN, α-ZEL, β-ZEL and ZOM-1 was performed on a 1100 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany), including a diode array detector (DAD) coupled to a QTrap mass spectrometer (AB Sciex, Foster City, USA) equipped with a heated nebuliser atmospheric-pressure chemical ionisation (APCI) source. Chromatographic separation was achieved at 25°C using an RP Gemini C18 column (150 × 4.6 mm i.d., 5 µm particle size, Phenomenex, Aschaffenburg, Germany) equipped with a 4 mm × 3 mm C18 security guard cartridge. The injection volume was 15 µl and a flow rate of 0.8 ml min^{-1} was used. Eluents were composed of methanol/water 20/80 (v/v; eluent A) and 90/10 (v/v; eluent B), both containing 5 mM ammonium acetate. The gradient started at 100% solvent A and continued with a linear increase to 90% eluent B from 0.1 to 2.0 min. 100% B was reached at 3 min and was held for a further 2.9 min. At 6.0 min the initial conditions were re-established and held for 2 min for re-equilibration. The

Table 2. Mass transitions and SRM parameters of investigated analytes.

Analyte	Precursor ion (*m/z*)	DP (V)	Product ions[a] (*m/z*)	CE[a] (eV)
DON	355.1	−16	59.1/265.0	−30/−12
DOM-1	339.1	−21	59.1/249.1	−40/−16
ZEN	317.1	−61	130.9/175.1	−40/−34
ZOM-1	351.2	−21	237.0/218.9	−16/−24
ZELs	319.2	−71	275.1/129.9	−22/−48

Notes: DON, deoxynivalenol; DOM-1, de-epoxy-deoxynivalenol; ZEN, zearalenone; ZOM-1, non-oestrogenic metabolite produced by the yeast *Trichosporon mycotoxinivorans*; ZELs, alpha- and beta-zearalenol; DP, declustering potential; CE, collision energy.
[a] Values are given in order quantifier ion, qualifier ion.

APCI interface was used in negative ion mode at 450°C with the following settings: curtain gas (CUR) 40 psi, nebuliser gas (GS1) 60 psi, auxiliary gas (GS2) 15 psi, corona discharge needle current (NC) −2 µA, collision-activated dissociation gas (CAD) 6 (arbitrary units, corresponding to the pressure of collision gas (nitrogen) in Q2), MRM dwell time 25 ms, pause between mass ranges 5 ms.

MS and MS/MS parameters (selected reaction monitoring mode (SRM) transitions, declustering potentials as well as collision energies) were optimised for all analytes in the negative electrospray ionisation (ESI) mode. The parameters of the optimised SRM transitions are summarised in Table 2. UV signals were recorded at 220 nm and 270 nm (for detection of toxins or product-related UV-absorbing compounds).

LC-MS/MS measurements to quantify HZEN (precursor ion: *m/z* 335.0, quantifier: *m/z* 149.0, qualifier: *m/z* 161.0) and DHZEN (precursor ion: *m/z* 291.1, quantifier: *m/z* 149.0, qualifier: *m/z* 161.0) were performed in the SRM mode on a 1290 Infinity ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies) coupled to a 4000 QTrap tandem mass spectrometer (AB Sciex) equipped with a Turbo V ESI source in negative ionisation mode. Chromatographic separation was carried out at a flow rate of 250 µl min^{-1} on a Kinetex C18 column (150 × 2.1 mm i.d., 2.6 µm particle size, Phenomenex) protected by a C18 security guard pre-column. The eluents contained 5 mM ammonium acetate and were composed of methanol/water 20/80 (v/v; eluent A) and 98/2 (v/v; eluent B), respectively. The injection volume was 5 µl and the gradient was set as follows: 0–0.1 min hold at 30% B; 0.1–8.9 min increase to 90% B; 8.9–9 min increase to 100% B, 9–11.9 min hold at 100% B; 11.9–12 min return to 30% B; 12–14 min 30% B for re-equilibration.

For external calibration, stock solutions of each single analyte (100 mg l^{-1}) were prepared in ACN or ACN/water (30/70, v/v). A multi-analyte stock solution was prepared by mixing the single analyte solutions of DON, DOM-1, ZEN, HZEN, DHZEN, α-ZEL, β-ZEL and ZOM-1, resulting in a concentration of 10 mg l^{-1}, respectively. This solution was further diluted in ACN/water (30/70, v/v) to obtain concentrations of 1, 3, 10, 30, 100, 300, 1000 and 3000 ng ml^{-1} for all analytes. The method was linear for DON and DOM-1 between 30 and 3000 ng ml^{-1} as well as between 1 and 1000 ng ml^{-1} for all other analytes. Only standards above the LOQ were used for quantification of the respective analytes.

In order to evaluate the effects of the matrix on mass spectrometric quantification, aerobic and anaerobic buffer solutions were each spiked at seven different concentration levels in a range of 1–1000 ng ml^{-1} for each analyte (termed matrix-matched standards). The matrix-matched standards as well as neat solvent standards were measured twice by LC-MS/MS and calibration curves were established. Signal suppression or enhancement (SSE) was calculated by dividing the slopes of the calibration curves gained for the matrix-matched standards by the slopes gained for liquid standards and multiplying by 100. SSE values < 100% indicated signal suppression, whereas SSE values > 100% revealed signal enhancement.

Limits of detection (LODs) and limits of quantification (LOQs) of each analyte were calculated at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively, based on matrix-matched calibration in aerobic and anaerobic buffer solutions.

Incubation experiments

Incubation experiments of DON and ZEN were performed simultaneously in buffer solutions in triplicate. Fifty milligrams of each product were added to 10 ml buffer solution (0.5%, w/v) containing 5 mg l^{-1} DON as well as 2 mg l^{-1} ZEN and incubated under aerobic or anaerobic conditions for 72 h at 37°C. The pH values of the incubated product batches were measured in the respective medium.

For aerobic incubation the suspensions were shaken at 200 rpm in Erlenmeyer flasks. The used buffer solution (pH 7) was composed of DSMZ 457 Brunner medium/vitamin solution (90/10, v/v). The vitamin solution which was added after autoclaving (at 121°C for 15 min) contained the following: biotin, 2 mg l^{-1}; folic acid, 2 mg l^{-1}; pyridoxine-HCl, 10 mg l^{-1}; thiamine-HCl × 2 H_2O, 5 mg l^{-1}; riboflavin, 5 mg l^{-1}; nicotinamide, 5 mg l^{-1};

D,L-pantotheinate, 5 mg l^{-1}; *p*-aminobenzoic acid, 5 mg l^{-1}; vitamin B_{12}, 0.1 mg l^{-1}; menadione, 100 mg l^{-1}; vitamin K_1, 22 mg l^{-1}; thioctic acid, 5 mg l^{-1}.

For anaerobic incubation a buffer solution (titrated to pH 6.8 with 4M sodium hydroxide solution), composed of minerals (K_2HPO_4, 0.45 g l^{-1}; KH_2PO_4, 0.45 g l^{-1}; $(NH_4)_2SO_4$, 0.45 g l^{-1}; NaCl, 0.9 g l^{-1}; $MgSO_4 \times 7\ H_2O$, 0.19 g l^{-1}; $CaCl_2 \times 2\ H_2O$, 0.23 g l^{-1}), vitamin solution (composition previously described; 10%, v/v), trace elements ($ZnSO_4 \times 7\ H_2O$, 50 µg l^{-1}; $MnCl_2 \times 7\ H_2O$, 15 µg l^{-1}; H_3BO_3, 150 µg l^{-1}; $CuCl_2 \times 2\ H_20$, 5 µg l^{-1}; $CoCl_2 \times 6\ H_2O$, 100 µg l^{-1}; $NiCl_2 \times 6\ H_2O$, 10 µg l^{-1}; $Na_2MoO_4 \times 2\ H_2O$, 15 µg l^{-1}), 5 mg l^{-1} hemin, 0.75 g l^{-1} L-cystein hydrochloride and 0.25 g l^{-1} Na_2S, was used. After preparation the buffer solution was autoclaved at 121°C for 15 min. Finally, 2.5% (v/v) of a 1M phosphate buffer (pH 6.9) was added by sterile filtration directly into the buffer medium. The product suspensions were incubated anaerobically under H_2 atmosphere in 25-ml glass flasks.

Sample aliquots were taken directly after addition of the products (0 h) and after 1, 3, 24, 48 and 72 h of incubation time. The same volume of ACN was added for microbial and/or enzymatic inactivation. Finally, the samples were clarified by centrifugation (2655 × *g*, 20°C, 10 min), the supernatants were transferred into glass vials and stored at −20°C until LC-UV-MS/MS measurement.

For the aerobic DON positive control, the aerobic DON-degrading bacterium was cultivated in DSMZ 830c R2A liquid medium for 24 h, transferred into the mineral medium prepared according to Ikunaga et al. (2011) and induced by repeated DON spiking of 10 mg l^{-1}. After 4 days of incubation, biomass from 10 ml of the fermentation broth was harvested by centrifugation (20,000 × *g*, 10 min, 10°C), washed and suspended in 10 ml aerobic buffer for further degradation experiments. Fifty milligrams of the BBSH 797 lyophilisate was used as anaerobic DON positive control. The ZEN positive control was prepared by adding 1 mg of the lyophilised lactonohydrolase to 10 ml of DON and ZEN containing buffer (0.01%, w/v) and incubated as described above.

In addition, negative controls of the respective liquid media containing only the toxins were incubated aerobically as well as anaerobically to prove toxin stability and absence of metabolites formed during incubation as well as to determine the toxin concentration at incubation start and over time. Negative controls containing solely the products in buffer were essential to subsequently differentiate whether chromatographic peaks not originating from DON and ZEN were related to the products or to the formation of new metabolites.

Data evaluation

For data analysis, Analyst 1.5.2 (AB Sciex) was used. Quantitative analysis was carried out on the basis of linear calibration curves (peak area versus concentration). The reduction of DON and ZEN (in %) during incubation experiments was calculated by dividing the DON or ZEN concentrations in samples taken after 1, 3, 24, 48 and 72 h of incubation by the DON or ZEN concentration in the samples taken directly after product addition (0 h) and multiplying by 100. Using three independent incubation experiments per product, the average values and standard deviations for each sampling time point were calculated. In the case of aerobic incubation, solvent evaporation was corrected prior to calculations by considering the concentration factor of the toxin in the respective negative control sample during incubation. DON or ZEN reduction was set to 0% in case of calculated negative reduction values.

Results and discussion

LC-UV-MS/MS analysis

Matrix-matched standards as well as neat solvent standards were measured twice by LC-MS/MS for evaluation of matrix effect and determination of LODs and LOQs. The observed average SSEs in aerobic buffer solution were: DON 94 ± 5%, DOM-1 96 ± 8%, ZEN 94 ± 3%, HZEN 86 ± 6%, DHZEN 95 ± 5%, α-ZEL 89 ± 11%, β-ZEL 98 ± 0% and ZOM-1 77 ± 3%, whereas average SSEs of 99 ± 4% for DON, 97 ± 7% for DOM-1, 90 ± 5% for ZEN, 92 ± 7% for HZEN, 106 ± 17% for DHZEN, 87 ± 21% for α-ZEL, 102 ± 5% for β-ZEL and 85 ± 0% for ZOM-1 in anaerobic buffer were calculated. As the observed matrix effects were between 77% and 106%, we did not correct the obtained results for matrix effects. In addition, as all results are given as toxin reduction in % correlated to the samples taken directly after product addition (0 h), matrix effects were compensated by this approach. LODs and LOQs in aerobic buffer solution were: DON 12 and 56 ng ml^{-1}, DOM-1 12 and 40 ng ml^{-1}, ZEN 19 and 62 ng ml^{-1}, HZEN 0.3 and 1.1 ng ml^{-1}, DHZEN 5.5 and 23 ng ml^{-1}, α-ZEL 49 and 189 ng ml^{-1}, β-ZEL 40 and 165 ng ml^{-1}, ZOM-1 48 and 155 ng ml^{-1}. In anaerobic buffer solution the following LODs and LOQs were yielded: DON 16 and 79 ng ml^{-1}, DOM-1 13 and 68 ng ml^{-1}, ZEN 14 and 46 ng ml^{-1}, HZEN 0.4 and 1.2 ng ml^{-1}, DHZEN 5.8 and 12 ng ml^{-1}, α-ZEL 34 and 117 ng ml^{-1}, β-ZEL 36 and 169 ng ml^{-1}, ZOM-1 41 and 148 ng ml^{-1}.

Incubation experiments

For many commercially available products *in vivo* mycotoxin reduction is claimed either by microbial or

enzymatic metabolisation of the toxins or by their irreversible adsorption. In the case of toxin metabolisation, formation of non-toxic metabolites or complete toxin degradation should be expected. Yet, the majority of manufacturers of such products provide only little or even no information regarding this aspect. However, generalised, vague or even non-scientific modes of action (like decomposition, "bio-inactivation" or "bioneutralisation") are often stated by the manufacturers (see Table 1) and mycotoxin-dependent differences in the mode of action are mostly not specified.

In our study, 20 commercially available products with the claim to detoxify DON and/or ZEN by metabolisation and additional adsorption were tested under aerobic and anaerobic conditions. Recently, testing conditions (pH value, toxin as well as binder concentration) for *in vitro* studies to demonstrate aflatoxin B_1-binding by bentonites were defined by an EURL-evaluated method (Commission Implementing Regulation (EU) No 1060/2013). In contrast, there are no official methods established that describe uniform efficacy testing conditions for feed additives claiming to detoxify DON or ZEN. Therefore, different experimental setups have been published. The methods used range from adsorption tests in aqueous (Galvano et al. 1998) or buffer solutions at different pH values (Avantaggiato et al. 2005; Sabater-Vilar et al. 2007) to incubation experiments promoting microbial growth (Bakutis et al. 2005) and *in vitro* models mimicking the gastrointestinal conditions of pigs (Döll et al. 2004; Avantaggiato et al. 2005; Kong et al. 2014).

In our study, a simple *in vitro* system was chosen to study the aerobic as well as anaerobic efficacy of feed additives claiming to detoxify DON and/or ZEN. An incubation temperature of 37°C was used to simulate *in vivo* conditions and an almost neutral pH value in buffer medium of universal composition was chosen to allow incubation of a broad range of microorganisms and/or enzymes. Experiments were performed for 0–72 h to establish conditions enabling mycotoxin degradation by aerobic and anaerobic microorganisms as well as by enzymes and/or mycotoxin adsorption. Time points of 48 and 72 h might be unrealistically long *in vivo*, but were included in our study to also monitor potentially slow growth of microorganisms under the conditions used.

Degradation experiments were performed in triplicate by weighing and incubating each product independently in three different flasks. The products were incubated for 72 h in buffer solution (pH 7; after product addition pH was in the range of 5.9–7.4) containing both toxins, DON (5 mg l^{-1}) and ZEN (2 mg l^{-1}), under aerobic as well as anaerobic conditions. Sample aliquots were taken directly after addition of the products (0 h) and after 1, 3, 24, 48 and 72 h of incubation time. Applying the described validated LC-MS/MS methods, besides DON and ZEN quantification, the possible formation of some known non-toxic metabolites such as DOM-1, ZOM-1, HZEN and DHZEN was monitored. Metabolites DOM-1 as well as HZEN and DHZEN were expected to be formed during incubation of the positive controls. α- and β-ZEL were measured as they are common metabolisation products of several microbial strains including *S. cerevisiae* (Scott et al. 1992), which was an ingredient of several products tested. Chromatographic UV data were recorded for screening of unknown UV-absorbing compounds indicating metabolisation. However, monitoring of formed metabolites was exclusively done at certain time points (sampling after 1, 3, 24, 48 and 72 h of incubation) and therefore some metabolites might have remained undetected.

The resulting DON and ZEN reductions are summarised in Table 3. The DON and ZEN concentration time courses (analysed by LC-MS/MS) as well as evaluation of metabolite formation (detected by LC-MS/MS and LC-UV) may allow differentiation between mycotoxin adsorption and metabolisation. In the case of adsorption, a fast toxin decrease within 1 h followed by stagnation in the absence of metabolites is typical, whereas a continuous and slower decrease as well as the formation of specific metabolites is characteristic for microbial toxin degradation. For enzymatic action the reaction depends on the specific enzymatic activity in the product and fast degradation might be observed.

Measurements of samples taken from the negative controls only containing the toxins in buffer solution demonstrated the stability of DON and ZEN for the entire incubation period.

