FACULTY OF
PHARMACEUTICAL SCIENCES

PHASE I AND PHASE II METABOLISM OF ALTERNARIA TOXINS

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A Master dissertation for the study program Master in Drug Development

Academic year: 2019 - 2020

GHENT
UNIVERSITY

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Department of Bioanalysis Ghent, Centre of Excellence in Mycotoxicology & Public Health.

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June 2, 2020

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PREAMBLE: IMPACT OF THE CORONAVIRUS

This master dissertation was written during the corona crisis of 2020 and the subsequent measurements had a drastic impact. After I arrived in Parma on February 2, I had my first meeting with my co-promotor Prof. Chiara Dall'Asta and dr. Luca Dellafiora on Wednesday 12 February. After a brief discussion of the subject of my master dissertation, I read literature from 13 to 21 February. The study was supposed to start from the biosynthesis of mycotoxin metabolites via biological reactors (*i.e.* liver microsomes), followed by purification and characterization of the produced metabolites via LC-MS. A substantial part of my work would have been aimed at optimizing the starting protocols of the microsomes reactions and metabolites purification by trial-and-error. Unfortunately, the University of Parma had to close due to the corona crisis starting from Monday 24 February. From then on, I was quarantined in Parma until I was obliged to return to Belgium on March 12. Since the labs of Ghent University were closed as well at the time of my return, the original plan for my master dissertation to optimize the protocols, could not be further developed. Consequently, an outline for a literature review was recorded on March 18 in consultation with my promotor Prof. Sarah De Saeger and dr. Marthe De Boevre.

'This preamble was written in consultation between the student and the promoter and approved by both.'

SUMMARY

Alternaria mycotoxins are potentially very toxic for human health. However, there is not much known about their metabolism and consequently their risk assessment. Therefore this literature review gives an update about what has been recently investigated about DON and ZEN, with the aim of helping research on the phase I and II metabolism of the emerging *Alternaria* toxins in the future.

The risk management of DON, ZEN, AOH and AME was discussed by direct and indirect risk assessment. Since chemical mixtures must be taken into account in the clarification of the toxicity, a Python algorithm regarding the indirect risk assessment of co-mixtures was developed. To assess the risks directly, a comparison was made between the commonly used matrices such as urine, faeces and blood and some newly developed techniques like Dried Blood Spot Sampling (DBS Sampling) and Volumetric Absorptive Microsampling (VAMS). The conclusion that this techniques could be promising for the determination and quantification of mycotoxins in future direct risk assessment, could be drawn.

Furthermore, an update was given on the latest developments of DON, ZEN, AOH and AME and their modified forms in *in vitro* and *in vivo* metabolism. In addition, some recently performed *in vitro* and *in vivo* studies were compared in the determination of the metabolism of T-2, HT-2 and ZEN and their masked forms, with the use of liver microsomes.

Further research into the *in vitro* metabolism via biological reactors (*i.e.* liver microsomes), is important. Considering, *in vitro* trials are less complex than *in vivo* trials, and follow The Three Rs principles, more *in vitro* studies should be carried out in the future. In this context, *in vitro* methods must be standardized internationally to compare the data and to upgrade our knowledge about mycotoxins and their metabolism.

SAMENVATTING

Alternaria mycotoxines zijn potentieel zeer giftig voor de menselijke gezondheid. Er is echter niet veel bekend over hun metabolisme en bijgevolg hun risicobeoordeling. Daarom geeft deze literatuurstudie een update over wat recentelijk over DON en ZEN werd onderzocht, met als doel het onderzoek naar de fase I en II metabolisme van de opkomende Alternaria toxines in de toekomst vooruit te helpen.

Het risicobeheer van DON, ZEN, AOH en AME werd besproken via directe en indirecte risicobeoordeling. Aangezien er bij de bepaling van de toxiciteit rekening moet worden gehouden met chemische mengsels, werd een Python-algoritme ontwikkeld voor de indirecte risicobeoordeling van co-mengsels. Om de risico's op directe wijze te beoordelen, werd een vergelijking gemaakt tussen de algemeen gebruikte matrices zoals urine, faeces en bloed en enkele nieuw ontwikkelde technieken zoals Dried Blood Spot Sampling (DBS Sampling) en Volumetric Absorptive Microsampling (VAMS). De conclusie is dat deze technieken veelbelovend kunnen zijn voor de bepaling en kwantificering van mycotoxinen in toekomstige directe risicobeoordeling.

Verder werd een update gegeven over de laatste ontwikkelingen van DON, ZEN, AOH en AME en hun gemodificeerde vormen in *in vitro* en *in vivo* metabolisme. Daarnaast werden enkele recent uitgevoerde *in vitro* en *in vivo* studies vergeleken voor de bepaling van het metabolisme van T-2, HT-2 en ZEN en hun gemaskeerde vormen, door gebruik te maken van levermicrosomen.

Verder onderzoek naar het *in vitro* metabolisme via biologische reactoren (*i.e.* levermicrosomen), is belangrijk. Aangezien *in vitro* studies minder complex zijn dan *in vivo* studies en ze voldoen aan het 3V-principe, zouden in de toekomst meer *in vitro* studies uitgevoerd moeten worden. In dit verband moeten *in vitro* methoden internationaal gestandaardiseerd worden om de gegevens te kunnen vergelijken en onze kennis over mycotoxines en hun metabolisme te verbeteren.

ACKNOWLEDGEMENTS

First of all, I would like to thank Prof. Sarah De Saeger for making this Erasmus experience in Parma possible. I would also like to thank her and dr. Marthe De Boevre for their support and very good follow-up. It was an adjustment to return to Belgium, but we were soon welcomed warmly by everyone of the Department of Bioanalysis.

I would also like to thank Prof. Chiara Dall'Asta and dr. Luca Dellafiora. We were warmly welcomed by them in their department in Parma and soon became part of their team. It is a pity that I have not been able to work with them any longer.

I am also very grateful for the support of my family. For this reason, I would like to thank my parents for their support, both in Parma and at home, for their good advice and for reading my thesis. In particular, I would like to praise my grandfather, whose advice was very useful, due to his keen interest in this subject.

Finally, I like to give my thanks to Celie with whom I have experienced this unique adventure, and to my other Erasmus friends for the support and unforgettable memories.

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LIST WITH ABBREVIATIONS

AME: Alternariol monomethyl ether

AOH: Alternariol

DBSs: Dried blood spots

DON: Deoxynivalenol

EFSA: European Food Safety Authority

HT-2: HT-2 toxin

IARC: International Agency for Research on Cancer

T-2: T-2 toxin

TDI: Tolerable daily intake

VAMS: Volumetric Absorptive Microsampling

ZEN: Zearalenone

α-ZEL: α-zearalenol

Phase I metabolites: oxidation, reduction, hydrolysis and epoxide hydrolysis reactions

Phase II metabolites: glucuronidation, sulphate conjugation, glutathione conjugation, acetylation, amino acid conjugation and methylation reactions.

1. INTRODUCTION

Mycotoxins are low-molecular-weight, secondary metabolites produced on plant tissue by phytopathogenic fungi. They can operate as insecticides, cooperate with fungi in the challenge of their ecological niche in nature and help to fight in plant defence to fungi [1]. The most important genera include *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* [2]. Mycotoxins are of a major public health concern, since they can enter the food chain worldwide and infect a wide number of crops and food commodities [3]. They cause toxic responses for both animals and humans, known as mycotoxicosis [4]. Carcinogenicity, hepatotoxicity, nephrotoxicity and endocrine disorders have been related to chronic exposure to low levels of mycotoxins [5]. Additionally, mycotoxins can cause metabolic and biochemical deficiencies, allergic reactions, immune diseases, reproductive deficiencies, fetal alterations and even death [6,7]. Depending on the toxin type, its metabolism, pharmacokinetics, accumulation and exposure conditions and the age, gender, immune system and health status of the exposed individual, the impact on human health varies [8].

Although mycotoxins are very toxic, not every compound produced by fungi is toxic; fungal metabolites with antibacterial effects, like Penicillium G and other small molecule metabolites (e.g. ethanol) are not considered mycotoxins [9,10]. Some mycotoxins are noxious to other microorganisms, like to other fungi and bacteria [11].

Fusarium mycotoxins are occurring most frequently [4,12,13,14] and their metabolism has been studied extensively. Therefore, this review will focus on zearalenone (ZEN) and deoxynivalenol (DON) of the *Fusarium* genus with the goal of using them as an example for the *Alternaria* mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME). The fact that this group of mycotoxins frequently occur in food, that they probably have genotoxic potential and consequently effects on human health, lead to an increasing interest in the genus *Alternaria*. Due to their abundance, occurrence and toxicity, *Alternaria* toxins belong to the so called “emerging” mycotoxins [3,15]. Since the available data is scarce, a proper risk assessment to human health and, consequently, specific regulations, is hindered [16,17].

2. OBJECTIVES

As previously mentioned (chapter 1), there is a great interest in *Alternaria* mycotoxins since they are potentially very toxic for human health. However, there is not much known about their metabolism and consequently their risk assessment. Therefore this literature review gives an update about what has been recently investigated about DON and ZEN, with the aim of helping research on the phase I and II metabolism of the emerging *Alternaria* toxins in the future.

Primarily, the risk management of DON, ZEN, AOH and AME was discussed by direct and indirect risk assessment. Since it is necessary that chemical mycotoxin mixtures are taken into account in future risk assessment, a Python algorithm regarding the indirect risk assessment of co-mixtures was developed. To assess the risks directly, a comparison was made between the commonly used matrices in animals and humans such as urine, faeces and blood and some newly developed techniques like Dried Blood Spot Sampling (DBS Sampling) and Volumetric Absorptive Microsampling (VAMS).

Furthermore, an update was given of the latest developments of DON, ZEN, AOH and AME and their modified forms in *in vitro* and *in vivo* metabolism. In addition, some recently performed *in vitro* and *in vivo* studies were compared in the determination of the metabolism of T-2, HT-2 and ZEN and their masked forms, with the use of liver microsomes in the *in vitro* studies.

The final aim of this thesis was to investigate the *in vitro* metabolism of mycotoxins via alternative methods such as bioreactor (*i.e.* liver microsomes) protocols. However, since no laboratory activities could be carried out, these protocols could not be further developed and improved.

3. DEOXYNIVALENOL

3.1. CHEMICAL STRUCTURE

(3α,7α)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, known as DON, belongs to the family of the trichothecenes and contains like all other trichothecenes, a 12,13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions (**Figure 3.1.**). Trichothecenes are classified in two groups, namely the macrocyclic and the nonmacrocyclic, depending on the presence of an ester-ether bridge between C-4 and C-15 or a macrocyclic ester. The nonmacrocyclic trichothecenes in succession can be subclassified into two groups, namely type A and type B, where type A has an ester or a hydrogen type side chain at the C-8 position and type B, a ketone. DON belongs to the latter type [18].

