Comprehensive REVIEWS in Food Science and Food Safety

Mycotoxin Biomarkers of Exposure: A Comprehensive Review

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Abstract: To date, the use of biomarkers has become generally accepted. Biomarker-driven research has been proposed as a successful method to assess the exposure to xenobiotics by using concentrations of the parent compounds and/or metabolites in biological matrices such as urine or blood. However, the identification and validation of biomarkers of exposure remain a challenge. Recent advances in high-resolution mass spectrometry along with new analytical (post-acquisition data-mining) techniques will improve the quality and output of the biomarker identification process. Chronic or even acute exposure to mycotoxins remains a daily fact, and therefore it is crucial that the mycotoxins' metabolism is unravelled so more knowledge on biomarkers in humans and animals is acquired. This review aims to provide the scientific community with a comprehensive overview of reported *in vitro* and *in vivo* mycotoxin metabolism studies in relation to biomarkers of exposure for deoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, diacetoxyscirpenol, ochratoxin A, citrinin, fumonisins, zearalenone, aflatoxins, and sterigmatocystin.

Keywords: biomarkers, exposure, human, *in vitro*, *in vivo*, metabolism, mycotoxin

Introduction

The term biomarker (that is, biological marker) refers to a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Atkinson et al., 2001). Specific well-characterized biomarkers have shown to predict relevant clinical outcomes across a variety of treatments and populations. Up to date, the use of biomarkers has become commonplace, and biomarker-driven research has been proposed as a successful method to assess the exposure to xenobiotics of individuals through an estimation of their metabolites in biological fluids (that is, *biomarker of exposure*). A *biomarker of effect* is measured through a biochemical, physiological, behavioral, or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease, while measuring an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance is called a *biomarker of susceptibility*. The chosen biological fluids to analyze biomarkers are urine or blood, although other options exist, including breast milk, hair, and feces (Sewram et al., 2001; Sewram, Mshicileli, Shephard, & Marasas, 2003; Tozzi et al., 2016). A challenge in biomarker-driven research is to identify, validate and prove the relevance of the biomarkers. Validity refers to the need to characterize a biomarker's effectiveness or utility as a surrogate endpoint, and relevance refers to a biomarker's ability to provide clinically relevant information on research questions and depends on the biomarker category (*exposure*, *effect*, or *susceptibility*).

In the past decade, mass spectrometry (MS) has played an important role in drug metabolite identification (Meyer & Maurer, 2012; Wen & Zhu, 2015). The development of high-resolution MS (HRMS) with more accuracy and stability, along with new techniques like post-acquisition data-mining based on mass defect filter (MDF), has improved the quality and output of the metabolite identification process (Meyer & Maurer, 2012). For this reason, the use of ultra-high-performance liquid chromatography (UHPLC)-HRMS has permitted to identify and detect new biomarkers (Wen & Zhu, 2015; Yang, Wang et al., 2015). Beside the advantage in structural elucidation, the ability of HRMS to record full-scan spectra without loss in mass accuracy is also used in untargeted analysis or screening (Lattanzio et al., 2012). The capability of HRMS to record full-scan spectra results in a theoretically unlimited number of compounds that can be detected simultaneously at low concentration levels (Lehner et al., 2011). Consequently, HRMS can be used as a screening method to simultaneously detect a large number of compounds, often belonging to different classes, at the same time (Malysheva et al., 2014; Maul et al., 2012; McCormick, Price, & Kurtzman, 2012; Mol, Van Dam, Zomer, & Mulder, 2011). This also offers the opportunity to detect and/or quantify emerging hazardous chemicals. The use of HRMS in mycotoxin-biomarker research is scarce, and therefore it should become more commonplace.

CRF3-2018-0019 Submitted 2/4/2018, Accepted 5/12/2018. *Authors Vidal, De Saeger, and De Boevre are with the Laboratory of Food Analysis, Dept. of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent Univ., Ghent, Belgium. Author Mengelers is with Dept. of Food Safety, National Inst. of Public Health and the Environment, Bilthoven, The Netherlands. Author Yang is with Inst. of Apicultural Research, Chinese Acad. of Agricultural Sciences, Key Laboratory of Bee Products for Quality and Safety Control, Laboratory of Risk Assessment for Quality and Safety of Bee Products, Bee Product Quality Supervision and Testing Center, Ministry of Agriculture, Beijing 100093, People's Republic of China. Direct inquiries to author Vidal (E-mail: arnau.vidalcorominas@ugent.be).*

doi: 10.1111/1541-4337.12367

Table 1–International Agency for Research on Cancer (IARC) classification for the mycotoxins present in this comprehensive review.

Group	Classification	Mycotoxins
1	Carcinogenic to humans	Aflatoxins
2A	Probably carcinogenic to humans	–
2B	Possibly carcinogenic to humans	Sterigmatocystin, fumonisins and ochratoxin A
3	Not classifiable as to its carcinogenicity to humans	Deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscirpenol, zearalenone, citrinin and fusarenon X
4	Probably not carcinogenic to humans	–

Mycotoxins are toxic fungal secondary metabolites, and they contaminate agricultural commodities during cultivation, harvesting, transport, processing, and storage (Moss, 1992). Many filamentous are toxigenic, and the most important producing genera are *Aspergillus*, *Fusarium*, and *Penicillium* (Pitt, Basilico, Abarca, & Lopez, 2000). These are globally distributed and are observed in a vast range of agricultural commodities and environments (Ostry, 1998).

Mycotoxins are present in a wide range of agricultural crops and they are common in cereals (rice, wheat, rye, barley, corn, soybeans, sorghum . . .), nuts (almonds, raisins . . .), and spices (pepper, cayenne . . .). Food processing will not completely remove mycotoxins (Schaarschmidt & Fauhl-Hassek, 2018), so mycotoxins are common in cereal-based foods (baking products, pasta, breakfast cereals), beverages (fruits, juices and purees, beer, and wine), and several animal products (dairy products, meat, eggs) (De Boevre et al., 2012; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Pitt et al., 2000).

Mycotoxin intake may lead to immune illnesses (Pierron, Alassane-Kpembi, & Oswald, 2016), metabolic and biochemical deficiencies, allergic manifestations (Norbäck et al., 2016; Schütze, Lehmann, Bönisch, Simon, & Polte, 2010), reduction of reproductive efficiency, and may also lead to teratogenicity, carcinogenicity, mutagenicity, and death (Hussein & Brasel, 2001). Moreover, the teratotoxic aflatoxins cross the placental barrier, consequently producing fetal alterations (Abdulrazzaq, Osman, & Ibrahim, 2002). Due to their high incidence and considerable toxicity, maximum levels have been set in foods to establish an effective food safety management aiming at a safe food supply, and guidelines have also been developed for the control of mycotoxins in animal feed (Alim, Iqbal, Selamat, & Ariño, 2016).

Mycotoxins as a group cannot be classified based on the adverse effects they induce or metabolism, as these vary according to the different physicochemical properties and they follow different metabolic pathways. There are many mycotoxins with a great diversity in their modes of action. The aflatoxins' exposure (Cousin, Riley, & Pestka, 2005) has been shown to alter renal function in addition to having potent hepatotoxic effects (International Agency for Research on Cancer (IARC) group 1) (WHO, 2002) (Table 1), and similar effects have been shown for sterigmatocystin (STERIG) (IARC, 1976) and citrinin (CIT) (IARC, 1993), all of them produced by toxigenic species of *Penicillium* and *Aspergillus* genera (Pitt et al., 2000). Furthermore, fumonisins (FB) and ochratoxin A (OTA) have both been classified as having clear evidence of carcinogenic activity, based on increased incidences of renal carcinomas (IARC group 2B) (WHO, 2002) (Table 1). Zearalenone (ZEN), a common mycotoxin present in cereals, stimulates the growth of human breast cancer cells, indicating its potential carcinogenicity to humans (Belhassen et al., 2015); however, IARC has assigned zearalenone to group three (IARC, 1993) (Table 1). Finally, trichothecenes are a large family of chemically-related mycotoxins, which are highly common in cereals and cereal products (Pinotti, Ottoboni, Giromini, Dell'Orto, & Cheli, 2016). Their abundant presence has caused large exposure levels. Exposure levels are above the tolerable daily intake (TDI) for deoxynivalenol (DON), the most common trichothecene, in many consumer groups (infants, toddlers and other children), and at high exposure in adolescents and adults, indicating a potential health concern (EFSA, 2017). Other trichothecenes, such as nivalenol (NIV), fusarenon-X (FUS-X), T-2 toxin (T-2), and diacetoxyscirpenol (DAS) showed large exposure levels through the approach to estimate exposure combining contamination data with consumption data. Information on exposure to other trichothecenes through biomarker analysis is scarce to nonexistent due to the lack of reliable biomarkers. In general, chronic exposure to trichothecenes causes reduced growth and adverse effects on thymus, spleen, heart, and liver (Aupanun, Poapolathep, Giorgi, Imsilp, & Poapolathep, 2017).

Nonetheless, dietary exposure or even acute exposure to mycotoxins remains a daily fact for humans and domesticated animals (Lewis et al., 2005; Ngindu et al., 1982; Wouters et al., 2013). Therefore, it is of crucial importance that the mycotoxins' metabolism and toxicokinetics are unravelled, and that more knowledge on mycotoxin biomarkers of exposure in humans and animals can be acquired. Understanding the metabolic pathways of mycotoxins in various species could enable researchers and public health officials to gain insight on how to assess the associated risks of mycotoxin exposure. In the late 1980s, one of the first applications of biomarkers of exposure to food chemicals was illustrated for aflatoxin B1 (AFB1) (Gan et al., 1988). Thus, the use of AFB1-albumin adducts as biomarkers of aflatoxin exposure was validated in human plasma (Wild et al., 1992; Wild, Jiang, Sabbioni, Chapot, & Montesano, 1990), and AFB1-N7-guanine adducts were validated in urine. AFB1-N7-guanine adducts provided a more adequate measure of acute short term (24 to 48 hr)-AFB1-exposure (Groopman et al., 1993; Groopman, Dematos, Egner, Lovehunt, & Kensler, 1992; Groopman, Roebuck, & Kensler, 1992). AFB1-lysine in plasma was validated as a reliable biomarker of chronic aflatoxin exposure using state-of-the-art isotope dilution HRMS (McMillan, 2018), and DON-glucuronides were validated in urine as a reliable biomarker of deoxynivalenol exposure (Vidal et al., 2018). However, up to date no more mycotoxin biomarkers of exposure were validated.

This review aims to provide the scientific community with a comprehensive overview of the existing *in vitro* and *in vivo* mycotoxin metabolism in relation to biomarkers of exposure for deoxynivalenol (DON), nivalenol (NIV), fusarenon-X (FUS-X), T-2 toxin (T-2), diacetoxyscirpenol (DAS), ochratoxin A (OTA), citrinin (CIT), fumonisins (FB), zearalenone (ZEN), aflatoxins (AF), and sterigmatocystin (STERIG).

Deoxynivalenol

DON is a secondary fungal metabolite produced by *Fusarium* species (Gilbert & Tekauz, 2000). DON is chemically classified as a type B trichothecene. DON is one of the most frequently occurring fungal contaminants of food and feed worldwide, and it is mainly observed in cereals and cereal-based products such as bread, pasta, or beer (Marin et al., 2013). In addition, modified DON or other DON forms have been found in the same type of matrices, namely deoxynivalenol-3-glucoside (DON-3-glucoside),

3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) (Edwards, 2009; Juan, Ritieni, & Manes, 2013; Simsek, Burgess, Whitney, Gu, & Qian, 2012; Yang, Geng, . . . 2013).

DON acts as a potent inhibitor of protein synthesis, stimulates the pro-inflammatory response, causes ribotoxic stress, cytotoxicity, and apoptosis, resulting in the impairment of multiple physiological functions, such as the intestinal barrier, growth, immune regulation, or reproduction (Rotter, Prelusky, & Pestka, 1996). Typical acute effects include nausea, vomiting, abdominal pain, diarrhea, headache, dizziness, or fever, and DON has been linked with animal and human gastroenteritis outbreaks (Pestka, 2010a, 2010b). Hence, DON is also called vomitoxin.

The absorption of DON is species-dependent, and ranges from 51% to 68%. Hence, Avantaggiato, Havenaar, and Visconti (2004) observed that approximately 51% of DON was absorbed, with 44% and 7% absorbed in the jejunum and ileum, respectively (Avantaggiato et al., 2004).

In vitro/*in vivo* metabolism

In vitro studies using liver microsomes demonstrated that DON is sensitive to metabolization as a large percentage of free DON (>75%) is converted to DON metabolites. DON metabolism is species-dependent, and significant differences have been observed among animals (rat, fish, cattle, and swine) and humans (Maul et al., 2015; Schwartz-Zimmermann et al., 2017) (Table 2). Deoxynivalenol-15-glucuronide (DON-15-glucuronide) was the predominant DON metabolite after DON-incubation in human liver microsomes, while deoxynivalenol-3-glucuronide (DON-3-glucuronide) was the main metabolite for all other studied animals (fish, rat, cattle, and swine) (Maul et al., 2015; Schwartz-Zimmermann et al., 2017). The second prevailing glucuronide was observed in lower levels (<10% compared to the first prevailing glucuronide) for all species and was species-dependent: DON-15-glucuronide in pigs (Maul et al., 2015), DON-3-glucuronide in humans (Maul et al., 2015; Schwartz-Zimmermann et al., 2017), iso-deoxynivalenol-3-glucuronide (iso-DON-3-glucuronide) in fish and bovine (Maul et al., 2015), and deoxynivalenol-8,15-hemiketal-8-glucuronide (DON-8,15-hemiketal-8-glucuronide) in rodents (Schwartz-Zimmermann et al., 2017; Uhlig, Ivanova, and Fæste, 2013). It is worth mentioning that DON-7-glucuronide was recently identified as iso-DON-3-glucuronide (Schwartz-Zimmermann et al., 2017).

While human liver microsomes had the capacity to only form 2 glucuronides (DON-15-glucuronide and DON-3-glucuronide), animal liver microsomes produced several different DON metabolites. Schwartz-Zimmermann et al. (2017) detected 6 different DON-metabolites after rat microsomal incubation: DON-3-glucuronide, DON-8,15-hemiketal-8-glucuronide, DON-15-glucuronide, iso-DON-3-glucuronide, iso-deoxynivalenol-8-glucuronide (iso-DON-8-glucuronide), and an unknown DON-glucuronide (Table 2). An inverted phenomenon was observed in intestinal human cells as a higher amount of DON-3-glucuronide was produced than DON-15-glucuronide. Moreover, iso-DON-3-glucuronides were detected in this specific type of cells (Maul et al., 2015).

Deepoxy-deoxynivalenol (DOM-1) was a catabolite of DON (Fuchs, Binder, Heidler, & Krska, 2002). *In vitro*, analogous products to the glucuronides formed upon microsomal incubations of DON were obtained for DOM-1. Human microsomal DOM-1-incubation revealed the presence of 2 metabolites, namely deepoxy-deoxynivalenol-15-glucuronide (DOM-15-glucuronide) and deepoxy-deoxynivalenol-3-glucuronide (DOM-3-glucuronide). While 6 other compounds were identified in rodents: DOM-3-glucuronide, DOM-15-glucuronide, 2 unknown DOM-glucuronides, iso-deepoxy-deoxynivalenol-glucuronide (iso-DOM-glucuronide), and iso-deepoxy-deoxynivalenol-8-glucuronide (iso-DOM-8-glucuronide) (Schwartz-Zimmermann et al., 2017).