In samples taken from the DON positive controls, complete DON reduction within 48 h of incubation was detected (aerobic – after 1 h: 22 ± 11%, after 3 h: 42 ± 8%, after 24 h: 89 ± 4%; anaerobic – after 1 h: 0 ± 3%, after 3 h: 3 ± 1%, after 24 h: 91 ± 3%). Furthermore, in case of the aerobic positive control two peaks of potentially new metabolites with retention times of 4.3 and 5.1 min (both with peak intensities > 10% compared to DON peak intensity) were observed by UV measurements at 220 nm. Equimolar DOM-1 formation was detected in samples taken from the anaerobic positive control (BBSH 797 lyophilisate). In samples taken from the ZEN positive controls (ZEN-degrading lactonohydrolase) a ZEN reduction of 88–100% was obtained after 3 h of incubation and equimolar HZEN and DHZEN formation was also detected.

In the case of DON, reduction values after 24 h of incubation are given in Table 3. Reduction after 1 and 3 h of incubation are not reported separately as no or only very minor DON reduction was encountered for all products. The sole exception was product no. 8 with a DON reduction of 31 ± 9% after 3 h under aerobic conditions. However, after 24 h no DON reduction was found for this product, indicating later release of the toxin due to very

Table 3. Resulting DON reduction (%) ± standard deviation (SD) (%) after 24 h and resulting ZEN reduction (%) ± SD (%) after 1, 3 and 24 h of incubation (three independent incubation experiments analysed).

		DON	ZEN		
Product no.	Condition	24 h	1 h	3 h	24 h
1	Aerobic	5 ± 6	46 ± 18	39 ± 17	53 ± 19
1	Anaerobic	5 ± 3	23 ± 9	36 ± 9	23 ± 30
2	Aerobic	3 ± 3	10 ± 11	3 ± 8	97 ± 4
2	Anaerobic	0 ± 14	0 ± 39	24 ± 17	N/A
3[a]	Aerobic	0 ± 11	0 ± 15	22 ± 21	52 ± 25
3[a]	Anaerobic	4 ± 4	N/A	N/A	N/A
4[a]	Aerobic	12 ± 8	9 ± 7	10 ± 9	63 ± 19
4[a]	Anaerobic	0 ± 7	9 ± 13	13 ± 6	5 ± 19
5[a]	Aerobic	6 ± 4	0 ± 3	1 ± 9	19 ± 9
5[a]	Anaerobic	3 ± 3	3 ± 10	2 ± 10	1 ± 13
6	Aerobic	6 ± 4	22 ± 2	29 ± 9	31 ± 12
6	Anaerobic	4 ± 5	10 ± 10	14 ± 8	2 ± 24
7	Aerobic	0 ± 4	22 ± 10	57 ± 13	22 ± 5
7	Anaerobic	4 ± 9	25 ± 6	44 ± 7	54 ± 12
8	Aerobic	0 ± 11	0 ± 9	45 ± 13	0 ± 10
8	Anaerobic	4 ± 1	0 ± 10	6 ± 16	0 ± 25
9	Aerobic	0 ± 3	0 ± 11	44 ± 16	0 ± 32
9	Anaerobic	5 ± 4	33 ± 24	2 ± 30	24 ± 15
10	Aerobic	0 ± 5	1 ± 17	N/A	61 ± 15
10	Anaerobic	3 ± 4	N/A	N/A	N/A
11	Aerobic	0 ± 5	21 ± 7	34 ± 27	37 ± 4
11	Anaerobic	6 ± 14	21 ± 15	36 ± 18	0 ± 26
12	Aerobic	3 ± 3	75 ± 3	97 ± 1	100 ± 0
12	Anaerobic	97 ± 1	28 ± 27	68 ± 13	100 ± 2
13	Aerobic	0 ± 13	6 ± 6	38 ± 28	56 ± 4
13	Anaerobic	3 ± 4	2 ± 25	4 ± 31	N/A
14	Aerobic	5 ± 4	22 ± 4	46 ± 17	57 ± 17
14	Anaerobic	3 ± 5	N/A	N/A	N/A
15	Aerobic	0 ± 11	23 ± 9	N/A	N/A
15	Anaerobic	3 ± 2	70 ± 5	35 ± 15	21 ± 6
16	Aerobic	4 ± 2	18 ± 11	23 ± 19	44 ± 3
16	Anaerobic	0 ± 4	N/A	4 ± 28	N/A
17	Aerobic	0 ± 3	14 ± 26	24 ± 17	41 ± 18
17	Anaerobic	3 ± 5	0 ± 38	11 ± 6	N/A
18	Aerobic	10 ± 5	4 ± 9	9 ± 12	37 ± 16
18	Anaerobic	0 ± 6	33 ± 23	N/A	18 ± 34
19	Aerobic	4 ± 0	28 ± 7	37 ± 8	34 ± 18
19	Anaerobic	3 ± 4	3 ± 31	4 ± 34	12 ± 16
20	Aerobic	5 ± 6	17 ± 13	22 ± 16	64 ± 14
20	Anaerobic	7 ± 2	N/A	0 ± 15	19 ± 29
DON PC[b]	Aerobic	89 ± 4	–	–	–
DON PC[c]	Anaerobic	91 ± 3	–	–	–
ZEN PC[d]	Aerobic	–	85 ± 6	100 ± 0	100 ± 0
ZEN PC[d]	Anaerobic	–	19 ± 12	88 ± 10	100 ± 0

Notes: DON, deoxynivalenol; ZEN, zearalenone; PC, positive control; N/A, not available, SD ≥ 40% (no sufficient repeatability).
[a] Two batches of product analysed and average values displayed; [b] aerobic DON-degrading bacterium; [c] lyophilisate of anaerobic strain BBSH 797; [d] ZEN-degrading lactonohydrolase.

weak binding. Reduction values after 48 and 72 h of incubation were not significantly different to the 24 h values for all products. The overall results of aerobic incubation showed a maximum DON reduction of 17 ± 8% (product no. 20) over the total incubation time of 72 h. During anaerobic incubation only one product (product no. 12, containing among others bentonite, inactivated yeast and BBSH 797) reduced the tested DON concentration by 97% within 24 h and an equimolar formation of DOM-1 was detected. Several studies have shown that DOM-1 is markedly less toxic than DON (Sundstøl Eriksen et al. 2004; Dänicke et al. 2010), which indicates detoxification. For all other anaerobically incubated products a maximum DON reduction of

11% ± 2% over the incubation time of 72 h was determined.

These findings of frequent failing in counteracting DON are in line with data from the literature. Several studies have shown that binder materials such as aluminosilicates, hydrated sodium aluminosilicates and yeast cell-wall based products (Galvano et al. 1998; Avantaggiato et al. 2005, 2007; Döll et al. 2005; Sabater-Vilar et al. 2007), as well as enzymatic or microbial products (Döll et al. 2004; Dänicke & Döll 2010; Kong et al. 2014) were ineffective in adsorbing and/or degrading DON.

In the case of ZEN, reduction values after 1, 3 and 24 h of incubation are given in Table 3. In contrast to the DON reduction data, high variations in the detected ZEN concentrations were observed between samples taken from the triplicate incubation experiments of the same product and analysed by LC-MS/MS. ZEN reduction results with a standard deviation of ≥ 40% are not reported. These high variations in ZEN concentrations were most probably not caused by matrix effects. This could be asserted on the one hand due to the determined negligible SSE values (according to method validation) and on the other hand to the fact that similar standard deviations were obtained for the matrix-independent UV signal at 274 nm. Moreover, in the same samples low variations of DON concentration were observed, proving good repeatability of the experimental setup. Thus, we presume that these high standard deviations are related to product inhomogeneity as well as different ZEN adsorption rates during incubation and sampling. During sampling, non-reproducible amounts of solids were taken, resulting in different ZEN concentrations after desorption.

The ZEN concentration time courses of all tested products were evaluated in order to suggest their possible mode of action. During aerobic incubation, for products no. 3 (batch 2), 5 (batch 1 and 2), 6, 8, 9 and 15 adsorption can be assumed, whereas the aerobic time courses of products no. 10, 11, 12, 16, 17 and 18 indicate degradation activity. Nevertheless, unclear mode of action during aerobic incubation remains for products no. 1, 2, 3 (batch 1), 4 (batch 1 and 2), 7, 13, 14, 19 and 20 because the time courses were neither representative for adsorption nor degradation. During anaerobic incubation, the low ZEN reduction obtained with products no. 2, 3 (batch 1 and 2), 4 (batch 1 and 2), 5 (batch 1 and 2), 6, 8 and 9 indicate their very limited action against this mycotoxin. The anaerobic time courses of products no. 1, 7, 11, 13, 17 and 18 indicate adsorption, whereas the anaerobic time course of product no. 12 suggests degradation. Unclear mode of action during anaerobic incubation remains for products no. 10, 14, 15, 16, 19 and 20.

In samples showing considerable mycotoxin reduction, formation of metabolites was expected in case of a present biotransformation activity. Therefore, in addition to monitoring of the known metabolites DOM-1, ZOM-1, α-ZEL, β-ZEL, HZEN and DHZEN by LC-MS/MS, chromatographic UV data were evaluated for detecting unknown metabolites. For samples showing a ZEN reduction of ≥ 40% over the total incubation period, as well as for samples showing possible ZEN biotransformation activity according to the concentration time curve, UV chromatograms at 274 nm (UV absorption maximum of ZEN and other possible ZEN-related metabolites) and 220 nm (UV absorption maximum of DON and some possible ZEN metabolites according to Kolf-Clauw et al. 2007) of the samples were evaluated. This evaluation was performed by comparison of samples containing the commercial product and the toxins with the respective samples of products incubated without toxins. Peaks additionally detected to ZEN and DON, representing potential (unknown) UV absorbing metabolites, are shown in Table 4, in case of detection in at least two of the three independent incubation experiments and in case of peak intensities > 10% compared to ZEN. Furthermore, the known ZEN metabolites included in the used LC-MS/MS methods (ZELs, HZEN and DHZEN) with % of ZEN conversion are given.

After an incubation of 24 h, a ZEN reduction ≥ 60% was obtained with five products under aerobic conditions (products no. 2, 4, 10, 12 and 20) and with one product under anaerobic conditions (product no. 12). The ZEN concentration time courses of those products indicated a degradation process involved for products no. 10 (aerobic) and 12 (aerobic and anaerobic). Product no. 10 (enzymatic degradation claimed) showed a ZEN reduction of 61 ± 15% after 24 h of aerobic incubation and we were able to detect one uncharacterised, putative metabolite with high intensity in the UV chromatograms. In contrast, no repeatable ZEN reduction after 24 h of anaerobic incubation was observed for this product, whereas three additional putative metabolites were detected. Without knowledge of the oestrogenic activities of these putative metabolites, detoxification cannot be stated.

Product no. 12 (which contains among others bentonite, inactivated yeast and BBSH 797 and manufacturer claims enzymatic degradation) was able to completely degrade ZEN to the less-toxic DHZEN and HZEN (the sum of the two metabolites was equimolar to the original ZEN concentration) under the applied aerobic as well as anaerobic conditions. Thus, these results indicate ZEN hydrolysis similar to the action of the enzyme lactonohydrolase to the metabolite DHZEN, which did not show potent oestrogenic activity in a human breast cancer MCF-7 cell proliferation assay (Kakeya et al. 2002).

Based on time courses of feed additives showing ZEN reduction ≥ 60% after 24 h, the possible modes of action were unclear for products no. 2, 4 and 20 during aerobic incubation. Product no. 2 (enzymatic degradation claimed) showed a ZEN reduction of 97 ± 4% after 24 h of aerobic

Table 4. ZEN metabolites included in the LC-MS/MS methods and additional peaks detected at 220 or 274 nm (three independent incubation experiments analysed).

Product no.	Condition	Known ZEN metabolites detected	Potential metabolites at 220 or 274 nm RT (min)
1	Aerobic	n.d.	n.d.
1	Anaerobic	n.d.	n.d.
2	Aerobic	α-ZEL (up to 11%)	n.d.
3, batch 1	Aerobic	α-ZEL (up to 16%), β-ZEL (traces)	4.3
3, batch 2	Aerobic	α-ZEL (traces), β-ZEL (up to 15%)	n.d.
4, batch 1	Aerobic	n.d.	4.6
4, batch 2	Aerobic	n.d.	3.8, 4.6
5, batch 1	Anaerobic	n.d.	n.d.
5, batch 2	Anaerobic	n.d.	n.d.
6	Aerobic	n.d.	n.d.
6	Anaerobic	n.d.	n.d.
7	Aerobic	n.d.	n.d.
7	Anaerobic	n.d.	4.8, 5.1
8	Aerobic	n.d.	n.d.
9	Aerobic	n.d.	4.3
9	Anaerobic	n.d.	n.d.
10	Aerobic	n.d.	4.3
10	Anaerobic	n.d.	3.8, 4.0, 4.4
11	Aerobic	n.d.	4.3
11	Anaerobic	α-ZEL (traces), β-ZEL (up to 67%)	n.d.
12	Aerobic	HZEN, DHZEN (up to 100%)	n.d.
12	Anaerobic	HZEN, DHZEN (up to 100%)	n.d.
13	Aerobic	α-ZEL (traces)	4.3
14	Aerobic	n.d.	4.7
15	Aerobic	n.d.	n.d.
15	Anaerobic	n.d.	n.d.
16	Aerobic	n.d.	n.d.
16	Anaerobic	n.d.	n.d.
17	Aerobic	n.d.	5.2, 5.3, 5.9
17	Anaerobic	α-ZEL (up to 15%), β-ZEL (traces)	n.d.
18	Aerobic	n.d.	4.5
18	Anaerobic	n.d.	n.d.
19	Aerobic	n.d.	4.6
19	Anaerobic	α-ZEL (traces), β-ZEL (up to 89%)	5.7
20	Aerobic	n.d.	n.d.
20	Anaerobic	n.d.	n.d.
ZEN PC[a]	Aerobic	HZEN, DHZEN	n.d.
ZEN PC[a]	Anaerobic	HZEN, DHZEN	n.d.

Notes: RT, retention time; ZEN, zearalenone; PC, positive control; n.d., not detected; α-ZEL, alpha-zearalenol; β-ZEL, beta-zearalenol; HZEN, hydrolysed zearalenone; DHZEN, decarboxylated hydrolysed zearalenone; traces, concentrations above the limit of detection, but below the limit of quantification.
[a] ZEN-degrading lactonohydrolase.

incubation and a ZEN conversion to α-ZEL of up to 11% (on molar basis) was observed. In contrast, during anaerobic incubation no repeatable ZEN reduction after 24 h was observed and no α-ZEL formation was detected. During aerobic incubation of product no. 4 (adsorption and enzymatic degradation claimed) a ZEN reduction of 63 ± 19% after 24 h was achieved and two additional, putative metabolites were detected. In contrast, during anaerobic incubation less ZEN reduction of 5 ± 19% after 24 h was observed for this feed additive. Product no. 20 (adsorption and biotransformation claimed) showed a ZEN reduction of 64 ± 14% after 24 h of aerobic incubation, whereas no additional peaks of putative metabolites were detected. Minor ZEN reduction of 19 ± 29% after 24 h of anaerobic incubation was observed and no metabolites were detected.

Reduction values after 48 and 72 h of incubation are not shown separately in Table 3 due to limited *in vivo* relevance. After an incubation of 72 h, a ZEN reduction ≥ 60% was obtained with 14 products under aerobic conditions (products no. 1, 2, 4, 7, 10–12, 14–20) and with three products under anaerobic conditions (products no. 10, 12 and 15).

In general, the data suggest that higher ZEN reduction values were mainly obtained during aerobic compared to anaerobic incubation, which indicates that on the one hand higher adsorption rates were achieved due to shaking, which was applied only during aerobic incubation. On

the other hand, microbial ingredients achieving ZEN degradation only under aerobic conditions are conceivable. However, due to the mainly anaerobic conditions in the gastrointestinal tract of animals, the application of exclusively aerobically degrading feed additives seems problematic and might not be effective. In contrast, the efficacy of mycotoxin adsorption is dependent on parameters such as the physical and structural properties of the binding material and the mycotoxins (Daković et al. 2005), but independent of the presence of oxygen in the gastrointestinal tract of mammals.

In some cases we were able to detect ZELs, although ZEN reduction remained below 60% during an incubation of 24 h. For product no. 11 (adsorption, enzymatic degradation and "bio-inactivation" claimed), a ZEN conversion up to 67% to β-ZEL during anaerobic incubation was detected. Due to only slightly lower oestrogenic properties of β-ZEL compared to ZEN, biotransformation to this metabolite only leads to minor detoxification. In addition, minor or no reproducible formation of the still oestrogenic metabolites α- and/or β-ZEL was observed during aerobic incubation of products no. 3 (adsorption and biotransformation claimed) and 13 (adsorption and enzymatic degradation claimed) as well as during anaerobic incubation of products no. 17 (adsorption and "bioneutralisation" claimed) and 19 (adsorption and biotransformation claimed).