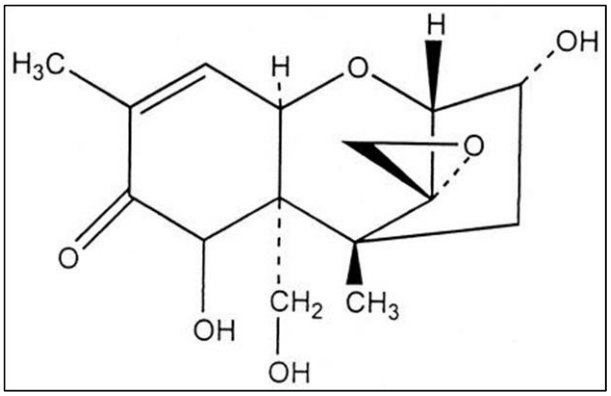


Figure 3.1.: Chemical structure of DON [18]

3.2. OCCURRENCE

DON, produced by the *Fusarium* species, *F. graminearum* and *F. culmorum*, is one of the most common mycotoxins in cereals with levels of occurrence in the order of hundreds of µg/kg [19,20,21,22). Besides cereals, it is frequently found in maize, rye, wheat, barley, oats, safflower seeds and mixed feeds [23]. Since DON appears more likely as a field than a storage contaminant and seasonal changes substantially affect the size of *Fusarium* infections, DON levels change from year-to-year. This makes it complicated to derive the typical levels of occurrence [22,24]. DON almost always co-occurs with zearalenone and other *Fusarium* toxins, like nivalenol, 3-acetyl DON,

fusarenone-X, moniliformin and fumonisins, what contributes to the problem of co-occurrence of mycotoxins [25].

3.3. TOXICITY

DON exerts its toxicity in various ways, like via inhibiting the DNA-, RNA- and protein synthesis by preventing chain elongation [26,27]. For that reason, it is responsible for damaging the eukaryotic cells of both animals and humans [26]. Furthermore, it causes emesis due to the possible interaction with the dopaminergic and serotonergic receptors in the area postrema. Consequences of this effect are loss of appetite and reduced feed intake which lead to growth retardation in swine [28]. This is why DON is occasionally called food refusal factor or vomitoxin [29,23] . Additionally, DON reduces the immunoglobin production and depletes the lymphocytes in the spleen, Peyer's patches and thymus which suppresses the immune system. More recent studies demonstrated an upregulation of pro-inflammatory cytokines by increasing the binding activities of transcription factors such as AP-1, NFκB and C/EBP [30,31]. This leads to an induction of the synthesis of TNFα, IL-6 and COX-2 [32,33] and stabilization of TNFα and IL-6 mRNA [32], and is the cause of DON's most important toxic effects.

Since some mycotoxins are linked to carcinogenic effects, the International Agency for Research on Cancer (IARC) has classified them in four groups. Group 1: carcinogenic to humans, based on sufficient human data; Group 2 (A/B): probably/possibly carcinogenic to humans, based on sufficient experimental or animal data, but limited human epidemiological information; Group 3: Not classifiable as to its carcinogenicity to humans. As experimental data or epidemiological information was missing at the time IARC did its evaluation, multiple mycotoxins like DON itself were classified in Group 3 (**Table 3.1.**) [34]. The Tolerable Daily Intake (TDI) of DON is set at 1.0 µg/kg [12].

Table 3.1.: Classification by the IARC [34]

Group 1	Carcinogenic to humans
Group 2A	Probably carcinogenic to humans
Group 2B	Possibly carcinogenic to humans
Group 3	Not classifiable as to its carcinogenicity to humans

4. ZEARALENONE

4.1. CHEMICAL STRUCTURE

6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-B-resorcyclic acid lactone (**Figure 4.1.**) was given the trivial name of zearalenone as a combination of ZEN's teleomorph, *Gibberella zeae*, resorcylic acid lactone, -ene, for the presence of the double bond and -one, for the presence of a ketone [35]. F-2 toxin was in early literature used as a synonym of ZEN as well [36,37].

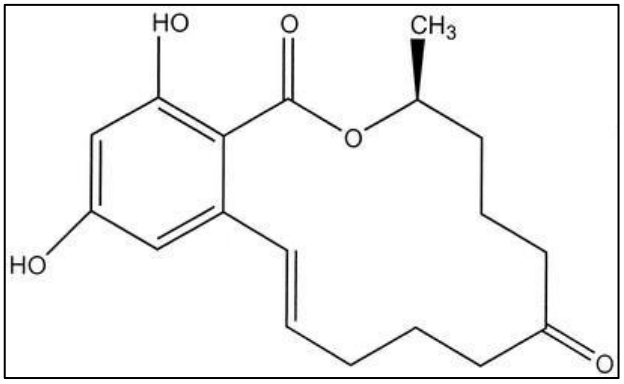


Figure 4.1.: Chemical structure of ZEN [18]

4.2. OCCURRENCE

ZEN, produced by the *Fusarium* species, *F. graminearum*, *F. culmorum* and *F. cerealis* [38], affects the whole human food chain through a range of food products including cereals, meat, milk, wine, beer, dried fruits and spices [12,39,40,41,42]. As discussed above (chapter 3.2.), ZEN almost always co-occurs with deoxynivalenol and more uncommonly with aflatoxins. ZEN is not removed during processing and its production is favoured by a high humidity and low temperature [43]. The European Commission formulated rules on maximum levels of ZEN in food to protect the health of consumers (Regulation 1881/2006). Depending on the food matrix, the levels varied between 20 and 200 mg kg^{-1} [17]. Since humans can also be exposed indirectly to ZEN, rules on the recommended levels of ZEN in feed were formulated as well. These levels varied between 100 and 3000 mg kg^{-1} [44].

4.3. TOXICITY

Although ZEN is biologically potent, it seldom is acutely toxic. For this reason, safety concerns of ZEN are mainly residing from its (sub)chronic toxicity. As ZEN and metabolites show structural similarity with 17β-estradiol, they are substrates for the estrogenic receptor (ER) in mammalian target cells with high binding affinity [45,46]. ZEN and its phase I metabolites are able to exert estrogenic effects, since the uterotrophic effect is considered as the endpoint for estrogenicity. The α-isomers show a much higher estrogenic potency than the β-isomers and even than ZEN, which is due to the orientation of the hydroxyl group on the aliphatic ring [47]. *In vivo*, α-ZEL is approximately 62 times more estrogen active than ZEN and almost as active as 17β-estradiol, the most potent endogenous estrogen. Zearalanone (ZAN), obtained by reducing the olefinic double bond of ZEN, shows no change in hormonal activity [48].

ZEN exerts an influence on the sex steroid hormones of mammalians via multiple mechanisms; it interferes in the feedback signal which mediates the production of FSH and LH in the pituitary gland, it inhibits the viability of the Leydig and granulosa cells which are in favour of the sex steroids production, it stimulates the leakage of electrons and increases the levels of free radicals, what contributes to oxidative stress, it induces apoptosis and influences the mitochondrial functions [49,50].

There are also indications that ZEN exposure can cause breast cancer via the potential role of α-zearalanol (α-ZAL), but specific information in this respect is lacking. As evidence of the carcinogenicity of ZEN is missing, ZEN is classified in group 3, “not classifiable as to its carcinogenicity to humans” of IARC, just like DON [5]. The TDI of ZEN is set at 0.25 µg/kg [12].

5. ALTERNARIOL & ALTERNARIOL MONOMETHYL ETHER

5.1. CHEMICAL STRUCTURE

Depending on their chemical structures, *Alternaria* toxins may be divided into five groups, of which the dibenzo-α-pyrones, including 3,7,9-Trihydroxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one or AOH (**Figure 5.1.**) and 3,7-Dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one or AME (**Figure 5.2.**) are the most important ones. Other *Alternaria* groups are the perylene quinones, tetramic acid derivatives, *A. alternata* f. sp. *lycopersici* toxins and miscellaneous structures as tentoxin (TEN), that have a cyclic tetrapeptide structure [3,51].

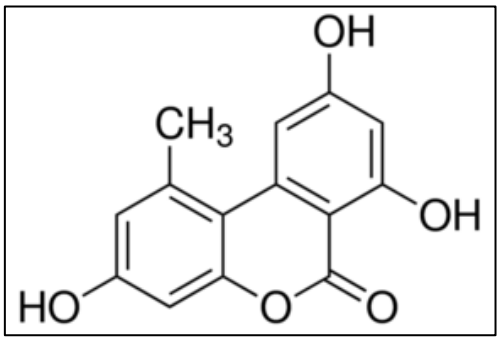


Fig. 5.1.: Chemical structure of AOH [52]

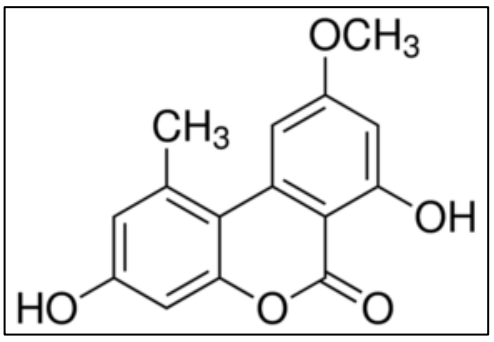


Fig. 5.2.: Chemical structure of AME [53]

5.2. OCCURRENCE

Although fewer studies have been carried out on the presence of *Alternaria* toxins in food than for the other mycotoxins, the interest is high [54]. Studies showed the presence of *Alternaria* toxins in different food products as cereals, vegetables and fruits and their juices, grains, seeds, herbs, nuts, oils and wine [16,55]. *Alternaria* toxins regularly occur simultaneously in food such as red peppers [56]. Reason is that AOH and AME [57], for example, share a similar biosynthesis pathway [58]. The highest concentrations of AOH were found in grains like oats and buckwheat, but AOH was also present in tomato based products as tomato sauce and puree. AME appeared to be abundant in oil and sesame seeds and in chest- and tree nuts, but was less present than AOH. Studies also revealed that children are the population group which is exposed to the largest concentrations of *Alternaria* toxins through their nutrition [59].

Despite the fact that *Alternaria* toxins are common in food and feed and their metabolites are toxic, there is not any regulation of their presence in food and feed worldwide. Because of this, maximum admissible limits should be established in the upcoming years.

5.3. TOXICITY

AOH and AME are considered to be among the most important *Alternaria* mycotoxins, since they cause harmful effects [51]. Some *in vitro* studies revealed that AOH and AME are genotoxic, mutagenic and carcinogenic [60,61]. They are able to induce DNA strand breaks at concentrations ≥ 1 μM [60], to perform clastogenic effects at ≥ 2.5 μM [59] and to act mutagenic at ≥ 10 μM [61]. They are also able to poison topoisomerase I and II (genotoxic effect) [60,63] and to reduce the human intestinal cells viability (HCT 116 and Caco-2) (cytotoxic effect). In addition, both AOH and AME can induce intracellular Reactive Oxygen Species (ROS), what is also contributing to their toxicity [64,65,66]. Although *Alternaria* mycotoxins were thought to be one of the causes of oesophageal cancer in humans, their carcinogenic effects have not yet been investigated in *in vivo* studies [67]).