Also, iso-deoxynivalenol (iso-DON) and iso-deepoxy-deoxynivalenol (iso-DOM) were identified in liver microsomes. In human liver microsomes, iso-DON was converted to iso-deoxynivalenol-15-glucuronide (iso-DON-15-glucuronide) and iso-deoxynivalenol-3-glucuronide (iso-DON-3-glucuronide), while in rats iso-DON-8-glucuronide, iso-DON-3-glucuronide, and iso-DON-15-glucuronide were formed through *in vitro* microsomal analysis. Regarding the incubation of iso-DOM in human liver microsomes, this resulted in iso-deepoxy-deoxynivalenol-15-glucuronide (iso-DOM-15-glucuronide) and iso-deepoxy-deoxynivalenol-3-glucuronide (iso-DOM-3-glucuronide), and in rat liver microsomes to iso-deepoxy-deoxynivalenol-8-glucuronide (iso-DOM-8-glucuronide), iso-DOM-3-glucuronide, and iso-DOM-15-glucuronide. However, in many cases, that is, human and rodents, the predominant compound revealed a 4-fold higher concentration than the other identified compounds: for rodents DON-3-glucuronide, DOM-3-glucuronide, iso-DON-8-glucuronide, and iso-DOM-8-glucuronide after incubation with DON, DOM-1, iso-DON, and iso-DOM, respectively. Regarding humans, DON-15-glucuronide, DOM-15-glucuronide, iso-DON-15-glucuronide, and iso-DOM-15-glucuronide were the predominant compounds after incubation with DON, DOM-1, iso-DON, and iso-DOM, respectively (Schwartz-Zimmermann et al., 2017).

Recently, DON-sulfates (deoxynivalenol-3-sulfate (DON-3-sulfate) and deoxynivalenol-15-sulfate (DON-15-sulfate) were identified after DON incubation in rat liver cells; however, they were only detected in female liver cells (Pestka, Clark, Schwartz-Zimmermann, & Berthiller, 2017). These compounds are therefore not suitable as reliable biomarkers.

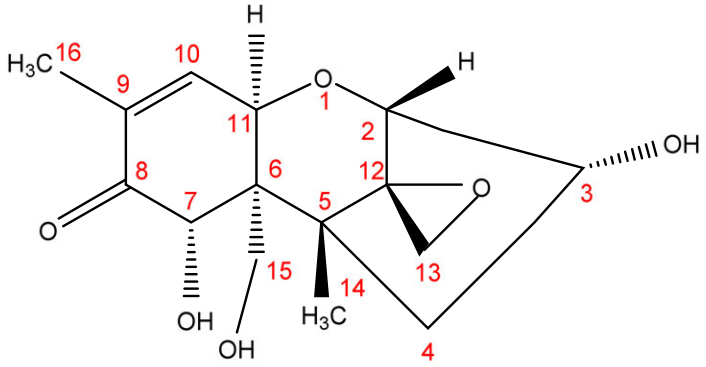
In addition, uridine-diphosphoglucuronyltransferases (UGT) have been tested with DON, and, although more than 20 UGT enzymes have been characterized up to now, only 12 are commercially available enabling an UGT reaction phenotyping. From the 12 available human recombinant UGTs, only UGT2B4 and UGT2B7 transformed DON in glucuronidated forms. UGT2B4 catalyzed the formation of DON-15-glucuronide, while UGT2B7 the DON-3-glucuronide (Maul et al., 2015).

In vivo studies in humans showed that DON glucuronides were the predominant compounds in urine, Heyndrickx et al. (2015) observed that more than 90% of the investigated human urine samples revealed DON or DON metabolites. The existing results concurred that most of the excreted DON was in the glucuronidated state (>75%) (Heyndrickx et al., 2015; Turner et al., 2011; Vidal, Cano-Sancho, Marin, Ramos, & Sanchis, 2016; Warth et al., 2012; Warth, Sulyok, Berthiller, Schuhmacher, & Krska, 2013), and DON-15-glucuronide was the predominant urinary excretion form in humans (Heyndrickx et al., 2015).

In contrast to humans, DON-3-glucuronide was the prevailing compound in rats and pigs (Nagl et al., 2014; Versilovskis et al., 2012). So, both results coincided with the *in vitro* observations (Table 2). Also, DON-sulfates (DON-3-sulfate and DON-15-sulfate) were identified in the urine from female rodents (Pestka et al., 2017). Concerning the gender difference, the DON excretion was higher in human females than human males due to a larger presence of glucuronidated forms (DON-15-glucuronide

Table 2–Identified deoxynivalenol (DON) metabolites *in vivo* and *in vitro*.

Chemical structure - Deoxynivalenol



$C_{15}H_{20}O_6$ Molecular Weight = 296.32

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
10-deepoxy-deoxynivalenol-1-sulfonate	10-DOM-1-sulfonate	$C_{15}H_{21}O_8S$	Rats	*In vivo*: urine	II	(Wan et al., 2014)
Deepoxy deoxynivalenol	DOM-1	$C_{15}H_{20}O_5$	Rats	*In vivo*: urine & feces	-	(Nagl et al., 2012)
			Cows	*In vivo*: urine & feces		(Cote, Dahlem, Yoshizawa, Swanson, & Buck, 1986)
			Swines	*In vivo*: urine & feces		
			Humans	*In vivo*: urine		(Nagl et al., 2014) (Vidal et al., 2018)
Deepoxy-deoxynivalenol-15-glucuronide	DOM-15-glucuronide	$C_{21}H_{28}O_{11}$	Humans, rats, swines, cows	*In vivo*: urine	II	(Schwartz-Zimmermann et al., 2017)
Deepoxy-deoxynivalenol-3-glucuronide	DOM-3-glucuronide	$C_{21}H_{28}O_{11}$	Humans, rats, cows	*In vivo*: urine	II	(Schwartz-Zimmermann et al., 2017)
Deoxynivalenol sulfonate 1	DON S1	$C_{15}H_{19}O_9S$	Rats	*In vivo*: feces	II	(Schwartz-Zimmermann et al., 2017)
Deoxynivalenol sulfonate 2	DON S2	$C_{15}H_{19}O_9S$	Rats	*In vivo*: feces	II	(Schwartz-Zimmermann et al., 2017)
Deoxynivalenol sulfonate 3	DON S3	$C_{15}H_{19}O_9S$	Rats	*In vivo*: feces	II	(Schwartz-Zimmermann et al., 2017)
Deoxynivalenol-15-glucuronide	DON-15-glucuronide	$C_{21}H_{28}O_{12}$	Humans	*In vivo*: urine	II	(Heyndrickx et al., 2015)
			Humans	*In vitro*: liver		(Schwartz-Zimmermann et al., 2017)
Deoxynivalenol-15-sulfate	DON-15-sulfate	$C_{15}H_{19}O_9S$	Rats	*In vivo*: urine	II	(Pestka et al., 2017)
			Rats	*In vitro*: liver		
Deoxynivalenol-3-glucuronide	DON-3-glucuronide	$C_{21}H_{28}O_{12}$	Humans	*In vivo*: urine	II	(Heyndrickx et al., 2015; Schwartz-Zimmermann et al., 2017)
			Rats, swines, cows, humans	*In vitro*: liver		Schwartz-Zimmermann et al. (2017)

(*Continued*)

Table 2–Continued.

Chemical structure - Deoxynivalenol						
Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Deoxynivalenol-3-sulfate	DON-3-sulfate	$C_{15}H_{19}O_9S$	Humans	*In vivo*: urine	II	(Warth et al., 2016)
			Chickens	*In vivo*: urine		(Wan et al., 2014)
			Chickens & turkeys	*In vivo*: urine		(Schwartz-Zimmermann et al., 2015)
			Rats	*In vivo*: urine		(Pestka et al., 2017)
Deoxynivalenol-8,15-hemiketal-8-glucuronide	DON-8,15-hemiketal-8-glucuronide	$C_{21}H_{29}O_{13}$	Rats	*In vitro*: liver	II	(Uhlig, Ivanova, & Fæste, 2016)
			Rats	*In vitro*: liver		(Schwartz-Zimmermann et al., 2017; Uhlig, Ivanova, & Fæste, 2013)
			Rats	*In vivo*: urine		
Iso-deepoxydeoxynivalenol	Iso-DOM	$C_{15}H_{20}O_5$	Bacterial strain BBSH 797	*In vitro*: incubation	-	(Fuchs et al., 2002)
Iso-deepoxy-deoxynivalenol-15-glucuronide	iso-DOM-15-glucuronide	$C_{21}H_{30}O_{11}$	Rats	*In vitro*: liver	II	(Schwartz-Zimmermann et al., 2017)
			Humans	*In vitro*: liver		
Iso-deepoxy deoxynivalenol-3-glucuronide	Iso-DOM-3-glucuronide	$C_{21}H_{30}O_{11}$	Rats & cows	*In vivo*: urine	-	(Uhlig et al., 2016)
Iso-deepoxy-deoxynivalenol-8-glucuronide	iso-DOM-8-glucuronide	$C_{21}H_{30}O_{11}$	Rats	*In vitro*: liver	II	(Schwartz-Zimmermann et al., 2017)
Iso-deoxynivalenol	Iso-DON	$C_{15}H_{20}O_6$	Rats	*In vivo*: urine	-	(Schwartz-Zimmermann et al., 2017)
Iso-deoxynivalenol-15-glucuronide	iso-DON-15-glucuronide	$C_{21}H_{30}O_{11}$	Rats & humans	*In vitro*: liver	II	(Schwartz-Zimmermann et al., 2017)
Iso-deoxynivalenol-3-glucuronide (previously deoxynivalenol-7-glucuronide)	iso-DON-3-glucuronide	$C_{21}H_{30}O_{11}$	Rats	*In vivo*: urine	II	(Schwartz-Zimmermann et al., 2017)
			Rats	*In vitro*: liver		
Iso-deoxynivalenol-8-glucuronide	iso-DON-8-glucuronide	$C_{21}H_{30}O_{11}$	Rats	*In vitro*: liver	II	(Schwartz-Zimmermann et al., 2017)

and DON-3-glucuronide) (Vidal et al., 2018). A rodent trial on DON exposure demonstrated that female animals had a higher UGT-enzyme expression than males, which suggested a gender difference in the response to DON (Pestka et al., 2017). Although there was a lower level of DON in male urine, larger DON concentrations in male feces were identified (Pestka et al., 2017). The analysis of DON in rats feces also permitted to identify 3 other DON-sulfonates (Schwartz-Zimmermann et al., 2014) (Table 2).

A small DOM-1 portion was identified in human urine after DON-exposure (Vidal et al., 2018), although DOM-1 was mainly excreted in pigs with the feces (Dänicke, Valenta, & Döll, 2004; Eriksen, Pettersson, Johnsen, & Lindberg, 2002). Nevertheless, due to the high absorption of DON by the small intestine, bacterial transformation of DON into DOM-1 could only be possible when reaching the colon. This explains why only a low percentage (<15%) of ingested DON was observed in the feces of monogastric animals (humans, pigs, rodents).

According to the authors' knowledge, there are no studies available on the microsomal incubation of DON-3-glucoside or acetylated DON. Regarding DON-3-glucoside, this toxin was almost completely converted to DON during rats' digestion in the small intestine and in the gut (Berthiller et al., 2011; Nagl et al., 2014; Versilovskis et al., 2012). For this reason, DON-3-glucoside caused a similar DON excretion profile (Broekaert et al., 2017), and DON-3-glucoside was scarcely detected in the urine of humans (Vidal et al., 2018) and pigs (Nagl et al., 2014). On the contrary, no hydrolysis was observed after oral administration of DON-3-glucoside to chickens (Broekaert et al., 2017). The total hydrolysis of DON-3-glucoside to DON in humans and other species is a point of concern. *In vitro* studies have reported that DON-3-glucoside was less toxic than DON (Pierron et al., 2016), however, the hydrolysis of DON-3-glucoside to DON in humans and in pigs indicated a decreased yet not negligible toxicological relevance of DON-3-glucoside.

Biomarkers of exposure

DON-exposure studies on biological samples have been widely conducted (EFSA, 2017). Heyndrickx et al. (2015) observed that more than 90% of the investigated human urines revealed occurrence of DON or DON metabolites. The existing results concurred that most of the excreted DON was in the glucuronidated state (>75%) (Heyndrickx et al., 2015; Turner et al., 2011; Vidal et al., 2016; Warth et al., 2013; Warth et al., 2012), and DON-15-glucuronide was the predominant urinary excretion form in humans (Heyndrickx et al., 2015). The available information of DON glucuronide forms in urine helped EFSA to conclude that the exposure estimates based on the biomarker analysis were within the same order of magnitude as the estimates based on the dietary intake (EFSA, 2017). However, there were several aspects which contributed to the uncertainty of the exposure estimates of DON by using biomarker data in urine: (a) individual variation in the extent of excretion (assumed to be 70%), (b) unknown contribution of 3-ADON, 15-ADON, and DON-3-glucoside, (c) time of urine collection, there was clear evidence that urinary DON excretion varies at different times of the day, and (d) different back-calculation methods (for example, whether or not corrected for molar mass) (EFSA, 2017). A recent toxicokinetic study of DON and DON-3-glucoside in humans answered some of the existing uncertainties of the exposure estimates of DON by using biomarkers of exposure in urine. The study permitted to elucidate the excretion rate of DON (64%) and DON-3-glucoside (58%), as well as the fast excretion rate of DON, suggesting to collect at least 16-hours urine to have a representative view on DON-consumption. These results enabled to validate DON-3-glucuronide and DON-15-glucuronide as suitable DON and DON-3-glucoside biomarkers in urine (Vidal et al., 2018).

In conclusion, the *in vitro* liver microsomal DON incubations permitted to discover a wide range of DON metabolites, however, animals produced more DON metabolites than humans. Humans only revealed the presence of glucuronidated forms. The analysis of urine confirmed the differences among species, however, DON glucuronides were the most common metabolites observed. DON glucuronides, especially DON-3-glucuronide and DON-15-glucuronide, have to be considered as DON biomarkers in urine.

Nivalenol

NIV is a mycotoxin mainly produced by *F. cerealis*, *F. poae*, *F. graminearum*, and *F. culmorum* (Langseth, Bernhoft, Rundberget, Kosiak, & Gareis, 1998; Prodi, Tonti, Nipoti, Pancaldi, & Pisi, 2009). NIV is chemically classified as a type B trichothecene. NIV is a field-contaminant, and has been observed in wheat (Trombete et al., 2016). NIV has demonstrated immunotoxicity, hematotoxicity, myelotoxicity, and developmental and reproductive toxicity. The IARC concluded that there was inadequate evidence of carcinogenicity of NIV in experimental animals, and that its carcinogenicity in humans was not classifiable (Group 3) (IARC, 1993). There have been reports of human toxicoses possibly linked it to the intake of trichothecene-contaminated food, but this could not be conclusively assigned to a specific toxin. More recently, exposure to dietary NIV has been associated with an increased incidence of oesophageal and gastric carcinomas in certain regions of China (Hsia, Wu, Li, Zhang, & Sun, 2004). NIV is fast absorbed, distributed and eliminated without accumulation in all vertebrates investigated (rodents, pigs and poultry) (Chain, 2017). The higher absorption of trichothecenes is caused by its higher hydro-solubility (Gonzalez-Arias, Marin, Sanchis, & Ramos, 2013).

In vitro/in vivo metabolism

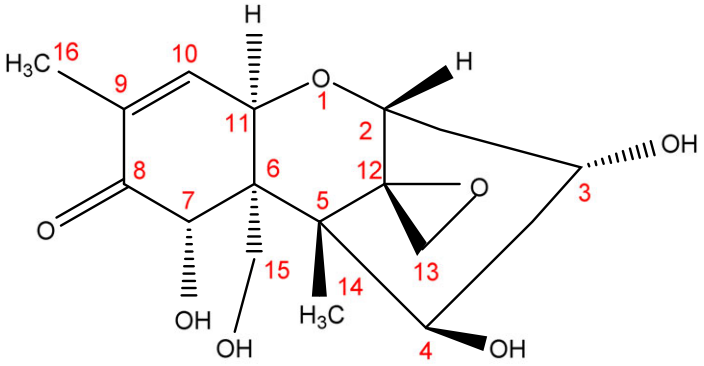
Despite the probable NIV presence in the human diet, the metabolism of NIV has been scarcely investigated. However, a similar behavior to DON could be expected due to their structural similarity. Especially NIV-glucuronides were expected, considering that glucuronides are the main DON-metabolites. Furthermore, NIV has been detected as a FUS-X metabolite during metabolism in rabbits, mice, broiler, and ducks (Poapolathep et al., 2008; Poapolathep, Sugita-Konishi, Doi, & Kumagai, 2003), which makes the exposure assessment through urinary analysis complex (Saengtienchai et al., 2014).

NIV was converted to deepoxy-nivalenol (deepoxy-NIV) by the intestinal microflora. Microflora of pigs showed an effective rate to transform NIV to deepoxy-NIV (>99%) after NIV incubation with pig feces after 48 hr (Hedman & Pettersson, 1997). So, the presence or absence of particular intestinal microflora could influence the extent to which an animal is sensitive to NIV, because the deepoxidated products were shown to be less toxic than the free NIV (Springler et al., 2017) (Table 3).

