universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Transfer, optimization, and in-house validation of a sensitive, stable-isotope assisted LC-MS/MS method for the quantification of mycotoxins“

verfasst von / submitted by

Patrick Windisch, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 659
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Lebensmittelchemie
Betreut von / Supervisor:	Assoc. Prof. Dipl.-Ing. Dr. Benedikt Warth, Bakk.techn.

Table of contents

Acknowledgments

First and foremost, I would like to thank Benedikt Warth, for providing me with the topic of, and giving me guidance during, this master's thesis, and also for offering me the opportunity to participate in an EU-wide ring trial. Next I would like to thank Dominik Braun for his outstanding co-supervision, during which he introduced me to the laboratory and, most importantly, to the mass spectrometer, and for always being available with help and advice when needed. Furthermore, I would like to thank the staff of the University of Vienna, employed at the Mass Spectrometry Center (MSC), for allowing me to use their equipment. Moreover, I would like to thank my parents Doris and Wolfgang and my girlfriend Melanie, who stood beside me all this time, sometimes had to endure my bad moods, and provided me with plenty of emotional support. Last but not least, I would like to say a big thank you to everyone, who accompanied through this stage of life, and which will now come to an end with this thesis.

Abstract

Mycotoxins are ubiquitous occurring food contaminants, which are considered to be harmful to humans. Some mycotoxins even have carcinogenic properties, calling for exposures of such to be as low as possible, since even miniscule amounts could be potentially harmful to humans and animals alike. In order to generate exposure data human biomonitoring (HBM) is used. In HBM studies biological samples such as urine are screened for the compounds of interest. Šarkanj *et al.* (2018, *Analytica Chimica Acta* 1019, pp. 84-92) developed a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the detection of multiple mycotoxins in urine. This was achieved through the combination of a general sample cleanup procedure, the use of isotopically labeled internal standards, enzymatic hydrolysis of conjugated toxins, and an optimized chromatographic separation. The aims of this master's thesis were the transfer of the aforementioned method to an in-house LC-MS/MS instrument, further optimization of the method, including the addition of various other analytes, the evaluation of the method's performance based on an in-house validation, and to test the method for a different biological matrix (serum). The results showed that a total of 22 mycotoxins and key metabolites were successfully validated in-house, which means that ten more mycotoxins and key metabolites were added to the previously published method. However, the optimized method was less sensitive compared to the method established by Šarkanj *et al.* (2018). In addition, some mycotoxins did not pass all in-house validation criteria, which had been validated previously (*e.g.* ochratoxin A). On the other hand, analysis of real-life urine samples showed that the optimized method is still sensitive enough to detect multiple mycotoxins, as shown in a proof-of-principle experiment including 15 unknown urine samples. Another aim of this thesis was to apply the optimized and validated LC-MS/MS method to serum. This preliminary screening revealed that the detection of analytes in the range of 15-500 ng/L is feasible. Although the optimized method is not as sensitive as the previously published method, it might still be valuable for the assessment of human mycotoxin exposure.

Zusammenfassung

Mykotoxine sind ubiquitär vorkommende Lebensmittelkontaminanten, welche als schädlich für den menschlichen Organismus eingestuft werden. Manche Mykotoxine besitzen karzinogene Eigenschaften, was bedingt, dass die Aufnahme derartiger Substanzen so gering wie möglich gehalten werden sollte, da bereits kleinste Mengen negative Auswirkungen auf Menschen und Tiere haben können. Zur Schaffung von Expositionsdaten wird Human-Biomonitoring (HBM) verwendet. Im Zuge von HBM-Studien werden biologische Proben, wie etwa Urin, auf unterschiedlichste Substanzen überprüft. Šarkanj *et al.* (2018, *Analytica Chimica Acta* 1019, SS. 84-92) haben eine sensitive Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS)-Methode zur Bestimmung mehrerer Mykotoxine in menschlichem Urin entwickelt. Die hohe Sensitivität wurde durch die Kombination einer allgemeinen Probenaufreinigungsprozedur mit der Verwendung von mit Stabilisotopen markierten internen Standards, einer enzymatischen Aufspaltung von konjugierten Mykotoxinen und optimierten Chromatographie-Bedingungen erreicht. Die Ziele dieser Masterarbeit waren der Transfer dieser Methode auf ein laborinternes LC-MS/MS-Instrument, die weitere Optimierung der Methode, die Erweiterung der Methode um weitere Analyten, die kritische Evaluierung der Methode anhand einer In-House-Validierung und der Versuch die Methode auf eine andere biologische Matrix (Blutserum) zu übertragen. Die Ergebnisse zeigten, dass insgesamt 22 Mykotoxine und Schlüssel-Metaboliten die In-House-Validierung erfolgreich überstanden haben, was bedeutet, dass die bereits publizierte Methode um zehn weitere Mykotoxine und Schlüssel-Metaboliten erweitert wurde. Jedoch erwies sich die optimierte Methode, verglichen mit der bereits etablierten Methode von Šarkanj *et al.* (2018) als weniger sensitiv. Zudem erfüllten manche Mykotoxine, die einer Validierung durch Šarkanj *et al.* (2018) standhielten, nicht alle In-House-Validierungskriterien (z.B. Ochratoxin A). Andererseits zeigte sich in einem Proof-of-Principle-Experiment, mit 15 unbekannten Urinproben, dass die optimierte Methode sensitiv genug ist, um damit verschiedenste Mykotoxine in, aus dem wirklichen Leben stammenden, Proben zu detektieren. Ein weiteres Ziel dieser Arbeit war die Anwendung der optimierten und validierten LC-MS/MS-Methode auf Blutserum. Dieses vorläufige Screening zeigte, dass die Analyten in einem Bereich von 15-500 ng/L detektiert werden können. Obwohl die optimierte Methode nicht so sensitiv ist wie die bereits publizierte Methode, könnte sie sich dennoch als wertvoll zur Abschätzung der humanen Mykotoxin-Exposition erweisen.

1 Introduction

The following subchapters provide an overview of the background of this thesis. First of all, various regulated and non-regulated mycotoxins, including their implications for human health, are discussed. In the succeeding subchapter the importance of human biomonitoring (HBM) is explained. The third subchapter deals with the possibilities and practical aspects of determining mycotoxin-exposure through the analysis of human samples. This subchapter is subsequently followed by the fourth and final subchapter, which highlights important method validation parameters.

1.1 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by filamentous microfungi (molds) of the phylum *Ascomycota* (Alshannaq and Yu, 2017). They are a group of chemically and structurally very diverse compounds and can lead to various adverse health effects in humans and animals alike (Slobodchikova *et al.*, 2019). So far over 300 different mycotoxins have been identified (Bellio *et al.*, 2016). These toxins can occur as a result of fungal growth and are frequently detected in contaminated foods and feeds, such as barley, maize, peanuts, rye, and wheat (Alshannaq and Yu, 2017; Sweeney and Dobson, 1998). Moreover, due to possible carry-overs from contaminated feed, they can also be detected in animal products, such as eggs, meat, and milk (Sweeney and Dobson, 1998). Most fungi, which cause frequent problematic contamination of comestible goods, belong to the fungal genera *Aspergillus*, *Fusarium*, and *Penicillium* (Marin *et al.*, 2013; Sweeney and Dobson, 1998). While *Aspergillus* and *Penicillium* species predominantly grow on food and feed during storage, *Fusarium* species more commonly infect crops during the plant's growth (Bennett and Klich, 2003). Recent studies have shown that 72-79 % of global feed samples and about 60-80 % of global food crops are contaminated with mycotoxins (Eskola *et al.*, 2019; Kovalsky *et al.*, 2016; Streit *et al.*, 2013). Furthermore, in over 20 % of analyzed food crops the detected concentrations were above the lower limits of the European Union's (EU's) guidance values for mycotoxins in food and feed. With continuously increasing global temperature, and therefore better growth conditions for certain fungi, these figures are expected to rise even higher (Eskola *et al.*, 2019).

When discussing diseases, which are caused by fungi, a distinction between the terms mycoses and mycotoxicoses has to be established. Direct growth of fungi on animal hosts leads to mycoses, whereas dermal, dietary, respiratory, and other forms of mycotoxin exposure lead to diseases, which are collectively referred to as mycotoxicoses. Symptoms of either can range from rather mild up to life-threatening (Bennett and Klich, 2003). The following subchapters of this thesis will depict the regulated and certain non-regulated mycotoxins, which, under certain circumstances, might cause mycotoxicoses.

1.1.1 Regulated mycotoxins

Even if many might pose a threat to human health, to date, only the following mycotoxins are actually regulated in the EU under Commission Regulation (EC) 1881/2006 and Commission Recommendation 2013/165/EU:

Table 1: Regulated and recommended maximum levels of mycotoxins for selected foodstuffs in the European Union based on Commission Regulation (EC) 1881/2006 and Commission Recommendation 2013/165/EU

Mycotoxins	Foodstuffs	Maximum levels (µg/kg)
Aflatoxins (sum of B_1, B_2, G_1 and G_2)	Groundnuts (peanuts), other oilseeds, and processed products thereof	4
	Maize and rice to be subjected to sorting or other physical treatment prior to human consumption	10
Aflatoxin M_1	Raw milk, heat-treated milk, and milk for milk-based products	0.05
	Infant formula and follow-up formula	0.025
Ochratoxin A	Unprocessed cereals	5
	Processed cereal-based foods	0.5
Patulin	Fruit juices	50
	Apple juice and solid apple products for infants	10
Deoxynivalenol	Unprocessed cereals	1,250
	Processed cereal-based foods	200
Zearalenone	Unprocessed cereals	100
	Processed cereal-based foods	20
Fumonisins (sum of B_1 and B_2)	Unprocessed maize	4,000
	Processed maize-based foods	200
T-2 and HT-2 toxin	Unprocessed cereals	200[a]
Citrinin	Food supplements based on rice fermented with red yeast (*Monascus purpureus*)	2,000

[a] guidance level, not recognized by law

Out of all regulated mycotoxins aflatoxins (AFs) present the highest risk to animal and human health. AFs are difuranocoumarins and produced by fungi of the genus *Aspergillus*, namely *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nominus*. The four main AFs are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Richard, 2007). They can commonly be detected in cereals (*e.g.* maize), nuts (*e.g.* Brazil nuts, pistachios), peanuts, and figs (Marin *et al.*, 2013). Although a carry-over might be possible, they are seldom found in meat and poultry (Armorini *et al.*, 2016). However, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) can be detected in milk and milk products derived from animals, which were fed with AF-contaminated feed during lactation. Across all AFs AFB_1 is the most toxic one. The International Agency for Research on Cancer (IARC) has classified it as a group 1 carcinogen, meaning that the compound is considered as carcinogenic to humans (IARC, 2012). This is due to the fact that AFs can be converted into their respective 8,9-epoxide forms, which are suspected to be responsible for the mutagenic properties of these toxins. The ultimately resulting 8,9-exoepoxides can bind to the N^7-guanine of the deoxyribonucleic acid (DNA). AFB_1 is especially prone to form such DNA-adducts. The liver is the predominant site of metabolism, which is why elevated exposure leads to hepatocellular carcinoma (Al-Jaal *et al.*, 2019; McMillan *et al.*, 2018). AFM_1's carcinogenic potential, on the other hand, is estimated to be about ten times lower than that of AFB_1. However, AFM_1 is classified as a group 2B carcinogen, meaning it is considered as possibly carcinogenic to humans (IARC, 2012).

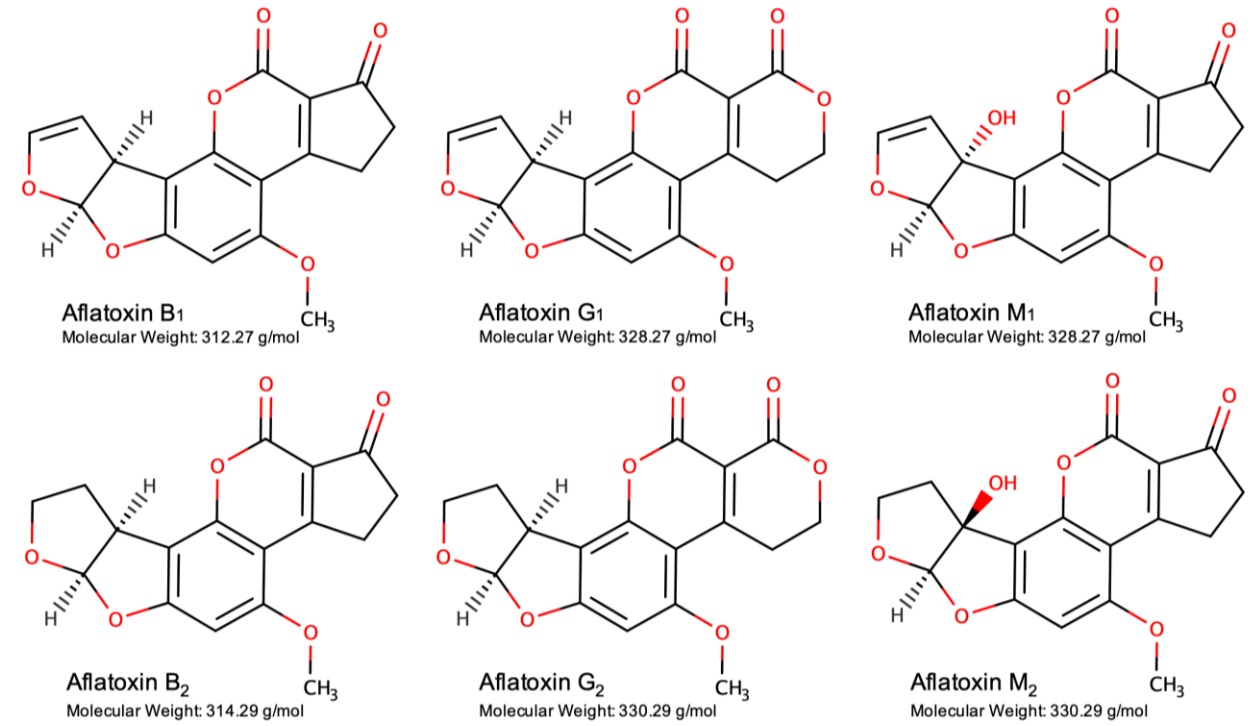


Figure 1: Regulated mycotoxins aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1, and non-regulated mycotoxin aflatoxin M_2

Ochratoxins (OTs) are toxic secondary metabolites produced by certain *Aspergillus* species, such as *Aspergillus ochraceus* and *Aspergillus niger*, and some *Penicillium* species, primarily by *Penicillium verrucosum*. There are three main types of OTs: ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC). While OTB and OTC are generally considered to be of lesser importance, OTA is said to be the most prominent one (Heussner and Bingle, 2015). It can commonly be detected in all kinds of cereals (*e.g.* barley, rye, wheat), making it a prominent contaminant in food and feed (Heussner and Bingle, 2015; Malir *et al.*, 2016). Different studies have shown that OTA might elicit various adverse health effects *in vitro* and *in vivo* alike. The key adverse health effect of an elevated exposure is believed to be carcinogenicity, which predominantly affects the kidneys (Dietrich *et al.*, 2005; Gekle *et al.*, 1998; Mally and Dekant, 2009). Various recent studies support the hypothesis that carcinogenesis is mediated via a non-DNA-reactive mode of action, involving mechanisms, which are primarily related to oxidative stress (Haighton *et al.*, 2012; Mally, 2012; Vettorazzi *et al.*, 2013). However, because of sufficient evidence suggesting the carcinogenicity of OTA in animal studies, but inadequate evidence supporting a carcinogenicity in humans, the IARC has categorized the compound as a group 2B carcinogen (IARC, 1993).

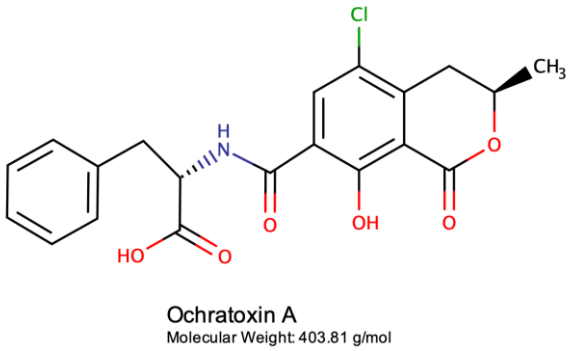


Figure 2: Regulated mycotoxin ochratoxin A

The mycotoxin patulin (PAT) is produced by over 60 different species of molds, from which *Penicillium expansum* is considered to be its main producer (Moake *et al.*, 2006). It can commonly be detected in apple and apple-based products (*e.g.* juice, jam). However, other fruits, such as pears and oranges, can also display various levels of contamination (Moukas *et al.*, 2008; Spadaro *et al.*, 2007; Torović *et al.*, 2017). Research has shown that over 20 % of the analyzed fruits and fruit products from European countries were tested positive for PAT. Although the detected concentrations were mostly below the maximum level for PAT and were, therefore, generally considered as safe, this means that the European population is frequently exposed to low doses of this toxin (Vidal *et al.*, 2019). Studies have shown that an elevated PAT exposure might lead to symptoms, such as intestinal inflammation and edema (Selmanoglu and Koçkaya, 2004; Singh *et al.*, 2018). Moreover, a chronic exposure was proven to be genotoxic,

immunotoxic, and teratogenic in various *in vitro* and *in vivo* studies (Alves *et al.*, 2000; Ciegler *et al.*, 1976; Llewellyn *et al.*, 1998). Yet, due to a lack of toxicological data regarding the adverse health effects of PAT in humans, the IARC has categorized the compound as a group 3 carcinogen, meaning that it is considered as not carcinogenic to humans (IARC, 1987).

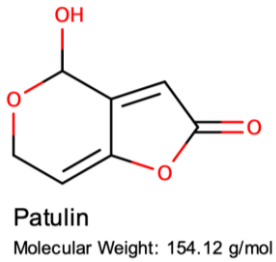


Figure 3: Regulated mycotoxin patulin

Trichothecenes are a big group of structurally related mycotoxins. Over 180 trichothecenes are known to date, which, depending on their functional groups, are divided into four types (A-D). However, only type A and B trichothecenes are relevant in feed and food. T-2 toxin (T-2) and HT-2 toxin (HT-2) are type A trichothecenes and produced by fungi of to the genus *Fusarium*. Major T-2- and HT-2-producing species are *Fusarium acuminatum*, *Fusarium nivale*, *Fusarium oxysporum*, *Fusarium poae*, *Fusarium solani*, and *Fusarium sporotrichioides*. T-2 and HT-2 are commonly detected in cereals, such as corn, rice, and wheat (Ok *et al.*, 2013). T-2 is one of the most toxic members of the trichothecene family, while also being the only compound, which can be absorbed directly through the skin (Adhikari *et al.*, 2017). Research suggests that it elicits cytotoxic and genotoxic properties (Horvatovich *et al.*, 2013; Shinozuka *et al.*, 1998). The modes of action are mainly related to inhibition of protein synthesis and oxidative stress. Moreover, it is also suspected to impair other enzymes such as the DNA polymerases (Adhikari *et al.*, 2017). In humans T-2-poisoning can lead to alimentary toxic aleukia, which is characterized by diarrhea, hemorrhagic inflammation, leukopenia, nausea, vomiting, and, in severe cases, even death (Lutsky and Mor, 1981). However, due to a lack of evidence regarding the carcinogenicity of T-2, the IARC has categorized the compound as a group 3 carcinogen (IARC, 1993).

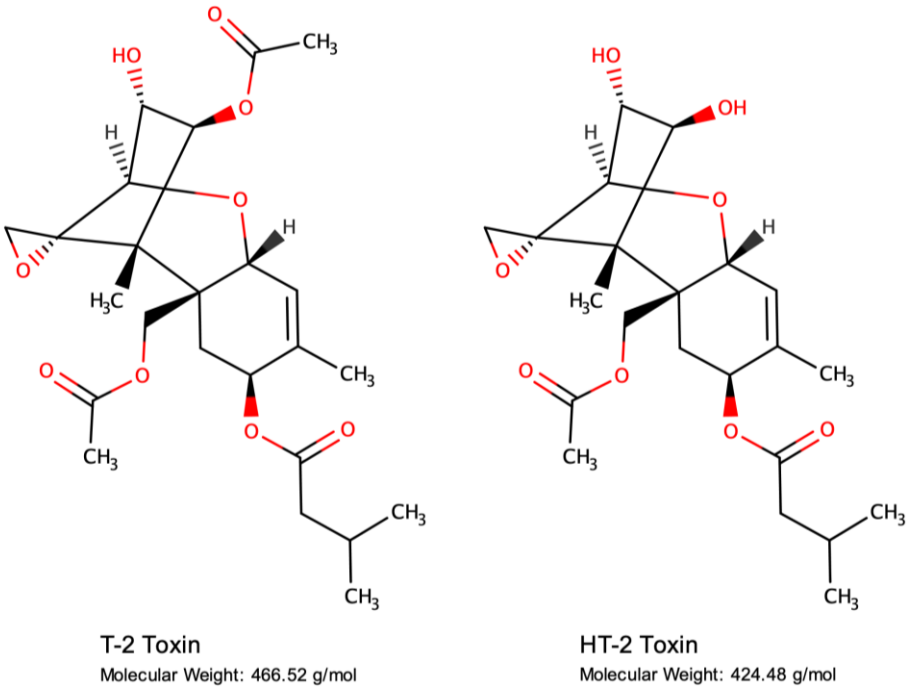


Figure 4: For regulation recommended, but not yet regulated, mycotoxins T-2 toxin and HT-2 toxin

Nivalenol (NIV), deoxynivalenol (DON), and fusarenon-X (FX) are all classified as type B trichothecenes (Aupanun *et al.*, 2017). DON is mainly produced by fungi of the genus *Fusarium*, especially *Fusarium graminearum* and *Fusarium culmorum (Zheng et al., 2017)*. It is the key representative for type B trichothecenes and can commonly be detected in food and feed, such as barley, corn, and wheat (Creppy, 2002; Malachová *et al.*, 2011; Zheng *et al.*, 2017). Research has shown that alongside DON and NIV contamination of crops, FX frequently occurs (IARC, 1993). Acute DON exposure is associated with two characteristic adverse health effects, which are anorexia and vomiting. Therefore, DON is also known as vomitoxin (Creppy, 2002). In extremely high doses, which are unlikely of ever being found in food and feed, however, it can even lead to a shock-like death (Sobrova *et al.*, 2010). Although it is one of the most well-studied trichothecenes, it is also among the least toxic ones. Due to the lack of evidence on the carcinogenicity of DON, the IARC has categorized the compound as a group 3 carcinogen (IARC, 1993).

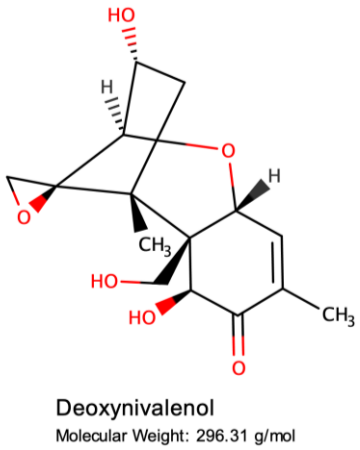


Figure 5: Regulated mycotoxin deoxynivalenol

Another major *Fusarium* mycotoxin is the resorcyclic acid lactone (RAL) zearalenone (ZEN) (D'Mello *et al.*, 1999; Dänicke and Winkler, 2015). It is mainly produced by *Fusarium cerealis*, *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium graminearum, and Fusarium semitectum* and can commonly be detected in cereals, such as barley, corn, oats, sorghum, rice, and wheat (Zinedine *et al.*, 2007). Although ZEN is the most abundant RAL in food and feed, others might be present as well. This includes key metabolites of ZEN, which are α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zearalanone (ZAN). Since ZEN is the most prominent RAL, its toxicity has been extensively studied (Dänicke and Winkler, 2015). It shares structural similarities with the human sex hormone 17β-estradiol (E_2). Therefore, it can bind to the estrogen receptors and is suspected to induce receptor-mediated gene expression (Vejdovszky *et al.*, 2017b). Following high chronic exposures E_2 was proven to lead to tumors *in vivo* (Andrade *et al.*, 2015). Although the estrogenic potential of ZEN is about 100-fold lower than that of E_2, it might still play an important role in the development of various estrogen-dependent cancers (*e.g.* breast cancer, prostate cancer) in humans (Ahamed *et al.*, 2001; Kowalska *et al.*, 2018; Vejdovszky *et al.*, 2017a). ZEN is also suspected to lead to abnormal growth rates and precocious puberty in girls (Massart *et al.*, 2008). Moreover, recently ZEN was proven to cross the human placental barrier, after which it can cause hormonal imbalances, that negatively affect the fetus during critical stages of development (Warth *et al.*, 2019). However, due to inadequate evidence regarding its carcinogenicity, the IARC has categorized it as a group 3 carcinogen (IARC, 1993).