Alternaria mycotoxins and several of their phase I and II metabolites show structural similarity with 17β-estradiol and are substrates for the estrogenic receptor (ER) as well [62,68]. Even though AME has the strongest estrogenic activity of the *Alternaria* mycotoxins, this activity will always be lower than ZEN's [68]. As a synergistic effect of the estrogenic potency of *Alternaria* toxins, the expression of the progesterone receptor is increased. This affects the hormone levels and leads to an increase of progesterone and estradiol production after exposure to AOH [69].

Both ZEN, AOH and AME have been pointed out as Endocrine-Disrupting Chemicals (EDCs), which are according to the World Health Organization and the United Nations Environment Programme (UNEP), "substances that alter one or more functions of the endocrine system and consequently cause adverse effects in an intact organism, its progeny and a (sub)population". EDCs would cause multiple health effects

during the fetal to adult life of an individual, depending on the timing and level of exposure. Of course it is most critical if exposure to EDCs occurs during human development. Considering humans are exposed to EDCs trough food intake, four categories can be listed: (1) compounds with the ability to bioaccumulate, (2) substances used for the production of foods, (3) chemicals present in food due to contact with materials, and (4) endocrine active compounds naturally present in food. Mycotoxins belong to the fourth category [66].

In addition to these genotoxic and endocrine-modulating effects, AOH and AME manage to modify the inborn immunity in both human epithelial cells and mouse macrophages [70].

Due to all these toxic effects of *Alternaria* mycotoxins and the possibility that they co-occur inducing a synergistic toxicity, they cause an emerging risk to human health. However, a proper risk assessment is still lacking due to the unavailability of appropriate *in vivo* toxicological data [71]. For this reason, no TDI for AOH and AME has been determined as well. Instead, the Treshold of Toxicological Concern (TTC) of 2.5 ng/kg body weight per day is used to evaluate the level of concern of *Alternaria* mycotoxins for human health [59]. This TTC is based on years of animal studies where structural and functional categories of chemicals were derived. *Alternaria* mycotoxins are not yet classified in one of the four groups of the IARC, but their intake attempts to be as limited as possible [34].

6. RISK ASSESSMENT

Humans are exposed to mycotoxins in different ways; through ingestion of contaminated food, such as cereals or through consumption of products of animal origin, such as eggs or milk, if the animals have previously been fed with contaminated feed [72,73,74]. In addition, humans can be exposed to mycotoxins by inhalation and dermal contact with contaminated dust or mold [75]. Consequently, understanding and controlling human and animal exposure to mycotoxins is a fundamental concern [8].

Controlling this exposure is very complicated, since mycotoxins are naturally occurring. As the growth of fungi and the subsequent production of mycotoxins depend on the weather conditions, it is hardly possible to avoid them. It is also difficult to reduce the existing mycotoxins in food and feed, since mycotoxins are very stable and resistant at high temperatures. Another issue is the existence of masked mycotoxins, which are toxins that bind to sugars, proteins and fats [76].

Risk evaluation of mycotoxins is based on prevention due to research into the behaviour between plants and fungi, and more specifically into the genome of fungi, fungicides and biocontrol on the field. By biocontrol, limiting the growth of mold on the field and during conservation, is meant. Furthermore, Good Agricultural Practices, selection of plant varieties and the use of prediction models are important factors in risk assessment. Transport and preservation under ideal conditions, performance of sufficient analyses and removal of moldy products need to be done [76].

Despite the fact that food is typically contaminated by multiple mycotoxins at the same time, current chemical risk assessment is based on the knowledge about and the exposure to a single food-related compound and consequently, on the individually caused harmful effect(s) [77,16]. It is noticeably that the simultaneous occurrence of mycotoxins and other bioactive food constituents can lead to combinatory, namely synergistic or antagonistic interactions and in this way may significantly change the final toxicological outcome. For this reason, it is necessary that risk assessment studies do not only depend on individual evidences, but reckon with the complexity of these

chemical mixtures to get an overall impression of the risk associated with the consumption of mycotoxin containing food [16].

According to the European Food Safety Authority (EFSA), chemical mixtures are defined as “several substances which may have combined effects on the body from their combined exposures”. Some methods for evaluating risks from combined exposure to groups of chemicals have been developed and EFSA and its European *partners want to expand these methods to combinations of chemicals in food. In this* way, EFSA conducted a study which investigated the opinions of the consumers. Results of the study revealed that consumers are well informed about artificial chemicals, although not about the chemicals that are naturally occurring. The knowledge of consumers about chemical mixtures and their risk assessment is scarce and they are very concerned about the effects of combined chemicals in food. Reason for this is that consumers have the perception that artificial chemicals cause a greater risk to human health than natural occurring substances [78]. To conclude, further investigations of the risks associated with chemical mixtures is necessary and consumers need to be better informed about the risks.

To investigate the exposure of humans to mycotoxins, there are two methods. The primary method is based on an external exposure and includes the analysis of food toxins and the combination of this knowledge with data on the consumption of food. The second method is, on the other hand, based on an internal exposure and includes biomonitoring of a biomarker in each fluid or tissue of humans [79,80].

6.1. INDIRECT RISK ASSESSMENT

As discussed above (chapter 6), indirect risk assessment is one of the two approaches in risk assessment and relies on the measurement of the mycotoxin contamination in a given food product and the subsequent comparison with the consumption of that food product. In this way, the mycotoxin exposure is determined by multiplying the contamination and the consumption (exposure = contamination x consumption).

Consequently, this exposure is compared to the Tolerable Daily Intake (TDI) of the mycotoxin species. The difference between Acceptable Daily Intake (ADI) and Tolerable Daily Intake (TDI) is that the ADI is used for food additives, while the TDI is used for contaminants, such as mycotoxins. The TDI is defined as “an estimate of the quantity of a chemical contaminant to which one may be exposed through environmental contamination, and which when found in food, can be ingested daily over a lifetime without posing a significant health risk” [81]. It is determined by the division of the No Observed Adverse Effect Level (NOAEL) by a safety factor. The NOAEL is “the highest daily dose that can be administered in a repeated-dose animal test without causing any observable toxic effects”. To extrapolate the NOAEL to the TDI, multiple safety factors must be taken into account as well. For instance, a safety factor of 10 is commonly used to correct for the differences between animals and humans, while another factor of 10 is used to account for the Inter-Individual Variability (IIV). This results in an overall safety factor of 100, implying that only one hundredth of the non-toxic dose in animals can be considered safe for humans (**Figure 6.1.**) [81].

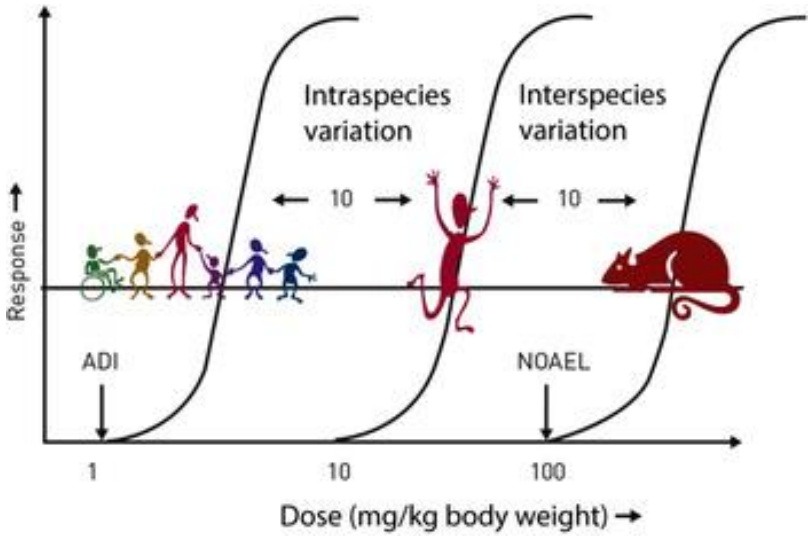


Figure 6.1.: Safety factors to extrapolate the NOAEL to the ADI. NOAEL: No Observed Adverse Effect Level; ADI: Acceptable Daily Intake [82].

In indirect risk management, a difference can be made between the way the risk analysis is done, namely by a deterministic or probabilistic analysis. When a deterministic analysis is carried out, the exposure to the parent and masked mycotoxins is determined by multiplying the mean mycotoxin concentrations by the mean,

maximum or P95-percentiles of the consumption data [83]. Deterministic estimations of exposures assume that all individuals consume the food at the same period of time and at a same level. In addition, the parent mycotoxins and their masked forms are considered to be continuously present at an average level. Since the deterministic analysis can cause an overestimation, an additional probabilistic analysis is often carried out to acknowledge a more detailed exposure assessment. In a probabilistic analysis, the estimated intake of the parent and masked mycotoxins is determined separately per food category. The total intake is then calculated by considering all concentration data of the food categories and the consumption data, while correcting for the intra person variability. Therefore, the sum of the means of the subcategories intake is not necessarily equal to the mean intake of the overall category [84].

Although indirect risk assessment is an important approach in risk management, it presents several disadvantages. The primary comes from the evaluation of the mycotoxin content in food, as they are not distributed uniformly, what makes correct sampling more difficult. Furthermore, some mycotoxins can be associated with elements of the matrix or can be biologically or chemically changed in the raw material [85]. For that reason, they cannot be detected during the analytical procedure, what results in an underestimated exposure level [86]. Additionally, it is complicated to acquire correct data on food consumption. Subsequently, the presence of these toxins in food does not undoubtedly indicate that humans have been exposed to them, since their bioavailability can differ depending on multiple aspects, as the composition and the treatment of the food [87] and the Inter-Individual Variability (IIV). For that reason, the execution of accurate risk assessments based on these data alone, is very complex.

In a study conducted by De Boevre et al. (2013), 174 cereal-based food products were analysed for the occurrence of several parent mycotoxins and their metabolites (chapter 7), namely deoxynivalenol (DON), 3-acetyldeoxynivalenol (3 ADON), 15-acetyldeoxynivalenol (15 ADON), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), T-2 toxin (T-2), HT-2-toxin (HT-2) and their respective masked forms, including deoxynivalenol-3-glucoside (DON3G), zearalenone-4-glucoside (ZEN4G), α-zearalenol-4-glucoside (α-ZEL4G), β-zearalenol-4-glucoside (β-ZEL4G) and zearalenone-4-sulfate (ZEN4S). The analysed cereal-based foods were fibre- and

bran-enriched bread, breakfast cereals, oatmeal and popcorn. During a period from April 2010 to October 2011, these products were analysed after they were collected in Belgian supermarkets according to an organized sampling plan. From a national representative food intake survey, the habitual consumption of all this cereal-based foods and the subsequent mean mycotoxin intake were estimated [84].

In agreement with the results of the probabilistic exposure analysis, the mean (and P95) mycotoxin intake for the sum of the DON-equivalents, ZEN-equivalents and the sum of HT-2- and T-2-toxins was 0.1162 (0.4047, P95), 0.0447 (0.1568, P95) and 0.0258 (0.0924, P95) µg kg^{-1} body weight day^{-1}, respectively. The TDI for deoxynivalenol, zearalenone and the sum of HT-2 and T-2, is 1.0, 0.25 and 0.1 µg kg^{-1} body weight day^{-1}, respectively, so the measured values were lower than the TDI. The absolute part of the Belgian population that exceeded the TDI for the cereal products was 0.85%, 2.75% and 4.11% respectively (**Table 6.1.**) [84].