Only little information is available on *in vivo* trials with NIV. Rats, which were orally administered with NIV, showed a predominant excretion of deepoxy-NIV in feces rather than in urine (Onji et al., 1989). Eriksen and Pettersson (2003) analyzed 10 human fecal samples, however, no NIV metabolites were detected, despite the fact that the human fecal microbiota was

Table 3–Identified nivalenol (NIV) metabolites *in vivo* and *in vitro*.

Chemical structure - Nivalenol



$C_{15}H_{20}O_7$ Molecular Weight = 312.32

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Deepoxy-nivalenol	Deepoxy-NIV	$C_{15}H_{20}O_6$	Swines	*In vitro*: feces	-	(Hedman & Pettersson, 1997)

able to metabolize trichothecenes to deepoxide-forms (Gratz, Duncan, & Richardson, 2013).

Biomarkers of exposure

Warth, Petchkongkaew, Sulyok, and Krska (2014) were not able to detect NIV in human urine, however, their applied method was able to determine only moderate-to-high NIV concentrations in the urine due to the limit of detection (LOD) used in their study (>4.0 ng/mL) (Warth et al., 2014). The nondetection of NIV could be assigned to the fact that NIV was probably predominantly excreted in the glucuronidated form, similar to DON (>90% DON-3-glucuronide and DON-15-glucuronide) (Heyndrickx et al., 2015).

To conclude, NIV has been barely studied in metabolic and toxicokinetic studies. Its high similitude with DON leads the authors to conclude that NIV-glucuronides are present, but not yet identified. Moreover, deepoxy-NIV is a predominant compound in feces.

Fusarenon-X

FUS-X is produced by different *Fusarium* species, and is chemically assigned as a type B trichothecene. FUS-X is mainly found in cereals, and co-occurs with DON and NIV, however in lower levels (Perkowski, Stachowiak, Kiecana, Goliński, & Chelkowski, 1997; Tolosa et al., 2017). The toxicity of FUS-X, however, has been found to be more potent than other type B-trichothecenes (Alassane-Kpembi et al., 2017). FUS-X mainly affects organs that have actively dividing cells such as hematopoietic tissues, spleen, and the thymus, and exerts intestinal inflammation, inhibits protein synthesis, induces apoptosis, and alters genetic material causing cell cycle delays, chromosomal aberrations, and sister chromatid exchanges (IARC, 1993). FUS-X is placed in group three by IARC because it is not classifiable as to its carcinogenicity in humans (IARC, 1993).

FUS-X is absorbed from the gastrointestinal tract of piglets (Saengtienchai et al., 2014), ducks (Poapolathep et al., 2008), broilers (Poapolathep et al., 2008), and rodents (Poapolathep et al., 2003), and its oral bioavailability was species-dependent: piglets (74%) (Saengtienchai et al., 2014), ducks (20%) (Poapolathep et al., 2008), and broilers (10%) (Poapolathep et al., 2003).

In vitro/*in vivo* metabolism

During *in vitro* microsomal trials, FUS-X was almost completely (>90%) converted to NIV (Saengtienchai et al., 2014). For this reason, special attention was attributed to FUS-X, and several metabolism studies were performed. Saengtienchai et al. (2014) found that 91% and 90% of FUS-X was converted to NIV after 60 min of incubation with liver and kidney piglet tissues, respectively. The same phenomenon was observed with liver microsomes in mice (94%, 60 min) (Poapolathep et al., 2003), rabbits (Ohta, Matsumoto, Ishii, & Ueno, 1978), broiler (94%, 45 min), and ducks (99%, 45 min) (Poapolathep et al., 2008). NIV was the predominant metabolite, however, approximately 10% FUS-X could be metabolized to other compounds such as glucuronides (Table 4).

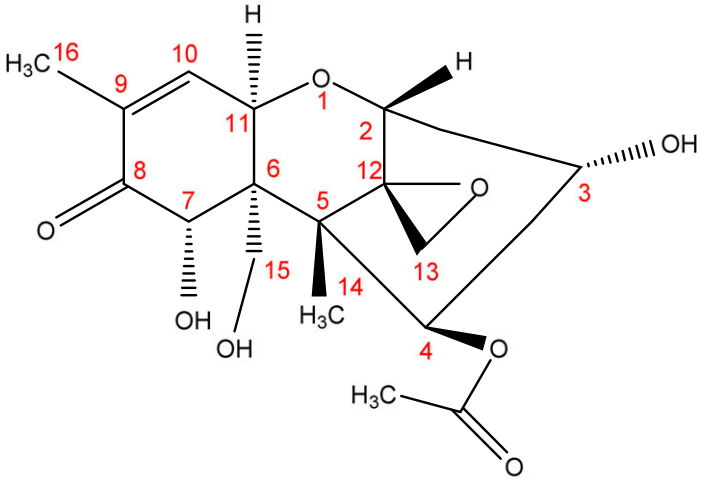
The published FUS-X *in vivo* results represent a similarity to those of *in vitro* results. Hence, a large amount of NIV was detected in the urine after FUS-X exposure in pigs (44%) (Saengtienchai et al., 2014), mice (90%) (Poapolathep et al., 2003), broilers, and ducks (76%) (Poapolathep et al., 2008). NIV was also present in feces of mice after oral administration of FUS-X (Poapolathep et al., 2003). Moreover, FUS-X was rapidly converted to NIV as it was found in broilers' and ducks' plasma after 10 min of oral FUS-X administration (Poapolathep et al., 2008), meaning that FUS-X was rapidly absorbed in the gastrointestinal tract.

Biomarkers of exposure

FUS-X was included in analytical methodologies for human urine (Heyndrickx et al., 2015; Huybrechts, Martins, Debongnie, Uhlig, & Callebaut, 2015; Rodriguez-Carrasco, Molto, Manes, & Berrada, 2014), however none were able to detect FUS-X. Taking into account the *in vivo* and *in vitro* results, it is clear that the evaluation of FUS-X after its exposure should focus on NIV. Also, regarding feces, NIV has been detected as a de-epoxidated form as other trichothecenes as DON to DOM-1 (Gratz et al., 2013). So, the presence or absence of particular intestinal microorganisms can affect the presence of other compounds in feces after FUS-X administration.

Table 4–Identified fusarenon-X (FUS-X) metabolites *in vivo* and *in vitro*.

Chemical structure - Fusarenon-X



$C_{17}H_{22}O_8$ Molecular Weight = 354.35

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Nivalenol	NIV	$C_{15}H_{20}O_7$	Duck	*In vitro*: liver	I	(Poapolathep et al., 2008)
			Broiler	*In vitro*: liver		(Ohta et al., 1978; Poapolathep et al., 2003)
			Rabit	*In vitro*: liver		
			Mice	*In vitro*: liver		(Saengtienchai et al., 2014)
			Pig	*In vitro*: liver		(Poapolathep et al., 2003)
			Pig	*In vivo*: urine		(Poapolathep et al., 2008)
			Mice	*In vivo*: urine & feces		
			Broiler	*In vivo*: plasma		
			Duck	*In vivo*: plasma		

In conclusion, FUS-X is highly converted to NIV (>90%) in liver and kidney, and NIV can be further metabolized (Table 4). FUS-X biomarker analysis should focus on quantification of NIV both in urine and in plasma. However, recent advances in HRMS could unravel new FUS-X metabolites.

T-2 Toxin

T-2 toxin (a type A trichothecene) is, as all trichothecenes, produced by various *Fusarium*, and it is detected in cereals and cereal-based products (Marin et al., 2013). T-2 toxin is a potent inhibitor of protein synthesis and mitochondrial function, and it shows immunosuppressive and cytotoxic effects. Moreover, toxic effects on skin and mucous membranes where attributed to T-2 toxin (Eriksen & Petterson, 2004; Sudakin, 2003; Visconti, Minervini, Lucivero, & Gambatesa, 1991). The toxicity of HT-2 toxin (HT-2) has been less investigated, because T-2 toxin is rapidly metabolized to HT-2 *in vivo*. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that the toxic effects of T-2 and HT-2 cannot be differentiated. IARC evaluated the experimental data on the carcinogenicity of T-2, and placed T-2 toxin in Group 3 (IARC, 1993). T-2 toxin is rapidly absorbed, as the other trichothecenes, and excreted in feces and urine (Chain, 2011).

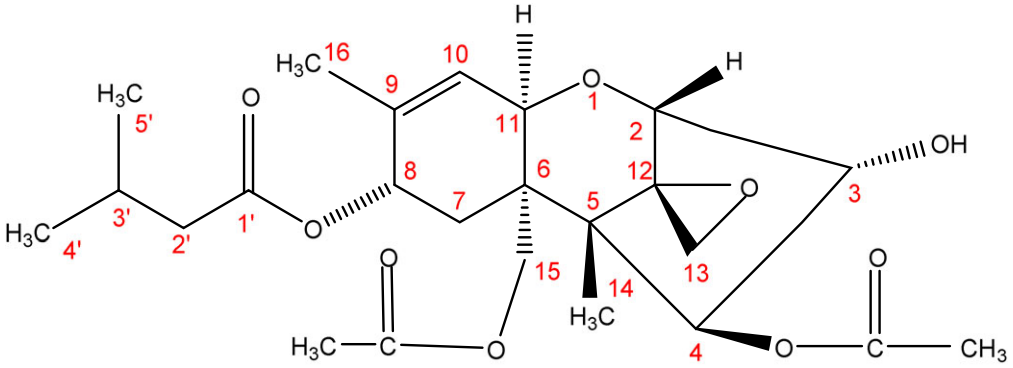
In vitro/*in vivo* metabolism

T-2 toxin has an extensive metabolization, and a large amount of T-2 metabolites have been identified *in vitro* (Table 5). The metabolic T-2 pattern was recently investigated using liver cells from different animals (chicken, rat, swine, goat, cow, and human). Four phase I (HT-2, neosolaniol (NEO), 3'-hydroxy-T-2 (3'-OH-T-2), and 3'-hydroxy-HT-2 (3'-OH-HT-2) and 3 phase II metabolites (T-2-3-glucuronide, HT-2-3-glucuronide, and HT-2-4-glucuronide) were observed in the *in vitro* systems Yang, S., De Boevre, M., et al., (2017). Owing to the different enzymatic systems, there were significant interspecies differences among the metabolic profiles of T-2 toxin (Kobayashi et al., 1987; Welsch & Humpf, 2012; Wu, Dohnal, Huang, Ku-ìa, & Yuan, 2010; Wu et al., 2011; Wu et al., 2014). The species variability contributed to different toxicological effects *in vitro* and intrinsic clearance among species (Li et al., 2011; Wu, Tu, Yuan, Yuan, & Wen, 2013; Wu et al., 2011). The T-2 concentration was mainly reduced *in vitro* (>55%) in all species studied species (human, swine, goat, rat, cow and chicken), but human, swine, and goat showed the largest T-2 concentration reduction (>95%), and chicken the lowest (approximately 55%). The latter phenomenon was also confirmed for other mycotoxins as OTA and DAS. *In vitro* cow microsomal analysis pinpointed the lowest T-2 degradation among mammalians, possibly because of its degradation by rumen microorganisms.

Despite the species differences, HT-2 was identified as the most common phase I metabolite *in vitro*. Then, hydroxylated products, such as 3'-OH-HT-2, were the second prevalent metabolites *in vitro* in human, swine, and goat, while for cow, rat, and chicken this was 3'-OH-T-2. Human liver cells were not able to produce more metabolites, however, chicken, rat, and cow were able to produce another metabolite, producing three metabolites (3'-OH-T-2 or 3'-OH-HT-2) Yang, S., De Boevre, M., (2017). Furthermore, chicken and cows liver cells were able to produce NEO.

Table 5–Identified T-2 toxin (T-2) metabolites *in vivo* and *in vitro*.

Chemical structure - T-2 toxin



$C_{24}H_{34}O_9$ Molecular Weight = 466.52

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
15-deacetylneosolaniol	15-deacetylneosolaniol	$C_{17}H_{24}O_7$	Rats	*In vivo*: urine & feces	II	Yang, Li, . . . (2013)
15-deacetyl-T-2	15-deacetyl-T-2	$C_{22}H_{32}O_8$	Rats	*In vivo*: urine & feces	II	Yang, Li, . . . (2013)
3′-7-dihydroxy-HT-2	3′-7-diOH-HT-2	$C_{22}H_{32}O_{10}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
3′-7-dihydroxy-HT-2 (isomer)	3′-7-diOH-HT-2 (isomer)	$C_{22}H_{32}O_{10}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
3′-7-dihydroxy-T-2	3′-7-diOH-T-2	$C_{24}H_{34}O_{11}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
3′-hydroxy-9-hydroxy-T-2	3′-OH-9-OH-T-2	$C_{24}H_{36}O_{11}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
3′-hydroxy-T-2-glucoside	3′-OH-T-2-glucoside	$C_{30}H_{44}O_{15}$	Rats Human	*In vitro*: liver *In vitro*: liver	I	Yang, S., Van Poucke, C., . . . (2017)
3-4-dihydroxy-T-2	3′,4′-di-OH-T-2	$C_{22}H_{32}O_8$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
3-4-dihydroxy-T-2 (isomer)	3′,4′-di-OH-T-2 (isomer)	$C_{22}H_{32}O_8$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
3-hydroxy-15-deacetyl-T-2	3-OH-15-deacetyl-T-2	$C_{22}H_{32}O_9$	Rats	In vivo: urine & feces	II	Yang, Li, . . . (2013)
3-hydroxy-HT-2(also known as T-2 triol)	3′-OH-HT-2	$C_{20}H_{30}O_7$	Rats, chickens, swines, goats, cows, humans. Chickens Rats Cows Chickens Humans	*In vitro*: liver *In vivo*: feces & bile *In vivo*: urine & feces *In vivo*: urine *In vivo*: plasma *In vivo*: milk	I	Yang, S., De Boevre, M. (2017) (Sun et al., 2015) (Rubert et al., 2014) (Yoshizawa, Sakamoto., Ayano, & Mirocha, 1982) (Sun et al., 2015)) (Rubert et al., 2014)
3-hydroxy-HT-2-3-sulfate	3′-OH-HT-2 3-SO_3H	$C_{24}H_{39}O_{11}S$	Chickens	*In vivo*: feces & bile	II	Yang, S., De Boevre, M. (2017)
3-hydroxy-T-2	3′-OH-T-2	$C_{24}H_{34}O_{10}$	Rats, chickens, swines, goats, cows, humans Chickens Rats Cows	*In vitro*: liver *In vivo*: feces & bile *In vivo*: urine & feces *In vivo*: urine	I	Yang, S., De Boevre, M. (2017) Yang, S., De Boevre, M. (2017) Yang, Li, . . . (2013); Yoshizawa, Sakamoto, & Kuwamura, 1985) (Yoshizawa et al., 1982)
3-hydroxy-T-2-3-sulfate	3′-OH-T-2 3-SO_3H	$C_{26}H_{41}O_{12}S$	Chickens	*In vivo*: feces & bile	II	Yang, S., De Boevre, M. (2017)
4′-carboxyl-3′-hydroxy-T-2	4′-COOH-3′-OH-T-2	$C_{17}H_{24}O_7$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4′-carboxyl-3′-hydroxy-T-2 (isomer)	4′-COOH-3′-OH-T-2 (isomer)	$C_{17}H_{24}O_7$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4′-carboxyl-HT-2	4′-COOH-HT-2	$C_{22}H_{32}O_8$	Chickens	*In vivo*: feces	I	Yang, S., De Boevre, M. (2017)
4′-carboxyl-HT-2 (isomer)	4′-COOH-HT-2 (isomer)	$C_{22}H_{32}O_8$	Chickens	*In vivo*: feces	I	Yang, S., De Boevre, M. (2017)
4′-carboxyl-T-2	4′-COOH-T-2	$C_{17}H_{24}O_7$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4′-carboxyl-T-2 (isomer)	4′-COOH-T-2 (isomer)	$C_{17}H_{24}O_7$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4′-hydroxy-T-2-glucoside	4′-OH-T-2-glucoside	$C_{30}H_{44}O_{15}$	Rats Human	*In vitro*: liver *In vitro*: liver	I	Yang, S., Van Poucke, C. (2017)
4′-hydroxy-T-2-glucoside (isomer)	4′-OH-T-2-glucoside (isomer)	$C_{30}H_{44}O_{15}$	Rats Human	*In vitro*: liver *In vitro*: liver	I	Yang, S., Van Poucke, C. (2017)
4-4-dihydroxy-T-2	4′,4′-di-OH-T-2	$C_{22}H_{32}O_8$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4-deacetylneosolaniol	4-deAc-NEO	$C_{17}H_{24}O_7$	Rats, chickens, swines, goats, cows, humans Chickens Rats	*In vitro*: liver *In vivo*: feces *In vivo*: urine & feces	II	Yang, S., De Boevre, M. (2017) Yang, S., De Boevre, M. (2017) Yang, Li, . . . (2013)

(*Continued*)

Table 5–Continued.

