Title: Metabolism of modified mycotoxins studied through in vitro and in vivo models: an overview

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METABOLISM OF MODIFIED MYCOTOXINS STUDIED THROUGH *IN VITRO* AND *IN VIVO* MODELS: AN OVERVIEW

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ABSTRACT

Mycotoxins are toxic, secondary metabolites produced by fungi. They occur in a wide variety of food and feed commodities, and are of major public health concern because they are the most hazardous of all food and feed contaminants in terms of chronic toxicity. In the past decades, it has become clear that in mycotoxin-contaminated commodities, many structurally related compounds generated by plant metabolism, fungi or food processing coexist with their free mycotoxins, defined as modified mycotoxins. These modified xenobiotics might endanger animal and human health as they are possibly hydrolysed into their free toxins in the digestive tract of mammals, and may consequently contribute to an unexpected high toxicity. As modified toxins represent an emerging issue, it is not a surprise that for most toxicological tests data are scarce to non-existent. Therefore, there is a need to elucidate the disposition and kinetics of both free and modified mycotoxins in mammals to correctly interpret occurrence data and biomonitoring results. This review emphasizes the current knowledge on the metabolism of modified mycotoxins using *in vitro* and *in vivo* models.

KEYWORDS: *in vitro, in vivo,* toxicology, metabolism, mycotoxins, modified mycotoxins, masked mycotoxins

MODIFIED MYCOTOXINS

Mycotoxins are toxic, low-molecular-weight, secondary metabolites produced by fungi. They function as insecticides, play a role in fighting against plant defence to the fungus, and they assist the fungus in some way to compete for their ecological niche in nature (Richard *et al.* 2007). Mycotoxins are of a major public health concern because they are the most hazardous of all food and feed contaminants in terms of chronic toxicity (Kuiper-Goodman 1998). Some mycotoxins are harmful to other micro-organisms such as other fungi or even bacteria, like the antibiotic penicillin, produced by *Penicillium chrysogenum* (Fleming 1929); others are potent carcinogens like aflatoxins. The most important fungal genera producing mycotoxins that are found in food products are *Aspergillus*, *Fusarium*, *Alternaria, Claviceps* and *Penicillium.*

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

The term *masked* mycotoxins was firstly introduced by Gareis *et al.* (1990) revealing the cleavage of zearalenone-14-glucoside (ZEN-14G) during digestion in swine (Gareis *et al.* 1990). The primary definition expressed *masked* mycotoxins as molecules, not detectable by standard routine analysis. Berthiller *et al.* (2013), however, suggested to use the term of *masked* mycotoxins, solely for the description of plant derivatives of mycotoxins (Berthiller *et al.* 2012). Continuing this line of reasoning, Rychlik *et al*. (2014) highlighted the necessity to systematize the nomenclature of mycotoxin conjugates, in order to avoid further misunderstanding (Rychlik *et al.* 2014). The term *modified* mycotoxins was introduced to describe all types of mycotoxins modifications on four levels. The first level distinguishes free mycotoxins from those being matrix-associated, chemically or biologically modified. Free mycotoxins are designated compounds, produced as secondary metabolites of microfungi causing disease and death in humans and other species (*e.g.* aflatoxins, patulin or zearalenone

(ZEN)). The mentioned definition of modified mycotoxins will allow systematizing nomenclature of mycotoxin-conjugates, and helps to uniform this topic in future.

The presence of modified mycotoxins was revealed in cereal-based food and feed for trichothecenes, myco-estrogens and fumonisins. More specifically deoxynivalenol-3-glucoside (DON-3G), zearalenone-14-glucoside (ZEN-14G), zearalenone-14-sulfate (ZEN-14S), α-zearalenol-14-glucoside (α-ZEL-14G), β-zearalenol-14-glucoside (β-ZEL-14G), T-2-toxin-3-glucoside (T-2-3G), HT-2-toxin-3-glucoside (HT-2-3G), diacetoxyscirpenol-3-glucoside (DAS-3G), nivalenol-3-glucoside (NIV-3G), mono-acetoxyscirpenol-3-glucoside (MAS-3G), neosolaniol-3-glucoside (NEO-3G) and fumonisins-esters (Berthiller *et al.* 2005; Berthiller *et al.* 2012; Dall'Asta *et al.* 2009a; Dall'Asta *et al.* 2009b; De Boevre *et al.* 2012; De Boevre *et al.* 2014; Nakagawa *et al.* 2013a; Nakagawa *et al.* 2011; Nakagawa *et al.* 2013b) were detected and confirmed.