Table 6.1.: The values (%) show the percentages of the population which exceeded the Tolerable Daily Intake (TDI) [84].

Mycotoxin	Fibre-enriched bread	Bran-enriched bread	Breakfast cereals	Oatmeal	Popcorn
DON	0.02	0.00	0.00	0.00	0.03
Sum DON-eq[a]	1.15	0.00	0.00	0.00	0.68
Sum T-2 and HT-2	16.60	1.89	0.00	0.02	0.00
ZEN	5.81	0.95	8.20	0.00	0.00
Sum ZEN-eq[b]	14.80	5.27	14.91	0.00	0.02

[a] Sum of DON-eq: 3 ADON, 15 ADON and DON3G; [b] Sum of ZEN-eq: ZEN4G, ZEN4S, α-ZEL, β-ZEL, α-ZEL4G and β-ZEL4G

From these results, it can be concluded that there is a small, but existing risk of exceeding the TDI of the mycotoxins. However, it must be borne in mind that only the results of cereal products were taken into account, even though *Alternaria* mycotoxins, for example, are also present in vegetables, fruit and their juices, nuts, seeds, herbs

and oils, as has already been said (chapter 5.2.) [16]. From this, it can be understood that when the full diet would have been examined, the risk of exceeding the TDI would most likely be higher.

This can potentially pose a health risk to people who consume mainly cereals, vegetables and fruit, such as vegetarians. Our current health aspect, which strongly promotes to eat less meat and more nuts and cereals, may also lead to an increase in mycotoxin intake and toxicity in the future.

As already mentioned above (chapter 6), risk assessment of mycotoxins is mainly based on the risk caused by only a single species, although it has already been proven that mycotoxins of the *Alternaria*, *Fusarium*, *Penicillium* and *Aspergillus* species are regularly occurring simultaneously in contaminated food. Although well documented, it is not yet routinely checked [16].

6.1.1. Python algorithm

In accordance to the course of Regulatory Affairs of health products, an algorithm, that calculates the individual risk of exceeding the TDI of mycotoxins, was designed. The purpose of this algorithm is to make a prediction about the total exposure to the most important and common mycotoxins, based on data such as gender, age, body weight, race, way of life, … This would be a major advantage, since, unlike the current method of risk assessment, a total picture of the exposure to mycotoxins from the entire diet is obtained. This could be a useful tool for people who focus on guiding the diet of patients, such as nutritionists and dieticians. In this way, they can give more specific advice on whether or not to adjust their patients' diet.

The algorithm is based on an indirect risk assessment, for the simple reason that direct risk assessment, based on urine, faeces or blood would cause more difficulties. Indirect risk assessment is, as previously mentioned (chapter 6.1.), based on the measurement of the mycotoxin content in a given food product and the comparison with the intake and this for all the consumed food products in the patient's

diet. Other influential factors such as gender (since the metabolism of some mycotoxins differs between men and women), age, body weight, race, way of life, … are also taken into account.

Table 6.2.: Mean concentrations of mycotoxins and their masked forms (µg/kg) in breakfast cereals and fibre-enriched bread [84].

Mycotoxin	Breakfast cereals	Fibre-enriched bread
Deoxynivalenol	44	34
3-acetyldeoxynivalenol	31	14
15-acetyldeoxynivalenol	10	9
Deoxynivalenol-3-glucoside	13	34
β-zearalenol	17	7
α-zearalenol	43	6
Zearalenone	76	29
Zearalenone-4-glucoside	39	15
Zearalenone-4-sulfate	23	4
β-zearalenol-4-glucoside	11	7
α-zearalenol-4-glucoside	11	3

Practical example *: based on data in **Table 6.2.**

Mycotoxin content in **breakfast cereals** [84]:
DON and equivalents: 98 µg/kg
ZEN and equivalents: 220 µg/kg

For an average portion of 30 g breakfast cereals per meal:
DON and equivalents: 2.94 µg
ZEN and equivalents: 6.6 µg

For, for example, a 50 kg female:
DON and equivalents: 0.0588 µg/kg
ZEN and equivalents: 0.132 µg/kg

Mycotoxin content in **fibre-enriched bread** [84]:
DON and equivalents: 91 µg/kg
ZEN and equivalents: 71 µg/kg

For an average portion of 134 g bread per meal:
DON and equivalents: 12.194 µg
ZEN and equivalents: 9.514 µg

For, for example, a 50 kg female:
DON and equivalents: 0.24388 µg/kg
ZEN and equivalents: 0.19028 µg/kg

Total mycotoxin content per mycotoxin species per day for a 50 kg female:
DON and equivalents: 0.30268 µg/kg
ZEN and equivalents: 0.32228 µg/kg

Tolerable Daily Intake (TDI) per mycotoxin species [84]:
DON: 1.0 µg/kg
ZEN: 0.25 µg/kg

if (daily intake of DON and equivalents greater than 1.0 µg/kg):
    diet adjustment
else:
    do not adjust diet *(see practical example)*

if (daily intake of ZEN and equivalents greater than 0.25 µg/kg):
    diet adjustment *(see practical example)*
else:
    do not adjust diet

*Repeat for the other mycotoxin species of the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera.

As discussed above (chapter 5.3.), the TDI of AOH and AME is not yet determined [59]. Consequently, additional compound-specific data is needed before this algorithm can be applied to this genus.

6.2. DIRECT RISK ASSESSMENT

Direct risk assessment is based on Human Biological Monitoring (HBM), which describes the risk at individual level. HBM is the analysis of mycotoxin biomarkers in body matrices as urine, faeces or blood [88]. Even though the direct and indirect method are complementary to each other, the direct method is more efficient in investigating human exposure [89,90] than the indirect method [91]. A great benefit is that the identification of the origin of contamination (ingestion of contaminated food or

inhalation of contaminated air) is not needed and that this approach is based on a single determination per person and in this way circumvents the problems correlated with food sampling and the collection of consumption data. As a result, biomonitoring should be performed worldwide to control mycotoxin exposure in humans [8].

6.2.1. Urine

Urine is one of the main biological matrices in toxicological research (chapter 7.2.). In comparison with blood, there are, depending on the component, usually metabolites in a higher concentration involved. As can be seen in **Table 6.3.**, urine has advantages and disadvantages as a toxicological matrix [92].

Table 6.3.: The advantages and disadvantages of urine as a toxicological matrix [89].

Advantages	Disadvantages
-A longer time window than blood (several days until one week, depending on the component)	-No definite link between the urinary concentration and the present effect -Privacy

6.2.1.1. Deoxynivalenol: urinary biomarkers of exposure

Urine as a biological matrix, has often been used to measure the DON-exposure [93], as it was revealed that more than 90% of the examined human urine showed occurrence of DON and its metabolites (chapter 7) [86]. In the study performed by Heyndrickx et al. (2015), the results proved that more than 75% of the excreted DON, was glucuronidated [86,94,95,96,97] and that DON-15-glucuronide was the primarily excretion product in urine of humans [86]. Based on this information, EFSA came to conclusion that exposure estimations, based on biomarker analysis, were within the same order of magnitude as the estimations based on alimentary consumption [93]. Nevertheless, there were still some uncertainties about the use of biomarkers of exposure in urine to estimate the DON-exposure; (1) the individual variation in the

eliminated amount, which was presumed to be 70%, (2) the unexplored contribution of DON-3-glucoside, 3-ADON and 15-ADON, (3) time of the day for urine collection and (4) various manners of back-calculation [93].

Some of these uncertainties were resolved in the recent study, conducted by Vidal et al. (2018). In this study, urine was used as a biological matrix to clear up the urinary excretion profile and metabolism of DON and its metabolite deoxynivalenol-3-glucoside (DON-3-glucoside). The urine of 20 volunteers was collected and analysed for the presence of the mycotoxins, DON, DON-3-glucoside, 3-ADON, 15-ADON, de-epoxy-deoxynivalenol (DOM-1), deoxynivalenol-3-glucuronide (DON-3-glucuronide) and deoxynivalenol-15-glucuronide (DON-15-glucuronide) [98].

According to the study schedule below (**Figure 6.2.**), the volunteers were restricted in ingesting cereals and cereal-based foods for four days. On the third day, they received a single DON-bolus of 1 µg/kg body weight and their urine was collected during 24 hours. After a washing-out period of two months, they received a second single bolus of 1 µg/kg body weight, this time of DON-3-glucuronide and once again, their urine was collected [98].

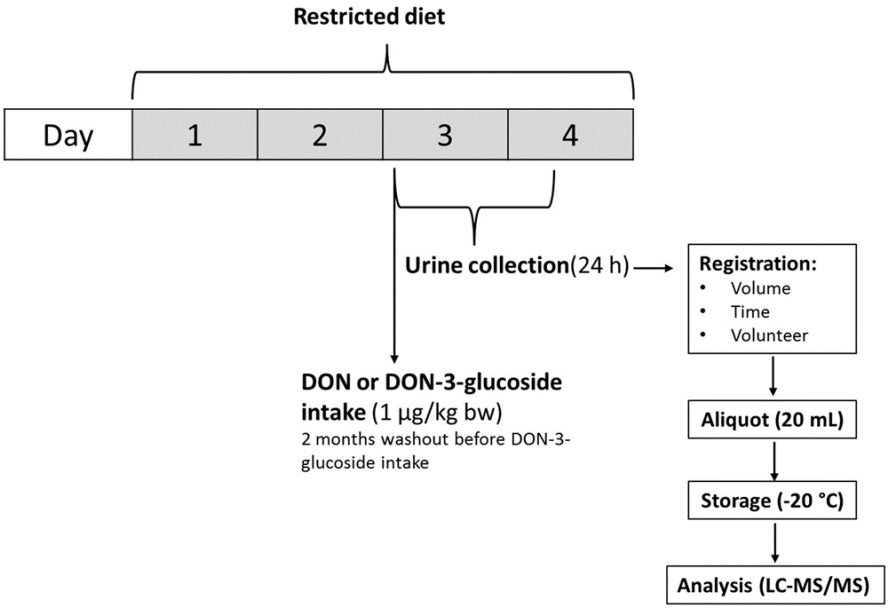


Figure 6.2.: Scheme of the study design [98].

In agreement with the results of this study, DON and DON-3-glucoside were rapidly absorbed, distributed, metabolised and excreted, with an excretion rate of 64% and 58%, respectively, based on information about their urinary biomarkers, DON-3-glucuronide and DON-15-glucuronide. Urine had been shown to be a trustworthy matrix, provided that the urine was collected for at least 16 hours. This to obtain representative results for the DON-consumption, since the use of morning urine is not sufficient to measure the DON exposure [98].

In conclusion, the analysis of urine confirms the dissimilarities between species as well, since animals tend to produce more DON metabolites than humans. DON glucuronides are the most examined metabolites in humans and particularly DON-3-glucuronide and DON-15-glucuronide need to be considered as biomarkers of DON in urine [99].