In vitro studies on enzymatic and/or microbial-based products for ZEN reduction are limited and demonstrated insufficient ZEN reduction (Döll et al. 2004). In contrast, vast adsorption of ZEN by mineral clays, yeast cell-wall derived products and commercial binders up to 88%, 77% and 99%, respectively, at a product concentration of 5 mg ml^{-1} and a pH of 8.0 was reported (Sabater-Vilar et al. 2007). High adsorption rates after 1 h of incubation were obtained, for example, anaerobically with product no. 15 (70 ± 5%), which claimed enzymatic degradation and contained *S. cer.* components. However, for this product a ZEN reduction of 21 ± 6% was found after 24 h of incubation, indicating later release of the toxin. Nevertheless, no or even negative *in vivo* activity of organophilic montmorillonite clays (Lemke et al. 2001) or esterified glucomannan derived from yeast cell-wall (Bursian et al. 2004) was observed, although *in vitro* binding of ZEN was proven. In addition, the toxicity of organoclays in a *Hydra vulgaris* toxicity bioassay was demonstrated (Marroquín-Cardona et al. 2009). Thus, *in vivo* evaluations of products mainly based on adsorption are essential prior to application in livestock.

In general, the *in vitro* activity of commercial products claiming to detoxify mycotoxins cannot be directly compared with the *in vivo* efficacy. Therefore, feeding trials using the target animal species are mandatory to prove *in vivo* detoxification. Moreover, poor *in vitro* activity does not necessarily implicate *in vivo* failure. While for instance *in vivo* data for one tested product (Kiyothong et al. 2012) is in agreement with our *in vitro* data for both toxins, another tested product shows reduction of deleterious effects caused by ZEN only *in vivo* (Brydl et al. 2014).

In conclusion, scientific studies on enzymatic or microbial products for DON and/or ZEN reduction are limited. Applying aerobic as well as anaerobic incubation at approx. pH 7, we were able to extent the current knowledge on commercially available feed additives with the claim to detoxify DON and/or ZEN. On the basis of the results from this study, it can be concluded that out of 20 aerobically and anaerobically tested products in total, only four products showed potential activity against DON and ZEN by biodegradation. In detail, only one product (product no. 12) was able to completely degrade DON to the non-toxic metabolite DOM-1 under the applied anaerobic conditions between 3 and 24 h of incubation. Concerning ZEN, only one of the tested products (product no. 12) completely degraded ZEN to the less-toxic DHZEN and HZEN under both applied conditions. Among further products showing a ZEN reduction ≥ 60% after 24 h (products no. 2, 4, 10 and 20), one produces the more oestrogenic metabolite α-ZEL (product no. 2, aerobically) and two products produce metabolites with unknown toxicity (product no. 4, aerobically; product no. 10, aerobically and anaerobically). As most of the tested products seem to be ineffective in degrading DON and/or ZEN under the applied conditions *in vitro*, critical assessment of any product claiming to degrade mycotoxins has to be performed *in vitro* as well as *in vivo*.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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RESEARCH ARTICLE

Hydrolysed fumonisin B_1 and *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1: stability and catabolic fate under simulated human gastrointestinal conditions

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Abstract

Food processing may induce thermal degradation of fumonisins in corn via Maillard-type reactions, or alkaline hydrolysis via loss of the two tricarballylic acid moieties. In the former case, *N*-(1-deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF) can be formed, while the latter derivative is called hydrolysed fumonisin B_1 (HFB_1). The aim of this study was to deepen the knowledge about the gastrointestinal stability of HFB_1 and NDF in humans. Due to the lack of standard, NDF was chemically synthesised and cleaned up in high purity to be used for further experiments. While NDF is already partially cleaved (about 41%) during simulated digestion, it remained rather stable towards human colon microflora. In contrast to this, HFB_1 is partially metabolised by the colon microflora to unknown compounds after 24 h of fermentation, as seen by a loss of about 22%. Concluding, the cleavage of NDF during digestion as well as the likely metabolisation of HFB_1 emphasise the need for animal trials to ascertain their toxicity *in vivo*.

Keywords

Bioavailability, food processing, fumonisins, gut microflora, mycotoxins

History

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Introduction

Fumonisins are fungal toxic secondary metabolites produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*. They are the main naturally occurring contaminants in corn and corn-based products. Fumonisins, with fumonisin B_1 (FB_1, Figure 1) as the most important toxin, have been widely studied because they represent a serious risk to animal and human health due to their toxicity and potential carcinogenicity. Their toxicity is caused by the structural similarity with the sphingoid bases sphinganine and sphingosine, which leads to the inhibition of the enzyme ceramide synthase, causing the disruption of the biosynthesis of sphingolipids. In addition, fumonisins are suspected to cause neural tube defects in humans (Marasas et al., 2004).

Fumonisins are relatively heat stable up to 100 °C, although it is known that food processing induces significant decrease of the detectable mycotoxins (Jackson et al., 1997): chemical degradation may take place via Maillard-type reactions at high temperature or hydrolysis via loss of the two tricarballylic acid moieties in the presence of alkali. The reduction in FB_1 content was probably due to the arising of some bound forms given by chemical interactions or reactions between FB_1 and reducing sugars to form stable Schiff bases (Lu et al., 1997). Among these derivatives, the formation of *N*-(1-deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF), due to Maillard-like reaction in thermally treated products was demonstrated (Voss et al., 2001). NDF was shown to be stable in non-aqueous media, while in aqueous systems it can be found in equilibrium with its precursor FB_1. For this reason, the isolation and separation from FB_1 are considered as extremely critical steps in NDF chemical characterisation (Poling et al., 2002). Several papers investigated the formation and stability of NDF during thermal treatment by using model systems (Lu et al., 2002; Poling et al., 2002), while fewer studies addressed its formation and occurrence in real products (Bullerman et al., 2008). Among thermal treatments, extrusion seems to mostly support the conversion of FB_1 to NDF, probably also on account of the strong moisture reduction occurring under these conditions.

Hydrolysed fumonisins are usually formed during alkaline process such as nixtamalisation for masa (tortilla flour) production: this treatment is widely used in South America, since tortilla flour still represents not only a very common ingredient in local food, but also a staple food for population living in rural area (Hamner & Tinker, 2014). Concerning the toxicity of fumonisin derivatives, almost nothing is known so far. Since it is supposed that the FB_1 primary amine group is responsible for most of its toxic activity, it could be reasonably hypothised that *N*-derivatives exert a lower toxicity compared to their precursor (Fernández-Surumay et al., 2004, 2005; Howard et al., 2002). Accordingly,

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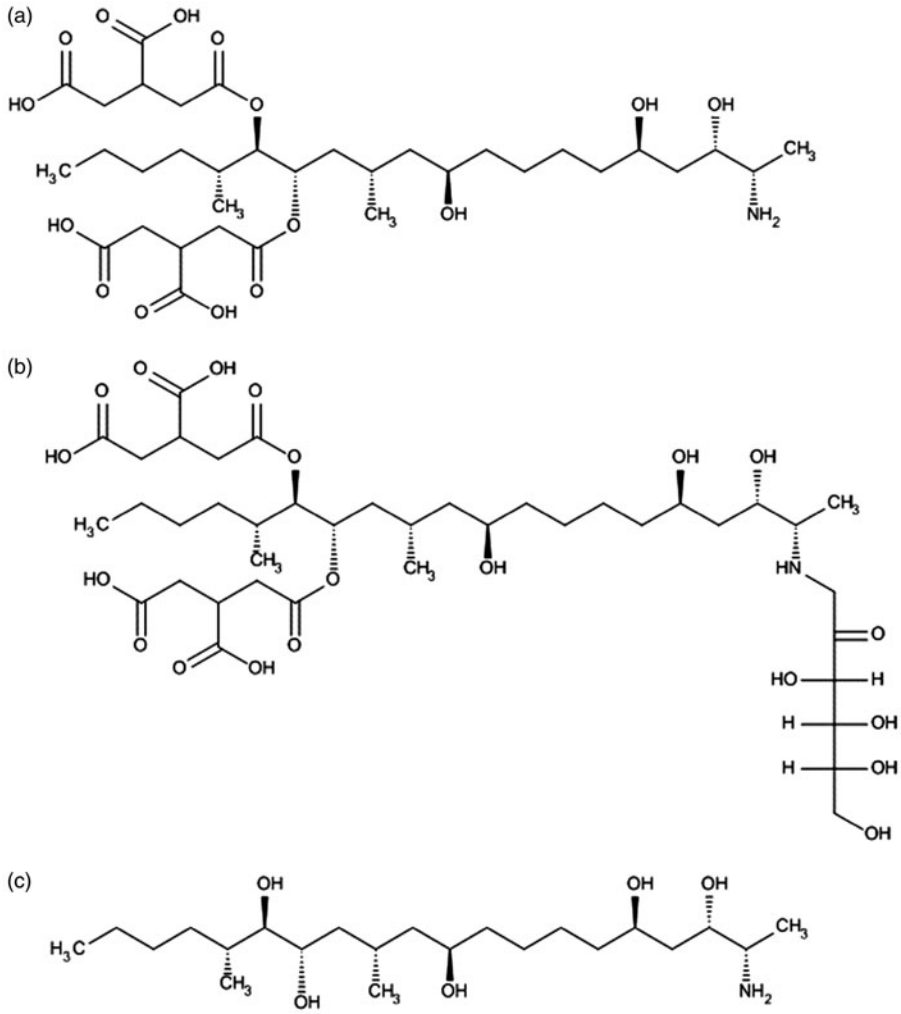

Figure 1. Chemical structure of (a) fumonisin B_1 (FB_1); (b) *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF) and (c) hydrolysed fumonisin B_1 (HFB_1) (Marvin Sketch v. 6.5.2, Marvin Suite, ChemAxon Kft., Budapest, Hungary).

heating treatments of corn during food and feed production could be considered as a possible detoxification route, when FB_1 is not released from its derivatives after ingestion.

As far as HFB_1 is concerned, in spite of a lower acute toxicity compared to FB_1, its higher absorption by the intestinal mucosa was reported (Caloni et al., 2002; Humpf et al., 1998). In contrast the lower toxicity of HFB_1 compared to FB_1 after oral administration in pigs was reported recently (Grenier et al., 2012). Other studies pinpointed the possible *in vivo* formation of cytotoxic *N*-acyl derivatives of HFB_1 and FB_1 (Harrer et al., 2013; Seiferlein et al., 2007).

Recently, Falavigna et al. (2012) reported the stability of NDF under digestive conditions using a simulated stomach model, but nothing is known about the effect exerted by human gut microflora. This study thus aimed at the investigation of the catabolic fate of NDF and HFB_1 under human gastrointestinal conditions, considering both the contribution due to the digestive enzymatic pool and the colonic microbiome. For this purpose, NDF was synthesised and purified with a sound improvement in respect to other methods reported in literature so far.

Materials and methods

Chemicals

Fumonisin B_1 was purchased from Romer Labs (Tulln, Austria), while D-glucose was purchased from Sigma-Aldrich (Stuttgart, Germany). Hydrolysed fumonisin (HFB_1) was prepared in our laboratory as reported by Dall'Asta et al. (2009). Bi-distilled water was produced in-house by an Alpha-Q system (Millipore, Marlborough, MA), while methanol and acetonitrile (both LC grade) were purchased from Sigma-Aldrich (Stuttgart, Germany).

All chemicals used for faecal fermentation, such as bile salts, calcium chloride, (+)-arabinogalactan, tryptone, yeast extract, buffered peptone water, casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, sodium hydrogen carbonate, potassium hydrogen phosphate, potassium chloride, sodium hydrogen phosphate, sodium chloride, magnesium sulphate monohydrate, guar gum and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO), while L-cysteine hydrochloride monohydrate and Fe(II)-sulfate heptahydrate were supplied by AppliChem (Darmstadt, Germany).

Synthesis of N-(1-deoxy-D-fructos-1-yl)-fumonisin B_1

A small-scale reaction was initially performed using 1.4 µmol of FB_1 and 56 µmol of D-glucose. The solvent free reaction mixture was heated in a reaction vial at 80 °C for 120 min. The mixture was then diluted in 500 µL of H_2O/CH_3CN (1/1, v/v) and NDF was separated from the reagents on immunoaffinity FUMONIPREP-cartridges (Romer Labs, Tulln, Austria). Unreacted FB_1 was eluted from the column with methanol, while the solution containing NDF was directly collected from the cartridge after the charging step. The reaction yield for NDF under the applied conditions was about 77%.

The reaction was then scaled-up as follows: in a reaction vial, a solvent free mixture of FB_1 (8.66 mg, 12 µmol) and D-glucose (86.45 mg, 480 µmol) was heated at 80 °C for 120 min in a heating

block, under a slight flow of nitrogen to avoid moisture formation. After a cooling step at room temperature, the mixture underwent the purification step as reported below. The reaction yield after the scale-up procedure and before purification was about 75%.

N-(1-deoxy-D-fructos-1-yl)-FB_1 purification on a preparative HPLC system

The reaction mixture was dissolved in bi-distilled water (4 mL). NDF was separated from reagents on a reversed phase XBridge C18 OBD preparative HPLC column (5 µm, 100 × 19 mm, Waters, Milford, MA) protected by a C18 security guard column (5 µm, 10 × 10 mm, Phenomenex, Aschaffenburg, Germany). An 1100 series preparative HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an automatic fraction collector from the same supplier and a Sedex LT-ELSD Evaporative Light Scattering Detector (Sedere, Alfortville, France) was used. Gradient elution was performed using water (A) and methanol (B) both containing 0.05% of aqueous ammonia as eluents. The initial conditions of 10% B were maintained for 1 min, then from 1 to 7 min eluent B was increased up to 70% and then up to 100% after another 0.2 min, maintaining this condition until 9 min. Then, within 0.2 min the initial conditions were restored and after that the column was reconditioned for 2.3 min, with a total run time of 11.5 min. The flow rate was 16 mL/min and the injection volume was 200 µL. Fractions were collected each 0.25 min from 3 to 6 min obtaining 12 different fractions.

All the separated fractions were analysed on an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap mass spectrometer (AB Sciex, Foster City, CA). Separation was achieved on a Luna HILIC column (5 µm, 150 × 3.0 mm, Phenomenex, Aschaffenburg, Germany) using a linear gradient from 95% to 60% CH_3CN (containing 0.1% acetic acid) within 8 min, a hold time of 3 min and subsequent re-equilibration for 4 min at a flow rate of 500 µL/min. FB_1 showed a retention time of 7.70 min, while NDF eluted at 8.42 min. MS parameters were as follows: ESI positive ionisation; source temperature 400 °C; capillary voltage 5 kV; curtain gas 25 psi; source gas one 30 psi, source gas two 65 psi; declustering potential 30 V. Detection was carried out using Q3 full scans (*m/z* 650–950); for both analytes $[M+H]^+$ ions were extracted (FB_1 *m/z* 722.2; NDF *m/z* 884.3). Pure NDF fractions were then pooled and evaporated to dryness on a rotary evaporator.

Application of the *in vitro* digestion assay

The *in vitro* digestion experiment was performed according to Falavigna et al. (2012), with slight modifications. Briefly, the main digestive juices were prepared by mixing the proper amounts of salts and enzymes according to the original protocol proposed by Versantvoort et al. (2005) and pre-heated at 37 °C before use. A volume of 200 µL of water/methanol (25/75 v/v) solution containing the two target compounds was transferred in a 4 mL septum vial and the solvent was evaporated under nitrogen. The remainders were taken up in 100 µL bi-distilled water each.

The *in vitro* digestion started by adding 75 µL of saliva to the target compounds. After an incubation step of 5 min at 37 °C, 150 µL of gastric juice was added and the mixture was incubated again for 2 h. At the end of the gastric step, 25 µL of 1 M bicarbonate solution, 150 µL of duodenal juice and 75 µL of bile were added and a final incubation step of 2 h was performed. In order to quench the enzymatic hydrolysis, 25 µL of acetonitrile was added and the whole volume was centrifuged for 10 min at 10 000 rpm, followed by direct LC-ESI-MS/MS analysis. The amount used for each tested compound is reported in Table 1.

Table 1. Amount of *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF), hydrolysed fumonisin B_1 (HFB_1) and fumonisin B_1 (FB_1) used for the experiments reported in this study.