It has become clear that modified mycotoxins could represent a potential additional risk for both animals and humans. However, despite the enormous progress, many research challenges remain since essential information is still lacking, and more studies are urgently needed to unravel the true modified mycotoxin problemacy. Nevertheless, most concerns are related to the potential health effects of conjugated forms of mycotoxins. Either direct toxic effects of the conjugated forms, or indirect toxicity via hydrolysis to their free forms can be attributed to modified mycotoxins. Only few reports indicated the possible toxic relevance of conjugated forms. In the near future, full-scale metabolism studies could reveal relevant toxicity data by incorporating modified mycotoxins *in vivo*.

IN VITRO **STUDIES**

To determine the impact of modified mycotoxins on human health, the scientific community requested to investigate the complete metabolic profile of these forms (European Food Safety Authority 2014). Up to now, data regarding the *in vivo* toxicological relevance of modified mycotoxins were lacking. As modified toxins represent an emerging issue, it is not a surprise that for most toxicological tests no data are available, such as genotoxicity, short-term and long-term toxicity including carcinogenicity, reproduction and developmental studies. As conjugation is known to be a detoxification process *in planta*, it seems likely that conjugated mycotoxins exhibit a lower acute toxicity compared to their free compounds. The accessibility and the high degree of complexity of *in vivo* trials limit the acquisition of information on health effects of specific processes. For this reason, *in vitro* batch incubations have been executed to circumvent these complexities. *In vitro* studies were designed to mimick natural

conditions during digestion, and to check the fate of modified mycotoxins during contact with stomach juices and interaction with human colonic microbiota. Recently, Berthiller *et al.* demonstrated the possible toxicological relevance of DON-3G by demonstrating that several lactic acid bacteria hydrolyse DON-3G during digestion *in vitro* (Berthiller *et al.* 2011). Acidic conditions, hydrolytic enzymes and intestinal bacteria, respectively, mimicking different stages of digestion, were included. DON-3G was found resistant to 0.2 M HCl for at least 24 h at 37 °C (pH 1.7), suggesting that the conjugated form will not be hydrolysed in the stomach of mammals. While human cytosolic β-glucosidase also had no effect, forty-seven different bacterial strains, isolated from the gut, were examined towards their ability to hydrolyse DON-3G. Fungal cellulase and cellobiase, and more specifically lactic acid bacteria, such as *Enterococcus durans, Enterococcus mundtii* or *Lactobacillus plantarum*, could cleave a significant portion of DON-3G.

Furthermore, De Nijs *et al.* (2012) investigated an *in vitro* static digestive simulation model mimicking the upper gastro-intestinal tract (saliva and stomach) and the lower gastro-intestinal tract (small intestine) (De Nijs *et al.* 2012). The transformation of DON-3G in the small intestine was assessed by adoption of Caco-2 cells in a Transwell® system. The authors likewise reported that deoxynivalenol (DON) was not released from DON-3G in the mammals' stomach and was resistant to human cytosolic β-glucosidase. There was no evidence of the release of DON from DON-3G spiked at a level of 2,778 μg DON-3G/kg food during the complete simulation protocol.

Besides DON, ZEN and its derivatives were also investigated. Recently, Dall'Erta *et al.* (2013) demonstrated that these modified forms were effectively deconjugated by human colonic microbiota, releasing their toxic aglucones and generating unidentified catabolites using an *in vitro* batch incubation experiment (Dall'Erta *et al.* 2013). The tremendous amount of different bacterial strains and associated enzymes in the human colon makes the gastrointestinal tract a remarkable bioreactor, able to chemically transform most of compounds ingested by humans. An *in vitro* digestion assay was performed for DON-3G, ZEN-14G and ZEN-14S by the use of four synthetic juices, namely saliva, gastric juice, duodenal juice and bile juice. After incubation in these phases the modified mycotoxins were fully recovered with amounts of 99.5%, 97.3% and 98.6%, respectively. Concurrently, an *in vitro* fecal fermentation assay was executed at time frames of 30 minutes and 24h. In contrast, when an in vitro fecal fermentation assay was conducted, the modified mycotoxin forms ZEN-14G and ZEN-14S were completely cleaved after 24h. Remarkably, ZEN, however, was only partially recovered in the fecal slurry which implied further degradation of ZEN in this

specific section of the digestive tract and the presence of unknown catabolites of ZEN. Regarding DON-3G, this conjugate was also not deglucosylated after 30 min. Complete degradation (90% DON) was observed in the fecal slurry after 24h. Discrepancies in the anatomy and gut microbiota were proven in this study as only traces of DOM-1 were found in humans.