6.2.1.2. Zearalenone: urinary biomarkers of exposure

In the same study conducted by Heyndrickx et al. (2015) (6.2.1.1.), ZEN, α-ZEL, β-ZEL, ZEN-14-glucuronide, α-ZEL-7-glucuronide, α-ZEL-14-glucuronide and β-ZEL-14-glucuronide were examined in the urine of humans [83]. Nevertheless, of α-ZEL and β-ZEL-14-glucuronide only traces were discovered (ZEN and its metabolites are discussed in chapter 7). In pigs, ZEN-metabolites in urine and faeces, are primarily checked 24 to 48 hours after ingestion [100], presumably due to the influence of the enterohepatic circulation. From this, it can be concluded that it is important to collect the urine at the accurate time to determine the exposure to ZEN [99].

The most prevalent metabolites of ZEN *in vitro* and *in vivo* are α-ZEL, β-ZEL 8-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide, and the primarily metabolic pathways are hydroxylation, reduction and glucuronidation [99].

In conclusion, even though ZEN is metabolised to multiple 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 [99].

6.2.2. Faeces

Besides urine [98,99], faeces is often used as a biological matrix in the risk assessment of mycotoxins [103,104] (chapter 7.2.). This type of matrix is often more complex than urine, due to the differences in metabolism and consequently, in metabolites between animal species. For this reason, faeces might seem to be a less relevant matrix at first sight, but nothing could be further than the truth. Faecal material namely is an interesting matrix to examine the unabsorbed material, what is relevant in the investigation of the metabolism of DON.

When an oral dose of DON was administered to polygastric animal species like sheep, between 54% and 75% was regained in the faeces as DON and DOM-1 [105]. The bacterial activity in the gastrointestinal system of sheep can transform DON into DOM-1, what results in higher levels of DON forms in their faeces than in their urine. Therefore, sheep and other ruminants are almost nonreactive to an acute oral DON-intoxication since they are able to lower the circulating amount of free DON to a large extent [106].

Mono-gastric animal species like humans, rodents and pigs, on the other hand, do not show a significant percentage of ingested DON in their faeces. This is due to the high absorption of DON in the small intestine, while conversion of DON to DOM-1 can only happen when the colon is reached [106,107,108]. In fact, it may be assumed that humans lack the proper microflora to induce the transformation into DOM-1, causing DOM-1 to be a minor and even negligible metabolic transformation product in humans [90].

However, this does not apply to all monogastric animals. For example, DON is mainly present in faecal samples of rats [109]. DON sulfonates, the main DON metabolites in rats are primarily excreted by faeces than in urine [110]. Since there is

already a lot of conscience on the metabolism of DON, Miró-Abella et al. (2018), succeeded to quantify the presence of DON and DOM-1 even at low concetration levels in faecal samples of rats [111].

For both urine and faeces, as important matrices in risk assessment, it can be concluded that detection and quantification of the sum of free and modified mycotoxins are fundamental for an adequate monitoring of the total levels of mycotoxins and thus for their influence on health [112].

6.2.3. Blood

Blood is by far the most relevant matrix in toxicological studies and in this way, it is often used to determine the concentration of the analyte. By blood, plasma or serum is meant, as the therapeutic intervals are set in these two matrices [92]. To collect plasma, a traditional venous collection method is used to withdraw blood [113]. Venepuncture is still considered as golden standard, since blood as a matrix offers a lot of information as there is a direct link between the concentration in blood and the effect. In comparison with urine, both the original components and the metabolites are present in blood [92]. Besides all these advantages, this matrix also has a lot of disadvantages, like large sample volumes, specified storage condition and controlled shipment. Furthermore, venipuncture is invasive and therefore needs qualified and trained technicians. All these factors contributed to the development of new sampling methods [113].

Microsampling is an alternative sampling which reduces the volume from milliliter to microliter and therefore is used for very small sample volumes. More comfort for the patient, simplified shipment and alignment with the 3Rs strategy (Reduce, Refine, Replace), are important advantages of this sampling method. Nowadays, there are two types of microsampling, namely Dried Blood Spot Sampling (DBS Sampling) and Volumetric Absorptive Microsampling (VAMS) [113].

6.2.3.1. Dried Blood Spot Sampling

'Dried Blood Spot Sampling' (DBS Sampling) is a microsampling technique in which blood is collected by a glass capillary via a skin prick (in heel or finger) or venous cannula which is then pipetted onto a specified substrate, like a celluloseor polymer-based filter paper. Finally, the sample is dried at 22 degrees for two to three hours over an open non-absorbent surface. However, this may vary depending on the filter paper used, the humidity and temperature [114]. This technique has seen a strong increase in the recent years, as it offers many advantages over classic venous sampling [92]. In **Table 6.4.**, the advantages and disadvantages of dried blood spots (DBSs) as a toxicological matrix are shown.

Table 6.4.: The advantages and disadvantages of dried blood spots as a toxicological matrix [92].

Advantages	**Disadvantages**
-Ease of sampling, enabling sampling at home -Cost-effective sampling, transport and storage of samples -Improved compound stability -Small blood volumes -Minimally invasive sampling -Reduced risk of infection -Simplification of sample preparation procedures -Suitability for automation of sample processing and analysis	-Correlation between venous and capillary blood concentrations -Adequate sampling -Contamination risk -Lack of sensitivity -Chromatographic effect and influence of the site of punching -Influence of spotted blood volume -Haematocrit effect

As can be seen in **Table 6.4.**, some of the major concerns with DBS are the homogeneity, the haematocrit effect and the influence of the spotted blood volume on the results [114]. The haematocrit (HCT), which is the volume percentage of the whole blood that contains red blood cells, influences the viscosity of blood and therefore, controls the volume of blood spreading onto a card [115]. Although this technique is

highly innovative, it was accepted slowly due to (1) sample manipulation, including card punching, (2) sample extraction and (3) handling prior to bioanalysis (**Figure 6.3.**) [116,117].

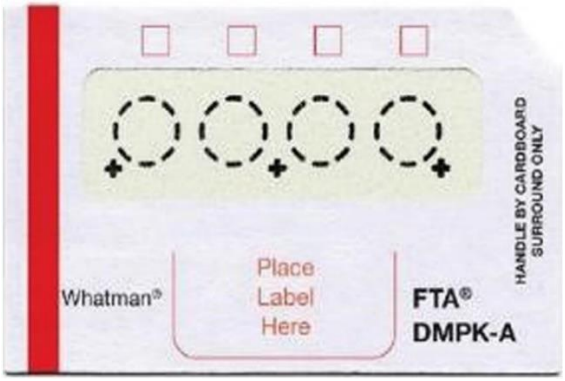


Figure 6.3.: DBS cards [113].

Figure 6.4.: Mitra® VAMS [113].

6.2.3.2. Volumetric Absorptive Microsampling

'Volumetric Absorptive Microsampling' (VAMS) is a new microsampling technique whose devices (Mitra®) circumvent almost all the problems related with classic venous sampling [116]. Benefits of VAMS are that regardless of the hematocrit, a consistent blood volume is obtained [116,117], and since a precise volume is absorbed on to the tip, the homogeneity of the sample is ensured [118]. The VAMS sampler is available in samplers of 10 µL and 20 µL for animals and 30 µL for humans. The device consists of an absorbent polymeric tip which collects a small, fixed volume of blood by capillary action, via skin prick (in heel or finger) for humans and tail vein for rodents. At the time of collection, the sampler is filled by holding the device at an angle of 45 degrees and dipping only the tip into blood drop, while allowing it to fill [116,117]. In conclusion, the greatest benefit of VAMS over DBS is that VAMS permits the precise and accurate collection of blood volumes for quantitative bioanalysis (**Figure 6.4.**) [117].

In a recent study conducted by Lauwers et al. (2019), DBSs were used for the first time as biological matrix in a multi-mycotoxin biomarker analysis in pigs and broiler chickens. The method was developed and validated for the determination of 23

mycotoxins and their phase I and II metabolites. The examined toxins were aflatoxins, ochratoxin A, several *Fusarium* mycotoxins and two groups of unregulated mycotoxins, namely *Alternaria* mycotoxins and *Fusarium* mycotoxins (enniatins and beauvericin). For most mycotoxins, there was no observed effect of variation in haematocrit and spotted blood volume. Furthermore, both the extraction of a standardized 8 mm disk and the entire blood spot can be used to determine mycotoxins in DBSs. As a fixed volume is not required, the 8 mm disk method was favoured. Ultimately, the DBSs were tested in a toxicokinetic and pilot exposure assessment study. The mycotoxin DBSs concentrations were compared to the plasma concentrations and a strong correlation ($r > 0.947$) was noted [119].

To that end, and considering all the advantages, DBS Sampling and VAMS could be promising techniques in the direct risk assessment of mycotoxins in the future.

7. MYCOTOXIN METABOLISM

As discussed above (chapter 1), mycotoxins cause a potential health risk for both animals and humans. However, not only the mycotoxins themselves are causing concerns, but also their metabolizing products. In the past decades, the existence of the modified mycotoxins was unravelled. This as a result of the discovery in the mid-80s, that in cases of mycotoxicosis, there was no correlation between the clinical observations in animals and the low mycotoxin content in the correspondent feed. It turned out that the possible hydrolysis of these undetected, conjugated mycotoxin forms into the free forms in the digestive tract of animals, was responsible for this unexpected, strong increase in toxicity [120]. Knowledge on the occurrence of these mycotoxin species is therefore absolutely necessary, to determine the amount of mycotoxins in food or feed [84].

The first definition of masked mycotoxins, introduced by Gareis et al. (1990), included that masked mycotoxins are molecules that cannot be detected by standard routine analytical techniques [120]. Years later, it was recommended by Berthiller et al (2013), to change that definition to only the plant derivatives of mycotoxins [121,122,123]. The term of modified mycotoxins was made known by Rychlik et al. (2014), to categorize the types of modifications of mycotoxins on four levels. The first level differentiates the free toxins, that are produced as secondary metabolites of mould to cause disease and death in animals and humans, from the modified and matrix-associated ones [85].

The modified mycotoxins include the “biologically” modified and the “chemically” modified mycotoxins. By “biologically” modified, modification, for example, by conjugation with a polar compound (mainly -glucoside, sulphate or even glutathione), is meant. Mycotoxins that belong to this category, fall under the “masked” mycotoxins as well. “Chemically” modified mycotoxins, on the other hand, are produced as a result of thermal or other sort of processes, that take place during the production of food and feed [85].

In addition to the modified mycotoxins, there are matrix-associated mycotoxins as well. These are toxins like the *Fumonisins*, that are linked to starch and oligosaccharides and are bound by covalent bonds or physically captured [85].

Nowadays, it has been proven that besides the free mycotoxins, the modified mycotoxins could also cause an additional health risk for animals and humans. There is mainly concern about the conjugated forms, as they are contaminating common foods and drinks (**Table 7.1.**) [124], and can be potentially toxic in two ways; directly toxic, in case of the conjugated form itself or indirectly toxic, after hydrolysis to their free forms [84].

Table 7.1.: Occurrence of modified mycotoxins in common foods and drinks [124].