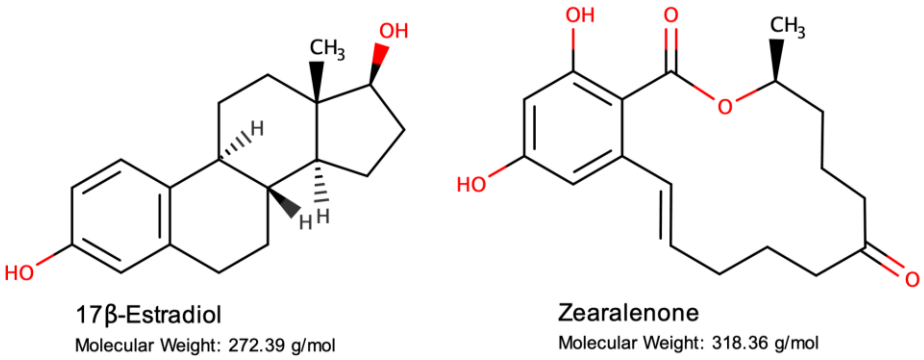


Figure 6: Human sex hormone 17β-estradiol and regulated mycotoxin zearalenone

Fumonisins are mycotoxins, which are also predominantly produced by fungi of the genus *Fusarium*. Main fumonisin-producing species are considered to be *Fusarium moniliforme* and *Fusarium proliferatum* (EFSA, 2005). Today there exist about 30 known fumonisin-analogues, which are divided into four groups: fumonisins A, B, C, and P (Ponce-García *et al.*, 2018). Out of all fumonisin analogues fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the most abundant ones (Rheeder *et al.*, 2002). They are frequently detected in crops, especially in corn and wheat, with FB_1 being the most prevalent one, and accounting for 70-80 % of total fumonisin B contamination (Marin *et al.*, 2013; Rheeder *et al.*, 2002). FB_1 is also presumed to be the most toxicologically relevant one. The main mode of action of fumonisins is suggested to be the disruption of the sphingolipid metabolism. Due to structural similarities with the sphingoid bases sphinganine and sphingosine they can inhibit the enzyme ceramide synthase, which disrupts the *de novo* biosynthesis of ceramide, and leads to an accumulation of sphinganine and sphingosine in the organism (Merrill *et al.*, 2001; Riley and Voss, 2006). These excess sphingolipids can subsequently induce cytotoxic, growth inhibitory, and proapoptotic effects (Merrill *et al.*, 2001). FB_1 was proven toxic to various organs of different animals, including kidney and liver (Voss *et al.*, 2007). Furthermore, fumonisins are suspected risk factors for liver and esophageal tumors in highly exposed individuals (Marasas, 2001). Therefore, the IARC has categorized both, FB_1 and FB_2, as group 2B carcinogens (IARC, 2002).

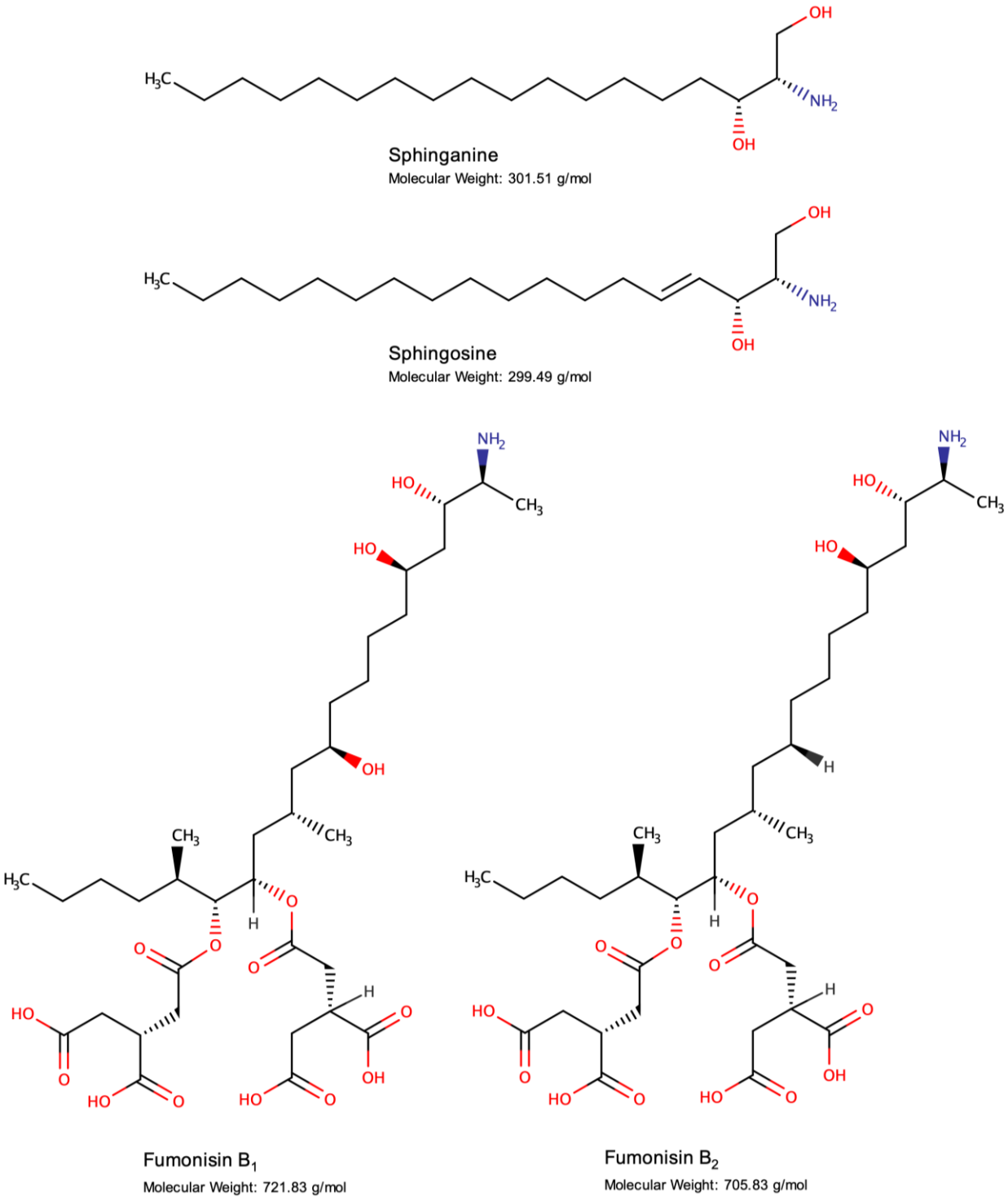


Figure 7: Sphingolipids sphinganine and sphingosine, and regulated mycotoxins Fumonisin B_1 and Fumonisin B_2

The polyketide mycotoxin citrinin (CIT) is produced by fungi of the genera *Aspergillus*, *Monascus*, and *Penicillium* (EFSA, 2012). Main CIT-producing fungal species are considered to be *Aspergillus oryzae*, *Penicillium citrinum*, and *Monascus purpureus* (Li *et al.*, 2017; Liang *et al.*, 2017; Sakai *et al.*, 2008). It can frequently be detected in cereals, such as barley, corn, rice, and wheat, but also in other food commodities, like fruits, vegetables, herbs, and spices (EFSA, 2012). CIT is a cytotoxic compound and can cause severe changes in normal mitochondria, ultimately leading to swelling and cell death (Chagas *et al.*, 1994). In animal studies it was shown to evoke cardiotoxic, hepatotoxic, and nephrotoxic effects (Krejci *et al.*, 1996; Rašić *et al.*, 2018; Wu *et al.*, 2013). There are numerous studies, which demonstrate its genotoxic effects *in vitro*. In one of these studies, for instance, CIT-treatment lead to increased chromosomal abnormalities, chromosome aberrations, and DNA-fragmentation (Bouslimi *et al.*, 2008; Knasmüller *et al.*, 2004; Liu *et al.*, 2003). However, due to a lack of evidence supporting its carcinogenic effects, the IARC has categorized the compound as a group 3 carcinogen (IARC, 1987).

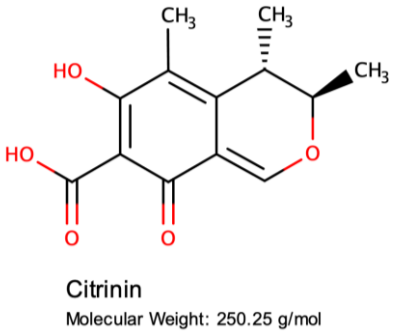


Figure 8: Regulated mycotoxin citrinin

1.1.2 Emerging and non-regulated mycotoxins

Although the aforementioned mycotoxins are regulated in the EU, there are many so-called emerging mycotoxins, which currently lack regulation. One of these unregulated mycotoxins is ochratoxin α (OTα). It is a major metabolite of OTA and formed after enzymatic hydrolysis of OTA through gut microorganisms. While OTα is less toxicologically potent than its parent compound, its monitoring might still be required in order to enable analysts to reach a conclusion regarding the total OTA-exposure of subjects (Kupski *et al.*, 2016). Although contamination levels are usually considered to be low, OTB and OTC can co-occur alongside OTA in food and feed. Research suggests that OTC is toxicologically more potent than OTB, but neither seems to be as potent as OTA. However, their contribution to OTA's toxicity is still unclear (Heussner and Bingle, 2015).

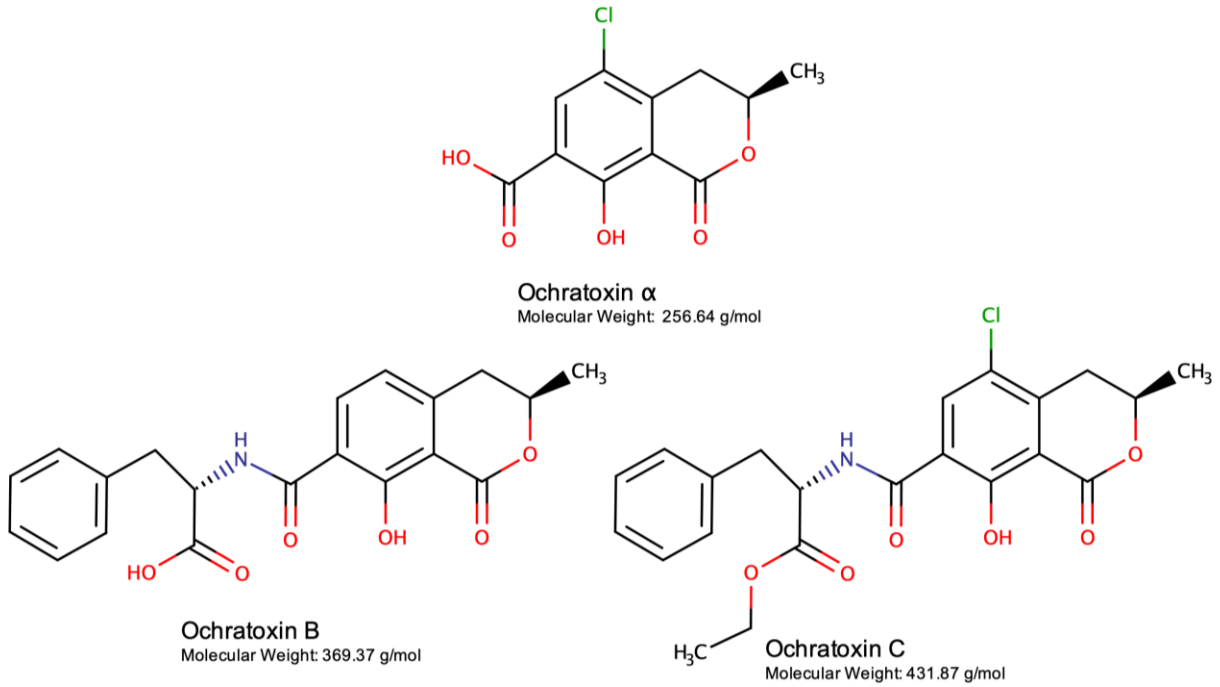


Figure 9: Non-regulated mycotoxins ochratoxin α, ochratoxin B, and ochratoxin C

Many AFs are currently regulated in the EU, however, others are not. One of these not yet regulated AFs is AFM_2, which is the hydroxy-metabolite of AFB_2. It can commonly be detected alongside AFM_1 in dairy products, such as milk and cheese (Bellio *et al.*, 2016; Pietri *et al.*, 2003). Moreover, AFM_2 was shown to be converted into AFM_1, making it a mycotoxin of toxicological concern (Dutton *et al.*, 1985). Like AFM_1, aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1) are also hydroxylated metabolites of AFB_1. While AFM_1 and AFQ_1 are structural isomers, AFP_1 can be seen as demethylated form of AFB_1 (Franco *et al.*, 1998). AFQ_1 is significantly less mutagenic than AFB_1 and other AFB_1-metabolites, making it an important detoxification biomarker (Coulombe *et al.*, 1982). Moreover, research suggests that the presence of urinary AFB_1, its metabolites AFM_1 or AFP_1, or the AFB_1-DNA-adduct aflatoxin-N^7-guanine (AFB-N^7-gua) can lead

to a four times higher risk of hepatocellular carcinoma, in comparison to individuals where these urinary mycotoxins are not detected (Qian *et al.*, 1994). Formation of AFB-N^7-gua is the most important step of AF-induced liver carcinogenesis, which makes it a powerful human biomarker (Groopman *et al.*, 1994). Another major AFB_1 metabolite is aflatoxicol (AFL). Although AFL was shown to be 18 times less toxic than AFB_1, AFL formation does hardly decrease its mutagenic potential, since AFB_1 and AFL can build the same DNA adduct (Bailey *et al.*, 1994; Coulombe *et al.*, 1982; Karabulut *et al.*, 2014; Theumer *et al.*, 2018). Furthermore, due to the fact that AFL can be reconverted into AFB_1, it can also act as an AFB_1 reservoir in the organism (Carvajal *et al.*, 2003). AFL was also shown to be the only AFB_1-metabolite capable of permeating the human placenta barrier (Partanen *et al.*, 2010). All of these circumstances make AFL a compound of strong analytic interest.

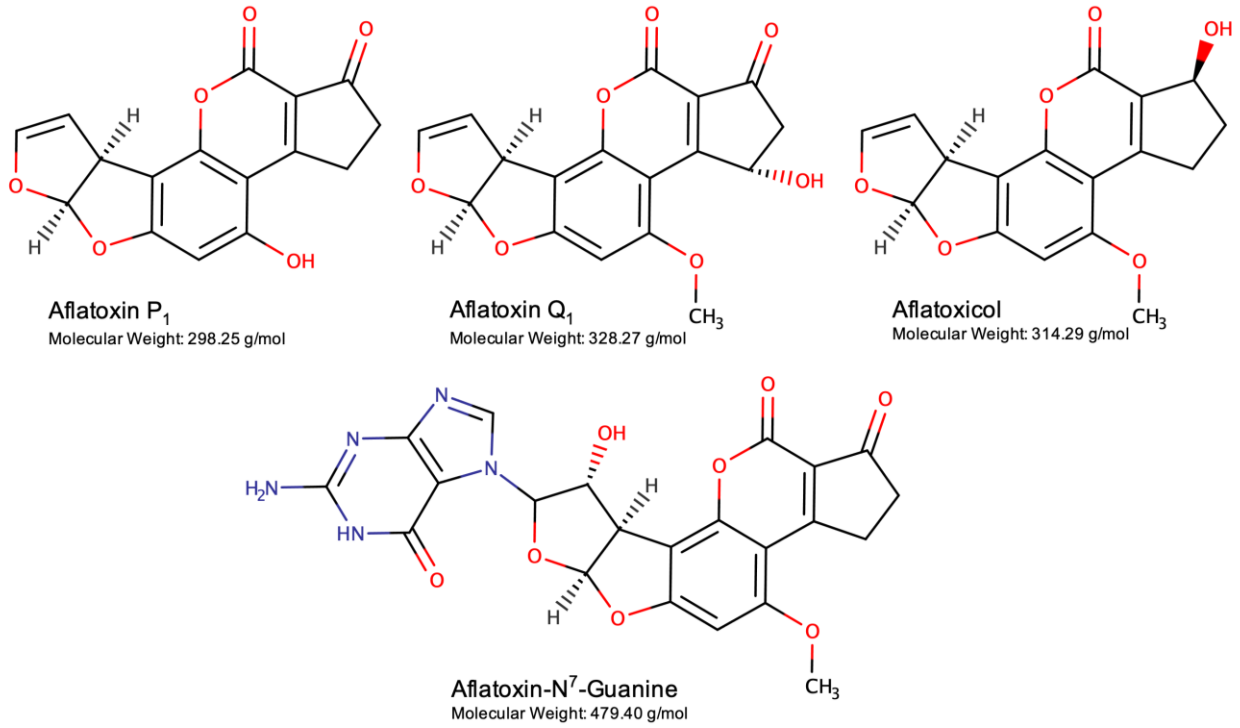


Figure 10: Non-regulated mycotoxins aflatoxin P_1, aflatoxin Q_1, aflatoxicol, and DNA-adduct aflatoxin-N^7-guanine

When discussing AFs one should not fail to mention the mycotoxin sterigmatocystin (STC), which is an AF precursor, and structurally closely related to AFB_1 (Gruber-Dorninger *et al.*, 2017). STC also possesses toxic properties. Various *in vitro* studies have shown that it elicits cytotoxic and genotoxic effects (Gao *et al.*, 2015; Liu *et al.*, 2014; Zouaoui *et al.*, 2016). One study has shown a significant increase in DNA strand breaks and intracellular markers for oxidative stress after STC exposure (Gao *et al.*, 2015). Moreover, several *in vivo* studies have demonstrated its toxic properties in various animals, which are similar to those of AFB_1. However, the compound's acute toxicity is considerably lower than that of AFB_1 (Abdel-Wahhab *et al.*, 2005; Kovalenko *et al.*,

2011; Sreemannarayana *et al.*, 1986). Based on the previously available data, the IARC has classified STC as a group 2B carcinogen (IARC, 1987). Considering that there are mycotoxins, which seem to be less carcinogenic than STC, but still regulated in the EU (*e.g.* CIT), this makes the compound worth monitoring (Ostry *et al.*, 2017).

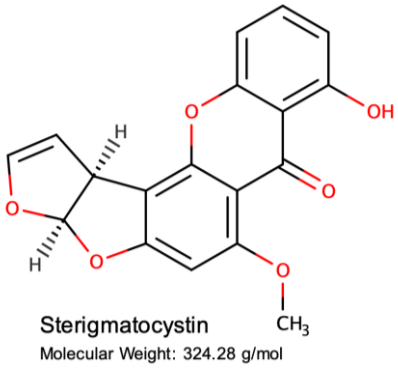


Figure 11: Non-regulated mycotoxin sterigmatocystin

Another prominent non-regulated mycotoxin is NIV. Although NIV does not occur as frequently in food and feed as DON, its toxic properties are about twice as high (IARC, 1993). Studies also suggest that NIV, like other trichothecenes, induces inhibitory effects on the immune system, protein synthesis, and DNA synthesis (Choi *et al.*, 2000; Sundstøl Eriksen *et al.*, 2004; Ueno *et al.*, 1968). However, data regarding the toxicity of NIV is still scarce (Bryła *et al.*, 2018). Another important emerging trichothecene is deepoxy-deoxynivalenol (DOM-1), which is a metabolite of DON. A recent study has suggested that the toxicity of DOM-1 is significantly lower than that of DON. This is partly attributed to the molecule's loss of its epoxide moiety. Yet, it was also shown that DOM-1 still retains some of DON's immune-modulatory effects (Pierron *et al.*, 2018). With the usage of certain bacteria of the *Coriobacteriaceae* family as feed additives, which can transform DON into DOM-1, the compound has recently gained relevance in feed (EFSA, 2013).

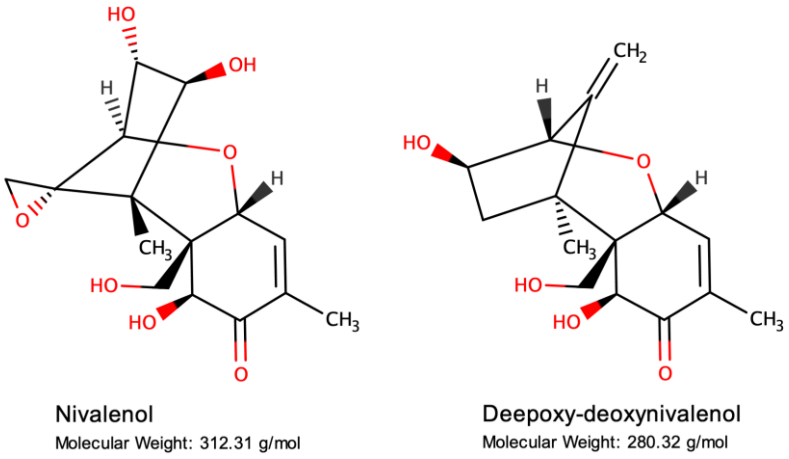


Figure 12: Non-regulated mycotoxins nivalenol and deepoxy-deoxynivalenol

Dihydrocitrinone (DHC) is a putative mycotoxin degradation product, which is formed after hydroxylation of CIT. It can be commonly detected in human blood and urine (Ali *et al.*, 2015; Degen *et al.*, 2018; Osteresch *et al.*, 2017). The genotoxic potential of DHC is about ten times lower than that of CIT, which is the reason why the metabolization is generally considered as a detoxification step (Föllmann *et al.*, 2014). However, in order to fully determine a subject's CIT exposure, individual DHC concentrations should be recorded as well (Degen *et al.*, 2018).

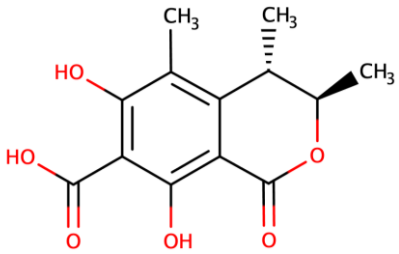


Figure 13: Non-regulated mycotoxin dihydrocitrinone

As it was already discussed previously ZAN, α-ZAL, β-ZAL, α-ZEL, and β-ZEL are major metabolites of ZEN. Studies have shown that, like ZEN, these compounds might elicit estrogenic properties as well (Frizzell *et al.*, 2011; Shier *et al.*, 2001; Vejdovszky *et al.*, 2017a). Out of all of these metabolites α-ZEL is suspected to be the most potent one, possessing an estrogenic effect, which is about 70 times higher than that its parent compound ZEN, and only slightly less potent than that of E_2 itself (Frizzell *et al.*, 2011; Vejdovszky *et al.*, 2017a). Apart from that, synthetic α-ZAL is marketed as an anabolic agent in livestock. While the EU has banned the use of such substances in food-producing animals since 1988, α-ZAL's application is still permitted in some countries around the world (*e.g.* United States of America (USA)) (Lega *et al.*, 2017).