	Digestion assay		Gut assay	
Target analyte	Amount	Mol	Amount	Mol
NDF	2.5 µg	2.8 nmol	1.8 µg	2.0 nmol
HFB_1	1.1 µg	2.5 nmol	0.8 µg	2.0 nmol
FB_1	1.7 µg	2.3 nmol	1.4 µg	2.1 nmol

Matrix effects may lead to ion suppression and thus a potentially lower response of both analytes during the MS/MS detection, resulting in misinterpretation of the stability of target analytes under gastrointestinal conditions. Therefore, a blank digestion sample was prepared by performing the *in vitro* digestion procedure as described above without the target compounds. Afterwards, the same amount of each target compound used for threated samples was dissolved in 500 µL of such blank sample containing acetonitrile (5%), centrifuged for 10 min at 10 000 rpm and analysed by LC-ESI-MS/MS.

Application of the *in vitro* human colonic fermentation assay

FB_1 derivatives underwent the human colonic fermentation assay as described by Dall'Erta et al. (2013). Fresh faeces were collected from three different healthy and non-smoking donors. Faeces were pooled to decrease inter-individual variability, then immediately stored in an anaerobic jar, mixed and weighted in order to obtain a 10% faecal solution in phosphate saline buffer (PBS). This buffer was previously prepared by mixing 1.6 g NaCl, 0.24 g anhydrous Na_2HPO_4, 0.04 g KH_2PO_4 and 0.04 g KCl in 200 mL of bi-distilled water and adjusting the pH to 7 by the addition of 1 N HCl.

Growth medium was prepared by weighting salts and enzymes as follows: 5.0 g soluble starch, 5.0 g peptone, 5.0 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 2 g pectin, 4.0 g mucin, 3.0 g casein, 2.0 g arabinogalactan, 1.5 g $NaHCO_3$, 0.69 g $MgSO_4 \cdot H_2O$, 1.0 g guar, 0.8 g L-cysteine $HCl \cdot H_2O$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salt, 0.08 g $CaCl_2$, 0.005 g $FeSO_4 \cdot 7H_2O$, 1 mL Tween80. After weighing, they were dissolved in bi-distilled water and the solution was then sterilised at 121 °C for 15 min. Each target compound was treated in triplicate, as follows. For each sample, 1.8 mL of growth medium and 1.8 mL of faecal solution were mixed and added to a proper amount of toxin derivative, as reported in Table 1. Each sample was then treated with a nitrogen flow in order to eliminate oxygen and allow fermentation under anaerobic condition. Samples were incubated in a water bath at 37 °C and mixed at 200 strokes/min. For t_0 samples, fermentations were immediately stopped by cooling to room temperature and adding 0.4 mL of CH_3CN. Samples were then collected after 30 min and 24 h, immediately centrifuged at 20 817 g for 10 min and stored at −80 °C until analysis. Blank control samples (mycotoxin not added) were also prepared and fermented.

LC-ESI-MS/MS analysis

LC-ESI-MS/MS analysis was performed by 2695 Alliance separation system (Waters Co., Milford, MA) coupled to a Quattro API triple-quadrupole mass spectrometer with an electrospray source (Micromass, Waters, Manchester, UK). Chromatographic conditions were as follows: the column was a XTerra C18 column (5 µm, 250 × 2.1 mm) (Waters, Milan, Italy);

the flow rate was 0.2 mL/min; the column temperature was set at 30 °C; the injection volume was 10 µL; gradient elution was performed by using bi-distilled water (eluent A) and acetonitrile (eluent B), both acidified with 0.2% formic acid: initial condition at 0% B, 0–5 min isocratic step, 5–30 min linear gradient to 100% B, 30–38 min isocratic step, 38–40 min linear gradient to 0% B and re-equilibration step at 0% B for 15 min (total analysis time: 55 min). MS parameters were the following: ESI + (positive ionisation mode); capillary voltage, 4.0 kV; cone voltage 50 V; extractor voltage, 2 V; source block temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow and desolvation gas flow (nitrogen), 50 and 700 L/h, respectively. Detection was achieved by using the multiple reaction monitoring (MRM) mode, by monitoring two transitions for FB_1, NDF, HFB_1 and partially hydrolysed FB_1 (pHFB_1), as follows: 722.4 → 334.4 and 722.4 → 352.4 for FB_1; 884.4 → 726.4 and 884.4 → 568.3 for NDF, 406.5 → 334.4 and 406.5 → 353.4 for HFB_1; 564.1 → 334.4 and 564.1 → 352.2 for pHFB_1. The first transition reported was used for quantification, while the second was chosen as qualifier. The limit of detection (LOD) was lower than 50 µg/L, expressed as signal-to-noise ratio 3:1, for all the considered compounds. Recovery in the faecal medium was evaluated at a spiking level of 500 µg/L for all the target compounds ($n = 3$), obtaining an average value of 93.1% for NDF, 95.4% for HFB_1, and 97.3% for FB_1.

Statistical analysis

Data obtained in the applied assays were compared by one-way ANOVA test followed by a *post-hoc* Tukey's test ($\alpha = 0.05$). Calculation was performed using the software SPSS v.21.0 (IBM SPSS Statistics, Milan, Italy).

Results

Synthesis and purification of N-(1-deoxy-D-fructos-1-yl)-FB_1

The small-scale synthesis was performed according to Falavigna et al. (2012). After the reaction, NDF was selectively separated from unreacted FB_1 using immunoaffinity chromatography. The calculated yield was 77%, in agreement with Poling et al. (2002). The same conditions were then replicated in a large-scale experiment aiming to obtain about 8 mg of NDF. The critical step during scale-up was the product clean up and isolation, since immunoaffinity chromatography cannot be successfully applied under these conditions. These cartridges are single-use columns only and show a very limited load capacity not feasible for a large scale purification. For these reasons, we opted to purify the product by semi-preparative HPLC.

Due to the strong structural and chemical similarity of FB_1 and NDF, the chromatographic parameters had to be optimised carefully to maximise peak separation. The best chromatographic conditions for separation were achieved using an alkaline mobile phase – in particular water and acetonitrile, both containing 0.05% ammonia – and a stationary phase able to support a wider upper range of pH. The presence of ammonia instead of formic or acetic acid in the mobile phase improved the separation between NDF and FB_1, on account of the structural differences between these compounds. In case of FB_1, a primary amine, a slight alkaline eluent allows for its complete protonation, whereas NDF is only partially positively charged at this pH (pH = 9). Fractions containing primarily NDF were pooled and re-injected for further purification. After this second purification process, all the collected fractions were pooled. NDF purity was then checked by LC-ESI-MS/MS, resulting in a purity degree of 96% compared to residual FB_1, which was considered a good level for performing the colonic fermentation assay.

In vitro stability of FB_1, hydrolysed FB_1 and N-(1-deoxy-D-fructos-1-yl)-FB_1 under gastrointestinal conditions

The stability under gastrointestinal condition of HFB_1 and NDF was tested *in vitro* and compared to data obtained for FB_1. Amounts of target compounds treated *in vitro* were reported in Table 1. Statistical comparison of the results obtain upon digestion with the control clearly indicate that HFB_1 is rather stable under the applied conditions ($p = 0.341$), while NDF and, at less extent, FB_1 are partially cleaved ($p = 0.000$ and $p = 0.021$, respectively). Data are reported in Figure 2.

Microbial transformation of FB_1, N-(1-deoxy-D-fructos-1-yl)-FB_1 and hydrolysed FB_1 in the simulated human gut

In this study, the catabolic fate of NDF and HFB_1 in a human gut microbiome assay was tested and compared to their precursor FB_1. Measurements were performed after 30 min and 24 h and the results were compared to the control (t_0). As reported in Figure 3, no significant differences were found in NDF and FB_1 after 24 h of fermentation, when statistically compared with the control sample ($p = 0.643$). On the other hand, a slight but significant decrease was recorded for HFB_1 ($p = 0.001$), suggesting the formation of still unknown metabolites. Further studies should be performed in order to identify these catabolites.

Discussion

Food products can be contaminated with fumonisins, among them the most important is FB_1, and their derivatives, such as HFB_1 and

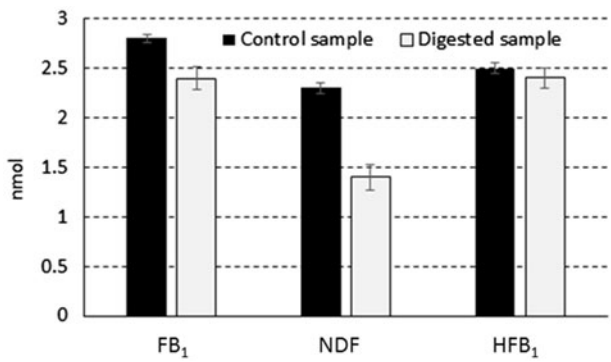

Figure 2. Stability of target compounds under human gastrointestinal simulated conditions. Data are expressed as molar amounts. FB_1, fumonisin B_1; NDF, *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1; HFB_1, hydrolysed fumonisin B_1.

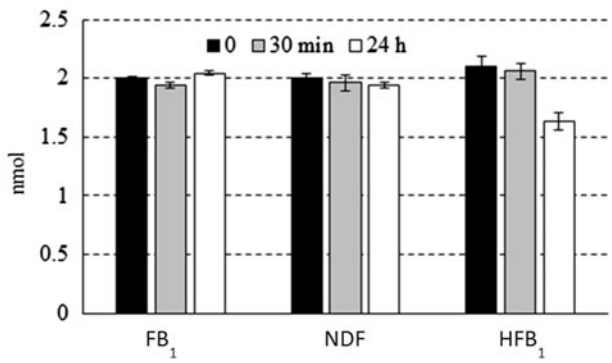

Figure 3. Stability of target compounds to 24 h of fermentation in the human microflora assay. Data are expressed as molar amounts. FB_1, fumonisin B_1; NDF, *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1; HFB_1, hydrolysed fumonisin B_1.

NDF, both formed upon processing. Despite a great number of studies performed in the last three decades, the toxicity exerted by FB_1 is still ambiguous: experimental evidence showed that, despite the low absorption and bioavailability of FB_1 after oral administration, toxic effects were recorded also after ingestion of low contaminated feed. This led to formulate the hypothesis that FB_1 derivatives may be present and preferentially absorbed then reconverted to the active forms in the body (Shier, 2000). However, the amount of FB_1 derivatives has not been considered in the regulatory limits fixed by food agencies for FB_1 due to the lack of data regarding their absorption and toxicity.

Few data are available about fumonisin absorption and transformation in humans: FB_1 is actually excreted mainly in faeces and, at lower amount, in urine (Riley et al., 2012), showing a toxicokinetic similar to that reported for animals. According to studies on farming animals, the majority of ingested FB_1 is excreted via faeces unchanged or after partial/total hydrolysis of the tricarballylic acid side chains (Shephard et al., 2007). In particular, Fodor et al. (2007) reported the biotransformation of FB_1 by the intestinal microbiome of pigs into $pHFB_1$ and HFB_1. It was recently proven that masked mycotoxins could be deconjugated by human colon microbiome, thus releasing their parent forms (Dall'Erta et al., 2013; Gratz et al., 2013). Since parent toxins could be absorbed in the intestine and return in blood circulation, this cleavage should be considered of toxicological relevance depending on the colonic absorption of the target compound (Dall'Erta et al., 2013). This study aimed to investigate the stability and the possible biotransformation of NDF, a major masked derivative of FB_1 occurring in processed food, HFB_1 and its parent compound.

Considering the results obtained upon *in vitro* digestion, HFB_1 was found to be rather stable under the applied conditions, while FB_1 was partially cleaved ($14.3 \pm 5.0\%$) to give partially and totally hydrolysed FB_1 ($pHFB_1$ and HFB_1, respectively). Similarly, NDF showed a significant decrease ($41.3 \pm 3.1\%$) in the digestion assay. This is in contrast with our previous results (Falavigna et al., 2012), as in that study NDF was found rather stable. A possible explanation is the use of raw reaction products, still containing significant levels of unreacted FB_1. To achieve the required sensitivity, the sample has been cleaned up on solid phase extraction columns, and this might bias the outcome. In the present study, NDF was of high purity and analyses were performed using state-of-the-art instrumentation, thus allowing to skip sample clean up. The NDF degradation products were not fully identified yet, thus further studies to characterise the cleavage are warranted. Regarding the human gut assay, FB_1 and NDF were found to be rather stable under the applied conditions. These results are in agreement with those studies reporting the recovery of FB_1 in faeces (Riley et al., 2012). Different results have been reported by Fodor et al. (2007), describing the degradation of FB_1 to HFB_1 by the intestinal microflora of pigs.

According to the experiment performed within this study, NDF was also found to be rather stable in the human gut assay. This finding is in contrast with previous works, since *in vivo* hydrolysis was proposed for rats (Hopmans et al., 1997). However, the inter- and intra-species variability as well as the active modulatory role played by gut microbiome should be taken into consideration. Hence, as often reported for other mycotoxins, metabolic conversion and gut biotransformation are strongly dependent on many factors, such as species and route of administration. In addition, factors such as the genetical background, health status, sex, diet and environment may significantly contribute to this variability.

Concerning HFB_1, a significant decrease (22.1 ± 2.4 %) was obtained in the human gut assay after 24 h of fermentation; this result suggests a conversion of the compound to one or more unknown metabolites. According to the literature, the possible formation of *N*-acyl derivatives should be taken into consideration, although no evidence has been found from the collected LC-MS/MS data (Humpf et al., 1998; Seiferlein et al., 2007). This degradation is in agreement with the reported absence of HFB_1 in urine and/or faeces from humans exposed to FB_1 contamination from the diet (Riley et al., 2012). On one hand, this lack of excretion can be explained considering that HFB_1 intake from food is far lower compared to the parent compound; on the other hand, the lower polarity of HFB_1 suggests that this compound can undergo absorption and biological transformation.

Data regarding HFB_1 toxicity are indeed controversial: Hendrich et al. (1993) reported significant toxicity in rats, although animals were fed with unpurified material. On the contrary, Grenier et al. (2012) reported that HFB_1 exerted a very low toxicity in pigs compared to its parent compound. Finally, Seiferlein et al. (2007) ascribed the possible toxicity in rats to the conversion to cytotoxic *N*-acyl-derivatives. Further studies should be performed to better address these possible metabolisation pathways and their inactivating/activating role.

A slightly different situation can be described for NDF. While NDF was stable during microbial fermentation, cleavage occurred already during digestion. In addition, since the intestine has been recognised as a possible target for fumonisin toxicity (Bouhet & Oswald, 2007), similar studies should be performed also for NDF to better investigate a possible toxic action.

Conclusion

In conclusion, this study investigates the stability and the possible metabolisation of NDF and HFB_1 compared to FB_1 in the human gastrointestinal tract. The applied *in vitro* assays covered both the digestion process and the fermentation occurring in the gut. Taking into account its low occurrence in food the potential toxicological relevance of NDF might be regarded as low. Nonetheless, the partial degradation occurring in the digestive tract should be considered since an additional amount of FB_1 and/or HFB_1 may be released. HFB_1 is partially metabolised by the colon microflora to unknown compounds after 24 h of fermentation. Although HFB_1 occurs at significant levels only in alkali-treated food, further studies should address the possible formation of *N*-acyl derivatives. A major advantage of the used *in vitro* models is their ease and speed compared to animal trials. While the latter are clearly needed to fully ascertain toxic effects of a multitude of substances and their derivatives, *in vitro* assays can provide crucial information about the worthiness and ultimately the need to sacrifice animals.

Declaration of interest

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. This article does not contain any studies with human or animal subjects.

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Publication #4

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The candidate performed the purification of the compounds by preparative HPLC and was responsible for the production and analysis of the experimental diets. Furthermore, the autor of this thesis contributed to the data evaluation and interpretation. The candidate and Veronika Nagl contributed equally to this study. Moreover, the author of this thesis wrote the manuscript together with Veronika Nagl and Franz Berthiller.

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Effects of orally administered fumonisin B_1 (FB_1), partially hydrolysed FB_1, hydrolysed FB_1 and *N*-(1-deoxy-D-fructos-1-yl) FB_1 on the sphingolipid metabolism in rats

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ABSTRACT

Fumonisin B_1 (FB_1) is a *Fusarium* mycotoxin frequently occurring in maize-based food and feed. Alkaline processing like nixtamalisation of maize generates partially and fully hydrolysed FB_1 ($pHFB_1$ and HFB_1) and thermal treatment in the presence of reducing sugars leads to formation of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF). The toxicity of these metabolites, in particular their effect on the sphingolipid metabolism, is either unknown or discussed controversially. We produced high purity FB_1, $pHFB_1$a+b, HFB_1 and NDF and fed them to male Sprague Dawley rats for three weeks. Once a week, urine and faeces samples were collected over 24 h and analysed for fumonisin metabolites as well as for the sphinganine (Sa) to sphingosine (So) ratio by validated LC–MS/MS based methods. While the latter was significantly increased in the FB_1 positive control group, the Sa/So ratios of the partially and fully hydrolysed fumonisins were indifferent from the negative control group. Although NDF was partly cleaved during digestion, the liberated amounts of FB_1 did not raise the Sa/So ratio. These results show that the investigated alkaline and thermal processing products of FB_1 were, at the tested concentrations, non-toxic for rats, and suggest that according food processing can reduce fumonisin toxicity for humans.