Modified fumonisins were evaluated using the same static digestion model (Dall'Asta *et al.* 2010; Falavigna *et al.* 2012). The various stages were reproduced with the exception of gut microbiota fermentation, and permeation of transport across the intestinal epithelium. The studies proved that upon digestion, hidden fumonisins (non-covalently, bound molecules) released their free forms, whereas bound fumonisins (covalently, bound particles) were rather stable during *in vitro* digestion (Falavigna *et al.* 2012). On the other hand, Dall'asta *et al.* (2010) reported an increased amount (30% to 50%) of total detectable fumonisins upon digestion of the food matrix (Dall'Asta *et al.* 2010). These data confirmed that gastrointestinal enzymes are able to destroy the matrix-fumonisin interactions, thus releasing the hidden forms. The occurrence of both hidden and bound fumonisins can be evaluated by indirect determination after alkaline hydrolysis, as these forms are completely cleaved to hydrolysed fumonisins. A tabulated summary of key findings from these *in vitro* studies with modified mycotoxins are pointed out in Table 1.

Table 1: tabulated summary of key findings from *in vitro* and *in vivo* studies with modified mycotoxins

(modified) mycotoxin	methodology	key finding	reference
		***IN VITRO* METABOLISM**	
Deoxynivalenol-3-glucoside	Static digestive simulation	1% to 62% conversion rate to DON with fungal cellulase, cellobiase and lactic acid bacteria	(Berthiller *et al.* 2011)
Deoxynivalenol-3-glucoside	Static digestive simulation	0% conversion rate to DON with salivary and gastric phase, and human cytosolic β-glucosidase	(De Nijs *et al.* 2012)
Deoxynivalenol-3-glucoside	Static batch incubation	0.5% conversion rate to DON with salivary, gastric, duodenal phase (*i.e.* 99.5% recovery of DON-3G)	(Dall'Erta *et al.* 2013)
	Fecal fermentation	90% conversion rate to DON with human colonic microbiota	(Dall'Erta *et al.* 2013)
Zearalenone-14-glucoside	Static batch incubation	2.7% conversion rate to ZEN with salivary, gastric, duodenal phase (*i.e.* 97.3% recovery of ZEN-14G)	(Dall'Erta *et al.* 2013)
	Fecal fermentation	40% conversion rate to ZEN with human colonic microbiota + 60% other ZEN-catabolites	(Dall'Erta *et al.* 2013)
Zearalenone-14-sulphate	Static batch incubation	1.4% conversion rate to ZEN with salivary, gastric, duodenal phase (*i.e.* 98.6% recovery of ZEN-14S)	(Dall'Erta *et al.* 2013)
	Fecal fermentation	40% conversion rate to ZEN with human colonic microbiota + 60% other ZEN-catabolites	(Dall'Erta *et al.* 2013)
Hidden fumonisins	Static batch incubation	70% to 99% conversion rate to free fumonisins under digestive conditions	(Dall'Asta *et al.* 2010)
Bound fumonisins	Static batch incubation	0% conversion rate to free fumonisins under digestive conditions	(Falavigna *et al.* 2012)
		***IN VIVO* METABOLISM**	
Deoxynivalenol-3-glucoside	*in vivo* – rats	2% to 3% conversion rate to DON in the gastric phase Small amounts of DON-3G in the small intestine (2% to 3%) and colon (1% to 2%)	(Versilovskis *et al.* 2012)
3-and 15-acetyldeoxynivalenol	*in vivo* – rats	11.4% (3-ADON) to 12.5% (15-ADON) conversion rate to DON in the gastric phase Small intestine: 0% of free 3-ADON and 15-ADON; 4.5% to 6.5% 3-ADON-glucuronide; 0.6% 15-ADON-glucuronide; high amount DON-3-glucuronide (*i.e.* deacetylation and glucuronidation)	(Versilovskis *et al.* 2012)
Zearalenone-14-glucoside	*in vivo* – rats	16% to 19% conversion rate to ZEN in the gastric phase Small amounts of ZEN-14G in the small intestine No confirmation of α-zearalenol, β-zearalenol or ZEN-glucuronides	(Versilovskis *et al.* 2012)
Deoxynivalenol-3-glucoside	*in vivo* – rats	Urine: recovery of 21% of DON-3G Faeces: major recovery of DON and deepoxy-deoxynivalenol, no explanation for remaining 79% DON-3G	(Nagl *et al.* 2012)
Deoxynivalenol-3-glucoside	*in vivo* – pigs	Urine: recovery of 85% DON and 40% of DON-3G Faeces: traces amounts of analysed mycotoxins	(Nagl *et al.* 2014a)
Hidden and bound fumonisins	no data available		