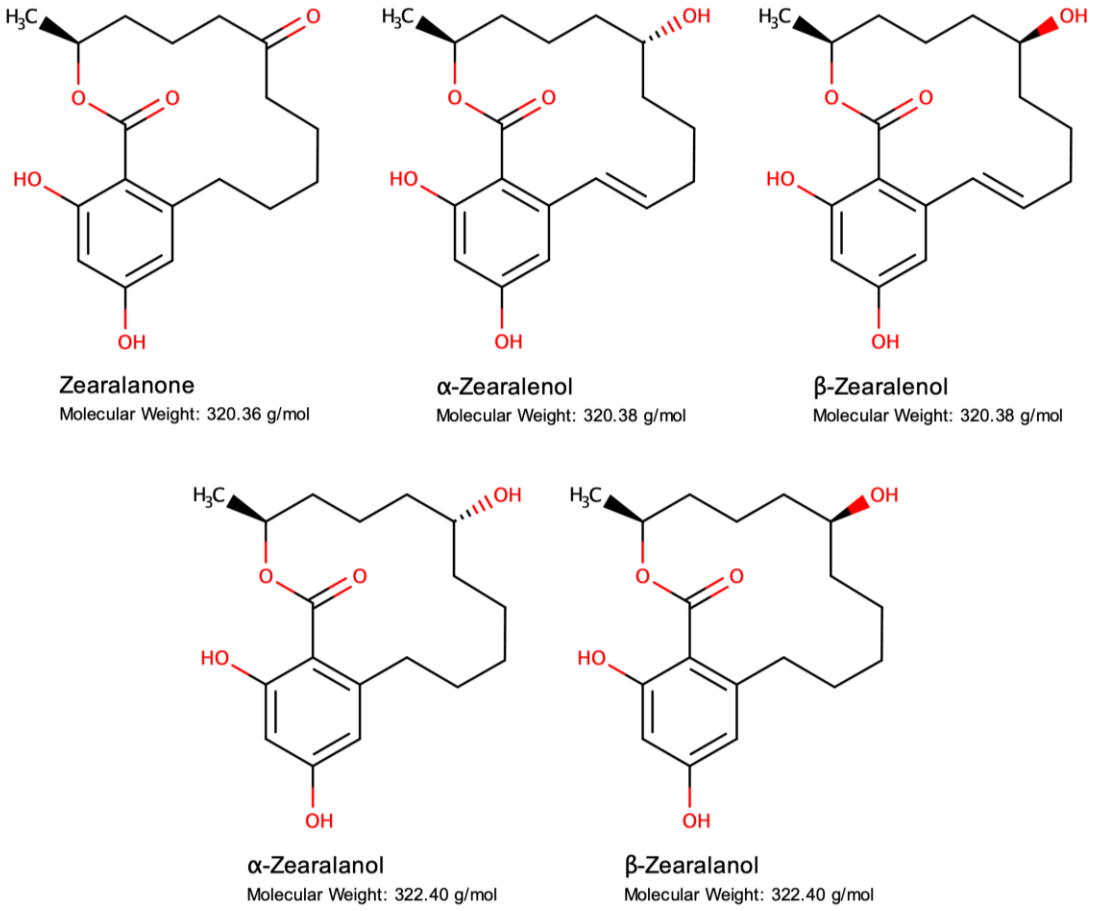


Figure 14: Non-regulated mycotoxins zearalanone, α-zearalanol, β-zearalanol, α-zearalenol, and β-zearalenol

There are also many non-regulated mycotoxins, which belong to the fungal genus *Alternaria*. Examples of such are alternariol (AOH), alternariol monomethyl ether (AME), and tentoxin (TEN), which are mainly produced by *Alternaria alternata* (EFSA, 2011). To date, still little is known about the toxicity of these compounds. However, recent studies have shown that AOH and AME elicit DNA-damaging effects, and might therefore induce tumors (Pahlke *et al.*, 2016; Tiessen *et al.*, 2017). Another study has shown that AOH and, to a lesser extent, AME potentially evoke immunotoxic effects as well (Grover and Lawrence, 2017). The chemical structure of TEN, on the other hand, differs greatly from the AOH and AME, which results in vastly different toxicological effects. In a recent *in vitro* study with HepaRG cells, TEN treatment led to an increased expression of several genes, that play a role in the induction of necrosis (Hessel-Pras *et al.*, 2019).

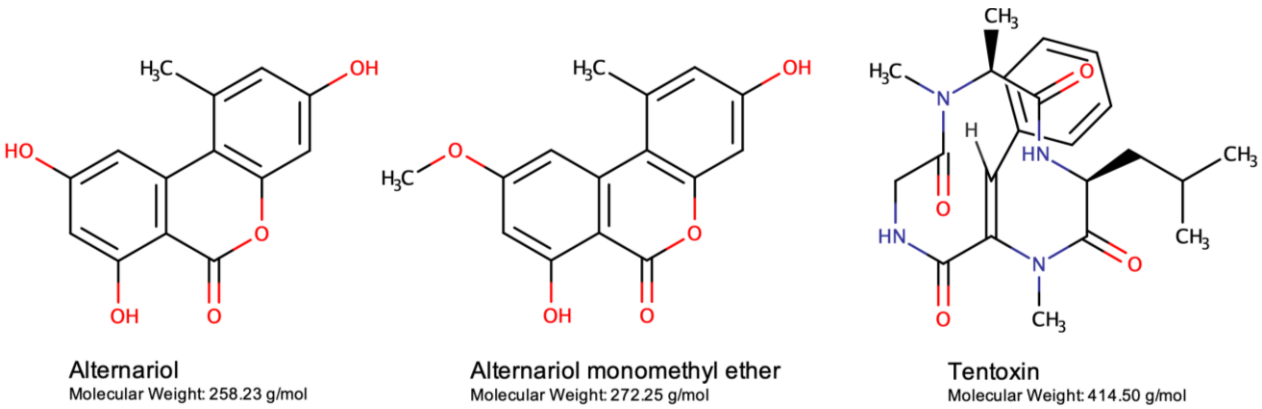


Figure 15: Non-regulated mycotoxins alternariol, alternariol monomethyl ether, and tentoxin

A big group of mycotoxins, which are not regulated to date, are the cyclic hexadepsipeptides. Members of this group are the enniatins (ENNs) and beauvericin (BEA) (Gruber-Dorninger *et al.*, 2017). BEA and the ENNs, especially enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), and enniatin B_1 ($ENNB_1$) can frequently be detected in food and feed (EFSA, 2014b; Tolosa *et al.*, 2019). The toxicity of these compounds is related to their ionophoric properties. Although they are toxic *in vitro* most *in vivo* data suggests low or no acute toxicity at all. Chronic toxicity data, however, is still lacking (Gruber-Dorninger *et al.*, 2017). A recent study has shown that these compounds can be frequently detected in breast milk (Braun *et al.*, 2020a). The follow-up study confirmed the first study's findings and also found little variation in concentration over an elongated time period. This suggests a low constant background exposure of individuals to these mycotoxins, beginning with birth (Braun *et al.*, 2020b).

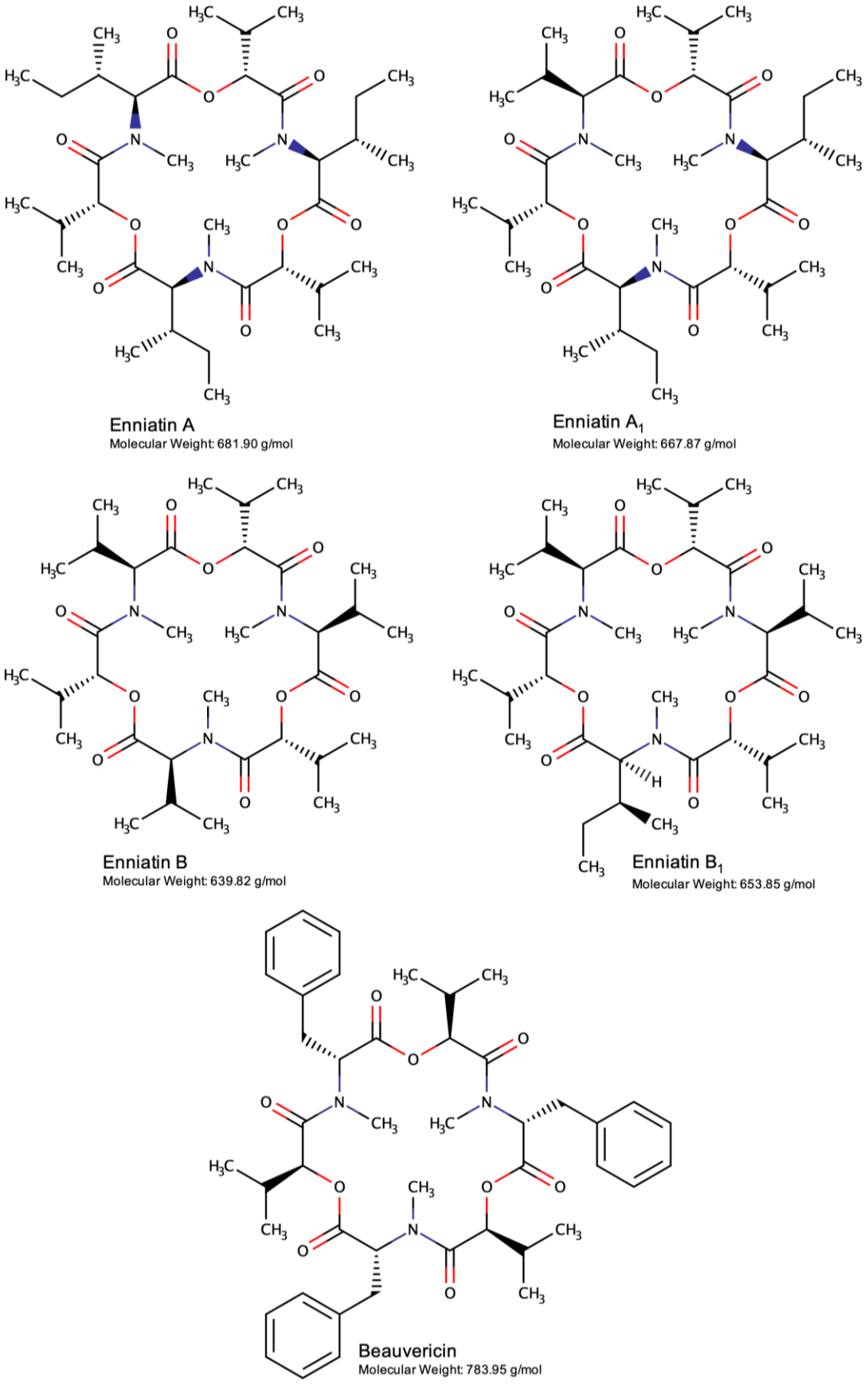


Figure 16: Non-regulated mycotoxins enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin

1.2 Human biomonitoring

1.2.1 General aspects

The aims of HBM are to determine human exposure to certain compounds by monitoring said compounds and/or their metabolites in human biological samples, mainly blood, breast milk, hair, saliva, and/or urine. HBM is a helpful tool in the assessment of total individual exposure to potentially toxic compounds. The thereby acquired data can be used to investigate how exposure to certain substances might ultimately affect human health, or assist in identifying risk groups for various diseases (Ali and Degen, 2019).

1.2.2 Human biomonitoring of mycotoxins

Humans mainly take up mycotoxins through oral ingestion. The total mycotoxin exposure of individuals, however, can be more diverse. One of the non-dietary mycotoxin exposure routes represents the inhalation of dust particles at the workplace, which might oftentimes be the case for mill workers. Therefore, mycotoxin exposure of occupationally exposed individuals can significantly differ from non-occupationally exposed individuals, resulting in a higher risk for certain mycotoxin-associated diseases (Ferri *et al.*, 2017; Viegas *et al.*, 2013; Viegas *et al.*, 2012). HBM of mycotoxins is a helpful tool in identifying these so-called high risk groups and HBM data is building an important foundation for the implementation of mycotoxin mitigation strategies (Ali and Degen, 2019).

The preferred matrix for HBM is urine, which is mainly due to the quick, easy, and non-invasive nature of the sampling procedure. Recent innovations in liquid chromatography tandem mass spectrometry (LC-MS/MS)-based analytical methods have made the simultaneous biomonitoring of multiple mycotoxins in urine more accessible (Ali and Degen, 2019; Braun *et al.*, 2020a; Šarkanj *et al.*, 2018).

1.3 Analytical determination of mycotoxins in urine

The ultra-sensitive analytical determination of multiple mycotoxins in biological matrices can be very challenging. Especially matrix interferences make ultra-trace analyses difficult. Here, sophisticated cleanup strategies were proven essential in order to create LC-MS/MS methods with best sensitivities (Braun *et al.*, 2020a; Šarkanj *et al.*, 2018). There are various sample treatment methods, which can be used in order to get rid of interfering matrix components and enable a more sensitive determination of mycotoxins in difficult biological matrices. The analytes of interest can, for instance, be extracted by means of salting-out assisted liquid-liquid extraction (SALLE). With SALLE analyte extraction is based on the compounds solubility in two different immiscible solvents, assisted by salting out, which allows efficient analyte extraction from aqueous samples (Mariño-Repizo *et al.*, 2018). Another important pretreatment procedure is the

mycotoxin extraction via immunoaffinity columns (IAC). IACs are packed with immobilized antibodies, which can selectively trap the target analytes. After matrix components are removed, the target analytes are eluted, and therefore, a clean extract, containing the compounds of interest, is obtained (Uchigashima *et al.*, 2012). In recent years, the so-called “dilute and shoot” approach was frequently applied, in which the sample is diluted with an extraction solvent, in order to decrease matrix effects (da Silva *et al.*, 2019). However, this method is usually lacking the sensitivity required for HBM studies (Escrivá *et al.*, 2017).

1.3.1 Solid phase extraction

Another cleanup method, called solid phase extraction (SPE), was proven as particularly useful for the extraction of multiple mycotoxins in HBM studies (Braun *et al.*, 2020a; Šarkanj *et al.*, 2018). SPE is based on chromatographic principles and involves the use of a sorbent material (stationary phase), which is usually packed into a column or supported by disks, the sample extract, and an elution solvent. There are two different strategies, which can be applied for SPE. The most common one is the “bind and elute” cleanup, where the sample extract is loaded onto the stationary phase, in order to trap the analytes of interest. These analytes are later eluted with the help of a strong solvent. The other method is based on the “trapping” strategy. Here, matrix contaminants are trapped by the sorbent material, while analytes of interest are not retained. Therefore, SPE can be used for two main purposes in order to ultimately enhance sensitivity. On the one hand for sample cleanup and, on the other hand, for preconcentration of the analytes of interest (Huertas-Pérez *et al.*, 2017).

The main steps involved in a SPE procedure are:

1. Conditioning/Equilibration of the cartridge
2. Loading of the sample
3. Washing step
4. Elution of the fractions (Waters, 2019)

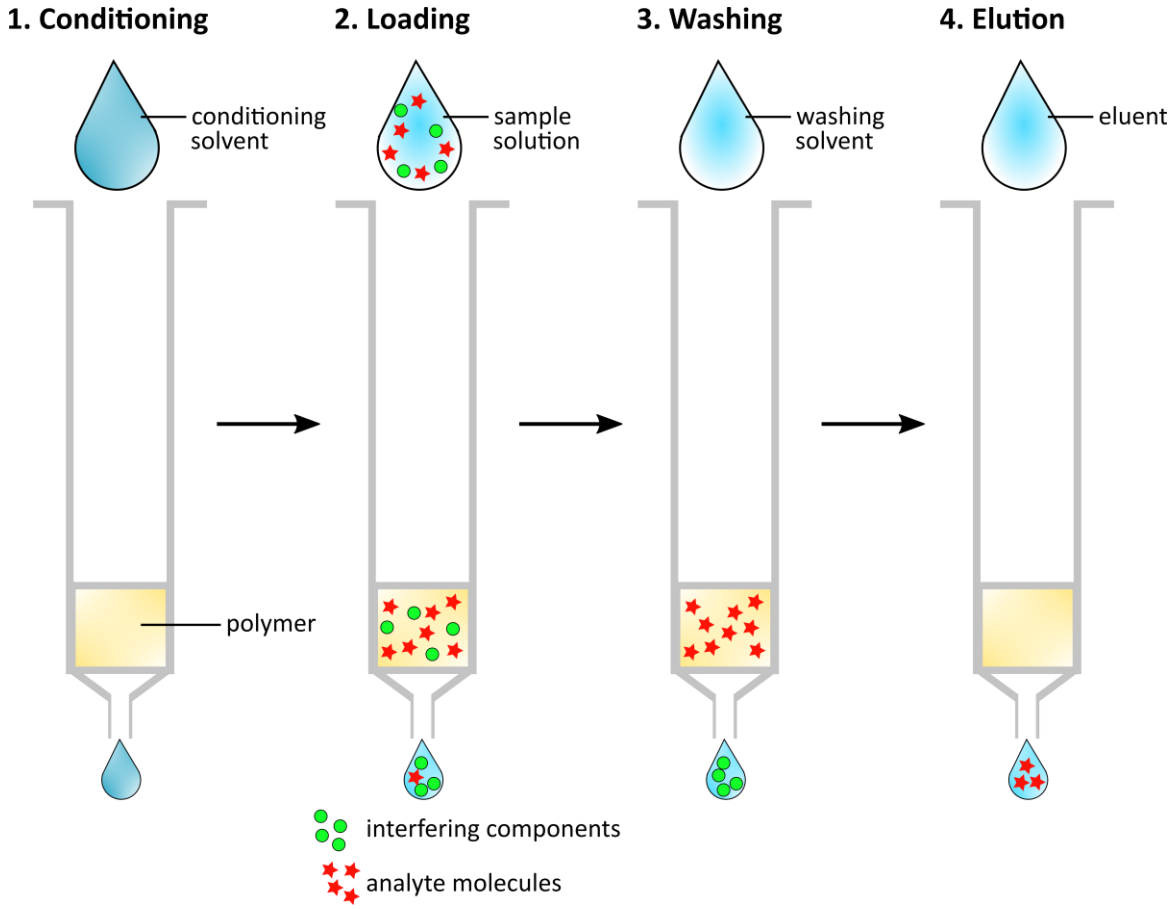


Figure 17: Illustration of a “bind and elute” solid phase extraction cleanup procedure

The classical SPE is usually performed in cartridges. Here the stationary phase is supported by disks. There are many different sorbents one can choose from (Huertas-Pérez *et al.*, 2017). One of the most widely used sorbent materials are hydrophilic lipophilic balance (HLB) polymers, which contain polar (*e.g.* pyrrolidone) as well as non-polar (*e.g.* benzene and aliphatic chains) groups. The sorbent can therefore interact with both, non-polar and polar, organic molecules (Brousmiche *et al.*, 2008). HLB polymers are commonly used for the analysis of environmental contaminants (Jeong *et al.*, 2017). Recently, SPE was proven effective in getting rid of unwanted matrix molecules prior to chromatographic separation and subsequent mass spectrometric determination of multiple mycotoxins in human biological samples (Braun *et al.*, 2020a; Šarkanj *et al.*, 2018).

1.3.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is one of the many chromatographic techniques for the separation and analysis of chemical mixtures. Almost universal applicability, incredible assay precision, and a wide range of commercially available columns and other equipment are the main reasons for HPLC oftentimes being superior to other separation techniques (Snyder *et al.*, 2010).

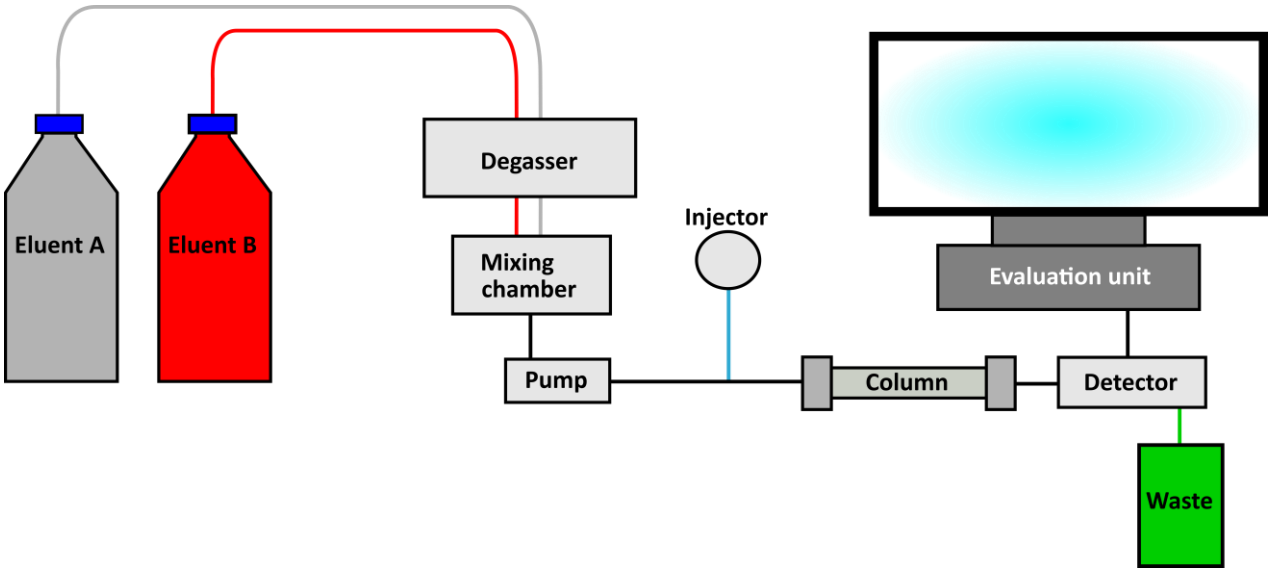


Figure 18: General schematic of a high-performance liquid chromatography system

The chromatographic process starts with the solvent (usually referred to as mobile phase or eluent) being pumped from the solvent reservoir in the direction of the detector. At the injection valve the sample is injected into the solvent flow. The eluent transports the sample to the column, where the chromatographic separation takes place. It is based on the affinity of the different analytes to the functional groups of the solid phase (usually referred to as stationary phase) and the interaction between the analytes with the mobile phase. Depending on that the analytes are separated and gradually reach the detector. The stronger the intermolecular interactions between an analyte and the stationary phase of the column the later that analyte arrives at the detector. Thereby resulting detector signals are then digitally plotted against time in order to generate the chromatogram (Snyder *et al.*, 2010).

There are many different experimental conditions, such as the stationary phase, flow rate, solvent, and temperature, which affect the chromatographic separation. The fundamental nature of the separation is mainly determined by the choice of stationary phase. It is a cylindrical tube, which is usually filled with small spherical particles (generally 1.5 to 5 µm in diameter) of porous silica. The inside of the pores is covered with the stationary phase. Reversed-phase chromatography (RPC) is the most widely used mode for sample analysis. It utilizes a nonpolar column in combination with a polar mobile phase (Snyder *et al.*, 2010).