1. Introduction

Fumonisins are mycotoxins mainly produced by *Fusarium verticillioides, Fusarium proliferatum* (Gelderblom et al., 1988) and *Aspergillus niger* (Frisvad et al., 2007). While several cereal species can be contaminated with fumonisins in principle, the major affected commodity is maize (Placinta et al., 1999). The most commonly occurring form within the family is fumonisin B_1 (FB_1, Fig. 1) and levels of up to several hundred mg/kg can be found in maize (Dutton, 1996). Animal diseases caused by the ingestion of FB_1 include equine leukoencephalomalacia and porcine pulmonary oedema. FB_1 is hepatotoxic in all tested species and nephrotoxic in a wide range of animal species, causing apoptosis in the affected tissues (reviewed by Voss et al., 2007). The main mode of action of fumonisins is the inhibition of ceramide synthase and the disruption of the sphingolipid metabolism due to their close similarity to the sphingoid bases sphinganine (Sa) and sphingosine (So) (Merrill et al., 1993a). The elevation of the free Sa/So ratio in urine closely reflected the changes that occurred in kidney of rats exposed to FB_1 (Riley et al., 1994) and was suggested as efficient biomarker of effect. To protect humans and animals numerous countries issued maximum or guidance levels for fumonisins in food and feed. For instance, within the European Union, maximum levels for the sum of fumonisin B_1 and B_2 are 0.2 mg/kg for baby food, 0.8 mg/kg for breakfast cereals or 1.0 mg/kg for maize intended for direct human consumption (European Commission, 2006a). Guidance levels for animals reflect the different toxicities towards different species, with pigs and horses among the most sensitive ones with 5 mg/kg (European Commission, 2006b).

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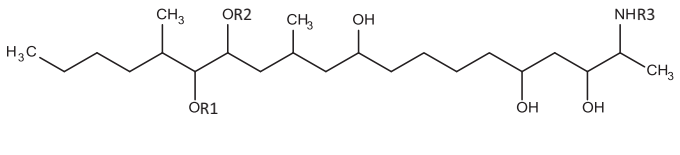

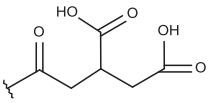

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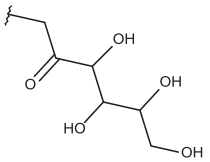

deoxy-D-fructos-1-yl

Analyte	R1	R2	R3
FB_1	TCA	TCA	H
$pHFB_1a$	TCA	H	H
$pHFB_1b$	H	TCA	H
HFB_1	H	H	H
NDF	TCA	TCA	deoxy-D-fructos-1-yl

Fig. 1. Structures of fumonisin B_1 (FB_1), partially hydrolysed fumonisin B_1 ($pHFB_1a$, $pHFB_1b$), hydrolysed fumonisin B_1 (HFB_1) and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF).

With a production of 873 million tonnes alone in 2012, maize is the most widely cultivated cereal in the world (FAO, 2014). Maize-based processed food for human nutrition includes cornbread, corn flakes, enchiladas, polenta, popcorn, porridge, quesadillas, steamed corn cobs, tacos and tortillas. Food processing, in particular thermal or alkaline treatment, can lead to derivatisation or cleavage of fumonisins (reviewed by Humpf and Voss, 2004). For instance, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF, Fig. 1) has been identified as thermal reaction product of FB_1 with glucose (Poling et al., 2002). NDF can further react to form *N*-(carboxymethyl) fumonisin B_1 (NCM-FB_1) (Howard et al., 1998). As the primary amino group of fumonisins is important for their toxic effects (Gelderblom et al., 1993), these derivatives are regarded as detoxification products (Howard et al., 2002). Still, these compounds might be considered as masked mycotoxins (reviewed by Berthiller et al., 2013), as they might be cleaved during digestion – liberating the precursor mycotoxin. Also alkaline treatment of maize – referred to as nixtamalisation – is extensively performed in several parts of the world, e.g. in Mexico. FB_1 is gradually hydrolysed in this process, as the tricarballylic acid esters at the C-14 and C-15 position are cleaved (Hendrich et al., 1993). The arising products are termed partially hydrolysed fumonisin B_1 ($pHFB_1a$ or $pHFB_1b$) as well as hydrolysed fumonisin B_1 (HFB_1) and are depicted in Fig. 1. The toxicity of $pHFB_1$ is poorly investigated and the sole *in vitro* study used rather resistant cell lines towards fumonisins, resulting in little decrease of cell viability even for FB_1 (Caloni et al., 2002). Toxicity trials in rodents and pig showed greatly reduced toxicity of HFB_1 compared to FB_1 when the mycotoxins were administered in feed (Collins et al., 2006; Grenier et al., 2012; Howard et al., 2002) or intraperitoneally (Voss et al., 2009). Reduced toxicity was manifested by no or only weak disruption of sphingolipid metabolism, no observed hepatic pathology in all species tested and no induction of neural tube defects in mice given HFB_1. While Gelderblom et al. (1993) showed no weight loss or induction of cancer by HFB_1, either, the authors noticed similar or even elevated cytotoxicity of HFB_1 compared to FB_1. In addition, feeding trials with fumonisin-containing, nixtamalised fungal culture material showed, depending on treatment conditions and on the starting concentration of intact fumonisins, complete or incomplete (Hendrich et al., 1993; Voss et al., 1996, 1998, 2013) reduction of toxicity. Partially hydrolysed fumonisins, which were suspected to be toxic and present in unknown concentrations in nixtamalised culture material used in early feeding trials, were speculated to be responsible for contradictory results (Hartinger and Moll, 2011). Another possible reason for partial toxicity of administered nixtamalised culture material might be the *in vivo* formation of *N*-acyl-HFB_1 (Seiferlein et al., 2007) and potential *N*-acyl-$pHFB_1$. HFB_1 can serve as substrate for ceramide synthase, and the resulting cytotoxic *N*-acyl-HFB_1 is a potent ceramide synthase inhibitor. If HFB_1 was present at sufficiently high concentration for this acylation reaction to occur to a relevant extent, toxicity might be explained.

The aim of the current study was to evaluate the effects of orally administered FB_1, $pHFB_1$, HFB_1 and NDF on sphingolipid metabolism in rats. Substances were prepared and added to cookies, which were fed to the rodents for three weeks. Urine and faeces were collected weekly for 24 h and biomarkers of exposure and a biomarker of effect were determined using validated LC–MS/MS methods. The study increases the current understanding about sphingolipid metabolism related toxicity and gastro-intestinal metabolism of fumonisin derivatives generated in alkaline or thermal food processing.

2. Materials and methods

2.1. Reagents

Acetonitrile (ACN), methanol (MeOH), glacial acetic acid (HAc) (all LC grade) and aqueous ammonia solution (25%, p.a.) were purchased from VWR International GmbH (Vienna, Austria). Petrol ether (40–60 °C, for chromatography) was purchased from Lactan Roth (Graz, Austria). Water was purified with a Purelab Ultra system (ELGA LabWater, Celle, Germany) after reverse osmosis. Ethyl acetate (EtAc, p.a.) was provided by Wagner & Munz GmbH (Vienna, Austria). All chemicals used for culture media, buffer solutions, as well as silica gel, Amberlite XAD-2 and 10% neutral buffered formalin were purchased from Sigma-Aldrich (Schnelldorf, Germany). Staining solutions for histopathological examinations (haematoxylin and eosin) were obtained from Morphisto (Frankfurt am Main, Germany). Liquid standards of FB_1, [^{13}C]-labelled FB_1, as well as fumonisins B_2 (FB_2) and B_3 (FB_3) were provided by Romer Labs GmbH (Tulln, Austria). Sa and So for calibration were purchased from Avanti Polar Lipids (Alabaster, AL, USA). HFB_1, $pHFB_1a$, $pHFB_1b$, NDF as well as a mixture of [^{13}C]-$pHFB_1a$ and [^{13}C]-$pHFB_1b$ were prepared and isolated on a preparative HPLC system according to Schwartz-Zimmermann et al. (in preparation) and Cirlini et al. (in press). [^{13}C]-labelled internal standards of HFB_1 and NDF were prepared from [^{13}C]-FB_1 in the same way at small scale. Single stock solutions of Sa, So, FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were prepared in a concentration range between 28 and 100 mg/l. Mixed stocks for preparation of calibration functions were prepared separately for the sphingolipids Sa and So and for all fumonisin analogues at a concentration of 3 mg/l.

2.2. Production and purification of the compounds

F. verticillioides M-3125 (Leslie et al., 1992) was selected as best FB_1 producing strain of our internal strain-database. An agar plug of a six-day old culture on modified Nirenberg-Agar (SNA) (Nirenberg, 1976) was used to inoculate liquid media. Incubation was performed at 25 °C and 70% humidity on a rotary shaker (200 rpm, GFL, Burgwedel, Germany) for 72 h. One millilitre of this fungal culture was added to each baby jar containing approx. 10 g of a mixture of autoclaved milled maize kernels and polenta. After incubation for 28 days (25 °C, 70% humidity) the maize-cultures contained 12.5 g/kg FB_1, 2.2 g/kg FB_2 and 1.3 g/kg FB_3 on average.

Each jar was extracted with 80 ml MeOH/water/HAc (49.5/49.5/1, v/v/v) using an Ultra-Turrax T25 (IKA-Werke, Staufen, Germany) to destroy the mycelium, followed by 90 min on a rotary shaker at room temperature. The extracts were pooled, evaporated to dryness and the remaining oil was dissolved in MeOH/water (25/75, v/v). Amberlite XAD-2 was pre-treated with water (washing), MeOH (conditioning) and MeOH/water (25/75, v/v, equilibration) for 15 min each before the dissolved oil was added. The batch was stirred for 30 min and filtered. Elution of the fumonisins from the residue was performed with the threefold amount of MeOH for 30 min. The filtered extract was evaporated to dryness and dissolved in EtAc/MeOH/HAc (60/39/1, v/v/v) before drying on silica gel. An approx. 700 × 40 mm glass column was packed with silica gel in EtAc/MeOH/HAc (75/24/1, v/v/v) and the fumonisin containing residue was added on top. The column was flushed with 5 l EtAc/MeOH/HAc (75/24/1, v/v/v) and thereafter the fumonisins were eluted with 5 l EtAc/MeOH/HAc (50/49/1, v/v/v) and 5 l EtAc/MeOH/HAc (25/74/1, v/v/v) and collected in two fractions per eluent. The fractions were evaporated to dryness and a preparative HPLC purification was performed for those fractions containing significant amounts of fumonisins. The isolation of all compounds was performed on a preparative 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Sedex LT-ELSD Model 85LT low temperature evaporative light scattering detector (Sedere, Alfortville, France). The column flow was split 1:60 (the minor part transferring into the ELSD and the main part moving to the fraction collector). Purification of FB_1 was

carried out using a Gemini NX C18-column (150 mm × 21.2 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) and a water–MeOH gradient containing 0.1% HAc. The relevant FB_1 fractions were evaporated to dryness, dissolved in water and further diluted for analysis. One FB_1 fraction with a purity of 97% was used for preparation of the cookies for the FB_1 group. Another FB_1 fraction was used for production of HFB_1. To this end, the FB_1 solution was diluted with water and hydrolysis was performed with 2 M KOH in the final solution. After 60 min the conversion was completed and the formed HFB_1 was extracted by liquid–liquid extraction (twice with ACN and twice with EtAc). All organic layers were combined, evaporated to dryness and dissolved in MeOH/water (30/70, v/v). The obtained HFB_1 solution was purified over a Gemini NX C18-column (150 mm × 21.2 mm, 5 µm) and a water–MeOH gradient containing 0.1% HAc using the preparative HPLC system. The relevant fraction was evaporated to dryness, dissolved in water and used for the preparation of the cookies for the HFB_1 group. $pHFB_1$a and $pHFB_1$b were synthesised by enzymatic hydrolysis of FB_1 and purified according to Schwartz-Zimmermann et al. (in preparation). NDF was synthesised and repeatedly purified as described by Cirlini et al. (in press). The collected fractions of each substance were pooled and evaporated to dryness on a rotary evaporator at 40 °C. The residues were re-dissolved in pure water. Stock solutions were prepared, quantified and stored at 4 °C until further usage.

2.3. Production and analysis of experimental diets

The experimental diets of the five treatment groups (negative control without toxin, FB_1, $pHFB_1$, HFB_1 and NDF) were produced in 1.5 kg batches. In general, the dough of each batch contained 1.2 kg rat feed previously checked for absence of fumonisins (ssniff R/M-H, Ssniff, Soest, Germany), 0.3 kg milled maize, 1 l water and amino acids in solid form (1.52 g/kg L-lysine-HCl, 0.26 g/kg DL-methionine, 0.76 g/kg L-threonine, 0.40 g/kg L-tryptophan and 0.94 g/kg L-valine). For the treatment groups, equimolar amounts of FB_1, $pHFB_1$a+b, HFB_1 and NDF were spiked into the water. Cookies were prepared, dried at 40 °C for three days and stored at –20 °C until 24 h prior to feeding. The analysis of the dried cookies was performed by twofold extraction of 5 g milled cookie with 20 ml each of ACN/water/HAc (79/20/1, v/v/v) for 90 min and LC–MS/MS analysis.

The stability of FB_1, $pHFB_1$a, $pHFB_1$b, HFB_1 and NDF during preparation and upon storage of cookies was investigated. To that end, cookies were prepared as described above (separately for $pHFB_1$a and $pHFB_1$b) and analysed after storage times of one week and one month, respectively, and at different temperatures (room temperature, 4 °C, –20 °C and –80 °C).

2.4. Feeding trial

Male Sprague Dawley rats (Hsd:Sprague Dawley SD, five weeks old, 110.8 ± 4.5 g) were purchased from Harlan Laboratories (Udine, Italy). Animals were housed pairwise in Macrolon type III cages (Ehret Life Science Solutions, Emmendingen, Germany) under constant environmental conditions (ventilated cabinets, Ehret Life Science Solutions; 23 ± 1 °C, 55% relative humidity, 12 h light/dark cycle). Enrichment was provided in form of cellulose tunnels (Bioscape, Emmendingen, Germany). Animals had free access to water for the whole duration of the experiment.

Using a randomised block design, rats were assigned to one of the following five groups (n = 4) according to their weight: negative control, FB_1, $pHFB_1$, HFB_1 and NDF. After an acclimatisation period of one week in which control diet was fed to all animals, treatment diets were provided *ad libitum* for three weeks. With exception of the negative control, diets contained 13.9 µmol/kg of the respective fumonisin derivative, corresponding to 10 mg/kg FB_1, 7.8 mg/kg $pHFB_1$ (3 mg/kg $pHFB_1$a, 4.8 mg/kg $pHFB_1$b), 5.6 mg/kg HFB_1 and 12.2 mg/kg NDF. On days 0, 7, 14 and 21, animals were weighed and kept individually in metabolic cages (Tecniplast, Hohenpeißenberg, Germany) for 24 h to collect urine and faeces. During sampling periods, feed was withdrawn to exclude contamination of excreta by diet derived fumonisins. Urine and faeces samples were volumetrically measured and weighed, respectively, and stored at –20 °C until further analysis. On day 22, animals were euthanised by CO_2 asphyxiation and kidney samples obtained post mortem were frozen at –80 °C.

The animal experiment was approved by the Institutional Ethics Committee and the national authority according to §26ff of the Austrian Law for Animal Experiments, Tierversuchsgesetz – TVG 2012 (BMVG 66.016/0006-II/3b/2013).

2.5. Sample preparation and LC–MS/MS analysis

The methods used for the determination of FB_1, HFB_1, $pHFB_1$a and $pHFB_1$b in urine and faeces samples as well as for the determination of Sa and So in urine samples were recently developed and validated by Schwartz-Zimmermann et al. (in preparation). The method for the determination of FB_1 and analogues in urine and faeces samples was additionally validated for analysis of NDF.