Chemical structure - T-2 toxin						
Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
4-hydroxy-HT-2	4′-OH-HT-2	$C_{22}H_{32}O_9$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
4-hydroxy-HT-2 (isomer)	4′-OH-HT-2 (isomer)	$C_{22}H_{32}O_9$	Chickens	*In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017)
7-hydroxy-HT-2	7-OH-HT-2	$C_{28}H_{42}O_{14}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
7-hydroxy-HT-2 (isomer)	7-OH-HT-2 (isomer)	$C_{28}H_{42}O_{14}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
9-hydroxyl-T-2	9-OH-T-2	$C_{24}H_{36}O_{10}$	Rats	*In vivo*: urine & feces	I	Yang, Li, . . . (2013)
De-epoxy-3′,7-dihydroxy-HT-2	De-epoxy-3′,7-diOH-HT-2	$C_{22}H_{32}O_9$	Rats	*In vivo*: urine & feces	-	Yang, Li, . . . (2013)
De-epoxy-3′-hydroxy-HT-2	De-epoxy-3′-OH-HT-2	$C_{22}H_{32}O_8$	Rats	*In vivo*: urine & feces	-	Yang, Li, . . . (2013)
De-epoxy-3′-hydroxy-T-2 triol	De-epoxy-3′-OH-T-2 triol	$C_{24}H_{34}O_9$	Rats	*In vivo*: urine	-	(Yoshizawa et al., 1985)
De-epoxy-HT-2	De-epoxy-HT-2	$C_{22}H_{32}O_7$	Rats	*In vivo*: urine & feces	-	Yang, Li, . . . (2013)
HT-2 toxin	HT-2	$C_{22}H_{32}O_8$	Rats, chickens, swines, goats, cows, humans Chickens Rats Humans	*In vitro*: liver *In vivo*: feces & bile *In vivo*: urine & feces *In vivo*: milk & urine	I	Yang, S., De Boevre, M. (2017)Yang, S., VAn Poucke, C. (2017)Yang, Li, . . . (2013)(Rubert et al., 2014) (Rodriguez-Carrasco et al., 2014)
HT-2-3-glucuronide	HT-2-3-glucuronide	$C_{28}H_{40}O_{14}$	Rats, chickens, swines, goats, cows, humans Chickens	*In vitro*: liver *In vivo*: feces	II	Yang, S., De Boevre, M. (2017) Yang, S., De Boevre, M. (2017)
HT-2-4-glucuronide	HT-2-4-glucuronide	$C_{28}H_{40}O_{14}$	Rats, chickens, swines, goats, cows, humans Chickens Humans	*In vitro*: liver *In vivo*: feces *In vivo*: urine	II	Yang, S., De Boevre, M. (2017) (Gerding, Cramer, & Humpf, 2014; Gerding et al., 2015) (Gerding et al., 2015)
HT-2-glucoside	HT-2-glucoside	$C_{28}H_{42}O_{13}$	Rats Human	In vitro: liver In vitro: liver	I	Yang, S., Van Poucke, C. (2017)
Neosolaniol	NEO	$C_{19}H_{26}O_8$	Rat, chickens, swines, goats, cows, humans Chickens	*In vitro*: liver *In vivo*: feces & bile	I	Yang, S., De Boevre, M. (2017) Yang, S., De Boevre, M. (2017)
Neosolaniol-3-glucoside	Neo-3-glucoside	$C_{25}H_{36}O_{13}$	Human	In vitro: liver	I	Yang, S., Van Poucke, C. (2017)
T-2 tetraol	T-2 tetraol	$C_{15}H_{22}O_6$	Chicken	In vivo: feces	I	(Visconti & Mirocha, 1985)
T-2-3-glucuronide	T-2-3-glucuronide	$C_{30}H_{42}O_{15}$	Rats, chickens, swines, goats, cows, humans Chickens	*In vitro*: liver *In vivo*: feces	II	Yang, S., De Boevre, M. (2017) Yang, S., De Boevre, M. (2017)

Phase II metabolism revealed different glucuronides: T-2-3-glucuronide, HT-2-3-glucuronide, and HT-2-4-glucuronide (Li et al., 2011; Wu et al., 2010; Yang, S., De Boevre, M. (2017). HT-2-3-glucuronide was the predominantly detected compound in human, rat, and swine liver cells, but also traces of HT-2-4-glucuronide and T-2-3-glucuronide were detected in the mentioned animals. Liver cells of ruminants, such as cows and goats, produced HT-2-4-glucuronide as the predominant compound in phase II, and both produced traces of HT-2-3-glucuronide and T-2-3-glucuronide. So, phase I and II investigations showed that the T-2 metabolic pathway consists of hydrolysis (HT-2 and NEO), hydroxylation (3'-OH-T-2 and 3'-OH-HT-2), and glucuronidation (T-2-3-glucuronide, HT-2-3-glucuronide, and HT-2-4-glucuronide).

T-2-glucoside, the modified form of T-2, was also tested *in vitro* with liver microsomes from rats and humans. However, the metabolization was much less extensive than T-2 toxin, and 5 new compounds were detected (HT-2-glucoside, neosolaniol-3-glucoside, 3'-O-H-T-2-glucoside, 4'-OH-T-2-glucoside and its isomer) Yang, S., Van Pucke, C. (2017). 3'-OH-T-2-glucoside was the major metabolite of T-2-glucoside Yang, S., Van Pucke, C. (2017). Major differences between rats and human cells were not detected, except for the presence of NEO-3-glucoside in human cells (Table 5).

The few existing *in vivo* findings correlated with the *in vitro* results: (1) a large amount (19 metabolites) of T-2 toxin metabolites were detected and (2) main metabolites were similar. Thus, 19 different metabolites were identified in the feces and bile of chickens, with 13 reported for the first time because HRMS (Q/TOF-MS), was used (Table 5) Yang, S., De Boevre, (2017).

As for DON and AF, differences among gender have been demonstrated for the profile of T-2 metabolites. Female rates were reported to excrete less T-2, and consequently, fewer T-2 metabolites (only 8 metabolites) were detected, while 18 metabolites were identified in male rats Yang, S., Van Pouck, C. (2017). The main T-2 metabolites for male rats were 3'-OH-HT-2, de-epoxy-3'-OH-HT-2, 3'-7-dihydroxy-HT-2, HT-2, 3'-OH-T-2, 4-deacetylneosolaniol, and 7-OH-HT-2. Female rats' predominant T-2 metabolites were HT-2, 3'-OH-HT-2, de-epoxy-3'-OH-HT-2, 3'-OH-T-2, 9-OH-T-2, and 4-deacetylneosolaniol sequentially Yang, S., Van Pouck, C. (2017). T-2-triol, T-2-tetraol, 3'-OH-T-2 triol, and deepoxy-3'-OH-T-2 triol have been also identified as prevailing compounds in chicken plasma and feces after HT-2 administration (Visconti & Mirocha. 1985; Sun et al., 2015). As proven in the *in vivo* trials, hydrolysis, hydroxylation, and de-epoxidation were the metabolic pathways of T-2 toxin.

The toxicokinetics of T2-glucoside were studied in broilers (Kongkapan, Giorgi, Poapolathep, Isariyodom, & Poapolathep, 2016). Significant discrepancies in toxicokinetic parameters between T-2 toxin and T-2-glucoside were present: a 5-times higher absorption for T-2-glucoside was observed, which indicated the flaw in assuming an equal toxicity of modified and free mycotoxins in risk assessment. Although no data are available on the intrinsic toxicity of T-2-glucoside, and given the high degree of structural similarity between DON and T-2, a similar decrease in

toxicity may reasonably be expected as DON-3-glucoside toxicity is much lower than its free form (Pierron et al., 2016). However, recently, the large transformation of DON-3-glucoside to DON during human digestion was proven (Vidal et al., 2018), and this conversion to the free form could also be expected for T-2 glucoside.

Biomarkers of exposure

The *in vitro* results suggest the use of HT-2 as T-2 biomarker (Wu et al., 2010). In addition, hydroxylated products and glucuronide forms could also be used as biomarkers for T-2 exposure. Hence, several studies detected T-2 and HT-2 in human urine (Gerding et al., 2015; Rodriguez-Carrasco et al., 2014). Due to the lack of commercial reference standards, other metabolites were scarcely analyzed in human urine, and only HT-2-4-glucuronide has been detected in human urine (Gerding et al., 2015). The new advances in HRMS should permit to detect more T-2 metabolites, especially the predominant metabolite, 3'-OH-HT-2.

In conclusion, HT-2 is the predominant compound during *in vitro* and *in vivo* studies, and should therefore be considered as the main T-2 biomarker in urine and in plasma. However, there are possibly more T-2 metabolites which have not yet been detected in human urine, however the low T-2 concentrations in food pose analytical challenges with identification in human urine.

Diacetoxyscirpenol

DAS is mainly produced by *Fusarium* species such as *F. langsethiae* and *F. poae* (Shams et al., 2011). DAS is a type A trichothecene and is structurally related to T-2. DAS is a natural contaminant of agricultural crops with a rather low prevalence and contamination level in food samples (JECFA, 2017). The main food group contributing to the occurrence of 4,15-DAS are cereals, and most reports are on sorghum, wheat, rice, and maize (Antonissen et al., 2014; Tittlemier, Gaba, & Chan, 2013; van Egmond, Schothorst, & Jonker, 2007; Xue et al., 2013). Like other trichothecenes, DAS exerts an amalgam of acute and chronic effects on humans and animals, such as immunotoxicity (Ayral, Dubech, Lebars, & Escoula, 1992), hematotoxicity (Rio, Lautraite, & ParentMassin, 1997), pulmonary disorders (Jesenska & Bernat, 1994), growth retardation (Kubena et al., 1997), and cardiovascular effects (Hussein & Brasel, 2001). In addition, several reports linked its toxicity to the inhibition of protein and DNA synthesis (Hassanane, Abdalla, Ei-Fiky, Amer, & Hamdy, 2000). As other trichothecenes, DAS is vastly absorbed from the gastrointestinal tract, and rapidly excreted through the urine and feces within 12 to 24 hr (Bauer, Bollwahn, Gareis, Gedek, & Heinritzi, 1985).

In vitro/*in vivo* metabolism

In vitro liver microsomal analysis identified 12 different DAS-metabolites. The metabolic profile was species-dependent (Table 6). Following metabolization in the liver cells, a fraction of free DAS remained after incubation, from 14% to 59%. Similar to other mycotoxins, human liver microsomes had a higher metabolization capacity for DAS as compared to other species (rats, chicken, swine, goat, and cow). The fraction of free DAS remained low in human cells (14%), followed by goat (15%), rat (29%), chicken (26%), cow (54%), and swine (59%) (Yang, De Boevre, et al., 2015). Differences among species were observed in the number of metabolites produced. Remarkably, human liver microsomes produced the lowest number of DAS metabolites for the identified metabolites, while they possessed a higher degradation capacity. However human liver microsomes could produce other types of metabolites which were not identified in that database. A similar pattern was observed for DON: strong metabolization, but a restricted number of produced metabolites. The metabolites produced by the human liver were 15-monoacetoxyscirpenol (15-MAS) (90%) > diacetoxyscirpenol-3-glucuronide (DAS-3-glucuronide), 15-monoacetoxyscirpenol-3-glucuronide (15-MAS-3-glucuronide) > NEO > 8β-hydroxy-diacetoxyscirpenol (8β-OH-DAS) > 7-hydroxy-diacetoxyscirpenol (7-OH-DAS) > 15-monoacetoxyscirpenol-4-glucuronide (15-MAS-4-glucuronide).

As in humans, 15-MAS was a common metabolite in all species, and its concentration was even larger than the free DAS in chicken, goat, and humans after liver cells incubation. Only in rats and in cows 15-MAS is not the prevalent metabolite, but more likely 7-OH-DAS (isomer) and DAS-3-glucuronide were, respectively (Yang, Wang, et al., 2015). Due to the 15-MAS predominance, this compound was also the first compound to be identified as a DAS metabolite in the past and it was after DAS incubation with rat and rabbit liver microsomes (Ohta et al., 1978). The low degradation of DAS in liver cows' cells could be related to the presence of bacteria and protozoa present in the rumen fluid, which showed their extensive capacity to completely transform DAS to 15-MAS, scirpentriol (SCP), deepoxy-15-monoacetoxyscirpenol (deepoxy-15-MAS), and deepoxy-scirpentriol (deepoxy-SCP) (Swanson et al., 1987). Although DAS-3-glucuronide was not analysed after microorganisms' incubation, the production of glucuronide compounds was not expected, contrary to liver cells incubation, where DAS-3-glucuronide was the predominant compound (Yang, De Boevre, et al., 2015).

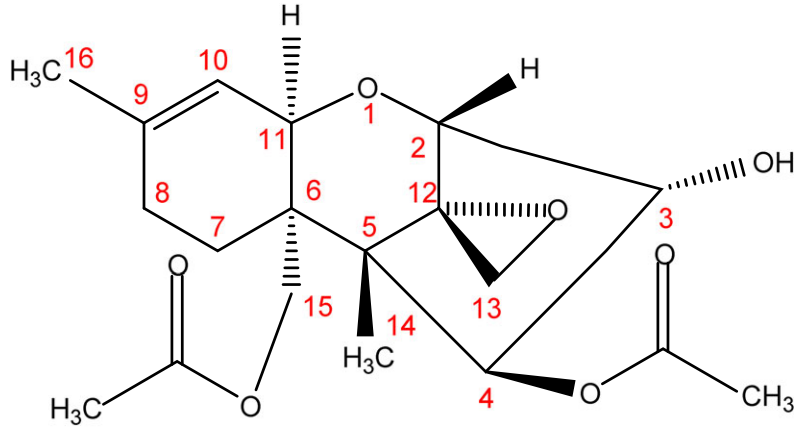
15-MAS and 7-OH-DAS (isomer) were considered as the predominant DAS metabolites in most species. Additionally, apart from chicken, glucuronidated forms have been detected as important DAS metabolites in liver cells' incubation (DAS-3-glucuronide, 15-MAS-3-glucuronide, and 15-MAS-4-glucuronide). DAS-3-glucuronide and 15-MAS-3-glucuronide were present in large amounts in different species (rat, swine, goat, cow, and human), while only few traces of 15-MAS-4-glucuronide were detected (Yang, De Boevre, et al., 2015).

Fecal incubation of DAS in rats and swine revealed the production of deepoxy products: deepoxy-15-MAS and deepoxy-SCP (Swanson, Helaszek, Buck, Rood, & Haschek, 1988). As the case for most trichothechenes, the deepoxy-forms were widely common after microorganisms' action. This suggested that deepoxidation was an important metabolic pathway for DAS (Swanson et al., 1988). Moreover, deepoxidation was suggested as an important metabolic detoxification in animals (Wu et al., 2013; Wu et al., 2010), next to hydrolyzation and conjugation (Wu et al., 2010).

Only few studies have focused on DAS during *in vivo* trials, but generally the outcome agreed with the *in vitro* results. 15-MAS was identified as the predominant DAS metabolite *in vivo* when urine and feces from rats and swine were analyzed (Sakamoto, Swanson, Buck, & Yoshizawa, 1986; Swanson et al., 1988; Yang, Wang, et al., 2015). In chicken, however, the 7-OH-DAS isomer was more prevalent next to 15-MAS (Yang, De Boevre, et al., 2015). 4-MAS, 7-OH-DAS, NEO, SCP, deepoxy-15-MAS, and deepoxy-SCP were identified in the rat urine (Swanson et al., 1988; Yang, De Boevre, et al., 2015), however, only 15-MAS, 7-OH-DAS and the deepoxy-conjugates (deepoxy-15-MAS and deepoxy-SCP) were verified in the feces (Swanson et al., 1988; Yang, De Boevre, et al., 2015).