Table 2: Different important high-performance liquid chromatography separation techniques according to Snyder *et al.* (2010)

Chromatographic Mode	Comment
Reversed-phase chromatography (RPC)	Nonpolar column (*e.g.* C_{18}) and polar mixture of water and organic solvent (*e.g.* acetonitrile or methanol) as mobile phase; RPC is the most widely used mode, especially for water-soluble analytes.
Normal-phase chromatography (NPC)	Polar column (*e.g.* unbonded silica) and mixture of less polar solvents (*e.g.* hexane with dichloromethane) as mobile phase; NPC is mainly used for water-insoluble samples and the separation of isomers.
Non-aqueous reversed-phase chromatography (NARP)	Nonpolar column (*e.g.* C_{18}) and mixture of organic solvents (*e.g.* acetonitrile with dichloromethane) as mobile phase; NARP is used for very non-polar analytes.
Hydrophilic interaction chromatography (HILIC)	Polar column (*e.g.* amide-bonded phase) and mixture of water with organic solvent (*e.g.* acetonitrile) as mobile phase; HILIC is used for highly polar analytes.
Ion-exchange chromatography (IEC)	Column contains charged groups, which can bind analyte ions of the opposite charge and usually an aqueous solution of salt with buffer as mobile phase; IEC is used in order to separate ionizable analytes (*e.g.* acids, bases, peptides, proteins).
Ion-pair chromatography (IPC)	RPC conditions, except that ion-pair reagent is added to mobile phase for interaction with sample ions of opposite charge; IPC is used for acids or bases, which are weakly retained in RPC.
Size-exclusion chromatography (SEC)	Inert column and either aqueous or organic mobile phase; SEC allows to separate analytes on the basis of molecular weight and is mainly used for large biomolecules or synthetic polymers.

Further factors, which play an important role for the chromatographic separation, are choice of solvent and temperature. In RPC the mobile phase usually is a mixture of water (H_2O) or aqueous buffer (eluent A) and an organic solvent (eluent B), such as acetonitrile (ACN) or methanol (MeOH). The composition of the mobile phase affects peak height, run time, and selectivity. An increase in column temperature often leads to a worse separation, while peak heights typically increase (Snyder *et al.*, 2010).

For the chromatographic process it is of further importance if the mobile phase composition remains unchanged (isocratic elution) or is deliberately changed over the duration of the

separation (gradient elution) (Snyder *et al.*, 2010). Generally speaking, isocratic elution is the preferred technique when the sample consists of less than ten analytes, which differ greatly in their affinity to the stationary phase, whereas gradient elution is used for chemical mixtures of ten or more analytes, since it provides a higher selectivity (Schellinger and Carr, 2006).

Due to the above mentioned benefits, HPLC is one of the most widely used analytical techniques today. This is especially due to the fact that it complements mass spectrometry in many ways, which makes a combination of these two analytical techniques essential for chemical analyses (Snyder *et al.*, 2010).

1.3.3 Mass spectrometry

Mass spectrometry is an important analytical tool with a wide range of scientific applications, which encompass the sequencing of biomolecules, cell research, material research, structural analyses of unknown substances, but also the quantification of known analytes in forensics, the environment or for the quality control of pharmaceutical drugs, food, and biological or synthetic polymers (Gross, 2011).

The basic principle behind mass spectrometry rests upon the ionization of inorganic or organic compounds, which are subsequently separated according to their respective mass-to-charge-ratio (*m/z*), and finally, measured by qualitative and quantitative detection of that specific *m/z*. Compounds can either be ionized thermally, through electric fields or by collision with high-energy electrons, ions, photons, high-energy neutral atoms, electronically excited atoms, cluster ions or electrostatically charged microdroplets. Ion separation can be achieved through the utilization of static or dynamic electric fields or magnetic fields. However, so-called “time-of-flight” mass analyzers also enable ion separation in a field-free region (Gross, 2011).

The basic setup of a mass spectrometer (MS) consists of an ion source, a mass analyzer, and a detector, which are all operated under high vacuum (Gross, 2011).

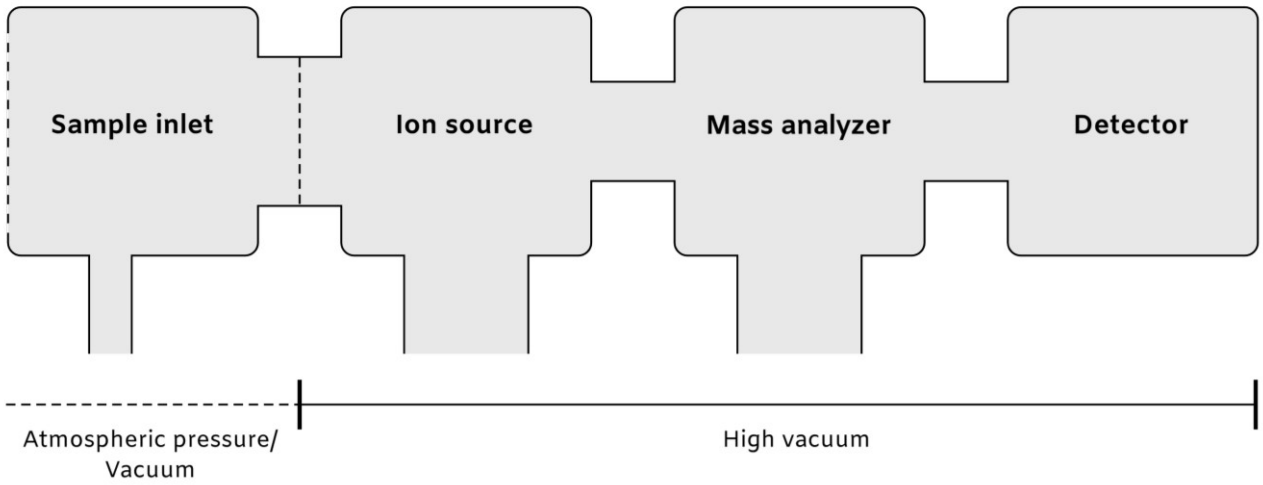


Figure 19: General schematic of a mass spectrometer

The unknown sample enters the MS through the sample inlet, which's essential function is to transfer the sample from atmospheric conditions to the high vacuum of the ion source. High vacuum is needed to prevent analytes from colliding with residual gas (*e.g.* air). However, there are ionization techniques, where the ion source is operated at atmospheric pressure (*e.g.* atmospheric-pressure chemical ionization (APCI), electrospray ionization (ESI)). After entering the ion source, the analytes are ionized and subsequently transported to the mass analyzer, where they are separated on the basis of their respective *m/z*. Finally, the analytes are registered by a detector and the mass spectra are recorded (Gross, 2011).

1.3.3.1 Setup of a triple-quadrupole mass spectrometer

A frequently used type of mass analyzer in mass spectrometry is the linear quadrupole. It consists of four cylindrically or hyperbolically shaped rod electrodes, which are arranged in a square structure. Each pair of opposing electrodes is electrically connected, and equal voltage is applied to either of the opposing electrodes. The voltage applied consists of a direct current and an alternating current. Ions, which enter the quadrupole and travel in the z-direction, are attracted to the oppositely charged electrodes. A periodical voltage is then applied to the rod electrodes, which results in a two-dimensional alternation of electrostatic attraction and repulsion of the ions. At a certain voltage ratio between the neighboring electrodes only ions of a certain *m/z* can travel through the quadrupole on a stable trajectory. Ions with unstable trajectories collide with the rod electrodes and are neutralized. This enables the selection of ions based on *m/z* (Gross, 2011).

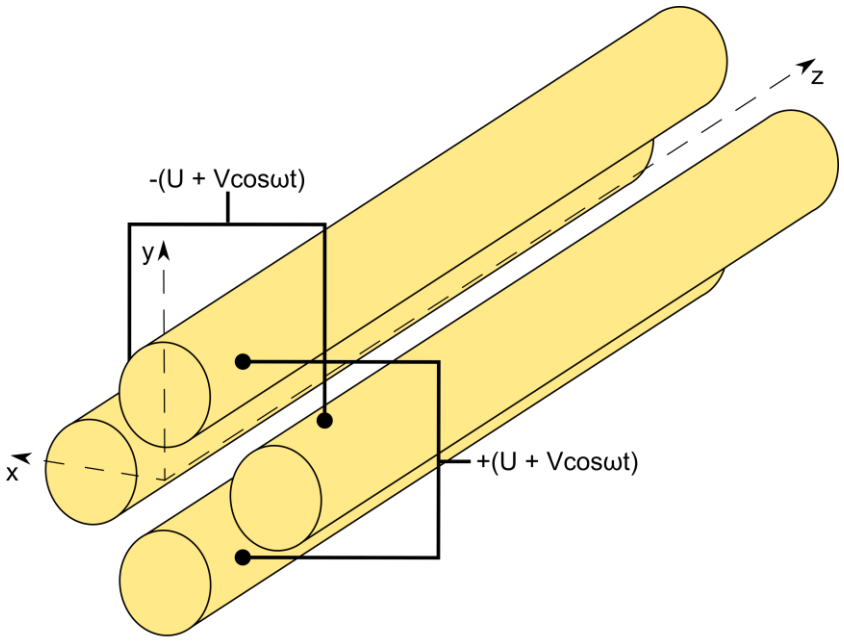


Figure 20: Schematic of a linear quadrupole mass analyzer
U = direct current, V = alternating current (radio frequency), ω = frequency

The quadrupole is commonly used in tandem mass spectrometry (MS/MS), with a popular MS/MS instrument being the triple-quadrupole mass spectrometer (QqQ). It is one of the most widely used analytical instruments and especially useful in routine analytics for the quantification of lower mass analytes. The instrument basically consists of an ion source, three different quadrupole mass analyzers (Q_1, q_2, Q_3), and a detector. After ionization, the sample analytes are transported to the Q_1, where only ions of a certain mass range can pass through. The preselected ions arrive in the q_2, which is most commonly used as a collision cell. In this “field-free region” the accelerated ions collide with a chemically inert collision gas (*e.g.* nitrogen gas), and are consequently fragmented. In newer instruments the q_2 is commonly replaced by a hexapole or octupole, leading to steeper potential wells, and as a result improving the ion current. The fragment ions ultimately reach the Q_3, where they are scanned (Gross, 2011).

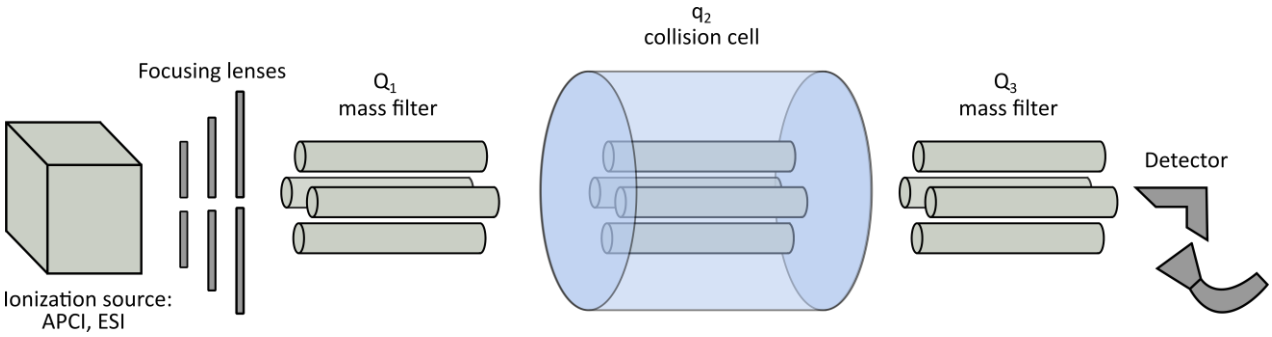


Figure 21: General schematic of a triple-quadrupole mass spectrometer
APCI = atmospheric-pressure chemical ionization, ESI = electrospray ionization

The QqQ can either be coupled with gas chromatography or liquid chromatography. In these assemblies it has become a standard analytical tool, which can be operated in various scan modes (Gross, 2011).

1.3.3.2 Scan modes of tandem mass spectrometry

There are various scan modes in which QqQs can be operated. The figure below shows the most relevant scan modes for when analyte quantification is required.

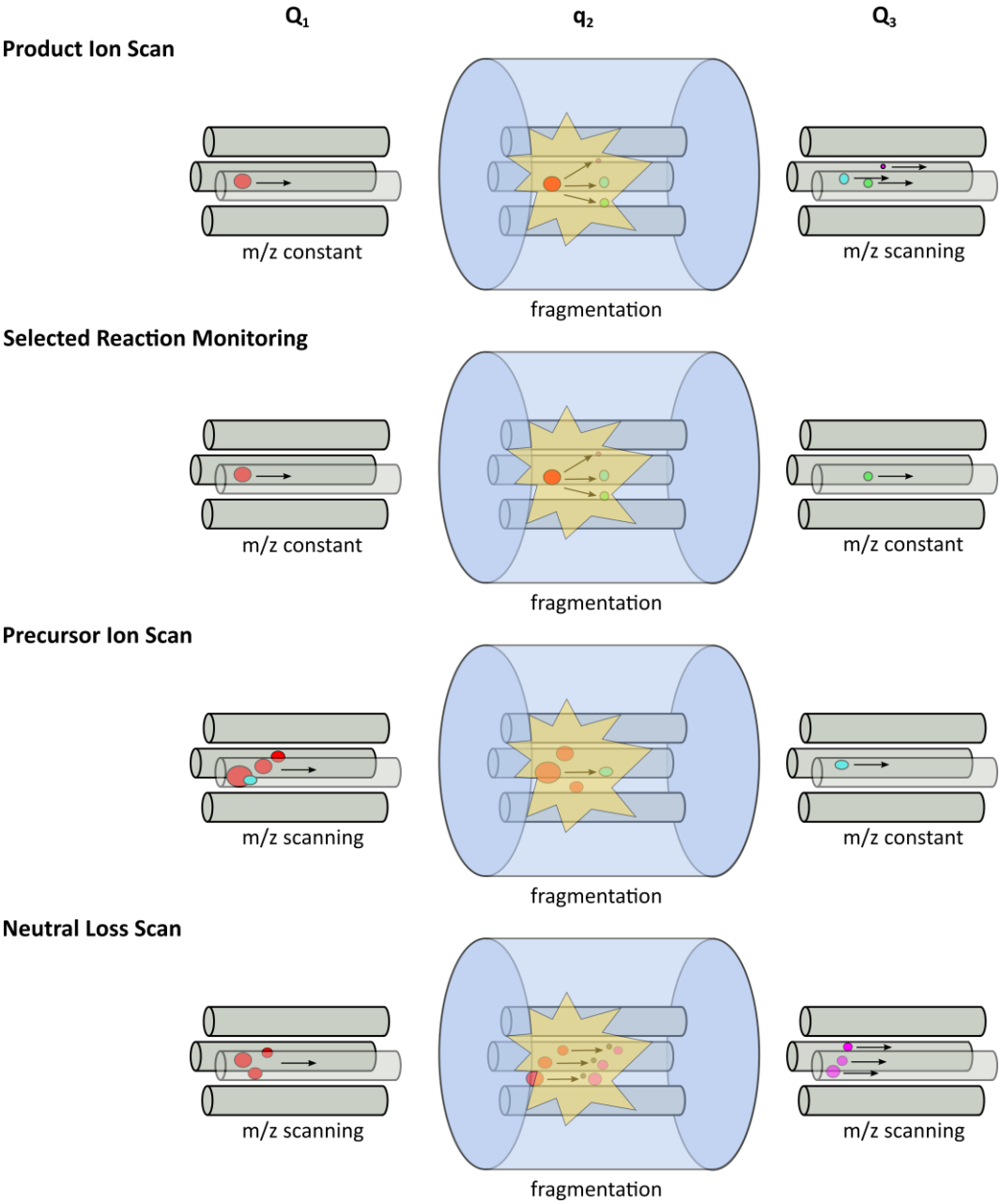


Figure 22: Different scan modes in tandem mass spectrometry
Q_1 = first quadrupole, q_2 = second quadrupole/collision cell, Q_3 = third quadrupole, *m/z* = mass-to-charge-ratio

Product Ion Scan: The precursor ion is selected in the first quadrupole (Q_1), fragmented in the second quadrupole (collision cell, q_2), and transferred into the third quadrupole (Q_3), where the fragments are subsequently scanned in order to generate a mass spectrum (Lin *et al.*, 2014).

Selected Reaction Monitoring (SRM): The precursor ion is selected in the Q_1, fragmented in the q_2, and, out of all fragment ions, a specific one is selected in the Q_3 (Lin *et al.*, 2014).

Multiple Reaction Monitoring (MRM): This mode is according to the same principle as SRM, with the distinction that two or more fragmentations take place during one cycle in order to further enhance the analyte selectivity of this scan mode (Gross, 2011).

Precursor Ion Scan: The Q_1 is scanned across a certain *m/z* range, the ions are fragmented in the q_2, and the Q_3 is held at a certain *m/z* in order to scan for a specific product ion (Lin *et al.*, 2014).

Neutral Loss Scan: The Q_1 is scanned across a certain *m/z* range. Ions, which enter the q_2, are fragmented and the resulting fragments are, once again, scanned across a certain *m/z* range in order to detect precursor ions, which underwent a specific neutral loss during fragmentation (Lin *et al.*, 2014).

1.3.3.3 Important parameters for compound optimization

The following subsections describe various critical parameters, which are commonly derived by using the automated compound optimization tool available in the software or by manual adjustment to achieve highest sensitivity. Terminologies used below are in accordance with the instrument vendor (AB Sciex) and might differ between MS providers.

Gas flows: There are various gas parameters that require optimization. First of all, there are the ion source gas 1 (GS1) and the ion source gas 2 (GS2). While the GS1 controls the nebulizer gas, which helps generating small spray droplets the GS2 controls the heater gas, which helps evaporating these spray droplets. Another important gas parameter is the curtain gas (CUR), which is needed in order to prevent contamination of the ion optics with sample molecules. For the CUR the highest possible value, where no loss of analyte sensitivity was observed, should be chosen. Finally, there is the collision gas (CAD) parameter. The CAD controls the pressure of the inert gas, which assists with the fragmentation of the precursor ions (AB SCIEX, 2017).

Temperature: The temperature parameter controls the temperature, which is transferred through the ceramic heaters of the ion source. This ion source- and gas-dependent parameter helps with the rapid evaporation of the ionspray droplets (AB SCIEX, 2017).

Ionspray voltage (ISV): The ISV is a source- and gas-dependent parameter, which means that it is influenced by the ion source used. It controls the voltage applied to the tip of the ionspray

needle, that ionizes the sample in the ion source. This parameter affects the ionspray stability and the sensitivity. The ISV can also be compound-dependent, which is why it should be optimized for each compound. (AB SCIEX, 2017). However, since the ISV is usually not changed during an analysis, a compromise for all analytes has to be found.

Declustering potential (DP): The DP is a pre-collision cell voltage. This compound-dependent parameter is the electrical potential difference between the ion injection orifice and the electrical grounding of the source and typically ranges from 20 V to 150 V in positive mode and from -20 V to -150 V in negative mode. A too low DP value can result in reduced ion intensities and potential interferences from solvent clusters, which might pile onto the analyte ions after entering the vacuum chamber. On the other hand, a too high DP value might cause direct analyte fragmentation in the ionization source of the MS (AB SCIEX, 2017).

Entrance potential (EP): The EP is another pre-collision cell voltage. This compound-dependent parameter is the electrical potential, which focuses and guides the ions through the high-pressure region, located prior to the Q_1. It is usually set to 10 V for positive ions and to -10 V for negative ions and has a negligible effect on compound optimization, which is the reason why it can be generally kept at the default values (AB SCIEX, 2017).

Collision energy (CE): The CE is the amount of energy, that the precursor ions receive during their acceleration into the q_2 and assists with fragmentation. This compound-dependent parameter controls the electrical potential difference between the region prior to the Q_1 and the q_2. The higher the CE, the greater the fragmentation. On the one hand, a too low CE entails inefficient fragmentation, on the other hand, a too high CE can cause excessive fragmentation. Both of these circumstances can lead to a significant reduction in sensitivity (AB SCIEX, 2017).

Cell exit potential (CXP): After fragmentation the fragment ions need to be transferred into the Q_3. This is achieved by the CXP, which focuses and accelerates the ions out of the q_2 and into the Q_3. The CXP is a compound-dependent parameter and controls the electrical potential difference between the focusing lenses and filter, positioned at the q_2 (AB SCIEX, 2017).

1.4 Method validation

Before an analytical method can be used in routine analysis, various validation parameters have to be fulfilled according to international requirements (EC, 2002). The relevant validation parameters for this thesis, regarding quantitative analytical methods, are depicted in the following subsections.

1.4.1 Specificity

The term specificity describes the ability to discriminate between the analyte and closely related compounds (*e.g.* degradation products, matrix constituents, isomers). It is assessed through the analysis of blank samples and blank samples fortified with substances, which are likely to interfere with the analyte. Detected interferences are further investigated whether they lead to false or hindered identification or significantly influence quantification. An appropriate specificity can be achieved through combination of an adequate cleanup procedure with a suitable chromatographic separation and mass spectrometric detection. However, possible interferences, which might arise from matrix components, have to be investigated (EC, 2002).

1.4.2 Signal suppression/enhancement

Effects of an analytical method, which are caused by all other components in a sample, except for the target analyte, are called matrix effects (Zhou *et al.*, 2017). They can be determined by calculating the signal suppression/enhancement (SSE):

$$\text{SSE (\%)} = 100 \times \frac{\text{area/slope (matrix-matched standard)}}{\text{area/slope (eluent-diluted standard)}}$$

A value below 100 % indicates signal suppression, while a value above 100 % indicates signal enhancement (Šarkanj *et al.*, 2018).

1.4.3 Trueness

This parameter describes the closeness of agreement between an average value and an accepted reference value, obtained from a big series of test results. The trueness can exclusively be determined with the use of a certified reference material (CRM). However, if there is no CRM available, the recovery must be determined instead (EC, 2002).

Table 3: Ranges of acceptance for the deviation of the experimentally determined recovery-corrected mass fraction from the certified value, depending on concentration, according to European Commission Decision 2002/657/EC

Mass fraction	Range
≤1 µg/kg	-50 % - +20 %
>1 µg/kg - 10 µg/kg	-30 % - +10 %
≥10 µg/kg	-20 % - +10 %

1.4.4 Recovery

The term recovery describes the percentage of the true concentration recovered from a substance during an analysis. This can be achieved by spiking numerous matrix-matched blank samples

around the minimum required performance limit or the permitted limit and analyzing them. The recovery is then calculated as follows:

$$\text{Recovery (\%)} = 100 \times \frac{\text{measured content}}{\text{fortification level}}$$

Validation results are only acceptable as long as the recovery is within the limits depicted in Table 3. When the recovery falls within these limits, a fixed correction factor can be applied to all samples, but, if the recovery lies outside of these bounds, the specific recovery factor for that batch must be used instead (EC, 2002).

1.4.5 Stability

Insufficient analyte stability or matrix constituent stabilities can lead to major deviations in the outcomes of analytical results. Analyte stability is usually well characterized for various storage conditions. However, if it is not known, it should be established in solution as well as in matrix (EC, 2002).

1.4.6 Calibration curve

One possibility of determining a substance in an unknown sample is by means of calibration curves. The calibration curve helps with the comparison of an unknown sample to a set of standard samples, whose concentrations are known. According to European Commission Decision 2002/657/EC the calibration curve should consist of at least five concentration levels (including zero), its working range, mathematical formula, and the goodness-of-fit (GOF) of the data to the curve should be specified. The GOF can be quantified via the calculation of the correlation coefficient (R^2) of the concentrations to their respective signal outputs (EC, 2002).

1.4.7 Repeatability

Repeatability conditions are usually considered, when independent test results are obtained by the same operator, using the same method, on identical test items, on the same equipment, in the same laboratory. Hence, the term ‘precision’ would not be entirely true and is therefore adjusted to the term ‘repeatability’. Intraday repeatability is determined by identically spiking numerous matrix-matched samples near the minimum required performance limit or the permitted limit, analyzing them, and calculating the analyte concentration, as well as the mean concentration, standard deviation, and coefficient of variation (relative standard deviation, RSD) of these fortified samples. To calculate the interday precision, the validation procedure must be repeated on at least three distinct days and the overall mean concentrations and RSDs for the fortified samples must be determined (EC, 2002).