For the determination of fumonisin analogues in urine 400 µl sample was mixed with 1200 µl acetone and 16 µl HAc. The mixture was shaken at room temperature for 10 min, sonicated in a water bath for 5 min and centrifuged (2655 × *g*, 20 °C, 10 min). The supernatants were evaporated to dryness under pressurised air and the residues were taken up in 300 µl ACN/water (30/70, v/v). Finally, the samples were clarified by centrifugation (2655 × *g*, 20 °C, 10 min), the supernatants were transferred into glass vials and stored at –20 °C until LC–MS/MS analysis. [^{13}C]-labelled recovery standards of FB_1, HFB_1 and NDF were spiked to each urine sample before work-up. In addition, a mixture of [^{13}C]-$pHFB_1$a+b was added to an aliquot of sample extract as internal standard prior to measurement for compensation of matrix effects (caused by co-eluting substances which affect ionisation) upon LC–MS/MS analysis.

The sample preparation method for determination of fumonisin analogues in faeces included threefold extraction of 300 mg of homogenised freeze dried faeces with 10 ml, 10 ml and 5 ml of ACN/water/formic acid (74/25/1, v/v/v) by shaking for 30 min, 20 min and 10 min, respectively. After centrifugation (2655 × *g*, 20 °C, 10 min), the supernatants were combined, vortexed, diluted 1:3 with extraction solvent and centrifuged again prior to LC–MS/MS analysis.

For analysis of Sa and So in urine samples 5 ml aliquots of urine were centrifuged at 2655 × *g* and 20 °C for 10 min. Subsequently, the pellet was extracted three times with EtAc. The organic phases were pooled, evaporated to dryness under pressurised air and the residues were taken up in 200 µl MeOH/water (80/20, v/v). The samples of the FB_1 group were diluted 1:5 with reconstitution solvent prior to LC–MS/MS analysis. For determination of Sa and So in kidney samples the tissue was homogenised in cold phosphate buffer (50 mM K_2HPO_4) using an Ultra-Turrax T25. MeOH/ACN (50/50, v/v) was added to 200 µl tissue homogenate for protein precipitation, followed by shaking and centrifugation of the mixture. The pellets were extracted with MeOH/water (80/20, v/v), the supernatants were pooled, evaporated to dryness under pressurised air and the residues were taken up in 200 µl MeOH/water (80/20, v/v). The samples of the FB_1 group were diluted 1:10 with reconstitution solvent prior to LC–MS/MS analysis.

LC–MS/MS analysis was performed in the selected reaction monitoring mode on a 1290 Infinity ultra-high performance liquid chromatography (UHPLC) system (Agilent Technologies) coupled to a 4000 QTrap tandem mass spectrometer (AB Sciex, Foster City, CA) equipped with a Turbo V electrospray ionisation (ESI) source in positive ionisation mode. Chromatographic separation was achieved at 25 °C on a reversed phase Gemini C18 column (150 × 4.6 mm, 5 µm particle size) equipped with a 4 mm × 3 mm C18 security guard cartridge. Additionally, enhanced product ion scan (EPI) measurements with a declustering potential of 89 V and collision energies of 35 eV were used to obtain mass spectra of possible NDF-related metabolites or synthesis by-products (*m/z* 726: partially hydrolysed NDF; *m/z* 568: hydrolysed NDF; *m/z* 780: NCM-FB_1; *m/z* 622: NCM-partially hydrolysed FB_1; *m/z* 464: NCM-hydrolysed FB_1).

2.6. Histopathological examinations

Liver and kidney samples obtained on day 22 were used for pathological scoring. Tissue was fixed in 10% neutral buffered formalin. Haematoxylin and eosin solution (H&E) was used to stain paraffin tissue sections (5 µm). Liver and kidney tissue was examined with a light microscope, randomly and without knowledge of the group allocation of the samples. Apoptotic cells were measured in the proximal convoluted tubules in the deep cortex and outer medulla. Pathological scoring was performed as described by Riley and Voss (2006).

2.7. Data evaluation

For MS data analysis Analyst 1.6.2 (AB Sciex) was used. Calibration functions for calculation of fumonisin concentrations in urine samples were established by linear regression of peak area ratios (analyte/internal standard) against analyte concentrations. As recoveries of extraction of $pHFB_1$a and $pHFB_1$b from urine were close to 100% and matrix effects were compensated by the internal standards, no further correction of the obtained concentrations was required. Due to addition of recovery standard prior to work-up, calculated concentrations of FB_1, HFB_1 and NDF were automatically corrected by the apparent recovery. Concentrations of fumonisins in faeces sample extracts were determined on the basis of linear neat solvent calibration functions. Correction by the apparent recoveries (86–92%) and the dilution factor yielded the concentrations in freeze-dried faeces. Concentrations of Sa and So were calculated from neat solvent calibration functions under consideration of the apparent recoveries (79 and 93%, respectively).

Limits of detection (LOD) and limits of quantification (LOQ) in urine and faeces were calculated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively, on the basis of matrix matched calibration functions. LODs and LOQs in urine were: FB_1 0.3 and 0.9 ng/ml, HFB_1 0.3 and 0.9 ng/ml, $pHFB_1$a 0.2 and 0.6 ng/ml, $pHFB_1$b 0.2 and 0.6 ng/ml, NDF 0.7 and 2.4 ng/ml. Consideration of the dilution factor yielded the following LODs and LOQs in freeze dried faeces: FB_1 310 and 940 ng/g, HFB_1 70 and 240 ng/g, $pHFB_1$a 100 and 290 ng/g, $pHFB_1$b 220 and 720 ng/g, NDF 220 and 840 ng/g.

Sample preparation of urine and faeces samples was performed in duplicate and average concentrations were calculated. In the case samples from one group showed concentrations below and above the LOQ, LOQ/2 was used for the former samples to calculate average values. The relative standard deviation of work-up and analysis was <20%. The total excreted amounts of FB_1, $pHFB_1$a, $pHFB_1$b, HFB_1 and NDF were calculated based on the volumes of urine and weights of faeces samples, respectively, which were collected from the individual rats per sampling day.

Statistical evaluation was performed using IBM SPSS Statistics 19.0. Comparison of means (body weight, Sa/So ratio) was carried out by analysis of variance with Bonferroni *post-hoc* test. Results were considered significant at $p < 0.05$. Statistics of pathological scoring was done with the Kruskal–Wallis test as nonparametric test

and Dunn's test was used for multiple comparisons. Results were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Purity of the compounds

FB_1, HFB_1, as well as $pHFB_1a$, $pHFB_1b$ and NDF were isolated by preparative HPLC in amounts between 30 and 140 mg. The achieved purity of FB_1 was 97.2% (impurities of 1.3% FB_3, 0.6% $pHFB_1a$ and 0.9% $pHFB_1b$), whereas the preparation of HFB_1 did not contain fumonisin related impurities. $pHFB_1a$ showed a purity of 73.2% ($pHFB_1b$ impurity of 26.8%), $pHFB_1b$ a purity of 93.0% ($pHFB_1a$ impurity of 7.0%). The purified NDF contained 2.5% of FB_1.

3.2. Stability test of used compounds during preparation of cookies

Upon preparation of cookies, the recovery for each compound was 70% or higher. However, partially hydrolysed fumonisins converted into each other during drying of the dough to a ratio of $pHFB_1b/pHFB_1a = 1.6$ which then remained stable during storage. Hence, only one group containing both partially hydrolysed fumonisins was included in the subsequent feeding trial. All other compounds (FB_1, HFB_1 and NDF) were stable during drying and storage.

3.3. Feeding trial and histopathological examinations

FB_1 is known to cause several adverse health effects in domestic and laboratory animals (reviewed by Voss et al., 2007). For the present study, the dose of administered FB_1 (and its analogues) as well as the duration of the trial were chosen on the basis of a pretrial in which cookies containing 10 and 50 mg/kg of FB_1 were fed to Sprague Dawley rats for 4 weeks. Significant elevation of the Sa/So ratio occurred already after one week of exposure in both treatment groups. Based on animal welfare considerations, on the EFSA guidance values for pig (5 mg/kg) and poultry feed (20 mg/kg) and on limited availability of pure fumonisin analogues, the main trial was performed with 10 mg/kg FB_1 and equimolar concentrations of fumonisin analogues in rat feed.

The different treatment diets had no statistical influence on the final body weight of the animals (data not shown). Likewise, hardly any histopathological effects (pathological scores <0.70 for all groups) were observed for liver and only mild effects were determined for kidney. In kidney, scores of microscopic effects were similar for the negative control group and the NDF group (both <0.60), slightly increased for the HFB1 (1.25 ± 0.46) and pHFB1 (0.88 ± 0.14) group and significantly elevated for the FB1 group (1.56 ± 0.13).

3.4. Fumonisin derivatives in faeces and urine

In the individual sampling periods, volumes of collected urine varied between 4 ml and 61.5 ml per rat, while amounts of freeze-dried faeces ranged from 0.3 g to 2.9 g. Therein, FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were determined by a validated LC–MS/MS based biomarker method. In faeces samples, concentrations of recovered FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF were between 4.1–43 μg/g, 1.1–13 μg/g, 1.1–23 μg/g, 0.9–39 μg/g and 27–88 μg/g, respectively. In urine, toxin concentrations exceeding the respective LOQ were only determined for FB_1 (up to 10 ng/ml) and NDF (up to 14 ng/ml). In Tables 1 and 2, the averages for the different treatment groups and sampling time points are shown. For better comparison, data are expressed in molar amounts.

In excreta of the negative control group, none of the fumonisin analogues included in our analytical method was detected. These results are in line with LC–MS/MS analysis of the used rodent diet prior to the start of the experiment which showed absence of all investigated fumonisin analogues as well as of FB_2 and FB_3 (LODs ≤ 7 μg/kg).

In contrast, considerable amounts of FB_1, $pHFB_1a$ and $pHFB_1b$ as well as traces of HFB_1 were detected in faeces of rats dosed with FB_1. In general, partially and completely hydrolysed fumonisins are the only metabolites described to occur after FB_1 exposure *in vivo*. It is assumed that formation of these metabolites takes place in the digestive tract, most likely realised by gut microbiota (Fodor et al., 2008; Shephard et al., 1995). The extent of FB_1 conversion varies

Table 1
Total amounts of FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF (mean values ± standard deviation (SD)) recovered in faeces of treated rats during a 24 h sampling period. Animals (n = 4) received either blank feed (negative control) or 13.9 μmol/kg diet of the respective fumonisin analogue *ad libitum*.

Group	Day	FB_1 ± SD (nmol)	$pHFB_1a$ ± SD (nmol)	$pHFB_1b$ ± SD (nmol)	HFB_1 ± SD (nmol)	NDF ± SD (nmol)
Negative control	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	n.d.	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	n.d.	n.d.
FB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	82.5 ± 19.3	3.57 ± 0.96	2.03 ± 1.14	Traces	n.d.
	14	71.4 ± 32.4	1.98 ± 1.15	1.53 ± 0.47	Traces	n.d.
	21	66.1 ± 36.0	1.48 ± 2.06	2.34 ± 1.82	Traces	n.d.
$pHFB_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	18.9 ± 10.1	32.3 ± 18.3	2.82 ± 1.78	n.d.
	14	Traces	32.5 ± 7.2	57.3 ± 15.0	4.69 ± 5.84	n.d.
	21	Traces	25.3 ± 7.0	46.9 ± 14.6	2.61 ± 1.65	n.d.
HFB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	84.4 ± 58.3	n.d.
	14	n.d.	n.d.	n.d.	90.4 ± 49.3	n.d.
	21	n.d.	n.d.	n.d.	60.9 ± 19.1	n.d.
NDF	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	33.9 ± 11.7	n.d.	n.d.	n.d.	63.8 ± 20.0
	14	26.9 ± 21.4	n.d.	n.d.	n.d.	67.2 ± 28.1
	21	23.6 ± 16.8	n.d.	n.d.	n.d.	96.4 ± 42.2

Notes: FB_1, fumonisin B_1; $pHFB_1a$ and $pHFB_1b$, partially hydrolysed fumonisin B_1; HFB_1, hydrolysed fumonisin B_1; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1; n.d., not detected (analyte concentration in all samples below the limit of detection); traces, at least one sample above the limit of detection, but below the limit of quantification.

Table 2
Total amounts of FB_1, $pHFB_1a$, $pHFB_1b$, HFB_1 and NDF (nmol, mean ± standard deviation (SD)) recovered in urine of treated rats during a 24 h sampling period. Animals (n = 4) received either blank feed (negative control) or 13.9 μmol/kg diet of the respective fumonisin analogue *ad libitum*.

Group	Day	FB_1 ± SD (nmol)	$pHFB_1a$ ± SD (nmol)	$pHFB_1b$ ± SD (nmol)	HFB_1 ± SD (nmol)	NDF ± SD (nmol)
Negative control	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	n.d.	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	n.d.	n.d.
FB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	0.11 ± 0.04	n.d.	n.d.	n.d.	n.d.
	14	0.07 ± 0.05	n.d.	n.d.	n.d.	n.d.
	21	0.11 ± 0.05	n.d.	n.d.	n.d.	n.d.
$pHFB_1$	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	Traces	Traces	n.d.	n.d.
	14	n.d.	Traces	Traces	n.d.	n.d.
	21	n.d.	n.d.	Traces	n.d.	n.d.
HFB_1	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	n.d.	n.d.	n.d.	Traces	n.d.
	14	n.d.	n.d.	n.d.	n.d.	n.d.
	21	n.d.	n.d.	n.d.	Traces	n.d.
NDF	0	n.d.	n.d.	n.d.	n.d.	n.d.
	7	Traces	n.d.	n.d.	n.d.	0.07 ± 0.04
	14	Traces	n.d.	n.d.	n.d.	0.08 ± 0.04
	21	Traces	n.d.	n.d.	n.d.	0.11 ± 0.06

Notes: FB_1, fumonisin B_1; $pHFB_1a$ and $pHFB_1b$, partially hydrolysed fumonisin B_1; HFB_1, hydrolysed fumonisin B_1; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1; n.d., not detected (analyte concentration in all samples below the limit of detection); traces, at least one sample above the limit of detection, but below the limit of quantification.

in dependence of factors like species, route of administration or individual differences (Fodor et al., 2008; Rice and Ross, 1994; Shephard et al., 1994a). In rats, the metabolisation pattern of FB_1 has not yet been completely elucidated. Shephard et al. (1992) reported the vast majority of orally administered FB_1 being excreted unmetabolised in faeces. In contrast, Hopmans et al. (1997) proposed that approximately 20% of the toxin dose undergoes metabolic transformation in the gastrointestinal tract. Unfortunately, details on the structure of produced metabolites were not presented in that study. Applying state-of-the-art analytical techniques, we were able to extend the current knowledge on FB_1 metabolisation based on hydrolysis in rats. In the present study, FB_1, $pHFB_1a$, $pHFB_1b$ and HFB_1 reflected 93.8 ± 2.4%, 3.1 ± 2.4%, 2.8 ± 1.4% and 0.3 ± 1.4%, respectively, of the total amount of detected FB_1 analogues in faeces of rats fed the FB_1 diet (average of all sampling time points). Quantifiable concentrations of $pHFB_1a$, $pHFB_1b$ and traces of HFB_1 could be found in faeces samples of all animals, albeit not at all sampling time points. Although a favoured formation of $pHFB_1a$ in comparison to $pHFB_1b$ was described in faeces of vervet monkeys ($pHFB_1a$:$pHFB_1b$, 83:17; Shephard et al., 1995), trans-esterification by intramolecular rearrangements was suggested to lead to a final equilibrium of 45:55 (Shephard et al., 1994b). In faeces of our rats, considerable variations of this proportion, reaching from 9:91 to 78:22, were observed between individual animals and different sampling time points. Seemingly, speed of analysis as well as individual differences in the gut microbiome have a strong influence on this ratio. In urine, comparably low levels of FB_1 were determined. The lack of urinary $pHFB_1a$, $pHFB_1b$ and HFB_1 points to a diminished relevance of hydrolysed fumonisins after oral FB_1 exposure in rats.

To the best of our knowledge, the metabolism of partially hydrolysed fumonisins has not been investigated *in vivo* so far. In rats dosed with $pHFB_1a$+b, the main faecal metabolites were found to be $pHFB_1a$ and $pHFB_1b$. Amounts of $pHFB_1b$ clearly exceeded those of $pHFB_1a$, leading to a proportion $pHFB_1a$:$pHFB_1b$ of 36:64 in the faeces samples. This ratio very much resembles the proportion of partially hydrolysed fumonisins in the provided diet ($pHFB_1a$:$pHFB_1b$, 38:62). In addition, HFB_1 and traces of FB_1 were detected in faeces samples of this group. The amount of HFB_1 generated from $pHFB_1a$+b was similar to the amount of $pHFB_1a$+b generated from FB_1, which indicates that intact and partially hydrolysed fumonisins are hydrolysed at similar rates. In urine of $pHFB_1$ dosed rats, only traces of $pHFB_1a$ und $pHFB_1b$ were found, thus indicating a low bioavailability of these analogues or further metabolisation.