Table 6–Identified diacetoxyscirpenol (DAS) metabolites *in vitro* and *in vivo*.

Chemical structure - Diacetoxyscirpenol



$C_{19}H_{26}O_7$ Molecular Weight = 366.41

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
15- monoacetoxyscirpenol-3-glucuronide	15-MAS-3-glucuronide	$C_{23}H_{32}O_{12}$	Rats, swines, goats cows, humans	*In vitro*: liver	II	(Yang et al., 2015)
15-monoacetoxyscirpenol	15-MAS	$C_{17}H_{24}O_6$	Rats, chickens, swines, goats, cows, humans Rats Chickens Swines	*In vitro*: liver *In vivo*: urine & feces *In vivo*: feces *In vivo*: plasma	I	(Yang et al., 2015) (Yang et al., 2015) (Sakamoto et al., 1986) (Yang et al., 2015) (Bauer et al., 1985)
15-monoacetoxyscirpenol-4-glucuronide	15-MAS-4-glucuronide	$C_{23}H_{32}O_{12}$	Swines, goats, cows, humans	*In vitro*: liver	II	(Yang et al., 2015)
4-monoacetoxyscirpenol	4-MAS	$C_{17}H_{24}O_6$	Rats Rats	*In vitro*: liver *In vivo*: urine	I	(Yang et al., 2015) (Yang et al., 2015)
7-hydroxy-diacetoxyscirpenol	7-OH-DAS	$C_{19}H_{26}O_8$	Rats, swines, goats, cows, humans	*In vitro*: liver	I	(Yang et al., 2015)
7-hydroxy-diacetoxyscirpenol (isomer)	7-OH-DAS (isomerr)	$C_{19}H_{26}O_8$	Rats, chickens, swines, goats, cows, humans Rats Chickens	*In vitro*: liver *In vivo*: urine & feces *In vivo*: feces	I	(Yang et al., 2015)
8β-hydroxy-diacetoxyscirpenol	8β-OH-DAS	$C_{19}H_{26}O_8$	Rats, swines, goats, cows, humans	*In vitro*: liver	I	(Yang et al., 2015)
Deepoxy-15-monoacetoxyscirpenol	Deepoxy-15-MAS	$C_{17}H_{24}O_6$	Rats, swines, cows Rats	*In vitro*: feces *In vivo*: urine & feces	I	(Swanson et al., 1988) (Sakamoto et al., 1986; Swanson et al., 1988)
Deepoxy-scirpentriol	Deepoxy-SCP	$C_{15}H_{22}O_5$	Rats, swines, cows Rats	*In vitro*: feces *In vivo*: urine & feces	I	(Swanson et al., 1988) (Sakamoto et al., 1986; Swanson et al., 1988)
diacetoxyscirpenol-3-glucuronide	DAS-3-glucuronide	$C_{25}H_{34}O_{13}$	Rats, swines, goats, cows, humans	*In vitro*: liver	II	(Yang et al., 2015)
Neosolaniol	NEO	$C_{19}H_{26}O_8$	Rats, swines, goats, cows, humans Rats	*In vitro*: liver *In vivo*: urine	I	(Yang et al., 2015)
Scirpentriol	SCP	$C_{15}H_{22}O_5$	Rats Swines Rats, swines, goats	*In vivo*: urine *In vivo*: plasma *In vitro*: liver	I	(Swanson et al., 1988) (Sakamoto et al., 1986) (Yang et al., 2015)

Biomarkers of exposure

Up to now, no urinary glucuronidated DAS was observed *in vivo*, which is in contrast to the *in vitro* results (DAS-3-glucuronide, 15-MAS-3-glucuronide, and 15-MAS-4-glucuronide). *In vivo* studies also detected SCP in pig plasma after oral administration of DAS, showing that metabolism via the intestinal tract is probable (Bauer et al., 1985; Sakamoto et al., 1986). DAS has been analyzed in human urine samples (Heyndrickx et al., 2015; Huybrechts et al., 2015; Rodriguez-Carrasco et al., 2014), but no study has revealed the presence of DAS. The evaluation of DAS exposure in humans should focus on the detection of 15-MAS, the main DAS metabolite after human liver cell incubations. More than 90% of DAS was converted to 15-MAS, emphasizing that the detection of DAS in human urine was obsolete and 15-MAS has to be used as DAS biomarker in urine for humans.

DAS was vastly and rapidly metabolized, and excreted within 12 to 24 hr (Bauer et al., 1985). Within these 12 hr after DAS administration the urine and feces revealed several compounds. The comparison between urine and feces from rats shows that DAS is mainly excreted in urine (>92%) compared to feces (Yang, De Boevre, et al., 2015). The remaining DAS metabolites identified in *in vitro* studies were not observed in urine or feces, which might be attributed to the lower amounts present. So, *in vivo* studies also confirmed that DAS underwent extensive metabolism via 4 different pathways, namely, hydrolyzation, hydroxylation, deepoxydation, and conjugation, but no conjugates have been observed *in vivo*.

In conclusion, DAS is metabolized in a wide range of metabolites, but its metabolism is species-dependent. Hydrolyzation, hydroxylation, deepoxydation, and conjugation are the main metabolic pathways resulting in 15-MAS, 7-OH-DAS, and deepoxy-forms. Future research needs to reveal if glucuronidated DAS forms can be assigned as relevant DAS biomarkers in urine and feces, however, there is no doubt that 15-MAS has to be the main DAS biomarker in urine and feces, and SCP in plasma.

Ochratoxin A

OTA is mainly produced by *Penicillium* and *Aspergillus* (Varga et al., 2013). OTA is observed in cereals and cereal-based products, coffee, grapes, and nuts (Marin et al., 2013). OTA exerts nephrotoxicity and possesses carcinogenic, teratogenic, immunotoxic, and neurotoxic properties ((SCF), 1998). This mycotoxin has been classified in group 2B as a possible human carcinogen by IARC (WHO, 2002). The amount of absorbed OTA was species-dependent, and, although the results present a high level of variability, humans showed to have the highest level of OTA absorption (62% to 100%) (Versantvoort, Oomen, Van De Kamp, Rompelberg, & Sips, 2005). Other species had a lower absorption level, namely pigs (66%), rabbits (56%), and chickens (40%) (Galtier, Alvinerie, & Charpenteau, 1981).

In vitro/*in vivo* metabolism

In vitro, OTA metabolization studies resulted in the presence of hydroxylated OTA-forms and ochratoxin B (OTB), the dechlorinated form of OTA (Table 7). However, the amount of produced metabolites was species-dependent. Human liver microsomes had a more noticeable amount of metabolites (>30%) compared to other species for similar OTA exposure (Yang et al., 2015), suggesting a larger catabolic capability to metabolize OTA through the liver. In human liver microsomes, and also from rat, swine, and goat, the main OTA-metabolite was (4R)-hydroxyochratoxin A (4(R)-OH-OTA), while its isomer (4S)-hydroxyochratoxin A (4(S)-OH-OTA) was the most common metabolite in cows. 7-hydroxyochratoxin A (7'-OH-OTA) was the most prevalent OTA metabolite in chickens (Yang et al., 2015), while 10-hydroxyochratoxin A (10-OH-OTA) was the most important metabolite in rabbits (Størmer, Støren, Hansen, Pedersen, & Aasen, 1983). Aside from the most common metabolites mentioned above, other compounds have been identified with different concentrations depending on animal species (Table 7). It is worth to mention that OTB was prevailing with a larger concentration (>50%) in human microsomes than in other species, pointing out that humans had a large dechlorination capacity (Yang et al., 2015). Rat liver microsomes did not provide satisfying information, as more than 85% of OTA remained unmetabolized, hinting that OTA was not primarily metabolized by the liver (Han et al., 2013).

Lately, *in vivo* studies have confirmed the *in vitro*-results that free OTA was more prevalent in urine and feces than OTA metabolites due to low level of metabolization. Some authors reported the presence of ochratoxin α (OTα), formed by the cleavage of the phenylalanine moiety of OTA, as a common metabolite in the feces and urine of animals (Muñoz, Blaszkewicz, & Degen, 2010; Storen, Holm, & Stormer, 1982).

However, phase II metabolites of OTA were also observed in biological fluids such as urine, serum, and milk. Glucuronidated conjugates of OTA were more likely to be present in animal and human urine, as after enzymatic hydrolysis of the urine samples the levels of OTA and OTα were considerably (up to 6-fold) higher (Muñoz, Cramer, Dopstadt, Humpf, & Degen, 2017; Pena, Seifrtova, Lino, Silveira, & Solich, 2006; Solfrizzo, Gambacorta, Lattanzio, Powers, & Visconti, 2011). Although there was clear evidence of OTA-glucuronides, no glucuronidated forms have been identified yet. Ochratoxin A-8-β-glucuronide (OTA-8-glucuronide) and open lactone-ochratoxin A-8-β-glucuronide (open lactone OTA-8-glucuronide) have been synthesized as reference standards, but they have not been observed in human urine. Probably this phenomenon was attributed to a low sensitivity and ionization efficiency revealed in spiked analysis by LC-MS/MS compared to OTA (Muñoz et al., 2017).

Biomarkers of exposure

Comparison of the *in vivo* and *in vitro* OTA-results was challenging as most *in vivo* investigations did not take into account the metabolites found in *in vitro* studies. Yang et al. (2015), however, analyzed urine and feces of rats and chickens, and they were able to find a similar pattern of OTA metabolites after liver microsomal incubation (Yang et al., 2015). Hydroxylated compounds found after liver cell incubations, especially 7-OH-OTA, (4R)-OH-OTA, and (4S)-OH-OTA, have been scarcely studied in biological fluids (Schaut et al., 2008). The analyses of these hydroxylated OTA-forms and OTB in human urine could increase the knowledge, and obtain a reliable pattern, of OTA excretion.

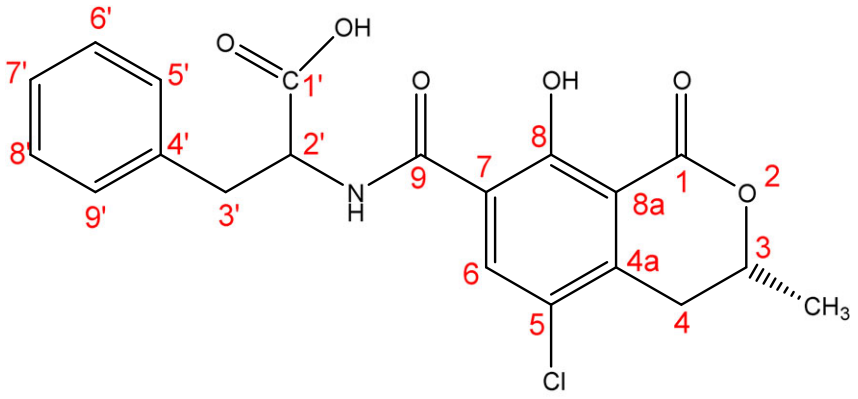
In conclusion, OTA, OTB, OTα, and their glucuronides are suggested to be the most prevailing fraction of total excreted OTA. The authors suggest to use these metabolites as OTA-biomarkers of exposure both in urine and in plasma.

Citrinin

CIT is produced by *Penicillium* and *Aspergillus* species (Deruiter, Jacyno, Davis, & Cutler, 1992; El-banna, Pitt, & Leistner, 1987) and mainly occurs in cereals and cereal-based products (Mornar, Sertić, & Nigović, 2013; Pleadin et al., 2017). The consumption of these food products is considered as the major contribution of dietary exposure to CIT, however occurrence data on CIT remain

Table 7–Identified ochratoxin A (OTA) metabolites *in vitro* and *in vivo*.

Chemical structure - Ochratoxin A



$C_{20}H_{18}ClNO_6$ Molecular Weight = 403.81

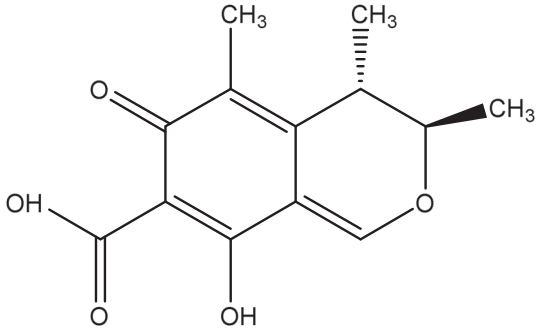
Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
(4R)-hydroxyochratoxin A	(4R)-OH-OTA	$C_{20}H_{18}ClNO_7$	Rats, chickens, swines, goats, cows, humans Rabbits Rats Chickens	*In vitro*: liver *In vitro*: liver *In vivo*: urine & feces *In vivo*: feces	I	(Yang et al., 2015) (Størmer et al., 1983) (Yang et al., 2015) (Yang et al., 2015)
(4S)-hydroxyochratoxin A	(4S)-OH-OTA	$C_{20}H_{18}ClNO_7$	Rats, chickens, swines, goats, cows, humans Rabbits Rats Chickens	*In vitro*: liver *In vitro*: liver *In vivo*: urine *In vivo*: feces	I	(Yang et al., 2015) (Størmer et al., 1983) (Yang et al., 2015) (Yang et al., 2015)
4(R)-hydroxyochratoxin B	4(R)-OH-OTB	$C_{20}H_{19}NO_7$	Chickens	*In vivo*: feces	I	(Yang et al., 2015)
4(S)-hydroxochratoxin B	4(S)-OH-OTB	$C_{20}H_{19}NO_7$	Chickens	*In vivo*: faeces	I	(Yang et al., 2015)
5-hydroxyochratoxin A	5'-OH-OTA	$C_{20}H_{18}ClNO_7$	Rats, chickens, swines, goats, cows, humans Rats Chickens	*In vitro*: liver *In vivo*: urine *In vivo*: feces	I	(Yang et al., 2015) (Yang et al., 2015) (Yang et al., 2015)
7-hydroxyochratoxin A	7'-OH-OTA	$C_{20}H_{18}ClNO_7$	Rats, chickens, swines, goats, cows, humans Rats Chickens	*In vitro*: liver *In vivo*: urine *In vivo*: feces	I	(Yang et al., 2015)
9-hydroxyochratoxin A	9'-OH-OTA	$C_{20}H_{18}ClNO_7$	Rats, chickens, swines, goats, cows, humans Rats Chickens	*In vitro*: liver *In vivo*: urine *In vivo*: feces	I	(Yang et al., 2015)
Ochratoxin A-8-β-glucuronide	OTA-8-glucuronide	$C_{26}H_{27}NO_{13}$	Chickens	*In vivo*: feces	II	(Bordini, Rossi, Ono, Hirooka, & Sataque Ono, 2017)
Ochratoxin alpha	OTα	$C_{11}H_9ClO_5$	Sheeps Humans Rats	*In vivo*: urine *In vivo*: plasma & plasma *In vivo*: urine & plasma	I	(Schaut et al., 2008) (Ali, Muñoz, & Degen, 2017) (Abbas, Blank, Wein, & Wolffram, 2013)
Ochratoxin B	OTB	$C_{20}H_{19}NO_6$	Rats, chickens, swines, goats, cows, humans Rats Chickens	*In vitro*: liver *In vivo*: urine & feces *In vivo*: feces	I	(Yang et al., 2015)
Open lactone-ochratoxin A-8- β-glucuronide	Lactone-OTA-8-glucuronide	$C_{26}H_{25}NO_{12}$	Chickens	*In vivo*: feces	II	(Bordini et al., 2017)

scarce. CIT affects the kidney function in different species, but it appears to be considerably less toxic than OTA. The mycotoxin results in necrosis of the distal tubule epithelium in the kidneys, alters the function, and degenerates processes of the renal tubules. Nonetheless, CIT is classified by IARC in group three because of its nonability to be carcinogenic to humans, and because of limited evidence in animals (IARC, 1993). To date, there are only legal requirements for food supplements based on red yeast rice powder (2 mg/kg) (212/2014/EC, 2014). CIT is rapidly absorbed and distributed, in particular to the liver and kidney (Constable, Hinchcliff, Done, & Grunberg, 2017). A recent CIT toxicokinetic study in humans showed that 40% of CIT was excreted in urine (Degen, Ali, & Gundert-Remy, 2018), so the CIT absorption was ≥40%.