Matrix	Technique[a, b]	Modified mycotoxin	Levels (μg/kg)/(μg/L)[c]
wheat	LC-MS/MS	Deoxynivalenol-3-glucoside	50–200 [121]
corn	LC-MS/MS	Deoxynivalenol-3-glucoside	<20 – 70 [121]
wheat	LC-MS/MS	Deoxynivalenol-3-glucoside	76–1070 [125]
corn	LC-MS/MS	Deoxynivalenol-3-glucoside	10–763 [125]
corn products	LC-MS/MS ELISA	Bound fumonisins	NR [126]
corn	LC-MS/MS	Hidden fumonisins	54–982 [127]
beer	LC-MS/MS	Deoxynivalenol-3-glucoside	<2.5–37 [128]
corn	LC-MS/MS	Total fumonisins	<25 – 44274 [129]
corn	ELISA	Total deoxynivalenol	120–750 [130]
malt	LC-MS/MS	Deoxynivalenol-3-glucoside	12.9–186 [131]

Table 7.1. (continued): Occurrence of modified mycotoxins in common foods and drinks [124].

baked goods	LC-MS/MS	Deoxynivalenol-3-glucoside	43–399 [131]
beer	LC-MS/MS	Deoxynivalenol-3-glucoside	6–82.1 [131]
beer	LC-MS/MS	Deoxynivalenol-3-glucoside	<3.5–81.3 [132]
corn	LC-MS/MS	Hidden fumonisins	NR [133]
durum wheat	LC-MS/MS	Deoxynivalenol-3-glucoside	<50 – 850 [134]

[a] LC-MS/MS - Liquid Chromatography with tandem mass spectrometry; [b] ELISA - Enzyme-Linked Immuno Sorbent Assay; [c] NR - Not reported: levels were reported only in charts.

As mentioned before, modified mycotoxins can be produced in different ways; by plants, microorganisms, processing and animals (**Figure 7.1.**). When modified mycotoxins are formed in the plant throughout processing or during digestion, they may cause a diminution of the general toxicity of the food (**Table 7.2.**) [124].

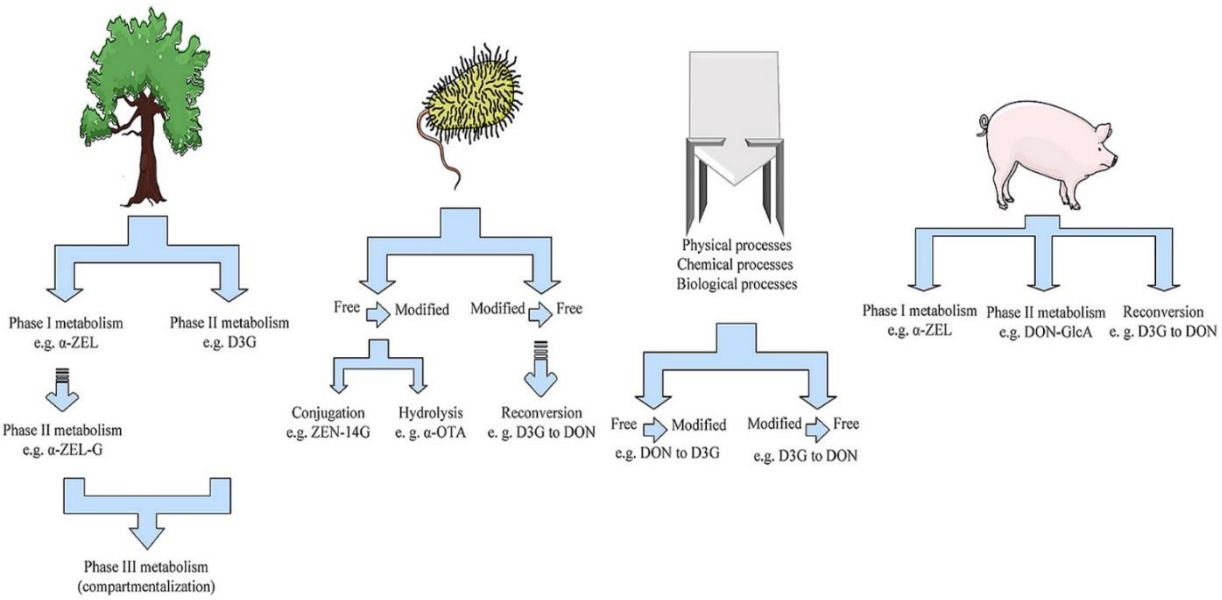


Figure 7.1.: Formation of modified mycotoxin by plants, microorganisms, processing and animals [124].

Table 7.2.: Cytotoxic effects of modified mycotoxins [124].

Modified mycotoxin	Cell line/system[a, b]	Exposure dose	Effects
nor DON A, B, and C	IHKE	100 µM	no toxic effects [135]
15-ADON	GES-1 cells	0.5 ppm	reduced the viability of the cell [136]
3-ADON	GES-1 cells	12 ppm	low reduction of cell viability [136]
D3G	GES-1 cells	12 ppm	not reduce cell viability [136]
DOM-3-sulfate	animal ribosomes	100 µM	not inhibit an *in vitro* translation assay [137]
DOM-15-sulfate	animal ribosomes	100 µM	not inhibit an *in vitro* translation assay [137]
14-(R)-ochratoxin	IHKE	50 nM	cytotoxic and apoptotic effects [138]
14-decarboxy-ochratoxin A	IHKE	10 µM	not cytotoxic effects [138]
14-(R)-ochratoxin	IHKE	50 µM	cytotoxic effects [139]
ochratoxin α amide	IHKE	50 µM	not reduce cell viability [139]
ochratoxin α	IHKE	50 µM	not reduce cell viability [139]

[a] IHKE - Immortalized Human Kidney Epithelial-1; [b] GES-1 - gastric epithelium cell line

Some of the major mycotoxins and their modified forms examined in food, are illustrated in **Table 7.3.** [124]. However, crucial data about modified mycotoxins is still incomplete and so more studies are necessary. By incorporating modified mycotoxins *in vivo*, upcoming studies could reveal relevant information about their toxicokinetics [84].

Table 7.3.: Major mycotoxins and their modified forms in food

Free mycotoxin	Major producing fungi	Matrix	Effects on health	Modified mycotoxin
DON	*Fusarium culmorum*; *Fusarium graminearum* [140,141]	feed [142], oat [143], cereals [144], maize [145]	in animal: reduced growth and weight gain, feed refusal and emesis, affects immune system and intestinal functions. In human: nausea, diarrhoea and vomiting [72,146,147,148]	DON-3-glucoside; DON-Hexitol; DON-S-cysteine; DON-S-cysteinyl-glycine; DON-glutathione; DON-di-hexoside;"DON-2H"-glutathione; DON-malonylglucoside; 15-acetyl-DON-3-glucoside; 3-Acetyl-DON; DON-3-sulfate; DON-15-sulfate; 3-epimer-DON; nor DON A, B and C; nor DON-3-glucoside A, B, C and D; DON-3-glucoside-lactone; de-epoxy DON; DON-glucuronide; de-epoxy DON-3-sulfate; de-epoxy DON-15-sulfate
ZEN	*Fusarium culmorum*; *Fusarium graminearum*; *Fusarium heterosporum* [149,73]	feed [142], cereals [150], breakfast cereals [151], maize [145]	precocious pubertal changes, fertility problems and hyper estrogenic [152,153,5]	ZEN-16-O-β-glucoside; ZEN-14-O-β-glucoside; α-zearalenol; β-zearalenol; α-zearalenol-glucoside; β-zearalenol-glucoside; ZEN-4-glucoside; ZEN-4-sulfate;malonyl-glucosides (ZEN-MalGlc, α-ZEN-MalGlc, β-zearalenol-MalGlc); di-hexose-(ZEN-DiHex,α- zearalenol-DiHex,β-zearalenol-DiHex); hexose–pentose disaccharides (ZENHexPent, α-zearalenol-HexPent, β-zearalenol-HexPent); tri-hexose conjugate (β-zearalenol-TriHex); α-zearalenol-sulfate; α-zearalenol; β-zearalenol
Ochratoxin A (OTA)	*Aspergillus* and *Penicillium* genera [154,155,156]	wine [154], coffee [157], cocoa [158], grapes [159], cereals [160]	immunosuppressive effect [161], teratogenic [162], carcinogenic [163], mutagenic [164], neurotoxic [165] and genotoxic [166]	ochratoxin α; 4S-hydroxyochratoxin A; 4R-hydroxyochratoxin A; hydroxyochratoxin A-β-glucoside; ochratoxin A methyl ester; Ochratoxinαamide; 14-decarboxy-ochratoxin A;. Ochratoxin A mono- and disaccharide esters
T-2 and HT-2	*Fusarium sporotrichioides* [167,168]	beer [169], corn, wheat, barley, oats [167]	inhibition protein synthesis and effects immunotoxins [148,170,171]	HT2 toxin-3-glucoside; T-2 toxin-α-glucoside; T-2 toxin-β-glucoside; 15-acetyl-T2-tetraol-glucoside; hydroxy-HT2-glucoside; hydroxy-HT2-malonyl-glucoside; T2-triol-glucoside; dehydro-HT2-glucoside; HT2-diglucoside; HT2-malonyl-glucoside; 3-acetyl-HT2; 3-acetyl-T2; feruloyl-T2; HT2-sulfate
Fumonisin	*Fusarium proliferatum*; *Fusarium verticillioides*; *Aspergillus niger* [172,173,174]	feed [142], beer [175], chili [176], cereal [144]	hepatotoxic, nephrotoxic [153], immunosuppressive [177], pulmonary edema in swine and leukoencephalomalacia in horses [178]	hidden fumonisins; N-(carboxymethyl) fumonisina B1; N-Acyl hydrolyzed fumonisin B1; bound hydrolyzed fumonisins

7.1. IN VITRO

Since modified mycotoxins represent an emerging issue, the scientific community demanded a complete investigation of their metabolic profile, with a view to determination of their impact [179].

Considering data on the toxicological pertinence of modified mycotoxins *in vivo* is still missing, there is no accessible data for most toxicological tests on genotoxicity, short-term and long-term toxicity as carcinogenicity, reproduction and developmental studies. It appears improbable that conjugated mycotoxins have a higher toxicity than their free forms, as conjugation is a detoxification pathway in plants. Given that *in vitro* trials respect The Three Rs (Replace, Reduce, Refine) principles and that they overcome many problems related to *in vivo* trials, such as complexity, inaccessibility and subsequently, limitation of information [84], they are promising for the future.

In vitro studies were constructed to mimic the natural conditions during digestion and to investigate the interaction between the modified mycotoxins and stomach juices and the colonic microbiota of humans [84]. These studies exist of an artificial contamination of the food matrix with the free mycotoxin, as a result of the lack of official standards and methodologies for detection. For now, the DON derivative, D3G, is the only standard that is commercially available.

In vitro studies have provided important information about the metabolism of the parent component and the subsequent formation of the modified mycotoxins and still do so.