In rats receiving the HFB_1 contaminated diet, exclusively HFB_1 was detected in faeces and urine. Previously, significant differences between HFB_1 concentrations in hydrolysed and non-hydrolysed faecal extracts of rats dosed with HFB_1 were observed (Hopmans et al., 1997). Authors therefore assumed an extensive metabolic conversion of this derivative. The high levels of faecally excreted HFB_1 in our experiment (similar levels compared to FB_1 treatment) do not corroborate these findings in rats. The presence of trace amounts of HFB_1 in urine of only one animal is notable, since a higher bioavailability of HFB_1 in comparison to FB_1 was suggested in former studies (Caloni et al., 2002; Dantzer et al., 1999; Hopmans et al., 1997). Metabolisation of HFB_1 after absorption from the gastrointestinal tract would explain the lack in urinary detected metabolites in our study. For example, the formation of *N*-acyl-metabolites was demonstrated after *intraperitoneal* HFB_1 administration (Seiferlein et al., 2007). If any such products were formed in our study, concentrations would have been too low to affect ceramide synthase activity. Furthermore, conjugation processes with sulphate (as proposed by Hopmans et al., 1997) or glucuronic acid are feasible. Thus, a future task will be the identification of such metabolites using high resolution mass spectrometry.

Masked mycotoxins like NDF pose a risk to human and animal health either by exerting biological effects on their own or by liberating their parent forms during mammalian digestion. Studies investigating the latter aspect came up with ambiguous results. While *in vitro* experiments showed that NDF is rather stable under conditions imitating the human (non-microbial) digestion (Falavigna et al., 2012), *in vivo* hydrolysis was proposed for rats (Hopmans et al., 1997). In the current experiment, NDF reflected the major faecal metabolite of animals exposed to the NDF contaminated diet. In addition, minor amounts of FB_1 were detected. The average proportion NDF:FB_1 (79.5:20.5) in faeces samples differed markedly from the one in the provided diet (97.5:2.5). We therefore conclude that faecally excreted FB_1 derives from partial cleavage of NDF in the gastrointestinal tract rather than from impurities in the feed. It can be

speculated that species specific differences in the gut microbiome play an important role regarding the extent of cleavage of this masked mycotoxin during digestion. Besides NDF and FB_1, no other NDF-related metabolites, in part described in the literature by Howard et al. (1998) and Poling et al. (2002) (partially hydrolysed NDF, hydrolysed NDF, NCM-FB_1, NCM-$pHFB_1$ and NCM-HFB_1), were detected in faeces samples. In urine of NDF exposed rats, NDF as well as traces of FB_1 were determined. The urinary excretion of intact NDF is contradictory to earlier findings, where the presence of merely metabolised NDF was suggested (Hopmans et al., 1997). Trace levels of urinary FB_1 indicate a very limited absorption of this toxin from the gastrointestinal tract.

In contrast to most of the previous toxicokinetic studies performed in rats (Dantzer et al., 1999; Hopmans et al., 1997; Shephard et al., 1992), we did not administer the toxins by gavage, but mixed them into the diet. To avoid bias in urine and faeces sample analysis due to diet derived fumonisins, feed was withdrawn for duration of the individual sampling periods. In consequence, excreted toxin amounts could not be correlated to amounts of ingested toxin and statements on the bioavailability of administered fumonisin analogues are accompanied by some uncertainties. Still, based on overall recovered toxin amounts in excreta, the proportion of analytes detected in faeces exceeded 99% in all treatment groups and on all sampling time points. Although biliary excretion of absorbed fumonisins after oral administration has to be taken into consideration to some minor extent (Dantzer et al., 1999; Shephard et al., 1994c), our data confirm the generally low bioavailability of fumonisins (reviewed by Voss et al., 2007). Further evidence for differences in the absorption rates of certain analogues (Dantzer et al., 1999; Hopmans et al., 1997) cannot be provided on basis of our results.

3.5. Sa/So ratios in urine and kidney

In animal models, the Sa/So ratio serves as specific biomarker for the evaluation of fumonisin toxicity (summarised by Riley et al., 2011). However, differences in organ sensitivity between species, strains and even sex have to be taken into consideration. For example, FB_1 exposure leads to increased Sa/So ratios in liver and plasma of pigs (Grenier et al., 2012), while in most rat strains (e.g. Sprague Dawley) the nephrotoxic effects of fumonisins are more prominent (Riley and Voss, 2006). Average Sa/So ratios in urine of the different treatment groups and sampling time points are provided in Table 3. Urinary Sa/So ratios of the negative control group did not increase over the whole duration of the trial. On the contrary, a disruption of sphingolipid metabolism in rats exposed to FB_1 was observed as early as day 7. Thereafter, levels of Sa/So stayed relatively constant, with Sa and So reaching absolute concentrations as high as 6100 ng/ml and 880 ng/ml in urine, respectively.

While the negative impact of FB_1 on sphingolipid metabolism has been extensively reviewed, reports on the *in vivo* toxicity of hydrolysis products of FB_1 are either controversial (in case of HFB_1) or non-existing at all (in case of $pHFB_1$a and $pHFB_1$b). Most of the studies on HFB_1 demonstrated a much lower toxicity in comparison to FB_1 (summarised by Humpf and Voss, 2004). Yet, certain effects of HFB_1 on the Sa/So ratio and on cancer promoting activity were observed in experiments where nixtamalised corn culture material was fed to rats (Hendrich et al., 1993; Voss et al., 1998). As possible reasons, the presence of matrix-bound fumonisins (Seiferlein et al., 2007) or partially hydrolysed fumonisins (Grenier et al., 2012) in administered culture material have been suggested. Burns et al. (2008) could indeed present evidence for interactions between FB_1 and the corn matrix during the nixtamalisation process. However, neither in the mentioned study, nor in a recent experiment conducted by Voss et al. (2013) significant impact of matrix-bound fumonisins on the sphingolipid metabolism in rats could be demonstrated. In contrast, only *in vitro* data are available concerning the toxicity of partially hydrolysed fumonisins (Caloni et al., 2002). Unfortunately, the cell line used in this study was rather insensitive to fumonisin exposure (independent of the tested analogue) and therefore conclusions on toxicity are limited. The two $pHFB_1$ variants might be expected to act as potent ceramide synthase inhibitors, because based on the results by Humpf et al. (1998), one TCA side chain should be enough to block the acyl-CoA binding site of ceramide synthase, as was reported for the *Alternaria alternata* f.sp. *lycopersici* AAL toxin (Abbas et al., 1994; Merrill et al., 1993b). In the present experiment, neither the exposition to HFB_1 nor to $pHFB_1$ induced significantly elevated Sa/So ratios in urine of treated animals. Based on these results, we suppose that HFB_1 and $pHFB_1$ are both significantly less toxic than FB_1. Admittedly, rats of the $pHFB_1$ group received a diet containing a mixture of $pHFB_1$a and $pHFB_1$b and hence, conclusions on toxicity of the individual forms are impaired. If one but not the other $pHFB_1$ variant is a ceramide synthase inhibitor, its concentration in the present study was too low to affect the sphingolipid biomarker. Nevertheless, no other impurities were detectable in the feed. Thus, our results do not confirm the assumption of partially hydrolysed fumonisins being the causative agent of negative health effects observed in former studies using nixtamalised corn material.

NDF, one of the major products formed by extrusion cooking of FB_1 in the presence of reducing sugars, is supposed to possess a lower toxicity than FB_1 due to blockage of the primary amino group (Howard et al., 2002). In the past, several studies aiming to evaluate the toxicity of reaction products of FB_1 and fructose/glucose have been performed (Fernández-Surumay et al., 2004, 2005; Howard et al., 2002; Liu et al., 2001; Lu et al., 1997). In general, a reduced toxicity of FB_1-glucose/fructose-adducts in comparison to FB_1 was reported (reviewed by Jackson et al., 2012). However, with exception of Howard et al. (2002), who fed NCM-FB_1 of high purity to rats, the reaction products in the administered diets were either not characterised or contained significant levels of unreacted FB_1. These drawbacks were discussed as reasons for toxicity signs still

Table 3
Urinary sphinganine-to-sphingosine ratios (Sa/So, mean ± standard deviation (SD)) of different treatment groups (n = 4) and sampling time points. Sa/So ratios with the same letter are not significantly different from each other (Bonferroni *post-hoc* test, $p < 0.05$).

Group	Sa/So d0 (mean ± SD)	Sa/So d7 (mean ± SD)	Sa/So d14 (mean ± SD)	Sa/So d21 (mean ± SD)
Negative control	0.17 ± 0.01	0.26 ± 0.12^b	0.26 ± 0.06^b	0.27 ± 0.11^b
FB_1	0.22 ± 0.07	6.10 ± 3.15^a	6.00 ± 1.57^a	6.68 ± 1.78^a
$pHFB_1$	0.16 ± 0.02	0.19 ± 0.04^b	0.16 ± 0.02^b	0.16 ± 0.03^b
HFB_1	0.17 ± 0.07	0.19 ± 0.02^b	0.17 ± 0.02^b	0.18 ± 0.04^b
NDF	0.18 ± 0.06	0.21 ± 0.03^b	0.23 ± 0.06^b	0.30 ± 0.08^b

Notes: FB_1, fumonisin B_1; $pHFB_1$a and $pHFB_1$b, partially hydrolysed fumonisin B_1; HFB_1, hydrolysed fumonisin B_1; NDF, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1.
Symbols "a" and "b" refer to statistical differences between groups (as indicated in the table description).

observable after administration of FB_1-glucose/fructose-adducts (e.g. enhanced tumour cytotoxicity, development of porcine pulmonary oedema, elevated Sa/So ratios) in some of these studies (Fernández-Surumay et al., 2004, 2005; Lu et al., 1997). In the present study, NDF of high purity, obtained by preparative HPLC purification and determined by LC–MS/MS methods, was used. In rats receiving the respective diet, no increase in urinary Sa/So ratios was detected. NDF itself had no effect on sphingolipid metabolism, and FB_1 was liberated from NDF at too low concentration or too far down the digestive tract to elicit toxicity. We therefore suggest that NDF is less toxic than FB_1 in rats.

Sa and So concentrations were also determined in kidney samples. Average Sa/So ratios in the negative control, FB_1, $pHFB_1$, HFB_1 and NDF group reached levels of 0.12 ± 0.01, 4.06 ± 0.63, 0.14 ± 0.03, 0.12 ± 0.01 and 0.23 ± 0.09, respectively. These results, demonstrating a statistically significant elevation of the Sa/So ratio only in kidney samples of rats exposed to FB_1, are in good agreement to data obtained for urine.

3.6. Conclusions

Partially hydrolysed FB_1, HFB_1 and NDF are major food and feed processing products of FB_1. In addition, as revealed in the present study, $pHFB_1$ and HFB_1 are natural hydrolysis products of FB_1 in rats, most likely formed by intestinal microorganisms. By producing high purity $pHFB_1$a+b, HFB_1 and NDF and feeding these substances to rats, we were able to extend the current knowledge on the toxicological relevance of these compounds. Whereas the Sa/So ratio in urine and kidney of the FB_1 group was tremendously elevated even after one week of exposure to 10 mg/kg FB_1 in the diet, neither $pHFB_1$ nor HFB_1 increased the Sa/So ratio. Analysis of fumonisin metabolites in rat faeces and urine by validated LC–MS/MS based methods indicated that NDF is partly cleaved to FB_1 during gastrointestinal passage. Yet, the liberated FB_1 was mainly excreted in faeces and did not raise the Sa/So ratio. Hence, all investigated fumonisin derivatives are of much lower toxicological relevance than FB_1. These insights into gastro-intestinal metabolism and toxicology of fumonisin derivatives are important contributions to food and feed safety.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Highlights

In this thesis, the following highlights regarding the detoxification of ergot alkaloids, deoxynivalenol, zearalenone and fumonisins have been achieved:

Detoxification of ergot alkaloids (publication #1)

- An LC-MS/MS method for the simultenous determination of ergot alkaloids and their selected metabolites has been developed and optimised.
- The characterisation and structure elucidation of unknown metabolites formed during microbial and enzymatic degradation of ergot alkaloids has been performed.
- The *in vitro* efficacy of the tested enzymes in regard to further developments of novel feed additives for *in vivo* detoxification of ergot alkaloids has been evaluated.

Detoxification of deoxynivalenol and zearalenone (publication #2)

- An *in vitro* assay to test the aerobic and anaerobic mycotoxin degradation ability of commercial feed addititives has been developed.
- The *in vitro* efficacy of numerous commercially available products claiming detoxification of deoxynivalenol and zearalenone by metabolisation and additional adsorption has been evaluated.
- The ineffectiveness of the majority of the tested products to detoxify these mycotoxins has been demonstrated.

Detoxification of fumonisins (publication #3 and #4)

- Chemically synthesised *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF) has been purified.
- The investigation of the catabolic fate of fully hydrolysed fumonisin B_1 (HFB_1) and NDF under human gastrointestinal conditions revealed that HFB_1 was partially metabolised during incubation with human faeces, while NDF was partially cleaved during incubation with digestive juices.
- The evaluation of the toxicological relevance of partially hydrolysed fumonisin B_1 ($pHFB_1$), HFB_1 and NDF in rats demonstrated that $pHFB_1$, HFB_1 and NDF had no effect on the sphingolipid ratio in rats, although NDF was partly cleaved to FB_1 during gastrointestinal passage.

Conclusion

Mycotoxins are common contaminants frequently occurring worldwide mainly in cereal- and fruit-based products. Due to their potential threat to human and animal health, suitable methods controlling mycotoxin exposure are required. Various strategies for counteracting mycotoxins in regard to food and feed contamination have been developed. Depending on the time point at which the mycotoxin is entering the food and feed chain, techniques range from the prevention of mycotoxin production on the field and during storage to methods addressing the elimination of the toxic compounds (including product decontamination by removing and destroying of the fungus and the toxin as well as *in vivo* detoxification). Pre-harvest techniques focus on the prevention of mycotoxin production on the field and include methods applying plant breeding and transgenic approaches, crop rotation, tillage procedures, chosen sowing date, chemical, biological and insect control as well as ideal harvesting date and climate factors. Strategies regarding the prevention of mycotoxin production during storage address storage conditions that do not promote fungal growth, cleaning of the used equipment and the application of antifungal agents. Post-harvest elimination methods can be divided into the decontamination of commodities and *in vivo* elimination techniques. Decontamination of infected commodities covers physical (separation and sorting, cleaning, washing, dehulling, milling, thermal treatment, irradiation and cold plasma) and chemical (oxidation, reduction, acidification and alkalisation) strategies. In contrast, biological *in vivo* elimination techniques are based on mycotoxin adsorption by feed additives resulting in reduced bioavailability as well as on the biotransformation and degradation of mycotoxins into less toxic compounds by microorganisms or isolated enzymes.

Pre-harvest strategies are promising approaches, but cost-intensively and more research is needed investigating further potentials, risks and applicability. In addition, their efficacy depends on various parameters that can hardly be influenced (e.g. weather) and therefore, mycotoxin contamination cannot fully be avoided. Consequently, more specific post-harvest techniques are required. Due to the chemically and biologically diversity of both fungi and mycotoxins, no single method has been found and developed that can equally counteract the majority of different potentially co-occurring mycotoxins, although certain strategies for the reduction of specific mycotoxins can be applied. In addition, adequate mycotoxin counteracting techniques have to fulfil various requirements. Besides their efficacy in reducing the load of mycotoxins, such methods have to guarantee that no toxic compounds are formed and that nutritional values and sensory properties should not be altered. In addition, mycotoxin counteracting processes have to be economically and technically feasible. Hence, only few methods are available and appropriable on an industrial scale. Furthermore, especially the efficacy of physical techniques is extremely variable and within the European Union chemical treatment of human food products for detoxification of mycotoxins is not allowed. Consequently, biological strategies aiming to detoxify mycotoxins *in vivo* after the consumption of contaminated commodities show the greatest promise of application. Several mycotoxin adsorbing materials traded

as feed additives have been repeatedly tested for their binding capacity against various mycotoxins *in vitro* as well as *in vivo*, whereas research regarding proper scientific evidence of the efficacy of microorganisms or enzymes to detoxify certain mycotoxins is still needed.

Therefore, the main objective of the presented thesis was to evaluate the effectiveness of different microbial interactions with certain mycotoxins *in vitro* and *in vivo* for possible detoxification. In particular, the efficacies of four different counteracting strategies for certain mycotoxins were investigated.