In vitro/*in vivo* metabolism

According to the authors' knowledge, CIT has not been investigated through *in vitro* metabolism studies. However, *in vivo* studies have revealed the presence of dihydro-citrinone (DH-CIT) in human urine from adults (mean, 752 pg/mL) (Heyndrickx et al., 2015), and is considered as the major metabolite (Table 8). Ali, Blaszkewicz, and Degen (2015) observed 84% DH-CIT in human urine from Germany, and approximately a three-fold higher level on average (0.1 ng/mL) than its free compound (0.03

Table 8–Identified citrinin (CIT) metabolites *in vivo*.

Chemical structure - Citrinin

$C_{13}H_{14}O_5$ Molecular Weight = 250.25

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Dihydrocitrinone	DH-CIT	$C_{13}H_{14}O_6$	Humans Rats	*In vivo*: urine *In vivo*: urine	I	(Heyndrickx et al., 2015) (Dunn et al., 1983)

ng/mL) which was present in 82% of the smaples (Ali et al., 2015). The ratio CIT/DH-CIT in urine was, however, highly variable from 0.75 to 11. Noteworthy, DH-CIT is less toxic than CIT (Föllmann, Behm, & Degen, 2014), so the formation of DH-CIT is considered as a detoxification reaction. The higher presence of DH-CIT than free CIT in human urine was similar in recently reported studies (Ali, Blaszkewicz, Alim, Hossain, & Degen, 2016; Degen et al., 2018; Föllmann, Ali, Blaszkewicz, & Degen, 2016; Heyndrickx et al., 2015). Consequently, as DH-CIT has a large presence as CIT metabolite in urine, it is essential to consider this metabolite in the evaluation of CIT exposure.

CIT is renally excreted after 20 to 22.5 hr of administration in humans. A maximal excretion pattern of CIT and DH-CIT is established after 22.5 hr in humans (Degen et al., 2018). DH-CIT was also identified in rats' urine, while CIT was not detected (Dunn et al., 1983), proving that DH-CIT is predominant in CIT rat metabolism. DH-CIT has been detected in human plasma (0.90 ng/mL) in even higher concentrations than in urine (0.78 ng/mL) (Degen et al., 2018). Experimental data regarding systemic effects in ruminants are not available. It is assumed that CIT is highly degraded and metabolized through the microbial activity in the forestomachs of ruminants (EFSA, 2012).

In conclusion, DH-CIT should be considered as the most relevant CIT metabolite in urine. However, the lack of information on other metabolites (for example, via *in vitro* or exposure studies) can lead to an underestimation of CIT-exposure as possibly relevant other CIT-biomarkers have not yet been identified.

Fumonisins (FB)

FB are fungal secondary metabolites produced by *F. verticillioides*, *F. proliferatum,* and *F. nygama* ((EFSA), 2005). FB are mainly observed in maize (Marin et al., 2013). FB cause a variety of negative effects in animals and humans, such as hepatotoxic, nephrotoxic, hepatocarcinogenic, and cytotoxicity in mammals (Norred, Wang, Yoo, Riley, & Merrill, 1992). Twelve FB-analogues have been described, with the most important being fumonisin B1 (FB1), fumonisin B2 (FB2), and fumonisin B3 (FB3). From a toxicological perspective, FB1 is the most important FB, and it has been classified in group 2B as possibly carcinogenic to humans by IARC (WHO, 2002), for this reason, JECFA recommended that FB exposure has to be reduced, particularly in areas where maize is the major dietary staple food (JECFA, 2017). The FB bioavailability in animals was low: <3.5% in pigs (Souto et al., 2017), 3.2% in turkey poults, and 2.3% in ducks (Vudathala, Prelusky, Ayroud, Trenholm, & Miller, 1994). This low absorption was caused by (1) the adsorption of FB to serum components, such as lipids or proteins, making the free toxin unavailable and/or (2) an extensive enterohepatic circulation of FB (Dilkin, Direito, Simas, Mallmann, & Corrêa, 2010).

In vitro/*in vivo* metabolism

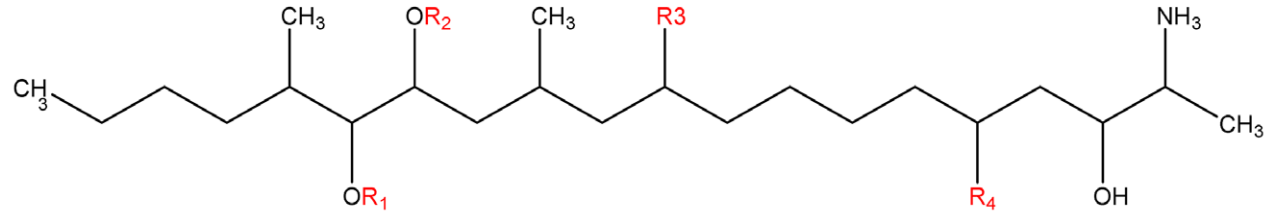
Little information exists on the FB metabolization *in vitro*, but FB seemed more stable than other *Fusarium* mycotoxins. Beside the high stability of FB during incubation, the formation of hydrolyzed forms (hydrolyzed FB1 (HFB1) and hydrolyzed FB2 (HFB2), N-acyl-fumonisin 1 (NAFB1), and N-acyl-hydrolysed fumonisin 1 (NAHFB1) in liver and kidney human cells was observed. Also, the FB-pattern was species-dependent. FB incubation pinpointed a high stability *in vitro* in pigs and cows. In pig liver microsomes, only traces of HFB1 and HFB2 were observed (Gazzotti et al., 2011), while in bovine liver microsomes FB1 was not degraded or transformed to other compounds (Spotti, Pompa, & Caloni, 2001). Also, in rat liver microsomes FB1 was scarcely metabolized (Merrill, Nikolova-Karakashian, Schmelz, Morgan, & Stewart, 1999). As in the case of pigs (Gazzotti et al., 2011), there was a small conversion rate of FB1 to HFB1 (<1%), indicating that this process was not important during metabolism (Fodor et al., 2007) (Table 9).

The fecal microbiota of pigs biotransforms FB1 to metabolites, such as *partially* hydrolyzed FB1 (pHFB1) (Fodor et al., 2007). The percentage of FB1 converted to pHFB1 was 46% and 49% after 48 and 72 hr of incubation, respectively. Also, a small amount of HFB1 was observed (Dang et al., 2017).

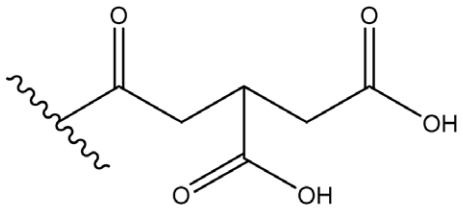
Human microsomes, however, showed a better ability to metabolise FB. NAFB1 and NAHFB1 were identified during incubation of FB1 and HFB1 with kidney human cells, respectively (Harrer, Laviad, Humpf, & Futerman, 2013). The amount of NAHFB1 was two times higher than the produced amount of

Table 9–Identified fumonisin (FB) metabolites *in vivo* and *in vitro*.

Chemical structure - Fumonisins



	R_1	R_2	R_3	R_4
FB1	TCA	TCA	OH	OH
FB2	TCA	TCA	H	OH
FB3	TCA	TCA	OH	H
pHFB1a	TCA	H	OH	OH
HFB1	H	H	OH	OH



Tricarballylic acid (TCA)

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Hydrolyzed FB1	HFB1	$C_{34}H_{59}NO_{13}$	Swines Humans	*In vitro*: liver *In vitro*: feces	I	(Gazzotti et al., 2011) (Cirlini et al., 2015; Hahn, et al., 2015)
Hydrolyzed FB2	HFB2	$C_{34}H_{59}NO_{12}$	Swines	*In vitro*: liver	I	(Gazzotti et al., 2011)
N-acyl-fumonisin 1	NAFB1	$C_{46}H_{85}NO_{13}$	Humans	*In vitro*: liver	I	(Harrer et al., 2013)
N-acyl-hydrolyzed fumonisin	NAHFB1	$C_{46}H_{81}NO_{14}$	Humans	*In vitro*: liver	I	(Harrer et al., 2013)
Partially hydrolyzed fumonisin B1	pHFB1a	$C_{40}H_{60}NO_{18}$	Swines Humans	*In vitro*: feces *In vitro*: feces	I	(Fodor et al., 2007) (Cirlini et al., 2015; Hahn, et al., 2015)

NAFB1 (Harrer et al., 2013). The higher production of NAHFB1 could be caused by the lack of accessibility to ceramide synthases (CerS)-active sites such as the tricarballylic acid moieties. It is noticeable that NAFB1 was significantly more cytotoxic than FB1 (Harrer et al., 2013). HFB1 was detected in the liver of weaned piglets fed with a FB1-diet indicating its dietary origin (Pagliuca et al., 2005). Recently, the *in vivo* metabolic conversion of FB1 to NAFB1 and HFB1 and NAFB1 was revealed in the kidney and liver of rats (Harrer, Humpf, & Voss, 2015), with a higher production of NAFB1 and NAHFB1 in the liver.

HFB1 and pHFB1 are present in foods as a result of alkaline processing like maize nixtamalization (De Girolamo, Lattanzio, Schena, Visconti, & Pascale, 2016). pHFB1 could be partially cleaved (>41%) during human digestion and new compounds were not identified. On the other hand it remains stable when incubated *in vitro* with human colon microflora (Cirlini et al., 2015). HFB1 was partially metabolized (22%) by the colon microflora to unknown compounds after 24 hr of fermentation (Cirlini et al., 2015; Hahn, et al., 2015).

Biomarkers of exposure

To evaluate the exposure to FB in experimental animal studies, the ratio of sphinganine to sphingosine in urine and serum was reported as a functional biomarker (Shephard, van der Westhuizen, & Sewram, 2007): however, it failed to yield the expected outcome with human studies due to low concentrations found (Shephard et al., 2007). *In vivo* studies agreed with the high FB stability in animals because FB had low metabolization. So, most of FB were detected in free FB form in urine and feces, and >90% free FB was found in pigs' feces (Prelusky, Trenholm, & Savard, 1994; Souto et al., 2017), while less than 3% was recovered in pigs' urine (Fodor et al., 2008; Gambacorta et al., 2013; Souto et al., 2017). The same phenomenon was observed in chickens (>97% of FB1 was recovered in chicken feces) (Vudathala et al., 1994).

Furthermore, FB1 has been detected in pooled hair (from 3 different individuals) with a 10-times higher concentration than in plasma and a 10-times lower concentration than in urine (Souto et al., 2017). FB1 has been also detected in the hair of primates, rats (Sewram et al., 2001) and humans (Bordin et al., 2015; Sewram et al., 2003). Differences in FB1 hair levels have been detected in various species, and humans had a higher FB1 accumulation (Bordin et al., 2015). The accumulation of FBs in hair evidenced that FB1 in hair could be used as a biomarker for a long-term dietary exposure studies.

Human urinary analysis focused on free FB with FB1 as the most prevalent next to FB2 and FB3 (Cirlini et al., 2017; De Santis, et al., 2017; Gerding et al., 2015; Gong, Shirima, Srey, Kimanya, & Routledge, 2015; Solfrizzo, Gambacorta, & Visconti, 2014; Torres et al., 2014). However the mean FB1 levels found in urine have been low, for instance 0.07 ng/mL (Cirlini et al., 2017), 0.4 ng/mL (De Santis, et al., 2017), 0.44 ng/mL (Gerding et al., 2015), 1.38 ng/mL (Gong, et al., 2015), and 0.05 ng/mL (Solfrizzo et al., 2014). Moreover, a statistically significant correlation between the estimated FB intake and the urinary FB1 levels based on the Pearson product correlation was observed ($r = 0.26$, $p = 2.13 \times 10^{-17}$, $n = 1229$) (Torres et al., 2014).

In conclusion, FB have low absorption and are mainly excreted via the fecal route. FB are highly stable in most species, and small fractions of HFB are detected as a result of FB metabolism. Human microsomes showed a higher ability to degrade FB (approximately 50%) to other compounds, such as HFB1, pHFB1, NAFB1, and NAHFB1, however, the level of FB detected in the human urine is low, and NAFB1 and NAHFB1 have been scarcely analyzed.

Zearalenone

ZEN is mainly produced by *F. graminearum*, *F. culmorum*, *F. equiseti*, and *F. verticilliodes* (EFSA, 2004, 2011, 2014, 2016). Cereals are susceptible to fungal infestation and subsequent ZEN contamination (Marin et al., 2013). ZEN has a powerful estrogenic activity as its hormonal action exceeds that of most other naturally-occurring nonsteroidal estrogens (Bennet & Klich, 2003). Fertility problems have been observed in laboratory animals (mice, rats, guinea pigs, hamsters, and rabbits), and in swine and sheep (Kuiper-Goodman, Scott, & Watanabe, 1987). ZEN may be an important etiologic agent of intoxication in infants or foetuses, which results in premature thelarche, pubarche, and breast enlargement (EFSA, 2011). Furthermore, ZEN stimulates the growth of human breast cancer cells, indicating its potential carcinogenicity to humans (Belhassen et al., 2015), however IARC assigned ZEN to group three (IARC, 1993).

Toxicokinetics of ZEN demonstrated that the mycotoxin is rapidly and extensively absorbed from the gastrointestinal tract (80% to 85% in pigs) (Biehl et al., 1993).

In vitro/in vivo metabolism

Numerous *in vitro* metabolism studies of ZEN in animals and humans have been carried out. The recent advances in HRMS permitted to detect new metabolites. Reduction of ZEN leads to the formation of α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases (HSDs), which were the first phase I metabolites of ZEN discovered (Zinedine, Soriano, Molto, & Manes, 2007). Both metabolites have different estrogenic activities in which α-ZEL is 92-fold more potent in comparison to ZEN, while β-ZEL is 2.5 times lower than ZEN (Drzymala et al., 2015; Molina-Molina et al., 2014). These toxicity differences give rise to the fact that the pathway to produce α-ZEL is considered as a toxic pathway, whereas the formation of β-ZEL is considered as a detoxifying pathway. *In vitro*, in rats, swine, goat, cows, and humans the yield of α-ZEL was much higher than β-ZEL (Ueberschär, Brezina, & Dänicke, 2016). However, in chickens the yield of α-ZEL was less or equal to β-ZEL. The reduction of ZEN is not only mediated by liver enzymes, but also by the intestinal mucosa and microbial flora. Unlike the reduction in the liver, β-ZEL is the most prevalent metabolite in intestinal microsomes (EFSA, 2011). Furthermore, Yang, S., Zhang, H. (2017) observed that hydrogenation and dehydrogenation of ZEN is a reversible process: ZEN, α-ZEL, and β-ZEL can be mutually transformed in the liver microsomes. 15-OH-ZEN and 8-OH-ZEN (isomer) are the other principal metabolites in all studied species (rats, chickens, goats, cows, swine and humans). The other metabolites, which are mainly hydroxylated forms, are only found *in vitro* in low amounts in most of the species (<5%) (rats, chickens, goats, cows, swine and humans). The hydroxylation sites of ZEN are positioned at C-2, C-3, C-4, C-5, C-8, C-9, C-10, C-13, and C-15 (Table 10).

Regarding phase II metabolites, 4 different glucuronides have been detected (ZEN-14-glucuronide, ZEN-16-glucuronide, ZEN-14,16-diglucuronide, and ZEN-14-2-glucuronide). ZEN-14-glucuronide is the predominant metabolite, followed by ZEN-16-glucuronide. While human liver microsomes reveal 2 glucuronides (ZEN-14-glucuronide and ZEN-16-glucuronide), ZEN-14,16-diglucuronide is only detected in swine and ZEN-14-2-glucuronide in goats and cows. Glucuronides do not possess any estrogenic activity, while ZEN sulfates have a low activity in comparison to ZEN (Drzymala et al., 2015).