The modified forms of ZEN, namely zearalenone-14-glucoside (ZEN-14G), α-zearalenol-14-glucoside (α-ZEL-14G) and β-zearalenol-14-glucoside (β-ZEL-14G) and the modified trichothecenes, namely deoxynivalenol-3-glucoside (D3G), nivalenol-3-glucoside (NIV-3G) and T-2 toxin-3-glucoside (T-2-3G), were found to be resistant to hydrolysis by artificial digestive juice. In an *in vitro* experiment, the epithelial cells were also not capable of hydrolysing these modified forms [180].

An artificial test that mimicked the digestion, to figure out the behaviour of T-2

toxin-glucosides anomers was executed by McCormick et al. (2015). The results were that, after an incubation with artificial saliva of humans, T-2 toxin α-glucoside and T-2 toxin β-glucoside remained untouched. When the behaviour of DON and ZEN glucosides were examined in digestion, analogous results were obtained. From this, it could be concluded that the transformation of the modified into the parent mycotoxin will presumably occur in other steps of the absorption, such as in stomach or intestine [181].

The same conclusion was obtained when the stability of D3G was tested in hydrochloric acid, enzymes, artificial stomach juice which contained pepsin, intestine juice which contained amylase, and intestinal bacteria. Under the examined conditions, both hydrochloric acid, the enzyme β-glucosidase, artificial stomach and intestine juice, showed no action against D3G. In the presence of cellulase, an intestine-enzyme of ruminants, on the other hand, a part of D3G was transformed into DON [122].

In the same study by Berthiller et al. (2011), it was tested if microorganisms could act synergistically in the gastrointestinal system of animals and humans and so could increase the DON-levels. *B. bifidum*, *B. longum*, *C. freundii*, *E. avium*, *E. coli*, *L. amylovorus*, *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. paracasei* and *L. rhamnosus* were incapable of transforming D3G into DON, while *E. cloacae*, *E. durans*, *E. faecium*, *E. mundtii*, *L. plantarum* and *B. adolescentis* transformed in an efficient way. *E. casseliflavus*, *E. faecalis* and *E. gallinarum* were able to transform, but to a limited extent. Consequently, not only the microorganisms themselves are able to convert, but also the probiotics in fermented products after ingestion [122].

Although some modified forms cannot be changed by the stomach environment, the lactic acid bacteria in the intestine, contrarily, manage to break the bonds between protein, sugar and toxin molecules. This causes the reoccurrence of toxicity, as the modified mycotoxin is transformed back into its free form. This is why, in toxicity studies, this cleavage must be taken into account, since a part of the parent component might be absorbed in the intestine [181,122].

Some bacteria of the human and animal microbiota produce hydrolytic enzymes and so are important in the transformation of modified mycotoxins. This enzymes can

break the toxin-glucosides bonds. β-glucosidase, which has the ability to hydrolyse and to transform along the absorption [182], can be produced by the species Oenococcus oeni [183] and Sphingomonas paucimobilis [184]. In this respect, the hydrolysis of ZEN-14G, α-ZEL-14G, β-ZEL-14G, D3G, NIV-3G and T-2-3G by the faecal microbiota of humans, was examined in the study performed by Gratz et al. (2017) (see above) [180].

Although the liver is regarded as the main bio activator of ZEN in multiple species [185], the systemic circulation can cause an additional increase in the levels of ZEN in the organism [124]. Other than the intestinal microbiota which is responsible for the transformation of modified mycotoxin to parent mycotoxin, whole blood, serum albumin, bovine plasma and foetal bovine serum (FBS), are able to hydrolyse ZEN-14G to ZEN as well, likewise their ability to convert ZEN to α-ZEL and β-ZEL. This is due to the presence of proteins, whose (pseudo) enzyme activity is responsible for this hydrolysis [186].

In the study conducted by Broekaert et al. (2015b), the absorption of 3-ADON and 15-ADON and their hydrolysis to DON were analysed in chickens and pigs [187], while the hydrolysis of D3G to DON was not noticed in broiler chickens. A far-reaching, pre-systemic hydrolysis of D3G to DON was noticed in plasma of pigs, but with a larger absorption of DON. In spite of the lower toxicity of D3G than DON, D3G is able to increase the DON levels and consequently, its toxic effects in organisms. This results display that the dissimilarities in absorption and metabolization between species must be taken into consideration and that the levels of modified DON must be examined [188].

Animals and humans are able to convert ZEN to α-ZEL, of which the latter has an estrogenic activity that is three to four times greater than the activity of its parent mycotoxin ZEN [189]. In addition, α-ZEL and ZEN can be conjugated with allyl isothiocyanate, which is a phytochemical compound in vegetables, acknowledged for forming adducts between the nucleophile groups of the mycotoxin with the central carbon of isothiocyanates, what lowers the mycotoxin levels. These conjugates proved to be less bio accessible at high levels of 30 µM, in contrary to lower levels of 15 µM, where the parent and modified mycotoxin were equally bio accessible. Contrarily, the

bioavailability of the conjugated forms was higher than their respective parent compound. For this reason, studies on the toxic effects of these conjugates still have to be executed [190].

7.2. IN VIVO

As discussed above (chapter 6.2.), urine, faeces and blood are important matrices to examine the metabolism of both free and modified mycotoxins *in vivo*.

In the survey conducted by Nagl et al. (2012), rats were treated with DON. In their urine, DON, deoxynivalenol-glucuronide (DON-GlcA) and de-epoxy deoxynivalenol (DOM-1) were found and DON-GlcA turned out to be the primary excreted metabolite. Although, when the rats received deoxynivalenol-3-glucoside (D3G), the toxins DON, DON-GlcA, DOM-1 and D3G were found in their urine. This results indicated that from the hydrolysis of D3G, DON was formed, then absorbed and finally metabolised to DON-GlcA. Nevertheless, in their faeces, only DON and DOM-1 were found after ingestion of DON. The formation of DOM-1 is due to the bacterial activity in the gastrointestinal system. On the other hand, DON-GlcA could, in contrary to urine, not be detected as a result of the activity of glucuronidase of bacteria in the intestine. This enzyme has been proven to be capable of breaking down the molecule. When rats received D3G instead of DON, the same metabolites could be observed in their faeces, although only remnants of D3G were found. As the largest part of this metabolite is broken down into DON, the toxicity of D3G is not comparable to DON's [191].

The exact same study was conducted, but now with pigs. After treatment of the pigs with DON; deoxynivalenol-3-glucuronide (DON-3-GlcA), deoxynivalenol-15-glucuronide (DON-15-GlcA) and DON were found in their urine. Although, when the pigs received D3G, the toxins DON, DON-3-GlcA, DON-15-GlcA and DOM-1 were found in their urine. In their faeces, however, the only compound that was detected after D3G ingestion, was DOM-1 [107].

In the study organised by Schwartz-Zimmermann et al. (2014), urine and faeces samples of rats were previously treated with DON and D3G. The sulfonates derivatives concentration in the urine was found to be lower than 1%, although the sulfonates of DON, DOM and D3G in faeces accounted for approximately 50% of the total quantity of DON or D3G that was administered to rats. These findings signify a low absorption of these metabolites in the digestive tract of the rat [110].

As to the use of urine and faeces as biological matrix for the investigation of ZEN-metabolism, the results in both matrices of pigs were rather similar, in contrary to DON. Zearalenone-14-glucoside (ZEN-14G), zearalenone-16-glucoside (ZEN-16G) and ZEN-14-sulfate were hydrolysed to ZEN and transformed into other unidentified metabolites in urine and faeces. Metabolism of the mycotoxin depends on the examined species, the mycotoxin administration, but also on the state and digestion of the individual. The role of modified ZEN on the general ZEN-toxicity has to be taken into account as well and so these derivatives must be included in the risk analysis and the determination of a tolerable limit of total ZEN [100].

The greatest danger of the presence of modified ZEN forms is that they can be transformed into their parent compound by the microbiota of the intestine. In an *in vitro* study executed by Gratz et al. (2017), was proven that modified ZEN and modified trichothecenes showed little absorption, since they were not efficiently transported through the epithelial monolayers of the intestine [180].

The mycotoxins DON, ochratoxin A (OTA), ZEN and citrinin in free form and their metabolites; ochratoxin α, 4-hydroxyochratoxin A and β-ZEL were analysed in samples of urine. These metabolized products occurred with their respectively free forms in sometimes even higher concentrations. This confirmed that these metabolites potentially cause detoxification and can be interested as biomarkers [112].

7.3. COMPARISON BETWEEN IN VITRO (liver microsomes) AND IN VIVO

Regarding the metabolism and consequently, the impact on health of T-2, HT-2 and their masked forms, multiple studies have been conducted recently. In the study

conducted by Yang et al. (2017), the *in vitro* metabolism of T-2 was determined in farm animals and humans, while the *in vivo* metabolism was determined in chickens. T-2 was incubated with the liver microsomes of animals and humans, and consequently, the phase I and phase II metabolites were examined. In total, four phase I and three glucuronide binding metabolites of T-2 were detected. The main compound in all the six tested species was HT-2, even though there were meaningful metabolic differences among the species. Furthermore, the *in vivo* metabolism of T-2 after ingestion was determined in chickens as well. Of the 18 metabolites that were discovered, 13 were discovered and reported for the first time. These newly detected metabolites were 3'-hydroxy-T-2 3-sulfate, 3'-hydroxy-HT-2 3-sulfate, 4'-hydroxy-HT-2, 3',4'-dihydroxy-HT-2, 4'-carboxyl-T-2, 4'-carboxyl-HT-2, 4'-carboxyl-4'-hydroxy-T-2 and their isomers, while 3'-hydroxy-HT-2, 4'-carboxyl-T-2, 3'-hydroxy-T-2, HT-2 and neosolaniol were the major metabolites. These results indicate that animals metabolize T-2 more than initially thought [192].

However, in another study performed by Yang et al. (2017), the *in vitro* metabolism of T-2 toxin-3-glucoside (T-2-3G) was examined in rats and humans and the *in vivo* metabolism in rats. Five metabolites were discovered after incubation with liver microsomes of rats and humans. The metabolic stability of T-2-3G was higher than of T-2 in rats and humans. Three metabolites were discovered when the influence of the intestinal microbiota of rats and humans on the metabolism of T-2-3G was examined. During incubation with fresh faeces, T-2-3G was transformed into T-2. Additionally, the *in vivo* metabolism of T-2-3G was examined in rats after digestion. The metabolites, T-2, HT-2 and 3'-OH-T2-3G were discovered in urine and faeces and it turned out that T-2-3G has little absorption and is primarily metabolized in the gastro-intestinal system of rats. In accordance with the results, the principal metabolic pathways of T-2-3G in mammals are hydroxylation (C-3' and C-4'), hydrolysis (C-4 and C-8) and deconjugation [193]).