1.4.8 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) are two very important terms and definitions to distinguish analyte detection and quantification. The LOD is defined as the smallest quantity of an analyte that can be detected with reasonable certainty for an analytical procedure (IUPAC, 1997). This means that an analyte can be detected, but not quantified. However, the LOQ is defined as the smallest quantity of an analyte that can be determined with acceptable repeatability and trueness (Eurachem, 2014). It allows for the detection and quantification of an analyte. LOD and LOQ are analyte- and matrix-dependent (EURL, 2016). There are various approaches on how these parameters can be determined. In this thesis, LOD and LOQ were calculated based on the signal-to-noise-ratio (S/N) of the chromatogram.

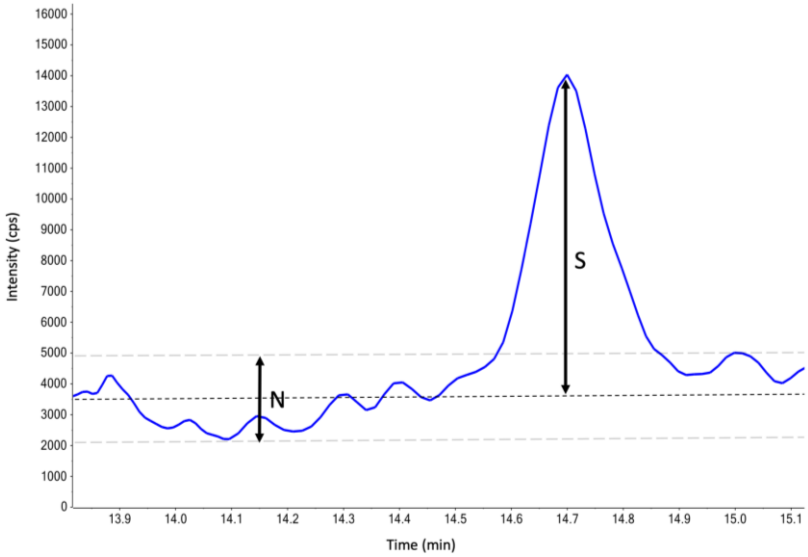
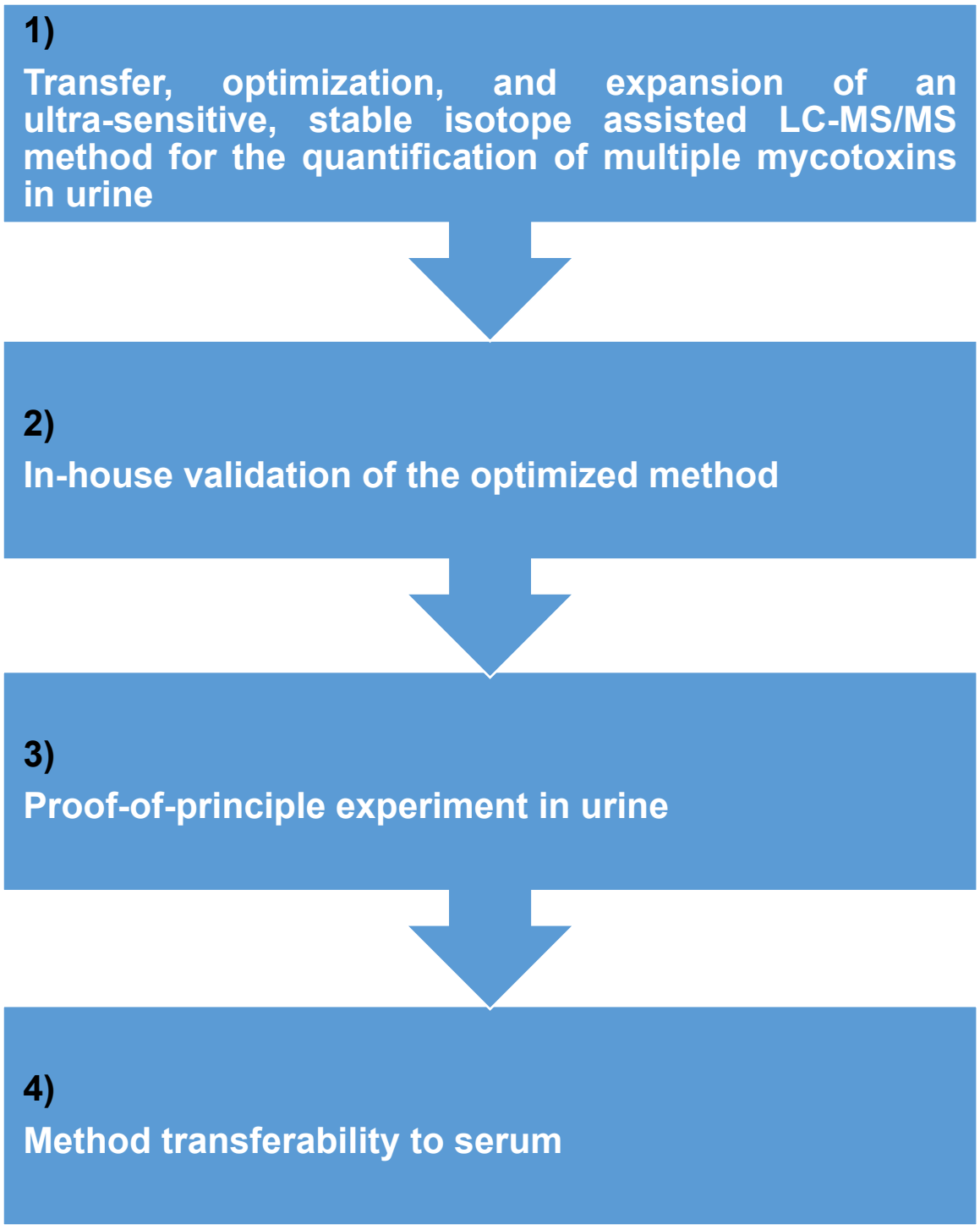


Figure 23: Estimation of signal-to-noise-ratio (S/N): S = 10,500 cps, N = 3,000 cps → S/N = 7
cps = ion counts per second, N = noise height, S = signal height

First, as shown in Figure 23, the baseline noise (N), close to the retention time of the analyte, is estimated. Thereafter the S/N is calculated by dividing the height of the signal (S) by half of the previously determined noise height (N/2). Based on the estimated S/N, LOD and LOQ are calculated. For LOD estimation a S/N of three and for LOQ estimation a S/N of six to ten is generally viewed as acceptable (EURL, 2016).

2 Thesis aims

The precise aims for this thesis were:



The original method was developed by Šarkanj *et al.* (2018, *Analytica Chimica Acta* 1019, pp. 84-92).

3 Materials and methods

3.1 Reagents, solvents, and chemicals

All chemicals used for the production of the buffer solution (potassium chloride (KCl, ≥99 %), potassium dihydrogen phosphate (KH_2PO_4, ≥99 %), sodium chloride (NaCl, ≥99.8 %), and di-sodium hydrogen phosphate dihydrate ($Na_2PO_4•H_2O$, ≥99.5 %)) were purchased from Carl Roth (Karlsruhe, Germany). Acetic acid (HAc), ACN, isopropanol, and MeOH, all LC-MS grade, were purchased from Honeywell (Seelze, Germany). H_2O, also LC-MS grade, was purchased from VWR (Darmstadt, Germany). For the de-glucuronidation of analytes β-glucuronidase (GUS, activity: 1,000,000-5,000,000 U/g protein) from *Escherichia coli* Type IX-A (G7396) was utilized, which was obtained from Sigma-Aldrich (Vienna, Austria). The isotopically labelled internal standards (ISs) $^{13}C_{17}$-aflatoxin M_1 ($^{13}C_{17}$-AFM_1), $^{13}C_{13}$-citrinin ($^{13}C_{13}$-CIT), $^{13}C_{15}$-deoxynivalenol ($^{13}C_{15}$-DON), $^{13}C_{15}$-nivalenol ($^{13}C_{15}$-NIV), $^{13}C_{34}$-fumonisin B_1 ($^{13}C_{34}$-FB_1), $^{13}C_{20}$-ochratoxin A ($^{13}C_{20}$-OTA), and $^{13}C_{18}$-zearalenone ($^{13}C_{18}$-ZEN) were purchased from Romer Labs (Tulln, Austria), while deuterated AOH ($^{2}H_4$-AOH) was synthetized at the Technical University of Munich, as described by Asam *et al.* (2009) and dissolved in neat ACN. Reference standards for AFB_1, AFB_2, AFG_1, AFG_2, STC, DON, DOM-1, NIV, FB_1, OTA, T-2, α-ZEL, β-ZEL, and ZEN were also supplied by Romer Labs (Tulln, Austria), AOH, ENNA, $ENNA_1$, ENNB, $ENNB_1$, tenuazonic acid (TeA), TEN, α-ZAL, and β-ZAL, and ZAN by Sigma-Aldrich (Vienna, Austria), and AFB-N^7-gua, AFM_1, AFM_2, AFP_1, AFQ_1, AFL, AME, BEA, CIT, OTα, and OTB by Toronto Research Chemicals (Ontario, Canada). Moreover, a DHC stock solution was kindly provided by Michael Sulyok (IFA-Tulln, Austria). Individual mycotoxin stock solutions were prepared in either neat ACN or MeOH, except for AFB-N^7-gua (ACN/H_2O, 1/1, v/v) and FB_1 (ACN/H_2O/HAc, 75/24/1, v/v/v) as described by Braun *et al.* (2018), Puntscher *et al.* (2018), and Preindl *et al.* (2019) and stored at -20 °C. These stock solutions were then either further diluted with neat ACN or directly used for the preparation of two multi-mycotoxin stock solutions:

- Stock solution A (15 mycotoxins and key metabolites): AME (75.0 ng/mL), AOH (1250 ng/mL), CIT (3750 ng/mL), DHC (1500 ng/mL), DON (5000 ng/mL), DOM-1 (2500 ng/mL), NIV (1250 ng/mL), FB_1 (500 ng/mL), TEN (250 ng/mL), α-ZEL (250 ng/mL), β-ZEL (250 ng/mL), ZEN (500 ng/mL), α-ZAL (1500 ng/mL), β-ZAL (1500 ng/mL), and ZAN (750 ng/mL)
- Stock solution B (15 mycotoxins and key metabolites): AFB_1 (150 ng/mL), AFB_2 (250 ng/mL), AFG_1 (175 ng/mL), AFG_2 (250 ng/mL), AFM_1 (175 ng/mL), AFM_2 (750 ng/mL), AFP_1 (750 ng/mL), AFQ_1 (750 ng/mL), AFL (1000 ng/mL),

AFB-N^7-gua (50.0 ng/mL), STC (12.5 ng/mL), OTA (50.0 ng/mL), OTB (50.0 ng/mL), OTα (250 ng/mL), and T-2 (7500 ng/mL)

For each of the stock solutions to amount to a final volume of 1 mL, missing volumes were compensated through the addition of dilution solvent (ACN/H_2O/HAc, 49.5/49.5/1, v/v/v). Both multi-mycotoxin stock solutions were utilized in order to create the highest working solution concentration level, which was subsequently used to prepare the five remaining working solutions, which were needed for the calibration curves and the spiking experiments.

3.2 Biological sampling

Since there is no CRM for mycotoxins in urine available, and in order to create adequate matrix-matched calibration curves and conduct the spiking experiments with smallest possible confounders, a blank urine needed to be generated. For this purpose, a 26-year-old, healthy, physically active, male volunteer from Austria was instructed to adhere to a special diet for three consecutive days. During this time period the study participant had to avoid the consumption of food, which is especially prone to mycotoxin contamination. The study participant's dietary protocols for each individual day are listed in Tables 4-6 on the following pages.

Table 4: Study participant's meal plan for day one of the dietary intervention

Meal	Meal components	Energy (kcal/100g)	Calculated energy intake (kcal/portion)
Breakfast	extra native olive oil	828[1]	83
	1 white onion	27[2]	24
	3 eggs	155[2]	225
	1 sweet pepper	19[2]	24
	½ cucumber	12[2]	19
	salt	-	-
Lunch	yogurt (3.6 % fat)	65[1]	402
	2 bananas	88[2]	203
	honey	302[1]	51
Dinner	extra native olive oil	828[1]	66
	salmon	202[2]	794
	white rice	354[1]	538
	butter	744[1]	119
	2 carrots	26[2]	54
	salt and pepper	-	-
		Sum (kcal/day)	2,602

[1] energy according to information provided on product label
[2] energy according to "Food Composition and Nutrition Tables" by Souci *et al.* (2008)

Since mycotoxins can be frequently detected in cereals and cereal products the study participant had to abstain from consuming any of these foodstuffs. This included day-to-day products, such as bread or cornflakes, but also beverages, such as beer or wine (Malachová *et al.*, 2011; Mariño-Repizo *et al.*, 2018; Ok *et al.*, 2013). However, since rice is considered to be the crop least susceptible to mycotoxin contamination, its consumption was permitted (Chulze, 2010). The study participant was also prohibited from consuming nuts, because of their commonly occurring contamination with AFs (Marin *et al.*, 2013). Although a carry-over of mycotoxins from animal feed to animal products can occur, carry-over rates for eggs are estimated to be insignificant (Ebrahem *et al.*, 2014; Tangni *et al.*, 2009). Moreover, mostly negligible amounts of mycotoxins can be detected in certain animal products, such as yogurt (Becker-Algeri *et al.*, 2016). Therefore, these and other animal products were introduced into the study participant's diet. The vegetable oil,

which was permitted for cooking, was extra native olive oil. Previous studies have found fewer rates of contamination when comparing olive oil to other vegetable oils (Cavaliere *et al.*, 2007; Zhao *et al.*, 2017). Although, due to the high content of unsaturated fatty acids present in extra native olive oil, under high frying temperatures it might undergo chemical changes, which can be detrimental to human health in the long term, it might not be the ideal choice for a cooking oil, a recent study found less mycotoxin contamination when comparing extra native olive oil to refined olive oil (Blasi *et al.*, 2018; Hidalgo-Ruiz *et al.*, 2019). Furthermore, most spices are especially prone to fungal infestation. Therefore, the study participant was allowed to solely season his food with black pepper and salt (Kabak and Dobson, 2017).

Table 5: Study participant's meal plan for day two of the dietary intervention

Meal	Meal components	Energy (kcal/100g)	Calculated energy intake (kcal/portion)
Breakfast	extra native olive oil	828[1]	41
	1 onion	27[2]	19
	3 eggs	155[2]	226
	1 sweet pepper	19[2]	21
	½ cucumber	12[2]	27
	salt	-	-
Lunch	yogurt (3.6 % fat)	65[1]	398
	2 oranges	42[2]	123
	honey	302[1]	60
Dinner	extra native olive oil	828[1]	66
	pork tenderloin	106[2]	470
	8 potatoes	68[1]	278
	kidney beans	113[1]	175
	crème fraîche	309[1]	269
	salt and pepper	-	-
		Sum (kcal/day)	2,173

[1] energy according to information provided on product label
[2] energy according to "Food Composition and Nutrition Tables" by Souci *et al.* (2008)

Important for a well-balanced diet is the inclusion of a variety of fruits and vegetables. Mycotoxins, however, can frequently be detected in apples, but less so in bananas and oranges (Moake *et*

al., 2006). Vegetables, which the study participant was allowed to eat, included carrots, cucumbers, onions, and sweet peppers. Since tomatoes are especially prone to *Alternaria* contamination, tomatoes and tomato products were excluded from the diet (Puntscher *et al.*, 2018). Legumes, on the other hand, typically show low levels of mycotoxin contamination, which is why chickpeas and kidney beans were added to the diet (Carballo *et al.*, 2018). Moreover, in order to introduce even more variation into the diet, fish and two different kinds of meat were consumed as well.

Table 6: Study participant's meal plan for day three of the dietary intervention (day of sampling)

Meal	Meal components	Energy (kcal/100g)	Calculated energy intake (kcal/portion)
Breakfast	extra native olive oil	828[1]	41
	1 onion	27[2]	26
	3 eggs	155[2]	222
	2 sweet peppers	19[2]	46
	½ cucumber	12[2]	23
	2 carrots	26[2]	22
	salt	-	-
Lunch	yogurt (3.6 % fat)	651	400
	3 bananas	882	387
	honey	3021	72
Dinner	extra native olive oil	828[1]	108
	chicken filet	145[2]	532
	chickpeas	131[1]	318
	crème fraîche	309[1]	241
	salt and pepper	-	-
		Sum (kcal/day)	2,438

[1] energy according to information provided on product label
[2] energy according to "Food Composition and Nutrition Tables" by Souci *et al.* (2008)

Additionally, in order to ensure a representative state of hydration, and, as a result of that, a realistic urine excretion, the study participant was instructed to drink two liters of Austrian tap water per day, for the duration of the dietary intervention.

On the third day of the dietary intervention the urine collection was conducted. The urine was gathered in a 2.5 L brown stained-glass bottle, which was flushed with midstream urine on the evening prior to urine collection. Urine collection started in the morning at 06:30 am and ended after a total collection period of 24 h. Exclusively midstream urine was collected, since it is deemed as adequate for urine analyses, because it is less contaminated with bacteria and, therefore, of higher validity (Massart *et al.*, 2008). After each individual urine sampling on that particular day the bottle, in which all daily samples were combined, was stored at 4 °C and, after the sampling period had ended, the bottle was stored at -21 °C overnight. On the next day the urine was aliquoted. The “non-spiked urine” aliquots, which were considered as a ‘potential blank’ sample, were stored at -21 °C until further usage.

3.3 Sample preparation

The sample preparation was mostly performed as described by Šarkanj *et al.* (2018). Urine samples were first thawed and allowed to reach room temperature. Then 1 mL aliquots were prepared, which were subsequently centrifuged at 5,600 rpm (2,940 x g) at room temperature for 2 min in a Universal Centrifuge Z326 (Hermle, Wehingen, Germany). Thereafter 500 µL supernatant was transferred into new micro-reaction tubes and 500 µL phosphate-buffered saline (PBS; 200 mM, pH = 7.4), containing 3000 U GUS, were added and samples were incubated on a Thriller® Thermoshaker-incubator (PEQLAB, Erlangen, Germany), which was set to 1000 rpm at 37 °C for 16 h, in order to cleave any mycotoxin glucuronides. The following day the SPE was performed using Oasis PRiME HLB® SPE columns (1 mL, 30 mg, Waters, Milford, Massachusetts, USA). The columns were conditioned with 1000 µL MeOH and equilibrated with 1000 µL H_2O. Thereafter the samples were loaded onto the columns and vacuum was applied, allowing the liquid to pass through the solid phase. The micro-reaction tubes were subsequently flushed with 500 µL H_2O, two times, in order to get out any analytes, that were potentially left in the tubes, and bring them onto the columns. This step simultaneously functioned as a washing step. Thereafter, mycotoxins were eluted into new micro-reaction tubes by adding 200 µL ACN thrice. The micro-reaction tubes, containing the eluates, were put into a CentriVap™ Benchtop Vacuum Concentrator (LABCONCO, Kansas City, Missouri, USA), which was set to 4 °C and linked to a CentriVap™ Cold Trap System (LABCONCO, Kansas City, Missouri, USA), and the ACN was evaporated at minimal pressure, overnight. The next day the samples were reconstituted in 490 µL dilution solvent (H_2O/ACN/HAc, 89.95/9.95/0.1, v/v/v), spiked with 10 µL isotopically labelled IS-mixture, and subsequently analyzed.

3.4 Equipment

Method optimization and validation, as well as, sample analysis experiments were performed with a QTrap® 6500+ LC-MS/MS system (AB SCIEX, Darmstadt, Germany), which was equipped with a Turbo V™ ESI source. The MS was coupled to an Agilent Technologies 1290 Infinity II UHPLC system (AB SCIEX, Darmstadt, Germany). Chromatographic separation was achieved with an Acquity UPLC HSS T3 column (1.8 µm, 2.1 x 100 mm, Waters, Vienna, Austria) preceded by a VanGuard pre-column (1.8 µm, 2.1 x 5 mm, Waters, Vienna, Austria).

3.5 Method development and optimization

The first aim of the method transfer was to figure out the sensitivity of the analytes without changing any major parameters reported, and by simply measuring the matrix-matched and solvent standards provided, by Šarkanj *et al*. In the next step matrix-matched standards and solvent standards were prepared containing 21 mycotoxins and key metabolites (AFB_1 (30.0 ng/mL), AFM_1 (30.0 ng/mL), AME (30.0 ng/mL), AOH (300 ng/mL), CIT (300 ng/mL), DHC (300 ng/mL), DON (1500 ng/mL), DOM-1 (1500 ng/mL), NIV (1500 ng/mL), EnnA (15.0 ng/mL), $EnnA_1$ (15.0 ng/mL), EnnB (15.0 ng/mL), $EnnB_1$ (15.0 ng/mL), BEA (15.0 ng/mL), FB_1 (300 ng/mL), OTA (30.0 ng/mL), OTα (1500 ng/mL), TeA (1500 ng/mL), ZEN (300 ng/mL), α-ZEL (300 ng/mL), and β-ZEL (300 ng/mL)). The first set of matrix-matched standards was prepared without GUS treatment, but measured with different injection volumes (5 µL and 10 µL) and at different source temperatures (450 °C and 550 °C).

Due to issues during the chromatographic separation, a new column of the same type was tested and the matrix-matched standards and solvent standards were remeasured, using that column with ACN + 0.1 % HAc and MeOH + 0.1 % HAc as eluent B, respectively. Moreover, different eluent flow rates were tested: 0.1 mL/min, 0.3 mL/min, and 0.6 mL/min with ACN + 0.1 % HAc and 0.1 mL/min and 0.2 mL/min with MeOH + 0.1 % HAc as eluent B. Moreover, certain analytes (CIT, DON, DOM-1, NIV, ENNA, ENNB, $ENNB_1$, BEA, and FB_1) were tuned. Tuning was performed via direct infusion of reference standards.

Upon further investigations into the method established by Šarkanj *et al.* (2018) it was discovered, that the time, the needle would be washed after injection, was set to 20 sec. However, Šarkanj *et al.* used a different HPLC system, and, when this was attempted with the in-house instrument, most analytes, which were detected previously, could not be detected any longer. Therefore, the needle washing time was changed back to its default value (3 sec).

During later periods of method optimization, a stock solution containing 15 more mycotoxins and key metabolites (AFB_2 (30.0 ng/mL), AFG_1 (30.0 ng/mL), AFG_2 (30.0 ng/mL), AFM_2 (300 ng/mL), AFP_1 (300 ng/mL), AFQ_1 (300 ng/mL), AFL (300 ng/mL), AFB-N^7-gua (300 ng/mL),

STC (30.0 ng/mL), OTB (30.0 ng/mL), T-2 (1500 ng/mL), TEN (300 ng/mL), ZAN (1500 ng/mL), α-ZAL (1500 ng/mL), and β-ZAL (1500 ng/mL)), which were added to the final method as well, was prepared. Therefore, new matrix-matched and solvent standards were prepared. Matrix-matched standards were additionally subjected to GUS from *Escherichia coli.* In order to find out, if a different enzyme-mixture might be better suited for purpose, an analysis comparing GUS from *Escherichia coli* in a PBS buffer (pH = 7.4) to GUS and arylsulfatase from *Helix pomatia* in a sodium acetate buffer (pH = 5.0) was performed.

Furthermore, Šarkanj *et al.* (2018) reported both gas parameters, GS1 and GS2, at 80 psi. During method optimization, however, the method's performance was critically assessed by testing different gas settings and 60 psi for GS1 and 40 psi for GS2 were deemed more suitable. Different needle heights (0 mm, 5 mm, and 10 mm) were tested as well in order to assess the impact on analyte sensitivity. Yet, in the end, needle height was adjusted to 2 mm, in order to introduce less matrix into the MS.