Based on the above-mentioned analysis of the metabolites, the major metabolic pathways of ZEN in animal and human liver microsomes are reduction, hydroxylation, and glucuronidation. The reduction of ZEN is mainly generated at the carboxyl of C-7, producing α-ZEL and β-ZEL. Hydroxylation occurs at C-13 and C-15 as well as the C-2, C-3, C-4, C-5, C-6, C-8, C-9, C-10 on the aliphatic ring. The binding sites for glucuronides are at C-14 and C-16.

In vivo studies demonstrated that the number of metabolites for ZEN are different from those observed *in vitro*. α-ZEL and β-ZEL were the most prevalent metabolites found *in vivo*, and some hydroxylated, glucuronidated, sulfated, and deepoxy-forms were detected in urine samples. Orally administered ZEN in rats and in chickens gave rise to the presence of 9 and 2 metabolites, respectively. In rats, α-ZEL and β-ZEL, as well as 7 hydroxylated metabolites (5-OH-ZEN, 4-OH-ZEN and its isomer, 9-OH-ZEN, 6-OH-ZEN & its isomer, and 15-OH-ZEN) were found Yang, S., Zhang, H. (2017).

Regarding phase II metabolites, the 3 glucuronides (ZEN-16-glucuronide, ZEN-14-glucuronide, ZEN-14,16-diglucuronide) were detected in rats and chickens Yang, S., Zhang, H. (2017). Furthermore, another 11 conjugates were identified in urine of rats, however, they were not clearly described Yang, S., Zhang, H. (2017). In detail, 7 different compounds were detected with the same accurate mass of m/z = 509, which was 176 Da higher than OH-ZEN, indicating that they were likely to be glucuronides of OH-ZEN. The retention times of these 7 metabolites were different, suggesting that they were structural isomers. Also, 4 different α-ZEL and β-ZEL-glucuronides in urine were detected, but the identification of their chemical composition is not yet established Yang, S., Zhang, H. (2017).

In vivo trials with pigs showed that ZEN was mainly recovered in feces (40%) compared to urine (26%) (Binder et al., 2017). Most of the detected ZEN in urine and feces was in its free form followed by ZEN-14-glucuronide and α-ZEL. α-ZEL was not detected in the urine, but ZEN-14-glucuronide was found in urine as the predominant metabolite followed by free ZEN.

In addition, ZEN is able to conjugate with sulfates. Three sulfated metabolites were detected in chicken feces, namely zearalenone-14-sulfate (ZEN-14-SO3H), α-zearalenone-14-sulfate (α-ZEL-14-SO3H), and β-zearalenone-14-sulfate (β-ZEL-14-SO3H) Yang, S., Zhang, H. (2017). *In vivo* results indicated that reduction, hydroxylation, and glucuronidation are the major metabolic pathways in rats, but reduction and sulfatation are the major ones in chickens Yang, S., Zhang, H. (2017).

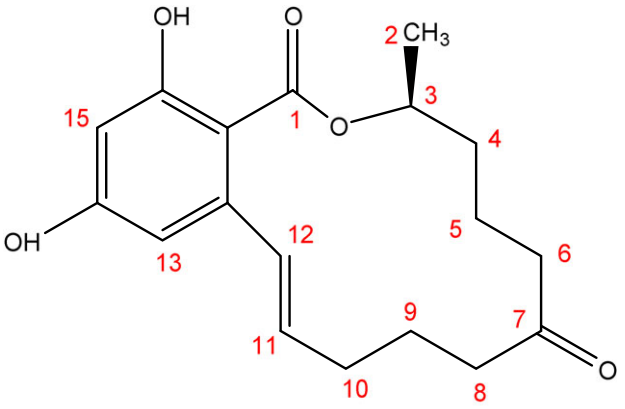
Biomarkers of exposure

Recently, Heyndrickx et al. (2015) analyzed ZEN, ZEN-14-glucuronide, α-ZEL, α-ZEL-7-glucuronide, α-ZEL-14-glucuronide, β-ZEL, and β-ZEL-14-glucuronide in human urine samples (Heyndrickx et al., 2015). However, only few traces of α-ZEL and β-ZEL-14-glucuronide were detected.

The occurrence of ZEN metabolites in urine and feces in swine is mainly observed 24 to 48 hr after administration (Binder

Table 10–Identified zearalenone (ZEN) metabolites *in vivo* and *in vitro*.

Chemical structure – Zearalenone



$C_{18}H_{22}O_5$ Molecular Weight = 318.36

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
10-hydroxy-zearalenone	10-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
13-hydroxy-zearalenone	13-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
15-hydroxy-zearalenone	15-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
2-hydroxy-zearalenone	2-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
3-hydroxy-zearalenone	3-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
4-hydroxy-zearalenone	4-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans Rats	*In vitro*: liver *In vivo*: urine	I	Yang, S., Zhang, H. (2017)
4-hydroxy-zearalenone (isomer)	4-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
5-hydroxy-zearalenone	5-OH-ZEN	$C_{18}H_{22}O_6$	Rats & chickens Rats	*In vitro*: liver *In vivo*: urine	I	Yang, S., Zhang, H. (2017)
5-hydroxy-zearalenone (isomer)	5-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
6-hydroxy-zearalenone	6-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
6-hydroxy-zearalenone (isomer)	6-OH-ZEN	$C_{18}H_{22}O_6$	Swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
8-hydroxy-zearalenone	8-OH-ZEN	$C_{18}H_{22}O_6$	Rats, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
8-hydroxy-zearalenone (isomer)	8-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
9-hydroxy-zearalenone	9-OH-ZEN	$C_{18}H_{22}O_6$	Chickens, goats, cows	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
9-hydroxy-zearalenone (isomer)	9-OH-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	I	Yang, S., Zhang, H. (2017)
deepoxy-zearalenone	Deepoxy-ZEN	$C_{18}H_{22}O_6$	Cows & humans	*In vitro*: liver	-	Yang, S., Zhang, H. (2017)
deepoxy-zearalenone (isomer)	Deepoxy-ZEN	$C_{18}H_{22}O_6$	Rats, chickens, swines, goats, cows, humans	*In vitro*: liver	-	Yang, S., Zhang, H. (2017)
Hydroxy-zearalenone-glucuronide	OH-ZEN-glucuronide	$C_{24}H_{30}O_{12}$	Rats	*In vivo*: urine & feces	II	Yang, S., Zhang, H. (2017)
Zearalenone-14,16-di-glucuronide	ZEN-14,16-di-glucuronide	$C_{30}H_{38}O_{17}$	Swines, goats, humans	*In vitro*: liver	II	Yang, S., Zhang, H. (2017)
Zearalenone-14-2-di-glucuronide	ZEN-14-2-di-glucuronide	$C_{30}H_{38}O_{17}$	Rats, goats, cows	*In vitro*: liver	II	Yang, S., Zhang, H. (2017)
Zearalenone-14-glucuronide	ZEN-14-glucuronide	$C_{24}H_{30}O_{11}$	Rats, chickens, swines, goats, cows, humans Swines Chicken & rats	*In vitro*: liver *In vivo*: feces *In vivo*: urine	II	Yang, S., Zhang, H. (2017) (Binder et al., 2017) Yang, S., Zhang, H. (2017)
Zearalenone-14-sulphate	ZEN-14-SO_3H	$C_{18}H_{22}SO_8$	Chickens	*In vivo*: feces	II	Yang, S., Zhang, H. (2017)
Zearalenone-16-glucuronide	ZEN-16-glucuronide	$C_{24}H_{30}O_{11}$	Rats, chickens, swines, goats, cows, humans Chicken & rats	*In vitro*: liver *In vivo*: urine	II	Yang, S., Zhang, H. (2017)
α-zearalenol / β-zearalenol	α-ZEL/β-ZEL	$C_{18}H_{24}O_5$	Rats, chickens, swines, goats, cows, humans Rats & chickens Humans Swines	*In vitro*: liver *In vivo*: urine *In vivo*: urine *In vivo*: urine	I	Yang, S., Zhang, H. (2017) (Heyndrickx et al., 2015) (Binder et al., 2017)

(*Continued*)

Table 10–Continued.

Chemical structure – Zearalenone						
Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
α-zearalenol/β-zearalenol-14-glucuronide	α-ZEL/β-ZEL-14-glucuronide	$C_{24}H_{32}O_{11}$	Rats & chickens	*In vivo*: urine & feces	II	Yang, S., Zhang, H. (2017)
α-zearalenol/β-zearalenol-14-sulphate	α-ZEL/β-ZEL-14-SO_3H	$C_{18}H_{24}SO_8$	Chickens	*In vivo*: feces	II	Yang, S., Zhang, H. (2017)
α-zearalenol/β-zearalenol-16-glucuronide	α-ZEL/β-ZEL-16-glucuronide	$C_{24}H_{32}O_{11}$	Rats & chickens Humans	*In vivo*: urine & feces *In vivo*: urine	II	Yang, S., Zhang, H. (2017) (Heyndrickx et al., 2015)

et al., 2017). Probably, this phenomenon is attributed to the enterohepatic circulation. This observation could indicate that the time of urine collection is of great importance to determine when ZEN exposure needs to be assessed.

In conclusion, reduction, hydroxylation, and glucuronidation are the major metabolic pathways of ZEN. α-ZEL, β-ZEL 8-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide are the predominant metabolites *in vitro* and *in vivo*. In addition, ZEN, α-ZEL, and β-ZEL can be mutually transformed in liver microsomes. Although ZEN is metabolized to an amalgam of ZEN forms, biomarker-analysis in urine should focus on free ZEN, α-ZEL, β-ZEL, and some of the most common hydroxylation and glucuronidation products like 8-OH-ZEN, 13-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide.

Aflatoxins

AF are the most widely studied mycotoxins with respect to their occurrence and toxicity to humans and animals. They are mainly produced by *A. flavus*, *A. parasiticus*, and *A. nomius*. AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) occur in a wide range of food commodities including cereals, nuts, spices, figs, and dried fruits (EFSA, 2007). AFB1 is the most important contaminant, and has been reported to be the most powerful natural carcinogen in mammals (Creppy, 2002). There is evidence that AFB1 is a major risk factor for hepatocellular carcinoma, therefore it is classified in group 1 by IARC (IARC, 2002). Moreover, AF cause a myriad of other effects: immunosuppresion, reduced growth rate, lowered milk and egg production, reduced reproductivity, reduced feed utilization and efficiency, and anemia (Do & Choi, 2007; McMillan, 2018). The absorption of AF, including AFB1, AFB2, AFG1, and AFG2, in pigs is defined as 46% (Avantaggiato, Havenaar, & Visconti, 2007).

In vitro/*in vivo* metabolism

Despite the toxic potential of AF, only very few studies have focused on their fate in *in vitro* studies. Moreover, the available data show a large variability, probably due to the diversity in the metabolism of AFB1 in different species. Nevertheless, aflatoxin M1 (AFM1) and aflatoxin Q1 (AFQ1) are the predominant metabolites in most of the animals and humans (Dhanasekaran, Shanmugapriya, Thajuddin, & Panneerselvam, 2011). AFQ1 was detected as the most prevalent metabolite in humans, with traces of AFM1 and aflatoxin B1 8,9-dihydrodiol (AFB1 8,9-dihydrodiol). Similar to AFB1, aflatoxin M1 8,9-dihydrodiol (AFM1 8,9-dihydrodiol) was formed when human liver microsomes were incubated with AFM1 (Neal, Eaton, Judah, & Verma, 1998) (Table 11). AFQ1 is also dominant in monkey and rat liver cells, whereas ducks produced aflatoxicol (AFL) as the predominant AFB1 metabolite (Lozano & Diaz, 2006). Aflatoxin P1 (AFP1) is observed in liver cells of monkeys, humans, and mice. Rabbit liver cells produce the highest quantity of AFB1 metabolites (AFQ1, AFL, AFB1 8,9-dihydrodiol, aflatoxin B1-glutathione conjugate (AFB1-GSH), AFM1, and AFB2a). Bovine hepatocytes predominantly metabolized AFB1 to AFM1, but also traces of AFB1-epoxide, AFB1-dihydrodiol, and AFB1-GSH conjugates were detected (Kuilman, Maas, & Fink-Gremmels, 2000). Detoxification of AFB1-8,9-epoxide and AFM1 in mammalian tissues is carried out via conjugation by glutathione (GSH), and is catalyzed by glutathione S-transferase (GST) (Langouët, Johnson, Guillouzo, & Guengerich, 1998; Massey, Stewart, Daniels, & Liu, 1995). Alternatively AFB1-8,9-epoxide is hydrolyzed to a dihydrodiol-form (Langouët et al., 1998; Massey et al., 1995). Bovine hepatocytes metabolized AFB1 to AFM1, but there were also measurable amounts (<0.01 ng/mL) of AFB1-epoxide, AFB1-dihydrodiol, and AFB1-GSH conjugates. Studies have pointed out that the GSH-GST detoxification mechanism is relatively low in humans compared to that of rats, mice, or rabbits (Edrington, Harvey, & Kubena, 1995; Massey et al., 1995). Further investigations also demonstrated variations in GST activity among species (Ball & Coulombe, 1991) and even within tissues of a single species (Larson, McMahon, & Wogan, 1993). Unlike other mycotoxins, the kidneys showed a lower activity to metabolize AF in all the studied species.

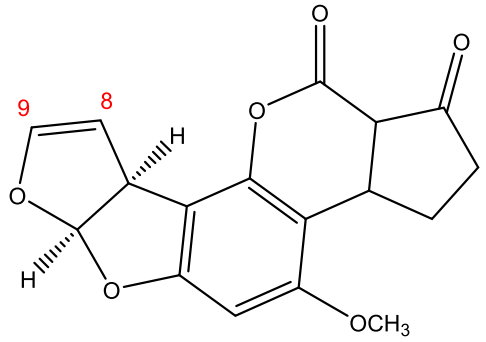
Differences between males and females were detected in the metabolism of AF, as is the case for DON and T-2. Male duck and turkey liver cells produced more aflatoxin B1-8,9-epoxide (AFBO) than females, and both produced AFB1-aldehyde reductase (AFAR). Male rats metabolized two to five times more AFB1 than females.

Based on the available *in vitro* studies, four metabolic pathways of AFB1 were identified: O-dealkylation to AFP1, keto-reduction to AFL, epoxidation to AFB1-8,9-epoxide, and hydroxylation to AFM1, AFP1, AFQ1, or AFB2a (Wu et al., 2009). Unlike other mycotoxins, conjugation with glutathione is a main route of detoxification. It is worth to mention that not all species are able to produce these conjugates.

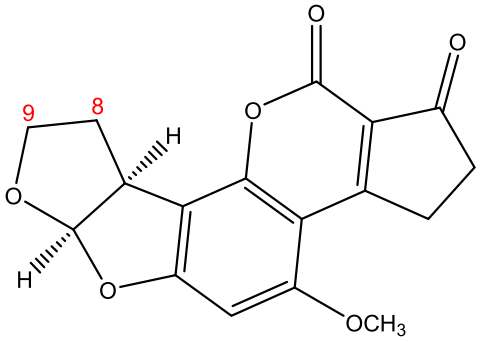
Only few *in vivo* trials were conducted in animals, and AFM1 and AFQ1 were the main compounds detected in urine, feces, and milk. However, differences have been detected among species. In the past, the ability to transform AFB1 to AFM1 in milk has been examined, demonstrating that the extent of carry-over (2.5% to 5.8%) was directly correlated to the milk-yield in cows (Britzi et al., 2013). A low AF carry-over (AFM1, 0.02% and AFM2, 0.31%) was reported in donkey milk after contaminated-feed administration (AFB1, 202 μg/kg and AFB2, 11 μg/kg, after 28 hr) (Tozzi et al., 2016). Goats showed the ability to excrete AFM1 with the milk, and the maximal concentration was found after 120 hr. Furthermore, traces of AFQ1 and AFL were detected in goat milk (Helferich, Baldwin, & Hsieh, 1986). Biotransformation of AFB1 in a cow's liver and the corresponding AFM1 levels in

Table 11–Identified aflatoxin (AF) metabolites *in vivo* and *in vitro*.