Recently, a *Rhodococcus erythropolis* strain MTHt3 (DSM 25948) capable of degrading ergopeptines has been isolated from soil and the responsible enzymes ErgA and ErgB have been identified and characterised by BIOMIN Holding GmbH. The alpha/beta-hydrolase ErgA is responsible for the first step of the ergopeptine degradation to ergine, while ErgB, an amidase, catalyses a further degradation of ergine to lysergic acid. In **publication #1** the determination and structure elucidation of unknown metabolites formed during this microbial and enzymatic degradation (by the MTHt3 strain, its lysate and the purified enzyme ErgA) of six different ergopeptines (ergocornine, ergocristine, α-ergocryptine, ergosine, ergotamine, ergovaline and the epimer ergotaminine) is described. Insights into the mechanism of the enzymatic degradation were provided by performing degradation experiments and LC-MS/MS measurements. For this purpose, an LC-MS/MS method for the determination of the ergopeptines, the intermediates and the products of the degradation reaction had to be developed and applied. The unknown metabolites were characterised and identified by using preparative HPLC for isolation, measurements of the optical rotation, ^{1}H-, ^{13}C- and 2D-NMR as well as LC-HR-MS analysis. As a result, in the first degradation step, catalysed by ErgA, two main groups of metabolisation products formed during the degradation of six different ergopeptines were identified: 1) diketopiperazines (cyclic dipeptides, DKPs, named 196, 210 and 244 metabolites) that are stable and probably non-toxic and 2) unstable ergine hydroxy carboxylic acids (intermediate metabolites termed 355 and 383 metabolites). Subsequently, the 355 and 383 metabolites are decomposed spontaneously to ergine and an oxocarboxylic acid (pyruvic and isopropylglyoxylic acid, respectively) due to their instability. The amidase ErgB, catalysing the second degradation step, is responsible for the cleavage of ergine to lysergic acid. The major deleterious effects of ergot alkaloids are based on their vasoconstrictive effects, which are 1000 times less pronounced with lysergic acid compared to ergovaline. In conclusion, the potential of the tested enzymes as feed additives to achieve safe *in vivo* detoxification of ergot alkaloids was evaluated by the characterisation of the degradation products and the enzymatic reaction. These are crucial and first steps in regard to a future development of novel feed additives for gastrointestinal detoxification of ergopeptines in farm animals. Most importantly, animal trials have to prove the efficacy and the safety of potential products.

Various feed additives claiming *in vivo* reduction and detoxification of mycotoxins are commercially available without a proper scientific evidence of their efficacy. Regarding their ingredients, mode of

action of the majority of those products is probably based on biodegradation or a combination of degradation and adsorption. The practical application and effectiveness of such products, especially in regard to DON and ZEN decontamination, has often been questioned and studies evaluation their performances are very limited. Within **publication #2** commercially available feed additives with the claim to detoxify DON and/or ZEN by metabolisation and additional adsorption were tested and evaluated *in vitro*. For this purpose, degradation experiments of DON and ZEN with 20 different products were performed separately under aerobic and anaerobic conditions for up to 72 h at pH 7.0 in triplicate. Defined incubation conditions were chosen in order to enable mycotoxin reduction by a broad range of stated product ingredients (agents such as microorganisms, enzymes or binders). By analysing the samples taken during incubation with LC-UV-MS/MS for DON, ZEN and related compounds, the ineffectiveness of the majority of the tested products to detoxify these mycotoxins was demonstrated. In detail, a complete DON reduction and the consequent formation of the non-toxic metabolite DOM-1 was exclusively achieved by one product under the applied anaerobic conditions, while for all other products a maximum DON reduction of 17% after 72 h of incubation was obtained. Concerning possible metabolisation of ZEN, with four products a ZEN reduction ≥ 60% after 24 h of incubation was observed, while only one product was able to degrade ZEN completely under aerobic and anaerobic conditions resulting in the formation of the less-toxic metabolites DHZEN and HZEN. In contrast, the incubation of six products with ZEN resulted in the formation of the still oestrogenic metabolites α- and/or β-ZEL and unknown metabolites with unidentified toxicity were detected during aerobic and anaerobic degradation with 10 and 3 products, respectively. In conclusion, to the best of our knowledge, publication #2 describes the most extensive study on the possible reduction of DON and ZEN by degrading enzymes or microorganisms. The proposed approach provides a first *in vitro* screening assay for testing the efficacy of products claiming mycotoxin detoxification and to verify manufacturers' claims. Our results demonstrate that numerous products currently on the market are ineffective in degrading DON and/or ZEN, at least under the applied conditions. Therefore, *in vitro* degradation experiments are essential to critically evaluate the claims of such products. Moreover, additional tests (in particular *in vivo* trials) are mandatory for a complete performance evaluation of the products.

Food processing of mycotoxin contaminated commodities may result in modified mycotoxin derivatives with unknown toxicological relevance. Especially for fumonisins, chemical modifications caused by thermal or alkaline food treatment have been reported. Therefore, the aim of **publication #3** was to investigate the stability and catabolic fate of HFB_1 and NDF under human gastrointestinal conditions compared to FB_1. HFB_1 represents a degradation product formed during alkaline treatment of food as well as during enzymatic degradation catalysed by a carboxylesterase used in a commercial feed additive. NDF is one major product formed by thermal food treatment and extrusion of fumonisin contaminated food or feed in the presence of glucose and liberation of the parent forms during mammalian digestion may occur. For this purpose, the stability of HFB_1 and NDF was tested by

applying an *in vitro* digestion and human colonic fermentation assay. To this end, NDF was chemically synthesised and purified using preparative HPLC for further experiments due to the lack of available analytical standards. As a result, a partial cleavage of NDF during simulated digestion was observed, while the stability of NDF towards human gut microbiota was demonstrated. For HFB_1 a partial metabolisation to unknown compounds after 24 h of incubation by gut microbiota was detected. In conclusion, the results of this study emphasise the need of animal experiments to investigate the toxicological relevance of those compounds *in vivo*.

In **publication #4** the toxicological relevance of orally administered FB_1, $pHFB_1$, HFB_1 and NDF in rats were investigated. In particular, the effects on the sphingolipid metabolism as well as a possible metabolisation of those fumonisin derivatives was evaluated and compared. To this end, target substances were prepared, purified, added to cookies and their stability was tested. The experimental diets (containing 13.9 µmol/kg of high purity FB_1, $pHFB_1$, HFB_1 and NDF, respectively) were fed to 20 male Sprague Dawley rats (n = 4 for each treatment group) for three weeks. The samples taken once a week (urine and faeces samples) as well as at the end of the trial (kidney samples) were analysed for possible metabolisation as well as for the Sa to So ratio by validated LC-MS/MS based methods. As a result, the Sa/So ratio was significantly increased in the FB_1 positive control group, while this biomarker of effect was indifferent from the negative control group for $pHFB_1$, HFB_1 and NDF. However, partial decomposition of NDF to FB_1 was observed in faeces samples, suggesting partial liberation during digestion *in vivo*. Nevertheless, all tested compounds were of lower toxicological relevance compared to FB_1 and food processing as well as enzymatic degradation may reduce fumonisin toxicity.

Three of the presented publications investigate the efficacy of biological strategies aiming to detoxify mycotoxins *in vivo* after the consumption of contaminated commodities and the current knowledge on novel and promising biological strategies was extended. In addition, the stability and toxicological relevance of derivatives formed during food processing was evaluated. Unknown metabolites formed during microbial and enzymatic degradation of ergot alkaloids were identified and characterised. In addition, the efficacy of commercially available feed additives claiming detoxification of DON and ZEN was tested *in vitro*. Furthermore, the catabolic fate of HFB_1 and NDF was investigated under human gastrointestinal conditions and the toxicological relevance of orally administered FB_1, $pHFB_1$, HFB_1 and NDF in rats was evaluated.

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Zinedine, A. and Mañes, J. (2009) Occurrence and legislation of mycotoxins in food and feed from Morocco. Food Control 20, 334-344.

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

Zöllner, P. and Mayer-Helm, B. (2006) Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionization mass spectrometry. J Chromatogr A 1136, 123-169.

Curriculum Vitae

I R E N E H A H N

Master of Science in Natural Sciences

P E R S O N A L D E T A I L S

Date of birth	August 16, 1984
Place of birth	St. Pölten, Lower Austria
Nationality	Austria

E D U C A T I O N

since 10/2011	**Doctoral Program in Technical Sciences** **Vienna University of Technology,** Austria Technical chemistry (E 786 800) CD Laboratory for Mycotoxin Metabolism at the IFA Tulln, BOKU (supervisors: Univ. Prof. DI Dr. Rudolf Krska and Assoc. Prof. Mag. Dr. Franz Berthiller)
09/2009 – 06/2011	**Master program Biotechnical Processes (569)** **University of Applied Sciences Wiener Neustadt, Tulln,** Austria Master of Science in Natural Sciences (passed with highest distinction) Master thesis I: „Determination of Fumonisin Biomarkers in Biological Samples", Mather thesis II: „Evaluation of Selected Fumonisin Biomarkers in Biological Samples" (supervisors: Univ. Prof. DI Dr. Rudolf Krska, Assoc. Prof. Mag. Dr. Franz Berthiller and Dr. Justyna Rechthaler)
09/2006 – 06/2009	**Bachelor program Biotechnical Processes (568)** **University of Applied Sciences Wiener Neustadt, Tulln,** Austria Bachelor of Science in Natural Sciences (passed with highest distinction) Bachelor thesis I: „Metabolism and Toxicity of Ergot Alkaloids", Bachelor thesis II: „Isolation and Purification of an Ergot Alkaloid Degrading Enzyme" (supervisor: Dr. Wulf-Dieter Moll)
10/2002 – 07/2006	**University of Vienna,** Austria **Field of study: Chemistry (A419)**

09/1994 – 05/2002	**Bundesgymnasium, Bundesrealgymnasium,** St. Pölten, Lower Austria, Austria Grammar school of general education General qualification for university entrance (passed with highest distinction): May 2002

GRANTS AND AWARDS

2011	**Award for best scientific master thesis** from the University of Applied Sciences Wiener Neustadt, Tulln
2010	**Grant for highest distinction** from the University of Applied Sciences Wiener Neustadt, Tulln
2007	**Grant for highest distinction** from the University of Applied Sciences Wiener Neustadt, Tulln

MEMBERSHIPS

since 2011	Member of the Austrian Chemical Society (**GÖCh**)
since 2011	Member of the Austrian Society for Analytical Chemistry (**ASAC**)

CONFERENCES

Oral presentations

(Bio-) Analytical Seminar, Tulln, Austria
4 March 2015
I. Hahn
Development and application of LC-MS/MS based biomarker methods to determine the toxicological impact of the mycotoxin fumonisin B_1 and its analogues on rats

30th International Symposium on Chromatography, Salzburg, Austria
14-18 September 2014
I. Hahn, V. Nagl, E. Varga, M. Cirlini, C. Schwarz, R. Krska, F. Berthiller, H. E. Schwartz-Zimmermann
Development and application of LC-MS/MS based biomarker methods to determine the toxicological impact of the mycotoxin fumonisin B_1 and its analogues on rats

10th ASAC JunganalytikerInnen Forum, Tulln, Austria
13-14 June 2014
I. Hahn, E. Kunz-Vekiru, M. Twarużek, J. Grajewski, R. Krska, F. Berthiller
In vitro testing and LC-UV-MS/MS evaluation of feed additives claiming to detoxify the mycotoxin deoxynivalenol

Seminar „Moderne Analytische Chemie“ (151.398) SS 2014, Institut für Chemische Technologien und Analytik, Technische Universität Wien, Vienna, Austria
8 May 2014
I. Hahn, M. Thamhesl, H. E. Schwartz-Zimmermann, V. Klingenbrunner, C. Hametner, G. Schatzmayr, W.-D. Moll, R. Krska, F. Berthiller
LC-MS/MS and LC-HR-MS characterization of metabolites formed by enzymatic degradation of ergot alkaloids

1st Campus Tulln Doc Day, Tulln, Austria
10 October 2013
I. Hahn, M. Thamhesl, V. Klingenbrunner, C. Hametner, R. Krska, G. Schatzmayr, W.-D. Moll, F. Berthiller, H. E. Schwartz-Zimmermann
Determination of metabolites formed by enzymatic degradation of ergot alkaloids

Mycotoxin Summer Talks, Tulln, Austria
4-5 July 2013
I. Hahn, M. Thamhesl, V. Klingenbrunner, C. Hametner, R. Krska, G. Schatzmayr, W.-D. Moll, F. Berthiller, H. E. Schwartz-Zimmermann
Determination and structure elucidation of ergot alkaloid metabolites formed by microbial and enzymatic degradation

ISM-MycoRed International Conference Europe, Martina Franca, Italy
27-31 May 2013
I. Schöner, M. Thamhesl, V. Klingenbrunner, C. Hametner, R. Krska, G. Schatzmayr, W.-D. Moll, F. Berthiller, H. E. Schwartz-Zimmermann
Determination and structure elucidation of ergot alkaloid metabolites formed by microbial and enzymatic degradation

(Bio-) Analytical Seminar, Tulln, Austria
23 January 2013
I. Schöner
Identification and characterization of metabolites formed by microbial and enzymatic degradation of ergot alkaloids

Scientific Evaluation of the Christian Doppler Laboratory for Mycotoxin Metabolism, Tulln, Austria
23 November 2012
I. Schöner
Identification and characterization of metabolites formed by microbial and enzymatic degradation of ergot alkaloids

(Bio-) Analytical Seminar, Tulln, Austria
12 October 2011
I. Schöner
Evaluation of selected fumonisin biomarkers in biological samples

7th ASAC JunganalytikerInnen Forum, Linz, Austria
25-26 September 2011
I. Schöner, B. Grenier, V. Slavik, O. Greitbauer, R. Krska, G. Schatzmayr, F. Berthiller, I. Oswald, W.-D. Moll, H. E. Schwartz
Evaluation of selected fumonisin biomarkers in biological samples

Poster presentations

World Mycotoxin Forum, Vienna, Austria
8-12 November 2014
I. Hahn, V. Nagl, H. E. Schwartz-Zimmermann, E. Varga, M. Cirlini, C. Schwarz, R. Krska, F. Berthiller
Effects of orally administered fumonisin B_1, partially hydrolysed fumonisin B_1, hydrolysed fumonisin B_1 and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 on the sphingolipid metabolism in rats

36th Mycotoxin-Workshop, Göttingen, Germany
16-18 June 2014
I. Hahn, E. Kunz-Vekiru, M. Twarużek, J. Grajewski, R. Krska, F. Berthiller
Aerobic and anaerobic *in vitro* testing of deoxynivalenol-detoxifying feed additives

ISM International Mycotoxin Conference, Beijing, China
19-23 May 2014
I. Hahn, E. Kunz-Vekiru, M. Twarużek, J. Grajewski, R. Krska, F. Berthiller
Aerobic and anaerobic *in vitro* evaluation of commercial feed additives claiming to detoxify deoxynivalenol

6th International Symposium on Recent Advances in Food Analysis (RAFA), Prague; Czech Republic
5-8 November 2013
I. Hahn, M. Thamhesl, V. Klingenbrunner, C. Hametner, R. Krska, G. Schatzmayr, W.-D. Moll, F. Berthiller, H. E. Schwartz-Zimmermann
Determination of metabolites formed by enzymatic degradation of ergot alkaloids

ANAKON der Gesellschaft Deutscher Chemiker - Fachgruppe Analytische Chemie, Essen, Germany
4-7 March 2013
I. Schöner, M. Thamhesl, V. Klingenbrunner, C. Hametner, R. Krska, G. Schatzmayr, W.-D. Moll, F. Berthiller, H. E. Schwartz-Zimmermann
Identification and characterization of metabolites formed by enzymatic degradation of ergot alkaloids

33rd Mycotoxin-Workshop, Freising, Germany
30 May-1 June 2011
I. Schöner, O. Greitbauer, C. Hametner, W.-D. Moll, R. Krska, F. Berthiller, H. E. Schwartz
Production of partially hydrolyzed fumonisins B_1as standards for determination of fumonisin biomarkers in biological samples

ANAKON der Gesellschaft Deutscher Chemiker - Fachgruppe Analytische Chemie, Zürich, Switzerland
22-27 March 2011
I. Schöner, B. Grenier, R. Krska, G. Schatzmayr, F. Berthiller, I. P. Oswald, W.-D. Moll, H. E. Schwartz
Determination of fumonisin $B_1$and its hydrolysis products in tissue samples of piglets

Mold-Meeting der ALVA-Fachgruppe Mikrobiologie & Molekularbiologie, Linz, Austria
2-3 December 2010
I. Schöner, B. Grenier, R. Krska, G. Schatzmayr, I. Oswald, W.-D. Moll, H. E. Schwartz
Determination of fumonisins and their hydrolysis products in pigs – part 1: tissue samples