Before the start of the validation experiments, new stock solutions, with adjusted concentrations, which were in accordance with the newly estimated LOQs, were prepared. These stock solutions are described in subchapter 3.1. of this thesis. Since, with the use of the optimized LC-MS/MS method, certain analytes (ENNA, $ENNA_1$, ENNB, $ENNB_1$, BEA, TeA) could not be sufficiently detected previously, these compounds were not included in the newly prepared stock solutions.

3.6 LC-MS/MS parameters and analysis

The final HPLC and MS parameters, that resulted from method development and optimization, are reported below and on the following pages.

3.6.1 HPLC parameters

In order to achieve optimal chromatographic separation, the same gradient was applied as reported by Šarkanj *et al.* (2018). For the aqueous eluent (eluent A) H_2O + 0.1 % HAc was used, while ACN + 0.1 % HAc was used for the organic eluent (eluent B), and the flow rate was set to 0.1 mL/min. Gradient elution began with an initial period of 2 min at 10 % eluent B, followed by a 13 min time span, in which the percentage of eluent B was linearly raised to 50 %. From minute 15.0 to minute 18.0 the eluent B content was, once again, linearly raised to 95 %. The gradient was held at this A/B for another 4 min, and, at minute 22.1, eluent B was rapidly decreased to 10 % and held there until minute 25.0, in order to allow column re-equilibration in the wake of the next injection.

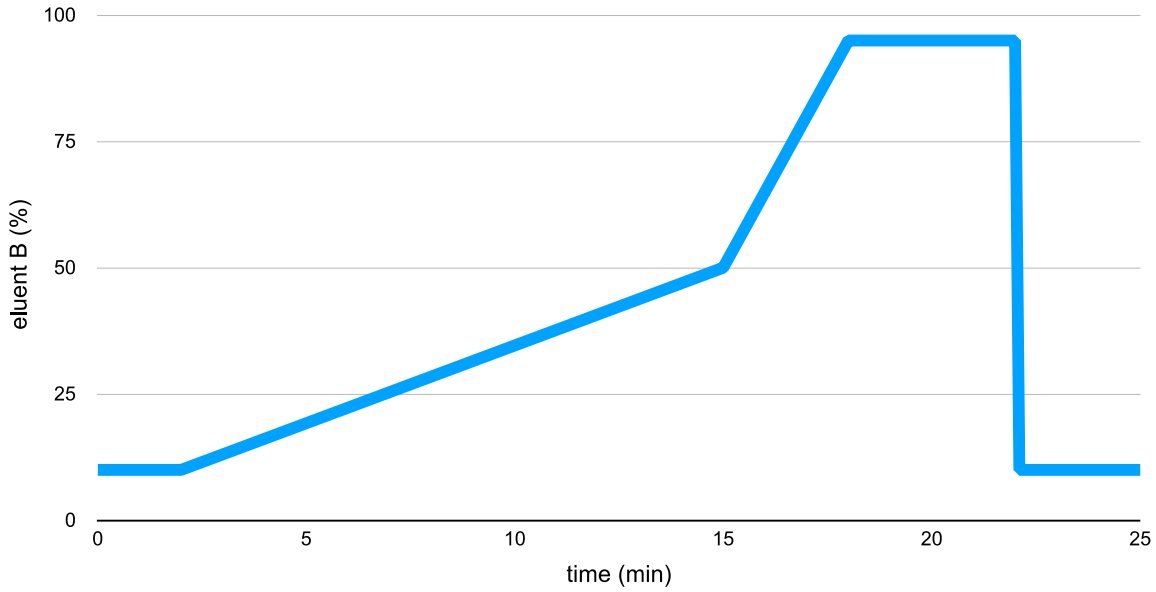


Figure 24: Gradient used for chromatographic separation in liquid chromatography tandem mass spectrometry experiments

All further important HPLC parameters are depicted in Table 7 on the following page.

Table 7: Important high-performance liquid chromatography parameters regarding pump (binary Pump), autosampler (multisampler), and column oven (column compartment)

Binary Pump		**Multisampler**		**Column compartment**	
Automatic Stroke Calculation A	Yes	Draw Speed	100 µL/min	Enable Analysis Temperature On - Left/Right	Yes
		Eject Speed	400 µL/min		
		Wait Time After Drawing	1.2 sec	Enable Analysis when Temperature is within - Left/Right	±0.8 °C
Stoptime Mode	No limit	Needle Wash	ACN/H_2O/HAc (49.95/49.95/0.1, v/v/v)		
Posttime Mode	Off			Temperature Equilibration Time - Left/Right	0.0 min
Stroke Mode	Synchronized	Needle Wash Mode	Flush Port		
Expected Mixer	No check	Duration	3 sec	Temperature Control Mode - Left/Right	Temperature set
		Injection volume	10 µL	Temperature - Left/Right	35.0 °C
		Overlap Injection Enabled	No	Enforce Column for Run Enabled	No
		Injection Valve to Bypass for Delay Volume Reduction	No	Stoptime Mode	As pump/injector
				Posttime Mode	Off
		Sample Flush-Out Factor	5.0	Enable Analysis when front door open	No
		Draw Position Offset	0.0	Position Switch After Run	Do not switch
		Use Vial/Well Bottom Sensing	Yes		
		Thermostat On	Yes		
		Temperature	7 °C		
		Stoptime Mode	No limit		
		Posttime Mode	Off		
		Rear Seal Wash	H_2O/isopropanol (80/20, v/v)		

ACN = acetonitrile, H_2O = water, HAc = acetic acid

3.6.2 MS parameters

MS parameters were generally in accordance with Šarkanj *et al.* (2018), except for some minor adjustments. For the first 2 min of the analysis eluent flow was directed to the waste container. From minute 2.0 to 24.0 the diverter valve was switched to the MS, which allowed the eluent flow to reach the ionization source. The eluent was redirected to the waste after 24.1 min and the overall chromatographic runtime was 25 min. The needle height of the Turbo V™ ESI source was adjusted to 2 mm, which positioned the needle further away from the orifice, as compared to default conditions (5 mm). Analytes were detected in basic scheduled MRM (sMRM) mode, with 120 sec MRM detection windows, and at least two different transitions were monitored for each analyte. Positive as well as negative ionization modes were run simultaneously with the application of fast polarity switching. The ISV was set to 4500 V for positive and to -4500 V for negative ionization mode, and the source temperature was set to 450 °C. Furthermore, the nitrogen gas parameters were adjusted as follows: CUR to 30 psi, CAD to high, GS1 to 60 psi, and GS2 to 40 psi. Additional important analyte-independent MS parameters are shown in Table 8 below.

Table 8: Important analyte-independent mass spectrometry parameters, including scheduled multiple reaction monitoring (sMRM) parameters, differential mobility spectrometry (DMS)

Target Scan Time (per sMRM experiment)	0.5 sec
DMS Off	Yes
Period Summary	
Duration	25.0 min
Delay Time	0 sec
Cycles	1500
Cycle Time	1.0 sec

Table 9: Optimized analyte-specific multiple reaction monitoring parameters, including retention time (RT), mass-to-charge-ratios (*m/z*) of precursor ion and product ions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP)

Analyte	RT	Precursor ion	Ion species	Product ion[a]	DP[a]	CE[a]	CXP[a]
	(min)	(*m/z*)		(*m/z*)	(V)	(V)	(V)
Aflatoxicol[b]	18.1	297.0	$[M-H_2O+H]^+$	269.1/115.1	71	29/83	12/14
Aflatoxin B_1[b]	17.6	313.0	$[M+H]^+$	241.0/213.0	106	49/61	14/16
Aflatoxin B_2[b]	16.7	315.0	$[M+H]^+$	243.0/203.0	125	53/49	16/12
Aflatoxin G_1[b]	16.7	329.1	$[M+H]^+$	200.0/243.1	86	59/39	12/14
Aflatoxin G_2[b]	15.8	331.1	$[M+H]^+$	313.2/245.2	111	35/43	18/14
Aflatoxin M_1[c]	14.7	329.1	$[M+H]^+$	273.2/229.1	91	35/59	16/12
$^{13}C_{17}$-Aflatoxin M_1[c]	14.7	346.0	$[M+H]^+$	288.2	91	35	16
Aflatoxin M_2[b]	13.8	331.0	$[M+H]^+$	259.0/241.0	96	33/57	16/14
Aflatoxin P_1[b]	14.6	299.1	$[M+H]^+$	270.7/215.1	126	35/38	18/11
Aflatoxin Q_1[b]	14.9	328.7	$[M+H]^+$	206.0/177.0	121	33/47	14/12
Aflatoxin-N^7-guanine[b]	11.0	480.0	$[M+H]^+$	152.1/135.0	46	23/60	10/14
Alternariol[d]	18.4	257.0	$[M-H]^-$	215.0/212.1	-110	-34/-38	-13/-12
$^2H_4$-Alternariol[c]	18.4	261.0	$[M-H]^-$	150.0	-110	-46	-5
Alternariol monomethyl ether[b]	20.7	271.1	$[M-H]^-$	256.0/227.0	-95	-32/-50	-13/-9
Citrinin[e]	19.0	251.0	$[M+H]^+$	233.1/205.1	36	23/37	14/18
$^{13}C_{13}$-Citrinin[c]	19.0	264.0	$[M+H]^+$	217.2	56	37	10
Dihydrocitrinone[b,c]	16.0	265.0	$[M-H]^-$	176.9/246.9	-30/-45	-34/-26	-13/-15
Deoxynivalenol[e]	8.5	354.9	$[M+CH_3COO]^-$	265.0/59.0	-30	-20/-58	-21/-27
$^{13}C_{15}$-Deoxynivalenol[c]	8.5	370.1	$[M+CH_3COO]^-$	278.8	-20	-22	-15
Deepoxy-deoxynivalenol[e]	10.2	280.9	$[M+H]^+$	109.1/137.1	36	21/19	12/10
Nivalenol[e]	5.7	371.0	$[M+CH_3COO]^-$	281.1/59.1/203.0	-20	-20/-40/-30	-19/-9/-13
$^{13}C_{15}$-Nivalenol[c]	5.7	386.0	$[M+CH_3COO]^-$	295.0	-75	-22	-15
Fumonisin B_1[b]	14.9	722.5	$[M+H]^+$	334.4/352.3	121	57/55	4/12
$^{13}C_{34}$-Fumonisin B_1[b]	14.9	756.3	$[M+H]^+$	356.3	130	46	10
Ochratoxin A[b]	20.6	404.0	$[M+H]^+$	239.0/102.0	91	37/105	16/14
$^{13}C_{20}$-Ochratoxin A[b]	20.6	424.0	$[M+H]^+$	250.0	51	33	12
Ochratoxin B[b]	19.5	370.1	$[M+H]^+$	205.0/103.1	86	33/77	12/16
Ochratoxin α[b]	15.0	254.9	$[M-H]^-$	166.9/123.0/110.9	-90	-36/-40/-44	-11/-17/-21
Sterigmatocystin[b]	21.1	325.1	$[M+H]^+$	281.1/310.2	96	51/35	16/18
T-2 toxin[f]	20.3	467.3	$[M+H]^+$	215.2/185.1	56	29/31	18/11
Tentoxin[b]	18.2	413.3	$[M-H]^-$	141.0/271.1	-105	-30/-24	-11/-15
Zearalanone[b]	20.7	319.1	$[M-H]^-$	107.0/161.0	-125	-40/-38	-13/-15
α-Zearalanol[b]	19.6	321.1	$[M-H]^-$	277.1/161.0	-120	-30/-38	-18/-9
β-Zearalanol[b]	18.8	321.2	$[M-H]^-$	277.1/303.1	-120	-30/-30	-18/-20
Zearalenone[c]	20.8	317.1	$[M-H]^-$	131.0/175.0	-110	-42/-34	-8/-13
$^{13}C_{18}$-Zearalenone[c]	20.8	335.2	$[M-H]^-$	185.1	-110	-34	-13
α-Zearalenol[b]	19.8	319.2	$[M-H]^-$	160.0/130.1	-115	-44/-50	-13/-20
β-Zearalenol[b]	19.0	319.1	$[M-H]^-$	160.0/130.1	-115	-44/-50	-13/-20

[a] values reported in following order: quantifying ion/qualifying ion/confirming ion
[b] values for precursor ion, product ions, DP, CE, and CXP adopted according to Braun *et al.* (2020a)
[c] values for precursor ion, product ions, DP, CE, and CXP adopted according to Šarkanj *et al.* (2018)
[d] values for precursor ion, product ions, DP, CE, and CXP adopted from old in-house tuning procedure
[e] values for precursor ion, product ions, DP, CE, and CXP acquired, while tuning, during method optimization
[f] values for product ions, DP, CE, and CXP adopted from ammonium-adduct according to Braun *et al.* (2020a) and mass downscaled to proton-adduct

3.7 Validation experiments

In-house validation was performed according to the European Commission Decision 2002/657/EC with minor modifications. During validation the following parameters were

determined: LOD, LOQ, linearity, repeatability (intraday precision, RSD_r), within-laboratory repeatability (interday precision, RSD_R), specificity, selectivity and recovery. Since there was no CRM for urine available the “non-spiked urine”, obtained as described in subchapter 3.2, was used for all validation experiments. Selectivity and trueness were estimated through calculation of the analyte extraction recoveries (R_Es), which were determined after spiking the ‘blank’ urine on two different levels (2x and 33x LOQ) with reference standards. LOD and LOQ were determined on the basis of these spikes as well, while LOD was identified on a S/N of three and LOQ on a S/N of six. Calibration curves for external calibration of each analyte consisted of at least five concentration levels (including zero; x/y = 0). In order to ensure sufficient linearity a R^2 ≥0.99 was deemed as acceptable. RSD_r and RSD_R were determined after analysis of samples on three days, within three different months, with three replicates per concentration level. Since, according to European Commission Decision 2002/657/EC, there are no fixed values for RSD_r when analyte concentrations are lower than 10 µg/kg and for RSD_R when analyte concentrations are below 100 µg/kg, RSDs ≤20 % were deemed acceptable for both RSD_r and RSD_R, respectively. Matrix effects were estimated through calculation of SSE.

Analytes were identified based on four criteria as described in European Commission Decision 2002/657/EC: retention time (RT), one precursor ion with two product ions, and their respective ion ratio. Moreover, in order to correct analyte concentrations for fluctuations during ionization, analytes, where specific isotopically labelled ISs were available (*e.g.* AFM_1 → $^{13}C_{17}$-AFM_1), were area corrected. For this purpose, an IS-mixture, that contained 8 isotopically labelled ISs ($^{13}C_{17}$-AFM_1, $^{2}H_4$-AOH, $^{13}C_{13}$-CIT, $^{13}C_{15}$-DON, $^{13}C_{15}$-NIV, $^{13}C_{34}$-FB_1, $^{13}C_{20}$-OTA, and $^{13}C_{18}$-ZEN), was added to all analyzed samples. IS-spiking was performed at previously determined LOQs, except for ^{13}C-ZEN, which was mistakenly spiked at the 2.7-fold amount. The ^{12}C-peak areas were then divided by the ^{13}C-peak areas and the resulting area ratios were subsequently used for all further calculations. Furthermore, other analytes, with close structural similarities (*e.g.* degradation products) to any of the ISs, were also area corrected with that IS (*e.g.* AFB_1 → $^{13}C_{17}$-AFM_1). Here, area ratios were used for most of the further calculations, except calculation of the respective SSEs. Certain analytes, however, which did not bear any close structural similarity to any of the ISs (T-2 and TEN), or could not be area corrected due to the estimated $^{13}C_{13}$-CIT-spiking level being too low (CIT and DHC), could not be corrected for any effects, that occurred during ionization. For these analytes, all calculations were conducted with the respective peak areas instead.

Moreover, a screening attempt was conducted, where the optimized LC-MS/MS method was applied to already extracted serum samples, which involved a matrix blank, matrix spikes (triplicates of each at a lower medium level and an upper medium level), matrix-matched calibration standards, and solvent calibration standards. The matrix-matched samples were

extracted in late 2018 by a staff member of the University of Vienna in accordance with the sample cleanup protocol developed by Preindl *et al.* (2019), with the exception, that samples were reconstituted in 100 % organic solvent (ACN/MeOH, 1/1, v/v) as opposed to 10 %. Solvent calibrants were prepared simultaneously, by the same person and with the same solvent, but were not subjected to the cleanup procedure. With the exception of DOM-1, the serum samples contained all mycotoxins, which were used for the method validation in urine and even a couple more (*e.g.* ENNs, BEA). Similar to the validation experiments in urine, parameters such as RT, ion ratio, R^2, R_E, RSD, SSE, LOD, and LOQ were assessed.

3.8 Data evaluation

For data acquisition and instrument control Analyst® software (version 1.7, AB SCIEX, Darmstadt, Germany) was used. Data evaluation was conducted with the SCIEX OS software package (version 1.6, AB SCIEX, Darmstadt, Germany). After the data evaluation in SCIEX OS was finished, results were transferred into Microsoft Excel (Redmond, Washington, USA), in which R^2, R_E, RSD_r, RSD_R, LOD, LOQ, and SSE were calculated. Moreover, for suspected outliers, Dixon's Q test (p=0.05) was performed.

4 Results and discussion

4.1 Method optimization

Since, during preliminary analyses, the sensitivities reported by Šarkanj *et al.* (2018) were not achieved, extensive method optimization was performed. Except for the centrifugation step being performed at 2,940 x g as opposed to 5,600 x g and the evaporation of ACN in a CentriVap™ Benchtop Vacuum Concentrator at 4 °C instead of under a nitrogen stream at room temperature, no further changes to the sample cleanup procedure, established by Šarkanj *et al.* (2018), were implemented. However, since, after a set of extracted samples was stored at -21 °C over the weekend, precipitation was observed, one attempt was conducted to further enhance sensitivity of the analytes. Therefore, the extracted samples were centrifuged at 13,500 rpm (17,110 rcf) at room temperature for 5 min. But, given that no significant improvements in sensitivity could be observed, no further changes to the cleanup protocol were implemented. Therefore, the method optimization mostly concerned the HPLC and MS parameters.

Others noticed a reduction in interfering signals and increase in analyte sensitivity after enzyme treatment (Cummings *et al.*, 2018; Qiao *et al.*, 2020), however, during this thesis, the opposite was observed. GUS treatment mostly lead to a major decrease in sensitivity. Enzymatic treatment mostly led to a major decrease in analyte sensitivity. Compared to *Escherichia coli*, the use of the enzyme-mixture from *Helix pomatia* did not seem to lead to significant sensitivity improvements, while the yield of cleaved mycotoxin conjugates was reduced to only about a third. Therefore, the *Escherichia coli* enzyme was used for sample treatment.

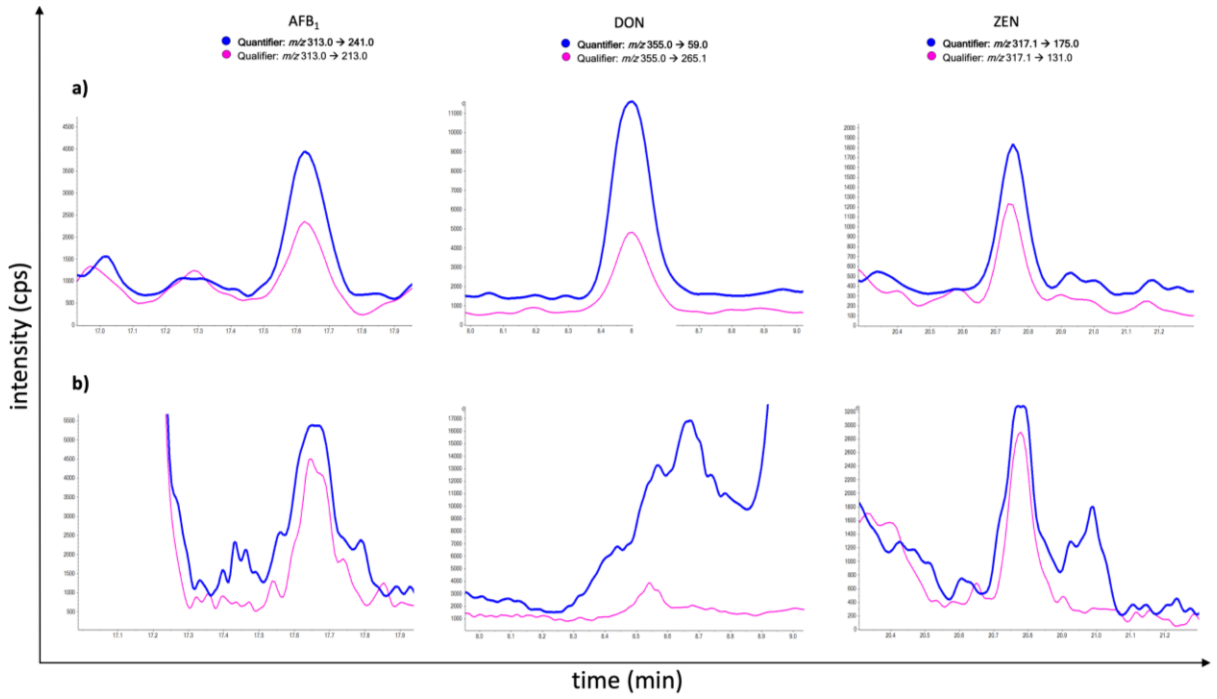


Figure 25: Illustration of key analytes aflatoxin B_1, deoxynivalenol, and zearalenone in matrix-matched samples before β-glucuronidase treatment a) and afterward b)
cps = ion counts per second

An injection volume of 5 µL was used in the early phase of method optimization in order to reduce the stress, which is put onto the column, and because recent studies have reported better results in different matrices when using lower injection volumes (Braun *et al.*, 2018; Fan *et al.*, 2019). However, since sensitivities were typically lacking by up to 10-fold and other analytes, such as CIT and DON, could not be detected at all, several parameters of the LC-MS/MS method were changed and thereafter tested. Although mycotoxins are generally stable compounds, matrix compounds might not be as stable. It was hypothesized that, since the samples provided by Šarkanj *et al.* were kept under storage conditions for a couple of years, they might be too old in order to achieve sufficient sensitivities with them.

When the needle washing time was set to 20 sec, as reported by Šarkanj *et al.* (2018), most analytes, which were detected previously, could not be detected anymore. However, Šarkanj *et al.* used a different LC system, which might execute the needle-washing step differently. In the in-house instrument the needle is flushed from below. It was hypothesized that such a long needle-washing step with needle-washing solution might act like a liquid-liquid extraction and flush most of the analytes out of the needle. This hypothesis was further proven, due to the fact, that, when the needle-washing time was changed back to its default value, the analytes could be detected again.

The column used for the first parts of method optimization was previously also used for breast milk analyses. A possible interference between a matrix with a higher fat content, such as breast

milk and a matrix with a higher water and salt content, such as urine that might lead to a decrease in sensitivity during urine analyses was suspected. Therefore, the old column was exchanged for a new one. With the use of the brand-new column and the data acquired from tuning CIT could finally be detected.

Sensitivity of some analytes (*e.g.* CIT, DON, NIV) was higher when MeOH + 0.1 % HAc was used. However, performance and sensitivity of most analytes, were better using ACN + 0.1 % HAc. Therefore, during another optimization attempt, 1 % of MeOH was added, as an additive, to ACN + 0.1 % HAc. However, compared to ACN + 0.1 % HAc, this did not improve the sensitivity for any of the analytes. Hence, in the final method ACN + 0.1 % HAc was used as eluent B. Different gradients and flow rates were also tested, but compared to the method used by Šarkanj *et al.* (2018) chromatographic separation and performance was reduced. Moreover, different flow rates with different gradients did not seem to greatly impact analyte sensitivities, but at high flow rates very polar compounds, such as NIV and even DON could no longer be detected, likely due to the possibility that these analytes were eluted together with the injection peak or directly eluted into the waste. On these grounds the gradient reported by Šarkanj *et al.* (2018) was used and the flow rate was changed back to 0.1 mL/min.