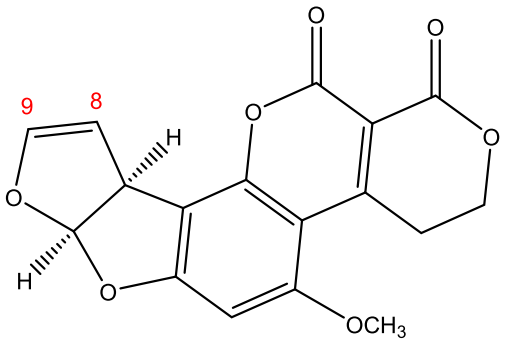
Chemical structure - Aflatoxins



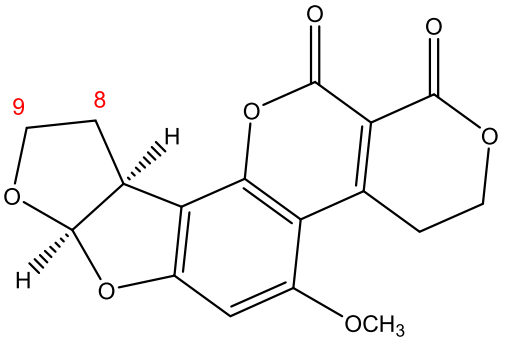
AFB1 = $C_{17}H_{12}O_6$ Molecular Weight = 312.27



AFB2 = $C_{17}H_{14}O_6$ Molecular Weight = 314.29



AFG1 = $C_{17}H_{12}O_7$ Molecular Weight = 328.27



AFG2 = $C_{17}H_{14}O_7$ Molecular Weight = 330.29

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Aflatoxicol	AFL	$C_{17}H_{14}O_6$	Cows Goats Rabbits, fish, swines, monkeys, rats, humans Chicken, turkey and ducks	*In vivo*: plasma & milk *In vivo*: milk, urine and feces *In vitro*: liver *In vitro*: liver	I	(Trucksess, Richard, Stoloff, McDonald, & Brumley, 1983) (Helferich et al., 1986) (Salhab & Edwards, 1977) (Lozano & Diaz, 2006)
Aflatoxin B1 8,9-dihydrodiol	AFB1 8,9-dihydrodiol	$C_{17}H_{14}O_8$	Humans	*In vitro*: liver	I	(Neal et al., 1998)
Aflatoxin B1-glutathione conjugate	AFB1-GSH	$C_{27}H_{29}N_3O_{13}S$	Bovines	*In vitro*: liver	II	(Kuilman et al., 2000)
Aflatoxin B1-8,9-epoxide	AFBO	$C_{17}H_{12}O_7$	Ducks & turkeys Rats Humans	*In vitro*: liver *In vitro*: liver *In vitro*: liver	-	(Lozano & Diaz, 2006) (Hayes, Judah, Mc Lellan, & Neal, 1991) (Johnson, Yamazaki, Shimada, Ueng, & Guengerich, 1997)
Aflatoxin B1-albumin	AFB1-albumin		Humans Rats	*In vivo*: plasma *In vivo*: plasma	II	(Turner et al., 2005) (Dirr & Schabort, 1986)
Aflatoxin B1-lysine	AFB1-lysine	$C_{23}H_{25}N_2O_8$	Humans Swines Rats	*In vivo*: plasma *In vivo*: plasma *In vivo*: plasma	II	(McMillan, 2018) (Di Gregorio et al., 2017) (Xue, Cai, Tang, & Wang, 2016)
Aflatoxin B-N7-guanine	AFB-N7 -guanine	$C_{22}H_{16}N_5O_7$	Humans Rats	*In vivo*: feces & urine *In vivo*: urine	II	(Mykkänen et al., 2005) (Groopman, Donahue, & Zhu, 1985)
Aflatoxin M1	AFM1	$C_{17}H_{12}O_7$	Cows Donkeys Humans Humans Rats Rats Goats	*In vivo*: milk *In vivo*: milk *In vivo*: urine & feces *In vivo*: milk *In vitro*: liver *In vivo*: urine *In vivo*: milk, urine and feces	I	(Britzi et al., 2013) (Tozzi et al., 2016) (Ferri et al., 2017) (Altun, Gurbuz, & Ayag, 2017) (Gurtoo & Motycka, 1976) (Groopman et al., 1985) (Helferich et al., 1986)

(*Continued*)

Table 11–Continued.

Chemical structure - Aflatoxins						
Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
Aflatoxin M1 8,9-dihydrodiol	AFM1 d8,9-dihydrodiol	$C_{17}H_{14}O_8$	Humans	*In vitro*: liver	I	(Neal et al., 1998)
Aflatoxin M2	AFM2	$C_{17}H_{14}O_7$	Donkeys Cows	*In vivo*: milk *In vivo*: milk	I	(Tozzi et al., 2016) (Sartori, de Mattos, de Moraes, & da Nobrega, 2015)
Aflatoxin P1	AFP1	$C_{16}H_{10}O_6$	Humans Rats	*In vivo*: urine & feces *In vivo*: urine	I	(Groopman et al., 1992) (Groopman et al., 1985)
Aflatoxin Q1	AFQ1	$C_{17}H_{12}O_7$	Rats Humans Goats	*In vitro*: liver *In vivo*: urine & feces *In vivo*: milk, urine and faeces	I	(Gurtoo & Motycka, 1976) (Mykkänen et al., 2005) (Helferich et al., 1986)
Aflatoxin B2a	AFB2a	$C_{17}H_{14}O_7$	Rabbits Humans	*In vitro*: liver *In vivo*: plasma	I	(Hatem, Hassab, Al-Rahman, El-Deeb, & El-Sayed Ahmed, 2005)

the milk depend on several factors, including milk-yield, the microsomal mixed function oxidase activity, and presence or absence of bacterial mastitis in the udder (Chopra et al., 1999). According to two studies, the normal carry-over was approximately 0.4% to 0.6%, and a daily AFB1 intake of 70 g in cows resulted in noncompliant levels in milk (regulatory limit, 0.05 μg/L AFM1). AFL was also detected in milk, which was probably a result of the ruminal microbial degradation (Chopra et al., 1999; Veldman, Meijs, Borggreve, & Heeres-Van Der Tol, 1992).

In humans, AFQ1, AFM1, and AFB-N7-guanine are the most prevalent metabolites of AFB1 in feces and urine. Moreover, AFQ1 is excreted in urine (mean, 10.4 ng of AFQ1/g) and feces (mean, 137.6 ng of AFQ1/g) at higher levels than AFM1 in urine (mean, 0.04 ng of AFM1/g) and feces (mean, 2.3 ng of AFM1/g) (Mykkänen et al., 2005). However, AFP1 and AFB-N7-guanine were major metabolites in urine in a Chinese investigation in humans (Groopman et al., 1992).

Biomarkers of exposure

In a human study from Gambia, AFG1, AFP1, AFQ1, and AFB-N7-guanine were the main metabolites, and AFB-N7-guanine was considered as most appropriate biomarker of acute exposure (Groopman et al., 1992). Regarding plasma analysis, aflatoxin-albumin (AF-albumin) adduct was the best available indicator of long-term exposure to AFB1 owing to the half-life of serum albumin (approximately 20 days) (Wild & Turner, 2002) (JECFA, 2017). Thus, it has been widely used as AF exposure biomarker in humans (Gong et al., 2002; Turner et al., 2000; Turner et al., 2005) and strong correlation ($r = 0.69$, $P < 0.0001$) between concentration of AF-albumin with AF intake was validated (Wild et al., 1992). However, the application of MS allowed to develop a 2.6-fold more specific technique compared to the use of ELISA for AF-albumin, through the analysis of aflatoxin B1-lysine (AFB1-lys) (McCoy et al., 2008; Scholl et al., 2006). Recently, McMillan et al. (2018) used LC-HRMS in combination with isotope dilution MS to quantify AFB1-lysine, which is the most reliable biomarker of chronic aflatoxin exposure (McMillan, 2018). This is the first report where HRMS is used to quantify aflatoxin biomarkers in a case-control study design, which is a promising research trend.

In conclusion, focus needs to be set towards the urinary analysis of AFB1, AFB2, AFG1, AFG2, and AFM1. Although AFM1 is a predominant compound of AFB1, other compounds should be considered to evaluate acute AF exposure, namely AFQ1, AFP1, and AFB-N7-guanine in urine. AFB1-lysine is the most reliable biomarker of chronic aflatoxin exposure in plasma. Moreover, more knowledge on AFB2, AFG1, and AFG2 metabolism is necessary.

Sterigmatocystin

STERIG is a biochemical precursor of aflatoxins and is produced by several *Aspergillus* species (Veršilovskis & de Saeger, 2010). STERIG-producing fungi are frequently isolated from different matrices, consequently STERIG is regularly detected in food, feed, but also in indoor environments, such as carpet and building materials (Malysheva et al., 2014; Veršilovskis & de Saeger, 2010). Multiple studies have shown that STERIG is carcinogenic in animals, and possibly carcinogenic to humans, therefore STERIG has been categorized as a type 2B carcinogen by IARC (IARC, 1976; Veršilovskis & de Saeger, 2010). This mycotoxin induces lung adenocarcinoma in mice and malignant transformations in human fetal lung tissue, which suggests that STERIG is a potent lung carcinogen (Cao, Wang, Zhang, & Sun, 2000; Fujii & Odashima, 1976; Huang et al., 2014; Xing et al., 2007). The absorption of STERIG is limited following oral exposure (EFSA, 2013). Although there is no available data on STERIG absorption in humans, the gastrointestinal STERIG absorption in monkey is not higher than 30% (Steyn & Thiel, 1976).

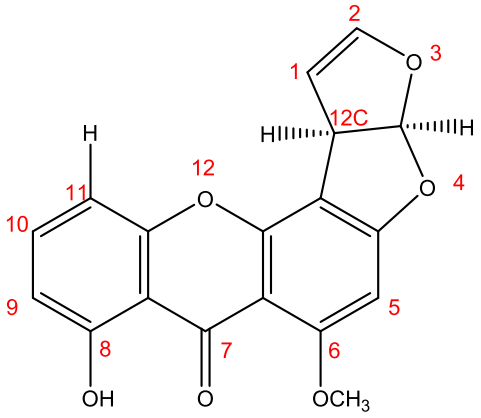
In vitro/*in vivo* metabolism

Little is known on the metabolism of STERIG. Only few studies pinpoint the presence of 8 STERIG-metabolites (Table 12). *In vitro*, 9-hydroxy-sterigmatocystin (9-OH-STERIG) has been identified as the predominant metabolite in both humans and rats (Pfeiffer, Fleck, & Metzler, 2014). Aromatic hydroxylation is the prevailing metabolization pathway, resulting in the presence of, for example, 12-hydroxy-sterigmatocystin (12-OH-STERIG) and 11-hydroxy-sterigmatocystin (11-OH-STERIG). Noteworthy, the hydroxylation at C-12 is analogous to the hydroxylation of AFB1 at position C-9a, which gives rise to AFM1, and represents a major pathway in the mammalian metabolism of AFB1.

In vivo studies have revealed an extensive metabolization of STERIG, consequently free STERIG was scarcely detected in the urine of animals (Fushimi et al., 2014). Fushimi et al. (2014) submitted goat urine to a β-glucuronidase treatment, and an increase of STERIG from < limit of detection (LOD) to 98 pg/mg of creatinine was shown after this enzymatic treatment. Glucuronidation

Table 12–Identified sterigmatocystin (STERIG) metabolites *in vitro*.

Chemical structure - Sterigmatocystin



$C_{18}H_{12}O_6$ Molecular Weight = 324.28

Metabolite	Abbreviation	Composition	Species	Method	Metabolic Phase	Ref
11-hydroxy-sterigmatocystin	11-OH-STERIG	$C_{18}H_{12}O_6$	Humans Rats	*In vitro*: liver	I	(Pfeiffer et al., 2014)
11,12c-dihydroxy-sterigmatocystin	11,12c-diOH-STERIG	$C_{18}H_{13}O_7$	Humans Rats		I	(Pfeiffer et al., 2014)
12c-hydroxy-sterigmatocystin	12c-OH-STERIG	$C_{18}H_{12}O_6$	Humans Rats		I	(Pfeiffer et al., 2014)
9-hydroxy-sterigmatocystin	9-OH-STERIG	$C_{18}H_{12}O_6$	Humans Rats		I	(Pfeiffer et al., 2014)
9,11-dihydroxy-sterigmatocystin	9,11-diOH-STERIG	$C_{18}H_{13}O_7$	Humans Rats		I	(Pfeiffer et al., 2014)
9,12c-dihydroxy-sterigmatocystin	9,12c-diOH-STERIG	$C_{18}H_{13}O_7$	Humans Rats		I	(Pfeiffer et al., 2014)
Sterigmatocystin-1,2-oxide	STERIG-1,2-oxide	$C_{18}H_{12}O_7$	Humans Rats		I	(Pfeiffer et al., 2014)
Sterigmatocystin-1,2-dihydrodiol	STERIG-1,2-dihydrodiol	$C_{18}H_{14}O_8$	Humans Rats		I	(Pfeiffer et al., 2014)

is the predominant pathway. The high amount of STERIG in glucuronidated form suggests that STERIG is not degraded in the rumen or stomach organs reaching the liver. This is in contrast to various other mycotoxins that are mainly inactivated by the rumen. However, information is lacking on the stereochemical position of the glucuronides, and therefore no identification has been established so far on these glucuronidated forms in human biological samples.

In conclusion, there is a lack of information about STERIG metabolites and more information is necessary regarding this compound. Moreover, STERIG glucuronides could be the predominant metabolites from STERIG.

Conclusion

The *in vitro* and *in vivo* investigations pinpoint similarities in the detection of mycotoxin biomarkers, however based on *in vitro* results more metabolites were identified than *in vivo* studies. This is probably related to the (nonphysiological) circumstances of *in vitro* systems and a dilution effect *in vivo*, and it confirms that a combination of *in vitro* and *in vivo* experiments is imperative to obtain a conclusive insight.

Interspecies differences in mycotoxin metabolism exist, and the human species shows the largest mycotoxin metabolization capacity. Ruminants are not able to metabolize some mycotoxins via the liver as compared to other species, probably because of the ruminal microorganisms' activity.

Free DON, DON-15-glucuronide, and DON-3-glucuronide are considered as DON-biomarkers of exposure in urine. NIV, on the contrary, requires more investigation to unravel its biomarker-profile. FUS-X is largely converted to NIV (>90%). HT-2 is the prevailing biomarker of T-2 toxin, but T-2 has different metabolites which have not yet been confirmed in human urine or feces. The biomarkers for DAS are 15-MAS, 7-OH-DAS, and deepoxy-DAS in urine and feces. OTA, OTα, and their glucuronides are the main metabolites in urine, so they should be considered as urinary biomarkers. Although there is only little information on CIT, DH-CIT is considered as CIT biomarker in urine. FB are stable, and mainly excreted through the feces; free FB and HFB are the potential FB-biomarkers. A cascade of ZEN metabolites makes the evaluation of ZEN-exposure complex; α-ZEL, β-ZEL, 8-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide are considered as ZEN biomarkers in urine. To evaluate acute AF exposure, AFQ1, AFP1, and AFB-N7-guanine should be considered in urine; AFB1-lysine is the most reliable biomarker of chronic aflatoxin exposure in plasma. STERIG is widely metabolized, however identification of *in vivo*-metabolites has not been successful so far.

Mycotoxin research enters to a new era with multitude biomarker-driven explorations. The use of state-of-the-art analytical techniques, such as HRMS, has enabled to reveal an amalgam of new mycotoxin metabolites. The latter is of crucial importance to assess multimycotoxin exposure and the consequent health risk in relation to both animal and human pathologies, such as different

types of carcinomas and malnutrition. The use of major databases of multi-mycotoxin-biomarkers and the access to large case-control biobanks (great amount of biological samples) will provide the scientific community with all the necessary tools to unravel the mycotoxin impact on animal and human health, including other factors such as environmental factors and anthropometrics. Finally, biomarker of exposure studies are expensive, time and recourse-consuming, for this reason it is of great importance that all levels of society get involved especially, in developing countries where the mycotoxin situation is more dramatic and where -through international collaborations- these countries can have access to these instrumentation (for example, MYTOX-SOUTH, 2018).

Acknowledgments

This review was compiled and financially supported in the framework of the Food Biomarker Alliance (FOOD-BALL)(foodmetabolome.org/) project (G0D4615N) which is part of the Joint Programming Initiative (JPI) on a healthy diet for a healthy live.

Author Contributions

AV, MM, SY, SDS, and MDB all contributed to writing the comprehensive review paper.

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