IN VIVO STUDIES

In vitro results allow to obtain a preliminary indication of the biotransformation and conversion of modified mycotoxins such as DON-3G, ZEN-14G, ZEN-14S, α-ZEL-14G, β-ZEL-14G and fumonisins-esters. *In vitro* trials are firstly elaborated to reduce animal experiments, which benefits the 3R-principle. Another limitation of the studies are the size and complexity of *in vivo* trials, and, most importantly, the limited amount of reference standards available.

To date, only few toxicokinetic and toxicodynamic investigations in mammals were performed, although required to further assess the risk of modified mycotoxins. The partial hydrolysis of modified mycotoxins during digestion in mammals will lead to an increased health risk. The role of the gut microbiota is becoming a key point for the investigation of xenobiotic bioactivity in humans and animals. To ascertain the statements regarding modified derivatives, a full-toxicokinetic metabolism study needs to be carried out, by the incorporation of mycotoxins in feed of species reliable as to extrapolation to humans, *i.e.* piglets. The pig has remarkable similarities with human beings with respect to the cardiovascular system, renal function, hepatic and gastrointestinal characteristics (Witkamp and Monshouwer 1998). So far, the effect of modified mycotoxins was investigated on rats (Nagl *et al.* 2012; Versilovskis *et al.* 2012) and pigs (Gareis *et al.* 1990; Nagl *et al.* 2014b).

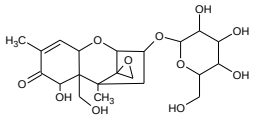
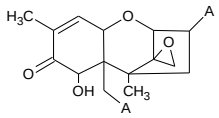
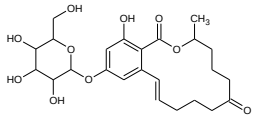
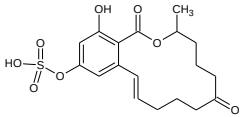
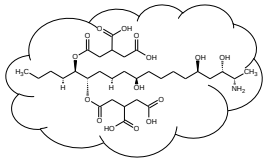
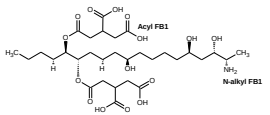
Recently, a study was executed on the metabolisation of glucosides of DON, acetylated DON (ADON) and ZEN-14G in rats (Versilovskis *et al.* 2012). After administration of ZEN-14G, ZEN was found in the stomach (16%-19%), suggesting the possibility of hydrolysis. This was also concluded for the acetylated DON-forms, where 11.4% (3ADON) and 12.5% (15-ADON) of free DON was observed. However, they could not detect DON (2%-3%) in the stomach of rats exposed to DON-3G, confirming the *in vitro* results (acidic hydrolysis) of Berthiller *et al.* (2011) (Berthiller *et al.* 2011). Small amounts of ZEN-14G were detectable in the small and large intestines suggesting that they were not fully hydrolyzed. Also, Versilovskis *et al.* (2012) reported the formation of glucuronidated acetyl-DON (Versilovskis *et al.* 2012). Rats were able to directly glucuronidate ADONs without deacetylation. Neither de-epoxydeoxynivalenol (DOM), α-zearalenol (α-ZEL) or β-zearalenol (β-ZEL) nor their glucuronides could, however, be quantified. Glucuronidated 3-acetyl-deoxynivalenol (3-ADON-Glu) accumulated in the small intestines, together with deoxynivalenol-3-glucuronide (DON-3Glu) in orally fed rats with 3- and 15-ADON. The large occurrence of α-ZEL (Videmann *et al.* 2012) and a sharp decrease of ZEN-14G in the small intestine suggested hydrolysis (Versilovskis *et al.* 2012), as a consequence, the total human exposure and risk

assessment to mycotoxins might be underestimated. ZEN is converted into α-ZEL and β-ZEL, which are subsequently conjugated with glucuronic acid and excreted in the urine of mammals (JECFA 2010; Zinedine *et al.* 2007). It appears likely that the toxic (estrogenic) effects of ZEN-14G equal that of ZEN in mammals.