It was observed that analyte sensitivities were generally better when samples were measured at a source temperature of 450 °C. Consequently, for almost all subsequent measurements, the source temperature was adjusted to this value. Nevertheless, still troubles with the detection of some analytes, including CIT and FB_1, occurred. In order to find out if CIT can be detected in urine eluents were prepared and samples analyzed according to the LC-MS/MS method, that was developed in-house by Braun *et al.* (2018). With the help of this method CIT was finally detected in negative ionization mode as MeOH-adduct as well as in positive ionization mode. However, the MeOH-adduct was far more sensitive. Another important method parameter, regarding the needle of the MS, is needle height. After it was adjusted to 10 mm (needle closer to the orifice) many analytes could not be detected any longer. This was probably due to the fact that the needle position was too low and the gas flow too weak to carry the analytes into the orifice of the MS.

Other analytes, like the ENNs and BEA, were included during method development. However, they could not be detected. The reason the ENNs and BEA were not detected might be due to the fact, that these very apolar compounds need a LC gradient with a very high organic content over a long time period, as described by Braun *et al.* (2018), in order to elute from the column. These analytes might also bind to glass vials or micro-reaction tubes if organic content in the sample is too low, which, in such small concentrations, as used for the respective calibration curves, might greatly affect the observed results. All in all, it has to be noted that reconstitution is a critical factor for these analytes.

Some analytes, which could be detected, were prone to carry-over (*e.g.* AME, CIT), especially after analysis of higher concentrated standards. In order to minimize carry-over for these compounds, five solvent blanks were measured, in between the highest calibration standard and the next sample.

Another curious observation, which was made, was that, after columns had been used for measuring urine samples for the first time, the initial pressure at the begin of further analyses had increased by about 30 bar. This was witnessed with the new column as well as the old one, which suggests that, this phenomenon is urine-specific. The pressure increase might be due to the transfer of salts (mainly calcium salts) onto the column (Hanai, 1991). Although most salts were likely removed during the centrifugation and transfer step of the sample cleanup procedure, it is possible that there were still some salts left.

4.2 Method validation

During in-house method validation the following parameters were investigated in detail: R^2, R_E, RSD_r, RSD_R, SSE, LOD, LOQ, and ion ratio. Out of the 30 mycotoxins and key metabolites, which were included in the final method validation, eight (AFG_2, AFB-N^7-gua, AOH, CIT, FB_1, OTA, OTB, and OTα) were not successfully validated, including four analytes (AOH, CIT, FB_1, and OTA), which completed the validation according to Šarkanj *et al.* (2018). For most of these analytes the lowest spiking level was too optimistically estimated in the initial assessment. Moreover, CIT and FB_1 showed very low R_Es and for other analytes matrix interferences were observed, which were less pronounced in the initial assessment (AFB-N^7-gua, OTB, OTα). In the matrix-matched samples AFG_2 had two interfering peaks in both MRM-chromatograms (quantifying ion and qualifying ion), which hindered proper quantification. R_Es for AFB-N^7-gua, OTA, OTB, OTα were acceptable, however, RSD_rs and RSD_Rs did not meet the validation criteria in full. For OTA, OTB, and OTα differences in analyte extraction were observed. Here, two of the three replicated spike samples per quantifiable spiking level were typically within a certain concentration range, while the third spike was merely at about half of that. These fluctuations did not seem to fit any pattern, but were repeatable within the specific validation run, and occurred across all validation runs. They even persisted after area correction. Moreover, the qualifier transitions of AFB-N^7-gua, OTB, and OTα could not be evaluated on the three lowest matrix-matched calibration levels due to matrix interferences. For AOH there was also an interference in both transitions observed, which was at a RT close to the actual analyte peak. In addition, validation was not successful due to insufficient R_Es, which were estimated below 10 % at both, low and high, spiking levels. It appears as though AOH was lost during the sample cleanup. Something similar was observed by Braun *et al.* (2020a), who reported a R_E of <6 %. Furthermore, CIT peaks were unusually broad and the noise observed for the respective chromatograms was rather high. However, since R_Es of low

and high spikes were not sufficient, it is unlikely that this noise superimposed the actual peak shapes to such a high degree. It seems like both, CIT and FB_1, were lost over the sample cleanup instead, which contradicts the findings by Šarkanj *et al.* (2018). However, due to the high interference in the CIT chromatograms there is no chance in verifying this hypothesis.

However, in total 22 mycotoxins and some of their key metabolites (AFL, AFB_1, AFB_2, AFG_1, AFM_1, AFM_2, AFP_1, AFQ_1, AME, DHC, DON, DOM-1, NIV, STC, T-2, TEN, ZAN, α-ZAL, β-ZAL, ZEN, α-ZEL, and β-ZEL) were successfully validated in-house. During the first validation run, however, an unexplained NIV contamination occurred in the second of the low matrix-matched spikes. Since the phenomenon did not repeat itself for any of the other analytes and the peak intensity for that particular spike was significantly higher than the peak intensity of the highest matrix-matched calibration standard it is unlikely that a sample mix-up had occurred. Moreover, the contamination was so prominent that it led to a carry-over during the analysis of the third spike, which is why these two spikes were excluded from the calculations of the method validation parameters. The analytes' RSD_rs were typically determined during the second validation run, except for the RSD_r of α-ZEL, which was obtained during the first validation run.

Previous LOQ estimations for AFQ_1 and T-2 were too low, which subsequently lead to the low spiking levels for these analytes being immeasurable. Therefore, these analytes were only successfully validated on the upper spiking level. STC was also only successfully validated on the upper spiking level, which was because of a lack in sensitivity during the first validation run, and probably occurred due to inefficient ionization. Although STC sensitivities during the other two method validation runs were significantly better, data from one validation run was missing, rendering the validation on the lower spiking level as unsuccessful.

Moreover, during comparison of matrix-matched samples to solvent samples and even during comparison of matrix-matched samples across different validation runs, for DHC a RT shift of up to ±0.4 min and for OTα a RT shift of up to ±0.2 min was observed. This phenomenon might have ensued due to minor variations in the pH values of the respective samples, that certain analytes are more susceptible to (Snyder *et al.*, 2010). Therefore, it is not recommended to set a narrower sMRM detection window.

Table 10: Performance characteristics for analytes that were not successfully validated in-house, including correlation coefficient (R^2), extraction recovery (R_E), interday precision (RSD_R), intraday precision (RSD_r), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	R^2	$R_E \pm RSD_R$		RSD_r		SSE[b]	LOD	LOQ	Ion ratio[c]
	spiking levels[a]	level 1	level 2	level 1	level 2				
	(ng/L)	(%)	(%)	(%)	(%)	(%)	(ng/L)	(ng/L)	
Aflatoxin G_2	-	200/3333	-	-	-	-	-	-	-	-
Aflatoxin-N^7-guanine	0.9993	40/667	-	51 ± 32	-	23	49	30	60	0.15
Alternariol	0.9977	1000/16667	-	-	-	-	79	190[d]	380[d]	1.66
Citrinin	0.9975	3000/50000	36 ± 25	32 ± 53	8	34	68	700	1400	0.11
Fumonisin B_1	0.9996	400/6667	35 ± 17	15 ± 36	-	24	88	70	140	0.98
Ochratoxin A	0.9980	40/667	58 ± 21	68 ± 36	20	52	96	20	40	0.33
Ochratoxin B	0.9977	40/667	-	73 ± 35	-	19	87	30	60	0.32
Ochratoxin α	0.9975	200/3333	-	90 ± 40	-	22	82	150	300	0.16

[a] spiking levels reported in following order: level 1/level 2
[b] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent
[c] calculated as the ratio of qualifying ion and quantifying ion
[d] calculated based on matrix-matched calibration standards

Table 11: Performance characteristics for analytes that were successfully validated in-house, including correlation coefficient (R^2), extraction recovery (R_E), interday precision (RSD_R), intraday precision (RSD_r), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	R^2	$R_E \pm RSD_R$		RSD_r		SSE[b]	LOD	LOQ	Ion ratio[c]
	spiking levels[a]	level 1	level 2	level 1	level 2				
	(ng/L)	(%)	(%)	(%)	(%)	(%)	(ng/L)	(ng/L)	
Aflatoxicol	0.9988	800/13333	87 ± 20	94 ± 11	14	6	69	170	340	0.81
Aflatoxin B_1	0.9995	120/2000	100 ± 18	97 ± 8	5	5	81	30	60	0.66
Aflatoxin B_2	0.9988	200/3333	79 ± 12	98 ± 8	10	6	92	45	90	0.52
Aflatoxin G_1	0.9993	140/2333	87 ± 13	97 ± 10	9	6	99	40	80	1.61
Aflatoxin M_1	0.9994	140/2333	96 ± 19	97 ± 6	2	4	97	30	60	0.55
Aflatoxin M_2	0.9995	600/10000	84 ± 11	97 ± 9	3	5	81	115	230	0.59
Aflatoxin P_1	0.9993	600/10000	74 ± 13	90 ± 8	4	5	66	110	220	0.32
Aflatoxin Q_1[d]	0.9995	600/10000	-	79 ± 8	-	5	100	1800	3600	0.69
Alternariol monomethyl ether	0.9989	60/1000	60 ± 14	66 ± 17[e]	8	18[e]	27	12	25	0.17
Dihydrocitrinone	0.9994	1200/20000	77 ± 14[e]	92 ± 11[e]	16	12	99	300	600	0.70
Deoxynivalenol	0.9998	4000/66667	86 ± 17	105 ± 13	6	7	92	950	1900	2.94
Deepoxy-deoxynivalenol	0.9993	2000/30000	71 ± 18	97 ± 13	20	9	37	470	940	0.96
Nivalenol	0.9999	1000/16667	103 ± 14[f]	94 ± 8	5	7	85	300	600	0.86
Sterigmatocystin[d]	0.9987	10/167	-	77 ± 11	-	14	95	5	10	0.85
T-2 toxin[d]	0.9991	6000/100000	-	92 ± 9	-	7	58	4600	9200	0.78
Tentoxin	0.9988	200/3333	89 ± 7	93 ± 7	3	4	42	45	90	0.56
Zearalanone	0.9996	600/10000	94 ± 14	103 ± 11	4	9	26	195	390	1.32
α-Zearalanol	0.9994	1200/20000	90 ± 17	85 ± 18[e]	20	14[d]	37	265	530	0.08
β-Zearalanol	0.9997	1200/20000	73 ± 18	98 ± 14[e]	15	19[d]	49	190	380	0.27
Zearalenone	0.9997	400/6667	80 ± 18	93 ± 11	8	12	101	65	130	1.20
α-Zearalenol	0.9993	200/3333	86 ± 19	88 ± 14	15	8	31	100	200	0.63
β-Zearalenol	0.9997	200/3333	82 ± 17	101 ± 19	18	13	56	65	130	0.65

[a] spiking levels reported in following order: level 1/level 2
[b] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent
[c] calculated as the ratio of qualifying ion and quantifying ion
[d] spiking level 1 was not successfully validated
[e] statistical outliers were removed
[f] outliers were removed due to supposed contamination
(For further information regarding the calibration ranges, calibration curves, and isotopically labelled internal standards used for area correction see Appendix pp. 92, 94.)

Out of all of the analytes that successfully completed the in-house validation AFP_1, AME, DOM-1, T-2, TEN, ZAN, α-ZAL, β-ZAL, α-ZEL and β-ZEL exhibited significant signal suppressions. Compared to solvent signals, in matrix, signals were suppressed by more than a third. The RALs (*e.g.* ZAN) seem to be especially affected. Signal suppressions occur due to the presence of undesired components (*e.g.* matrix compounds), which co-elute during the chromatographic separation and suppress the ionization process. Although SSE is not solely determined by matrix effects, it can be reasoned, that matrix greatly affects the ionization of these compounds (Gosetti *et al.*, 2010). None of the analytes, that successfully completed in-house validation, exhibited significant signal enhancements.

LOQs ranged from 10 ng/L (STC) to 9200 ng/L (T-2). However, the sensitivity of the original method could not be reproduced. On average analyte LOQ values were 28 times higher compared to the published method. This might be due to the fact that Šarkanj *et al.* used a different “non-spiked urine”. Besides exhibiting very strong matrix effects, urine can also be very variable, which might also affect analyte sensitivities (Schlittenbauer *et al.*, 2015). In addition to that, a mistake during later runs of method optimization, stretching over the duration of the validation experiments, regarding the first step of sample cleanup, happened where samples were centrifuged at 5,600 rpm (2,940 x g) instead of 5,600 x g. The error was only discovered at a later time, after method validation was already completed. However, given that an additional centrifugation step at 13,500 rpm (17,110 rcf) for 5 min did not improve the analyte sensitivities, this circumstance might not have a big impact on the method’s overall sensitivity. Moreover, the single-analyte stock solutions, which were used for the preparation of the multi-analyte stock solutions, were not prepared freshly. Instead, they were prepared about two years ago. However, since mycotoxins were proven to be rather stable compounds and because these stock solutions were opened once or twice in total, and otherwise stored at -21 °C, only a minor impact on analyte sensitivities is to be expected (Kiseleva *et al.*, 2020).

However, as already mentioned previously, during comparison of untreated urine to GUS-treated urine, there was a big sensitivity difference observed (up to 20-fold). Based on the observed differences, it may be likely that Šarkanj *et al.* did not treat their samples matrix-matched samples with GUS. These variations are indicated by *e.g.* interfering peaks in the MRM-chromatogram of AFM_1. Šarkanj *et al.* (2018) reported that in matrix-matched samples two prominent peaks were observed, which are eluting close to this analyte. Therefore, the authors subsequently had to optimize the HPLC gradient in such a way, that AFM_1 elutes in between of the two interfering peaks. In the results presented in this thesis, interfering peaks were only observed in untreated urine. However, when urine samples were subjected to GUS treatment, the first interfering peak disappeared and the intensity of the second one was reduced (Figure 26).

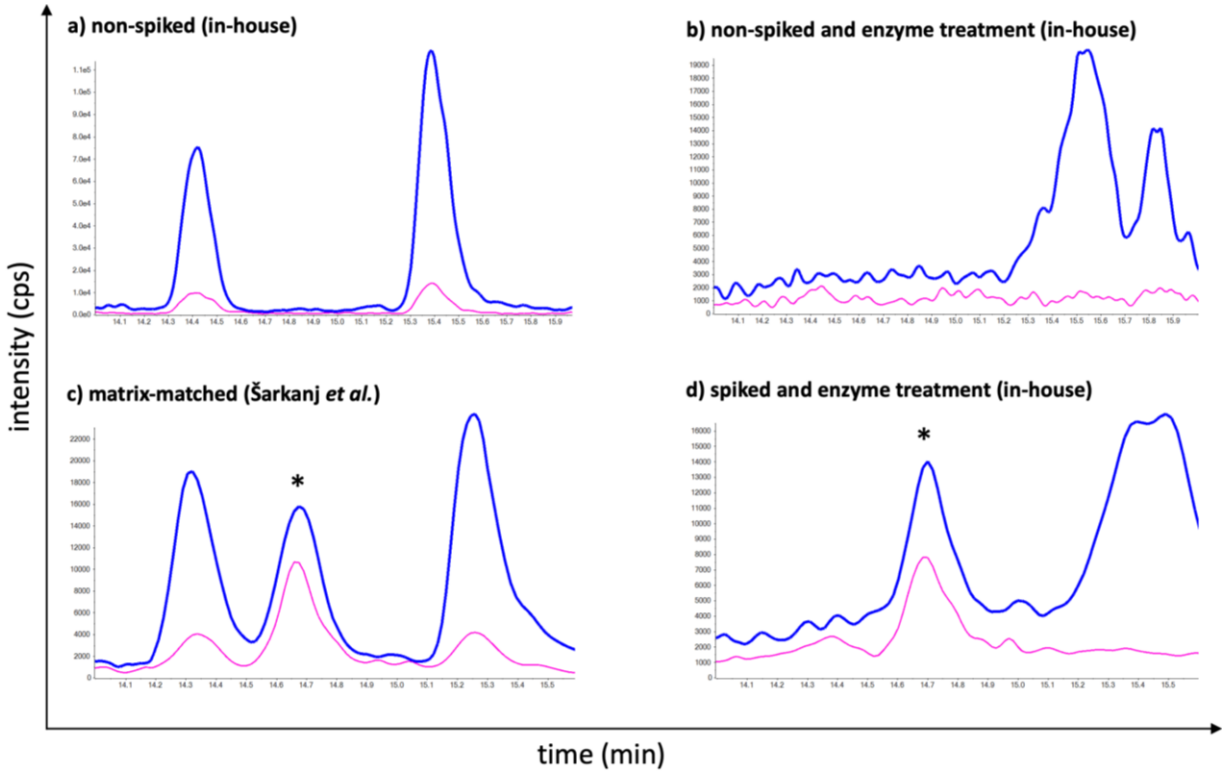


Figure 26: Demonstration of interfering peaks in a non-spiked urine sample without a) and with β-glucuronidase treatment b), in a remeasured matrix-matched sample provided by Šarkanj *et al.* (100 ng/L) c), and a matrix-matched sample using the non-spiked urine collected in this thesis spiked at a similar concentration (70 ng/L). The quantifying ion is given in blue, qualifying ion is depicted in pink, and analyte aflatoxin M_1 is tagged (*).

Similar results, as shown in Figure 26, were observed in a proof-of-principle experiment (see subchapter 4.3). These findings indicate that the interfering molecules are prone to GUS treatment and may, therefore, be glucuronides too. But, since the enzyme is extracted from a rather crude mixture, other enzymes might be present as well, which could potentially cleave the interfering molecules. However, no attempts were made to identify these unknown compounds.

Comparing the matrix-matched standard set, provided by Šarkanj *et al*., with matrix-matched samples prepared in-house, the estimated LOQs were different. After the evaluation of the sensitivities in both calibration sets, several LOQ values in samples provided by Šarkanj *et al*. (*e.g.* DON, DOM-1, NIV) matched with the results reported by Šarkanj *et al.* (2018). This suggests GUS treatment and sample properties have a major influence on sensitivity.

During method optimization it was observed that, although the subject, who provided the ‘blank’ urine, adhered to a special diet, the “non-spiked urine” was still contaminated. Results of the validation experiments showed that the urine was contaminated with AME (43±18 ng/L), OTA (<LOQ), α-ZAL (850±80 ng/L), and β-ZAL (420±110 ng/L).

The standard deviations for AME and β-ZAL were rather high, which is likely due to the fact that contamination, for both of these analytes, was close to their respective LOQs and, therefore, it might have happened that more noise or less of the peak area was integrated during the evaluation of the different validation runs.

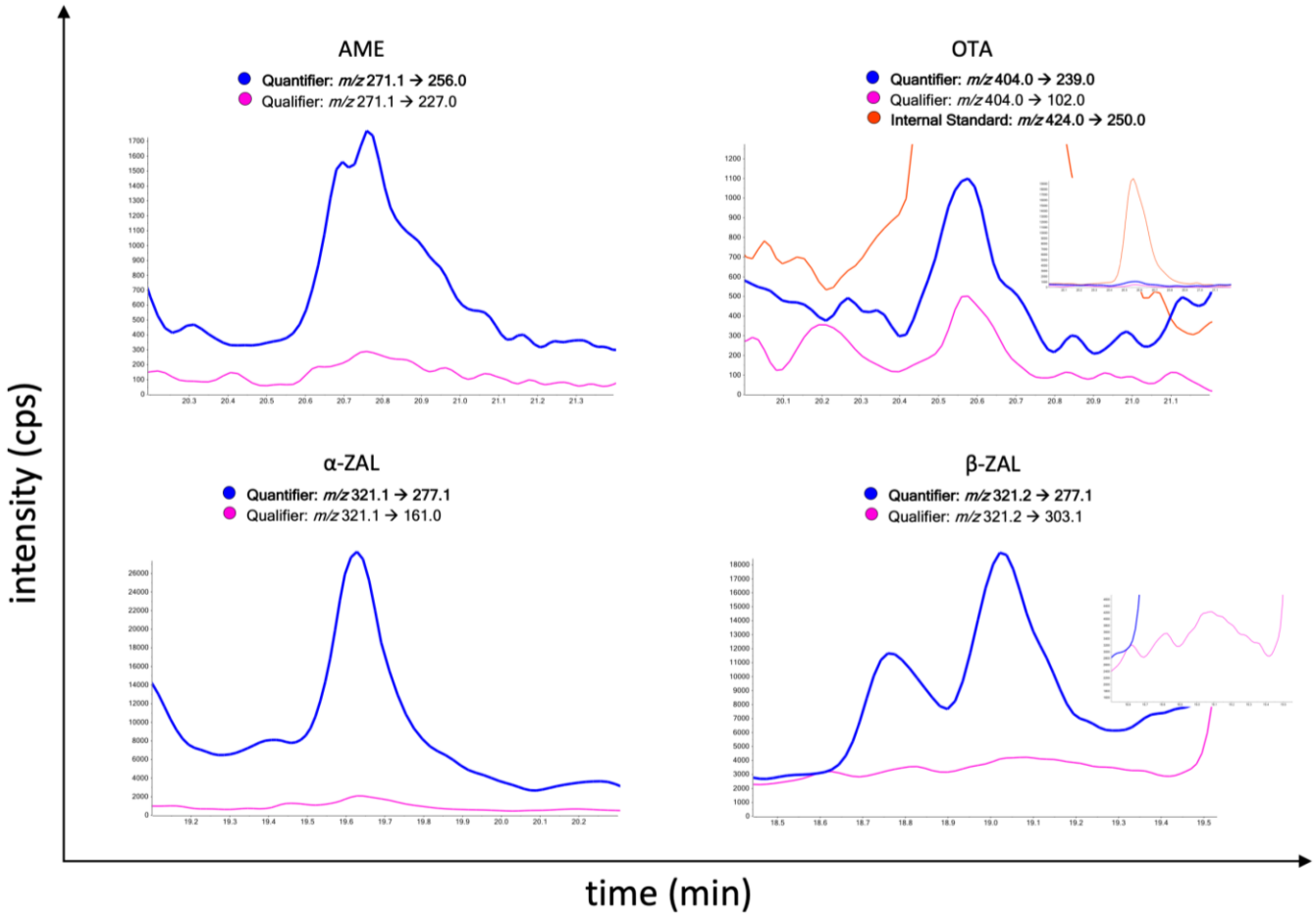


Figure 27: Mycotoxins detected in "non-spiked urine"
AME = alternariol monomethyl ether, OTA = ochratoxin A, α-ZAL = α-zearalanol, β-ZEL = β-zearalenol*;*
cps = ion counts per second, *m/z* = mass-to-charge-ratio

The occurrence of AME in this urine sample was potentially due to the consumption of potatoes during day two of the dietary protocol (Sun *et al.*, 2019). Potatoes were cooked, but since AME appears to be stable under high temperatures and is almost fully excreted after 24 h of ingestion, a contamination, through this source of exposure, seems plausible (Estiarte *et al.*, 2018; Puntscher *et al.*, 2019a). Moreover, similar levels of AME contamination in urine have been reported recently (Liu *et al.*, 2020). OTA is typically found in cereals, which is why most, except for rice, were excluded from the diet (Heussner and Bingle, 2015). However, OTA contamination still occurred, but since rice is the crop least susceptible to fungal growth, an exposure through rice seems unlikely (Chulze, 2010). The occurrence in urine is likely due to OTA's strong affinity to serum albumin, which it can bind to after absorption and results in a very long half-life. It can take a few days to up to one month for the absorbed OTA to be fully excreted again (Kőszegi and Poór, 2016). Therefore, it is entirely possible that exposure took place before the dietary intervention had even begun.