Risk assessment of HT-2 toxin is still a problem, since there is not sufficient information about the toxin. In the recent study conducted by Yang et al. (2018), rats were administered orally and intravenous 1.0 mg/kg body weight of HT-2 to examine the toxicokinetic properties. In plasma, HT-2 itself could not be discovered after oral ingestion, while its hydroxylated metabolite, 3'-OH HT-2 was analysed. In contrary to

after oral ingestion, HT-2, its 3'-hydroxylated product and glucuronide derivative, 3-GlcA HT-2 could be detected in plasma after intravenous administration and the latter proved to be the prevalent metabolite. Additionally, liver microsomes were used to examine the metabolization of HT-2 after HT-2 was missing in plasma, resulting in the discovery of eight phase I and three phase II metabolites. The major metabolic pathways were hydroxylation, hydrolysis and glucuronidation. Hydroxylation appeared to be the most important pathway and is mediated by the cytochrome P450 enzyme, 3A4. Finally, significant dissimilarities in metabolization among species were detected as well [194].

Not only for HT-2, but also for ZEN and metabolites there is a problem concerning their risk evaluation. This is due to the unavailibilty of reference points as metabolic and toxicokinetic data are lacking. This is why, Yang et al. (2019), investigated the toxicokinetic properties of alpha-zearalenol (α-ZEL) and its masked form, α-zearalenol-14 glucoside (α-ZEL-14G) in rats, and the comparative biotransformation from different animals and humans in liver microsomes. This research is important, as α-ZEL and α-ZEL-14G are much more estrogenically active than the parent compound ZEN itself. In this study, the metabolic and toxicokinetic profiles, and phenotyping of α-ZEL and α-ZEL-14G were explored. In plasma, both α-ZEL and α-ZEL-14G could not be discovered after oral ingestion, although both could be converted to multiple metabolites after intravenous administration. When α-ZEL-14G is entirely hydrolysed to α-ZEL, the toxicity of α-ZEL increases. Futhermore, for α-ZEL, 31 phase I and 10 phase II metabolites were analysed, and hydroxylation, dehydrogenation and glucuronidation turned out to be the principal metabolic pathways. At the same time, 9 phase I and 5 phase II metabolites of α-ZEL-14G were analysed, and α-ZEL-14G's principal metabolic pathways were deglycosylation, reduction, hydroxylation and glucuronidation. Additionally, the metabolism of α-ZEL and α-ZEL-14G was examined in the liver microsomes of rats, chickens, pigs, goats, cows and humans and significant dissimilarities among these species were noticed. The results of phenotyping studies showed that α-ZEL and α-ZEL-14G were mediated by the enzymes, CYP 3A4, 2C8 and 1A2 and the deglycosylation of α-ZEL-14G was mediated by carboxylesterase 1 (CES-1) and carboxylesterase 2 (CES-2). This information offers prospects for future risk assessment of mycotoxins and their modified forms [195].

Regardless of the fact that not every modified mycotoxin shows toxicity, more studies on the metabolism of mycotoxins and their modified forms need to be performed. In this way, the influence of modified forms on risk evaluation and tolerable limits in food can be determined.

8. EXPERIMENTS IN THE FRAMEWORK OF ERASMUS PARMA

The initial setup of this thesis was to optimize the protocols (Department of Food and Drug, University of Parma) of microsomes reactions and to purify the metabolites by trial-and-error. The study was supposed to start from the biosynthesis of mycotoxin metabolites via biological reactors (*i.e.* liver microsomes), followed by purification and characterization of the produced metabolites via LC-MS(/MS). The mycotoxins intended to be used were mainly ZEN and DON, with the aim of making a proof-of-principle concept so it could be further applied in the future for the *Alternaria* mycotoxins, AOH and AME. Some non-optimized starting protocols were provided, but as no laboratory activities could be performed, they could not be optimized through trial-and-error. This will require further development in the future.

8.1. PROTOCOL 1: PURIFICATION OF ZEN-GLUCURONIDE (Incubation with microsomes- FC, 29/02/2020)

- Solvent:
  - Ice cold methanol (-20 °C)
- Procedure:
  - Put 10 mL of ice-cold methanol in each of the 3 round-bottom flasks containing the dry crude extract.
  - Shake the flasks with rotational movements to resuspend the gelatinous substance (containing glucuronides).
    - If necessary, put the flasks in the ultrasonic bath for 1 minute to resuspend the whole crude extract.
  - Transfer the 30 mL in a 50 mL falcon tube
  - Put the falcon tube at -20 °C for 1h.
  - Centrifuge at max speed for 30 min (4°C)
  - Transfer the supernatant in a new falcon tube and store at -20 °C (This solution contains the glucuronides).
    - A certain amount of glucuronides is still present in the pellet. To recover the glucuronides from the pellet:

- Add 10 mL of ice-cold methanol in the falcon containing the pellet
- Vortex and put the falcon tube in the ultrasonic bath for 15 min (put some ice in the ultrasonic bath to avoid the increase of temperature!)
- Centrifuge at max speed for 30 min (4°C)
- Transfer the supernatant in a new falcon tube and store at -20 °C (This solution contains glucuronides).
    - Don't mix the content of the two falcon tubes. Keep them separated from each other!

- Isolation of ZEN-glucuronide by semi-preparative HPLC
    - Method? (It still needs to be developed!)
    - Injection volume: 500 µL

8.2. PROTOCOL 2: PURIFICATION OF ZEN-GLUCURONIDE

After incubation of ZEN with microsomes (and subsequent protein precipitation steps), check the crude extract by LC-MS/MS analysis to confirm the successful synthesis of ZEN-glucuronide.

- Dilute the crude extract with methanol (dilution factor to be defined) and put 500 µL into a vial.

LC-MS/MS analysis

Instrument: UHPLC Ultimate 3000 separation module (Dionex, Sunnyvale, CA, USA), coupled with a TSQ Vantage triple quadrupole (Thermo Fisher, Waltham, MA, USA) equipped with an ESI interface.

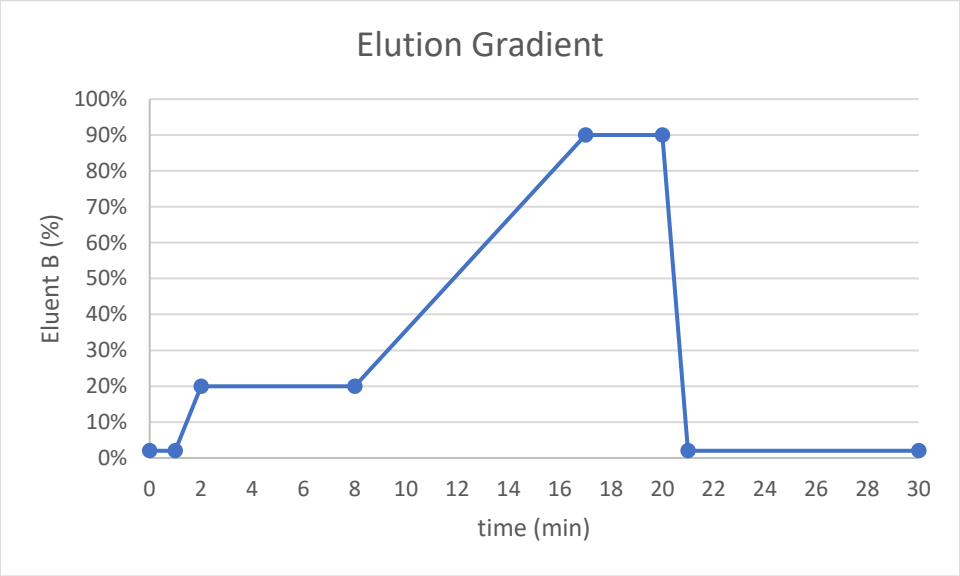
Column for the separation of the analytes: RP-C18 EVO Kinetex column (2.6 µ, 100A; 100 × 2.10 mm) from Phenomenex (Torrance, CA, USA).

Chromatographic conditions:

- *Eluents → A:* ammonium acetate 5 mM in bi-distilled water (+0.2% of acetic acid); *B:* methanol (+0.2% of acetic acid)

- Elution gradient →

Time (min)	Eluent A	Eluent B
0	98%	2%
1	98%	2%
2	80%	20%
8	80%	20%
17	10%	90%
20	10%	90%
21	98%	2%
30	98%	2%



- Column temperature: 40 °C
- Flow: 0.35 mL/min
- Volume of injection: 4 µL

MS conditions:

- Ion-monitoring mode: negative ion mode
- Spray voltage: 3500 V
- Capillary temperature: 270 °C
- Vaporizer temperature: 200 °C
- Sheath gas flow: 50 units
- Auxiliary gas flow: 5 units

- Detection of the analytes in SRM modality (Single Reaction Monitoring)
    - Transitions to be monitored:

Compound	**Precursor Ion (m/z)**	**Product Ions (m/z)**
ZEN	317 [M-H]-	175/131
ZEN-14GlcA	493.1 [M-H]-	317/175

After confirming the presence of the glucuronide, proceed with the purification step.

Purification of ZEN-glucuronide (Semipreparative HPLC)

Instrument: HPLC system? (It still needs to be developed!) equipped with a UV analyzer

Column: Synergi 4u Fusion-RP 80A (Size: 150 x 10.00 mm – 4 µm) (P/No. 00F-4424-N0)

Wavelength: λ = 265 nm

Volume of injection: 500 µL

- Collect separately the two major peaks (corresponding to ZEN-14GlcA and ZEN)
- Confirm the successful collection of ZEN-14GlcA by LC-MS/MS analysis (If purification was successful, you should no longer find ZEN in the purified fraction containing the glucuronide)
- Possibly, confirm the chemical structure of the collected glucuronide by NMR? (It still needs to be developed!)
- Determination of the purity: by calculating the percentage of the peak area in relation to total area of peaks.

9. CONCLUSION AND FUTURE PERSPECTIVES

Although *Alternaria* toxins could cause serious health effects, their occurrence in food is still not regulated due to the lack of toxicological and exposure data. Clarification of their toxicity is necessary to record a TDI and to determine their IARC-classification. In this way, their risk assessment will be more reliable and with a lower degree of uncertainty. The effects of chemical mixtures of *Alternaria* mycotoxins must hereby be taken into account, since the analysis of a single mycotoxin may not be enough to explain many cases. As there is not much known about co-mixtures of mycotoxins in general, this should be extended to other mycotoxins as well.

The best way to reduce the harms of mycotoxins is prevention through the management of risks in the first links of the food chain and through Good Agricultural Practices. Nevertheless, since it is not possible to completely free food from mycotoxin contamination, techniques as DBSs and VAMS could be promising for the determination and quantification of mycotoxins in future direct risk assessment.

With respect to the mycotoxin metabolism, further research into the *in vitro* metabolism via biological reactors (*i.e.* liver microsomes), is important. In this respect, it is valuable to optimize and validate the protocols of the Department of Food and Drug of the University of Parma in the future. Considering *in vivo* trials are complex, subsequent data on the toxicological pertinence of modified mycotoxins *in vivo* is still missing. Taking these facts and the fact that *in vitro* trials follow The Three Rs principles into account, more *in vitro* studies should be carried out in the future to avoid these problems. In this context, *in vitro* methods must be standardized internationally to compare the data and to upgrade our knowledge about mycotoxins and their metabolism. To facilitate these studies current efforts are being made to optimize *in vitro* techniques by introducing innovative technologies such as organ-on-a-chip (OOC).

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