Nagl *et al.* (2012) reported that DON-3G is partly bioavailable in rats (Nagl *et al.* 2012). Six rats received water, DON (2.0 mg/kg body weight (bw)) and the equimolar amount of DON-3G (3.1 mg/kg bw) by gavage on day 1, 8 and 15, respectively. Urine and faeces were collected and analysed for DON-3G, DON, deoxynivalenol-glucuronide (DON-Glu) and DOM by a validated LC-MS/MS method. Whereas only 21% of the administered DON-3G could be recovered, the majority was recovered as DON and DOM in faeces. The authors concluded that digestion of DON-3G occurred in the distal part of the intestines. However no explanation was available for the 79% of the DON-3G which was not recovered. The authors concluded that DON-3G present in food and feed seems to have a significantly lower toxic equivalency compared to DON. However, due to the differences regarding the gastrointestinal anatomy and physiology and gut microbial composition and metabolic activity, the bioavailability and metabolisation may exhibit species dependent properties and should be experimentally determined (Nagl *et al.* 2012). Therefore, according to previous assumptions, DON-3G might be cleaved during digestion in pigs (Nagl *et al.* 2014a). Four piglets received water *ad libitum*, the modified mycotoxin DON-3G (116 mg/kg bw) and the equimolar amount of DON (75 mg/kg bw) by gavage on day 1, 5 and 9. Additionally, 15.5 mg DON-3G/kg bw were intravenously administered on day 13. Urine and faeces were collected and analysed for DON, DON-3G, DON-3Glu, deoxynivalenol-15-glucuronide (DON-15-Glu) and DOM by UHPLC–MS/MS. After oral application of DON and DON-3G, 84.8% ± 9.7% and 40.3% ± 8.5% of the given dose were detected in the urine. In faeces, just trace amounts of metabolites were found. The data indicate that DON-3G is nearly completely hydrolysed in the gastro-intestinal tract of pigs, while the toxin seems to be rather stable after systemic absorption (Nagl *et al.* 2014a).

Data regarding *in vivo* experiments on fumonisins in combination with fumonisin-esters are non-existent. A tabulated summary of key findings from these *in vivo* studies with modified mycotoxins are pointed out in Table 1. In Figure 1 a hypothetical graph is represented with indicating conditions resulting in the release of free mycotoxins from modified forms.

Figure 1: graph with indicating conditions resulting in the release of free mycotoxins from (modified) forms

	deoxynivalenol-3-glucoside*	**3-and 15-acetyl deoxynivalenol***	**zearalenone-14-glucoside***	**zearalenone-14-sulphate****	**Hidden fumonisins****	**Bound fumonisins****
						
Saliva	no change	no change	no change	no change	no change	no change
Stomach	no change	11% DON (3-ADON 13% DON (15-ADON)	max. 19% ZEN	no change	no change	no change
Small intestine	max. 5% DON-3G	0% 3-ADON 0% 15-ADON	small amounts ZEN-14G	no change	no change	no change
Large intestine	max. 2% DON-3G faeces: DON + DOM	deacetylation + glucuronidation	faeces: 40% ZEN faeces: 60% catabolites	faeces: 40% ZEN faeces: 60% catabolites	max. 99% fumonisins	no change

* based on *in vitro* and *in vivo* analysis; ** based on *in vitro* analysis; deoxynivalenol=DON; deepoxy-deoxynivalenol=DOM; deoxynivalenol-3-glucoside=DON-3G; 3-and 15-acetyl deoxynivalenol=3-ADON, 15-ADON; zearalenone-14-glucoside=ZEN-14G; zearalenone-14-sulphate=ZEN-14S

CONCLUSION

Scientific insights in toxicological fields have contributed greatly to the current knowledge of chemical hazards in food. Nevertheless, most concerns are related to the potential health effects of free mycotoxins and their modified forms. The metabolic fate of modified mycotoxin forms remains an unrevealed matter. Only a few reports focused on *in vitro* and *in vivo* metabolism studies of modified mycotoxins, and indicated the possible toxic relevance of these forms. The fraction of modified mycotoxin forms in food and feed is generally lower than their free forms. So, the toxic effects of contaminated food and feed in humans and animalia are caused by the free mycotoxins. However, based on the reported scientific results the sharp decreases of the modified forms in the small intestines suggest hydrolysis, as a consequence, the total exposure and risk assessment to mycotoxins might be underestimated. To confirm the statements made, repeated full-scale metabolism studies in relevant animal species could reveal relevant toxicity data by incorporating modified mycotoxins in food and feed.

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