Although rice might be relatively resistant to mycotoxin contamination, ZEN can sometimes be detected (Palumbo *et al.*, 2020). It is possible that the consumed rice was contaminated with ZEN, which subsequently lead to its degradation products being excreted, due to the fact, that ZEN is metabolized after absorption. It is primarily degraded to α-ZEL and β-ZEL, which are subjected to further reductions to α-ZAL and β-ZAL, and finally partially conjugated and excreted with urine (Zinedine *et al.*, 2007). Belhassen *et al.* (2014) detected similar concentrations of α-ZAL in the urine of Tunisian women, but only found one positive sample for β-ZAL, below the LOQ value (1000 ng/L). Compared to the calculated LOQ value in this thesis their reported LOQ was 2.6 times higher, which might explain these differences in occurrence. Due to contamination of the "non-spiked urine", method validation parameters for the affected compounds were calculated by means of standard addition.

4.3 Proof-of-principle exposure experiments

First morning urine samples were provided by three female volunteers from Austria over a time period of five consecutive days and were analyzed using the established and validated LC-MS/MS method. Socio-demographic data for all mothers was recorded. The women were between 25 and 32 years old and had a normal body mass index. While all mothers earned a medium wage, two women had a higher level of education (university degree) and none of the women adhered to a special diet (*e.g.* vegetarian). Over the course of the study the women maintained their regular, mixed European, diets. The study was approved by the Ethics Committee of Lower Austria. In order to minimize bias and maximize the validity of the results, the samples were blinded prior to analysis. The final results for the unblinded samples are shown in Table 12.

Table 12: Recovery-corrected results for mycotoxins and key metabolites detected in urine obtained from three Austrian nursing mothers on five consecutive days

	Day	Analyte (ng/L)							
		AME	**CIT**	**DON**	**OTA**	**α-ZAL**	**ZEN**	**α-ZEL**	**β-ZEL**
Mother #1	1	132	**n.d.**	45200	<LOQ	1850	642	<LOQ	<LOQ
	2	73	<LOQ	87600	<LOQ	1120	575	<LOQ	<LOQ
	3	49	**n.d.**	9000	<LOQ	600	242	<LOQ	**n.d.**
	4	33	<LOQ	11700	<LOQ	1130	229	<LOQ	**n.d.**
	5	**n.d.**	**n.d.**	12800	<LOQ	**n.d.**	<LOQ	**n.d.**	**n.d.**
Mother #2	1	77	**n.d.**	8500	**n.d.**	2470	<LOQ	**n.d.**	**n.d.**
	2	37	**n.d.**	4100	**n.d.**	2330	<LOQ	**n.d.**	**n.d.**
	3	100	**n.d.**	12900	<LOQ	2590	<LOQ	**n.d.**	**n.d.**
	4	<LOQ	**n.d.**	16500	**n.d.**	4760	165	**n.d.**	**n.d.**
	5	89	**n.d.**	21600	<LOQ	5230	169	**n.d.**	**n.d.**
Mother #3	1	<LOQ	<LOQ	21300	**n.d.**	**n.d.**	**n.d.**	**n.d.**	<LOQ
	2	**n.d.**	**n.d.**	11800	<LOQ	**n.d.**	**n.d.**	**n.d.**	**n.d.**
	3	**n.d.**	**n.d.**	35300	<LOQ	**n.d.**	**n.d.**	**n.d.**	**n.d.**
	4	44	<LOQ	11000	**n.d.**	**n.d.**	<LOQ	**n.d.**	**n.d.**
	5	59	**n.d.**	10000	<LOQ	**n.d.**	**n.d.**	**n.d.**	**n.d.**

AME = alternariol monomethyl ether, CIT = citrinin, DON = deoxynivalenol, OTA = ochratoxin A, α-ZAL = α-zearalanol, ZEN = zearalenone, α-ZEL = α-zearalenol, β-ZEL = β-zearalenol;
LOQ = limit of quantification, n.d. = not detected

The results above show that, despite the sensitivities reported by Šarkanj *et al.* (2018) were generally not achieved, the method was still sensitive enough for the determination of various mycotoxins in urine. Mycotoxins detected were DON (100 %), AME (80 %), ZEN (73 %), OTA (67 %), α-ZAL (60 %), CIT (27 %), α-ZEL (27 %), and β-ZEL (20 %). Although some of the samples, contaminated with CIT or OTA, were at concentration levels above the previously estimated LOQs, results were still reported as "<LOQ", since these analytes failed the in-house validation experiments and a R_E correction of these results was not performed. At least two co-occurring mycotoxins were detected across all samples. The study cohort, however, was far too small in order to perform any statistical tests. Especially interesting was the frequent occurrence of the, currently unregulated, mycotoxin AME, which is in accordance with the findings in breast milk and suggests a frequent background exposure (Braun *et al.*, 2020b). The high abundancies of DON, OTA, and ZEN are probably due to frequent consumption of cereals and cereal products, which reflect the typical characteristics of the Central European Diet (Duś-Żuchowska *et al.*, 2018). Moreover, urine, which was contaminated with ZEN, was also commonly contaminated with its degradation products α-ZEL, β-ZEL, and α-ZAL. No β-ZAL and ZAN were detected, which is probably due to low conversion rates of α-ZAL to β-ZAL and ZAN (Belhassen *et al.*, 2014).

Results for ZEN were in a similar concentration range as the mean value (750 ng/L) reported in a cohort from northern Nigeria (n = 120). In that group ZEN was detected in 81 % of urine samples, which is similar to the findings of the proof-of-principle experiment. Values obtained for DON, however, differed greatly. In the cohort from northern Nigeria DON was reported less frequently (19 %) and concentrations were significantly lower (maximum value = 6220 ng/L) (Šarkanj *et al.*, 2018). Results obtained for DON closely reflect the findings reported in a cohort of pregnant women from Croatia (n = 40). Here, free DON was reported almost as often (76%) and in similarly high concentrations as in this study (Šarkanj *et al.*, 2013). However, due to the dilution through liquid intake, urinary mycotoxin concentrations alone have an uncertain meaning for human health. In order to make the results, reported in Table 12, more comparable to different studies, one would also need to account for different levels of hydration through creatinine correction of these values. Furthermore, other factors, such as age, gender, and race, also affect the urinary creatinine excretion and should be taken into account as well (Jain, 2016).

For two of the mycotoxins, which were detected and quantified in the urine samples, tolerable daily intakes (TDIs) are established (DON and its modified forms = 1 µg/kg bodyweight (bw)/d and ZEN = 0.25 µg/kg bw/d) (EFSA, 2014a, 2017). TDIs are the estimated amounts of a compound in food or drinking water, which can be taken in by humans for a lifetime, without an elevated risk for adverse health effects (EFSA, n.d.).

In order to be able to relate the results to the respective TDIs, the total DON and ZEN intakes of the participants were calculated. For this estimation the urinary excretion rates for DON (68 %) and ZEN (9 %), as reported by Warth *et al.* (2013), were chosen and the bw of the study subjects was fixed at 60 kg.

Daily DON intakes values from 0.1 µg/kg bw/d to 2.1 µg/kg bw/d. Although, according to these estimations, Mother #1 exceeded the group TDI during two separate days of this five-day-trial this is no cause for concern, yet. Since daily DON intakes, for the remaining three days, were significantly below the group TDI, the woman is not expected to suffer from any adverse health effects in relation to the substance.

Total daily ZEN intakes ranged from 0.01 µg/kg bw/d (LOQ/2) to 0.12 µg/kg bw/d, which means that none of the women exceeded the TDI at any time point during the five-day-trial. However, it is unclear if the ZEN metabolites, that were present in the urine samples, resulted from ZEN exposure.

Moreover, a comparison of the urinary mycotoxin patterns of the different women showed that, compared to Mother #1 and #2, the urine of Mother #3 seemed to be a lot less contaminated with ZEN and its metabolites. However, investigation of dietary habits and respective food analysis to examine a potential association to excretion patterns would be necessary in the future.

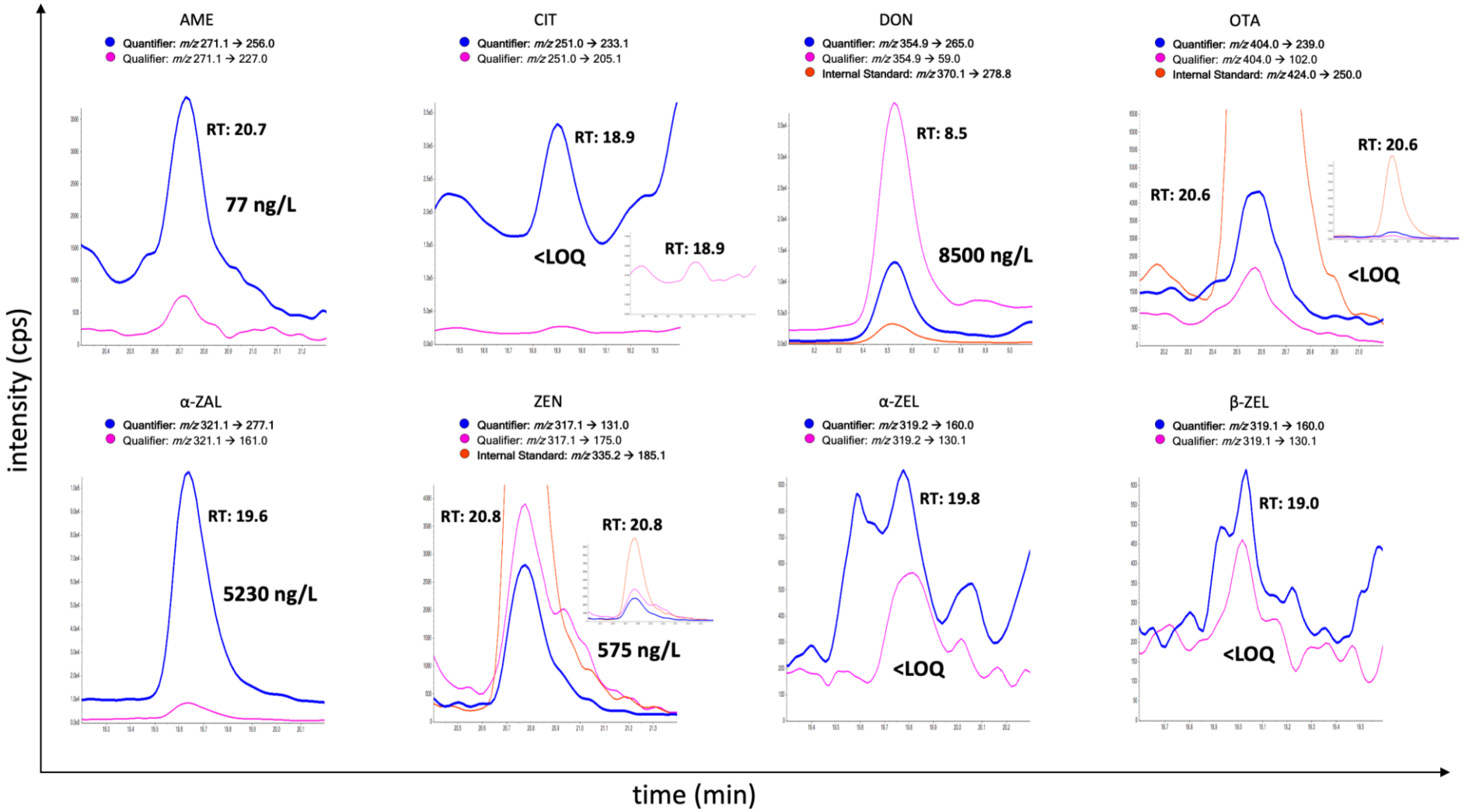


Figure 28: MRM-chromatograms for mycotoxins (citrinin (CIT), deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEN)) and mycotoxin metabolites (alternariol monomethyl ether (AME), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL)) detected in unknown urine samples (n = 15) obtained from three study subjects.
cps = ion counts per second, RT = retention time, *m/z* = mass-to-charge-ratio

4.4 Screening in serum

Certain mycotoxins (*e.g.* OTA) are not as quickly excreted as others. In order to determine real-time mycotoxin exposure other matrices, such as serum, have to be analyzed. Almost all analytes could be detected in matrix-matched serum samples, except for CIT, DHC, and T-2. However, many analytes could not be quantified due to insufficient peak shapes (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFP_1, AFQ_1, DON, NIV, OTα). For these analytes either very broad peaks or double peaks were observed. The unusual peak shapes probably resulted from the fact that samples were reconstituted in 100 % organic solvent (ACN/MeOH, 1/1, v/v), however, LC starting conditions of the mixed eluents were in 10 % organic content. The difference between the organic content of the eluent until minute 19 of the chromatographic separation and the sample, combined with a relatively high injection volume (10 µL), likely resulted in peak broadening and disturbed separation efficiency (Snyder *et al.*, 2010). This is further indicated by the observation that peak shapes improved over the duration of the respective analyses and, therefore, with higher organic content. Regular peak shapes were observed starting from minute 19 (95 % mixed-eluent organic content).

However, this issue can easily be resolved through evaporation and reconstitution of the samples in 10 % organic solvent. Samples were originally reconstituted in exclusively organic solvent, because it was observed previously that R_Es for the ENNs and BEA were lacking if samples were reconstituted in less than 75 % organic solvent.

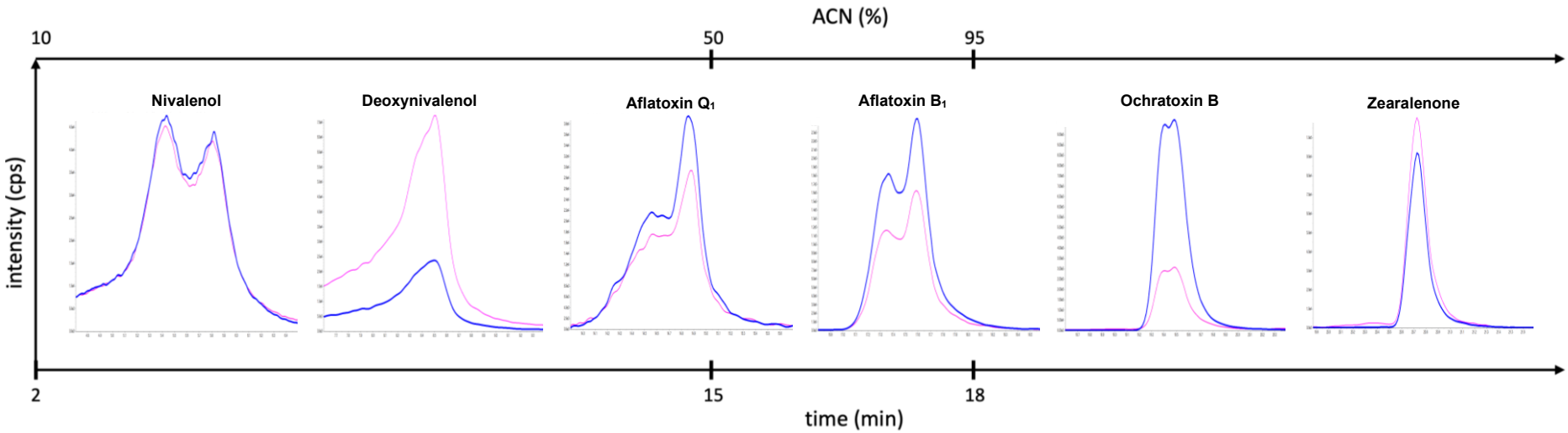


Figure 29: Illustration of peak shape improvement over the duration of sample analysis, depending on the content of acetonitrile (ACN) in the eluent-mixture. The quantifying ion is shown in blue, while qualifying ion is depicted in pink.
cps = ion counts per second

Overall, three analytes (AFL, AFB-N^7-gua and FB_1) did not fulfill typical validation criteria in this first screening attempt. For example, FB_1 showed insufficient linearity (R^2 <0.99) and highly variable peak areas between solvent and matrix-matched calibrants, which lead to an abnormally high SSE. The R_Es of the lower spiking level for AFL slightly exceeded the permitted deviation of +10 % and the R_Es for both spiking levels of AFB-N^7-gua exceeded the permitted deviation of -30 %.

However, besides differences in sample reconstitution, twelve mycotoxins and key metabolites met all validation criteria in this screening attempt. Since spiking levels for most isotopically labelled ISs were either too low or showed insufficient peak shapes, they could not be used for area correction of three of these mycotoxins and key metabolites (OTA, OTB, and STC). However, $^{2}H_{4}$-AOH and $^{13}C_{18}$-ZEN met the required criteria and were used for area correction of the eight remaining mycotoxins and key metabolites (AOH, AME, ZAN, α-ZAL, β-ZAL, ZEN, α-ZEL, and β-ZEL). Additionally, since the 'blank' serum, which was used for the matrix-matched calibration and spiking experiments, was contaminated with OTA (192 ng/L) and β-ZAL (<LOQ) method validation parameters for these analytes were calculated by means of standard addition. The OTA concentration of contaminated "non-spiked serum" was significantly higher in comparison with contaminated "non-spiked urine". However, due to OTA's albumin-binding capabilities, this is not surprising (Kőszegi and Poór, 2016). Moreover, "non-spiked serum" was obtained by pooling samples, which were collected from different study participants from the USA. The results for analytes, which successfully completed the screening attempt in serum, are shown in Table 14 on the next page.

Table 13: Performance characteristics for analytes that did not successfully complete the screening attempt in serum including retention time (RT), correlation coefficient (R^2), extraction recovery (R_E), relative standard deviation (RSD), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	RT	R^2		R_E ± RSD		SSE[b]	LOD	LOQ	
			spiking levels[a]	level 1	level 2				Ion ratio[c]
	(min)		(ng/L)	(%)	(%)	(%)	(ng/L)	(ng/L)	
Aflatoxicol	18.0	0.9999	1200/3000	112 ± 11	96 ± 6	54	150	300	0.82
Aflatoxin B_1[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin B_2[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin G_1[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin G_2[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin M_1[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin M_2[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin P_1[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin Q_1[d]	-	-	240/600	-	-	-	-	-	-
Aflatoxin-N^7-guanine	11.0	0.9996	1200/3000	68 ± 4	65 ± 9	42	15	30	0.15
Citrinin[e]	-	-	240/600	-	-	-	-	-	-
Dihydrocitrinone[e]	-	-	480/1200	-	-	-	-	-	-
Deoxynivalenol[d]	-	-	3600/9000	-	-	-	-	-	-
Nivalenol[d]	-	-	6400/16000	-	-	-	-	-	-
Fumonisin B_1	14.9	0.9845	6000/15000	34 ± 13	34 ± 13	4979	25	50	0.98
Ochratoxin α[d]	-	-	799/2000	-	-	-	-	-	-
T-2 toxin[e]	-	-	480/1200	-	-	-	-	-	-

[a] spiking levels reported in following order: level 1/level 2
[b] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent
[c] calculated as the ratio of qualifying ion and quantifying ion
[d] screening attempt not successfully completed due to insufficient peak shapes
[e] analyte not detected

Table 14: Performance characteristics for analytes that successfully completed the screening attempt in serum including retention time (RT), correlation coefficient (R^2), extraction recovery (R_E), relative standard deviation (RSD), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	RT	R^2		R_E ± RSD		SSE[b]	LOD	LOQ	
			spiking levels[a]	level 1	level 2				Ion ratio[c]
	(min)		(ng/L)	(%)	(%)	(%)	(ng/L)	(ng/L)	
Alternariol	18.3	0.9997	480/1200	103 ± 7	109 ± 4	98	30	60	1.75
Alternariol monomethyl ether	20.7	0.9998	480/1200	98 ± 12	102 ± 7	22	10	20	0.17
Ochratoxin A	20.5	0.9997	480/1200	98 ± 9	81 ± 8	26	10	20	0.34
Ochratoxin B	19.5	0.9997	480/1200	114 ± 10	93 ± 7	34	30	60	0.28
Sterigmatocystin	21.1	0.9983	120/300	87 ± 3[d]	73 ± 7	14	8	15	0.85
Tentoxin	18.2	0.9999	480/1200	89 ± 12	98 ± 3	62	15	30	0.60
Zearalanone	20.6	0.9995	480/1200	90 ± 9	102 ± 8	20	90	180	1.36
α-Zearalanol	19.6	0.9992	640/1600	96 ± 6	104 ± 2	24	150	300	0.09
β-Zearalanol	18.8	0.9994	640/1600	112 ± 9	106 ± 1	31	250	500	0.24
Zearalenone	20.7	0.9995	480/1200	95 ± 6	94 ± 5	82	100	200	1.34
α-Zearalenol	19.7	0.9993	640/1600	94 ± 13	95 ± 3	22	80	160	0.63
β-Zearalenol	19.0	0.9989	640/1600	94 ± 10	89 ± 4	26	80	160	0.63

[a] spiking levels reported in following order: level 1/level 2
[b] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent
[c] calculated as the ratio of qualifying ion and quantifying ion
[d] statistical outlier was removed
(For further information regarding the calibration ranges, calibration curves, and isotopically labelled internal standards used for area correction see Appendix pp. 93-94.)

LODs and LOQs were estimated based on the lower spiking levels. However, since spiking levels for many of the analytes were rather high the above listed LODs and LOQs should generally be regarded as an initial approximation. Nevertheless, the results indicate that the optimized LC-MS/MS method can also be used for the sensitive multi-mycotoxin determination in serum.

4.5 Limitations and challenges

Urine is a very diverse matrix and several matrix interferences make the sensitive detection of multiple analytes very challenging (Schlittenbauer *et al.*, 2015). Although the established method was proven sensitive enough for the quantification of multiple mycotoxins in unknown samples, its analyte sensitivities are still lacking compared to those reported by Šarkanj *et al.* (2018). This might mostly be due to the enzymatic treatment, which cannot simply be left out. Depending on the compound, rather large amounts of the initially ingested mycotoxin may be excreted as glucuronides (Muñoz *et al.*, 2017; Puntscher *et al.*, 2019b; Warth *et al.*, 2013). A major limitation was the lack of a CRM for mycotoxins in urine, which would have enabled the exclusion of sample variability. Some of the analytes, which were sufficiently recovered in the study of Šarkanj *et al.* (2018) (AOH, CIT, FB_1, and OTA), were not efficiently extracted in this work. Moreover, other mycotoxins, like the very apolar ENNs and BEA could not be detected, which might be due to the low organic solvent content in the reconstituted samples. All in all, the method transfer and optimization was successful, however, sensitivity was 28 times lower compared to the published method of Šarkanj *et al.* In contrast, several new analytes were added and, on average, the method was proven to be about seven times more sensitive than a previously developed “dilute and shoot” approach (Warth *et al.*, 2012).

4.6 Recommendations to further enhance sensitivity of the analytes

There are various approaches of how certain analytes’ sensitivities can be increased and many analytes, which might be added to method in the future. Even if the impact of centrifugation on analyte sensitivities at 2,940 x g instead of 5,600 x g might be minimal, samples should still be centrifuged at 5,600 x g in order to make sure that as many, potentially interfering, matrix components as possible are removed. Moreover, there is a possibility that the method might perform better at injection volumes below 5 µL. On one hand, lower injection volumes entail lower analyte amounts on the column, but also fewer interfering matrix. While most analytes were detected more sensitively at a source temperature of 450 °C, a source temperature of 550 °C was shown to be better suited for AFG_1 and AFQ_1. Certain analytes could be detected more sensitively when MeOH + 0.1 % HAc as eluent B was used. This included AME, DON, DOM-1, and, especially, CIT. For CIT the use of this particular eluent allowed for its MeOH-adduct being measured, which seemed to be almost 100-fold more sensitive compared to the proton-adduct. Moreover, if properly tuned, the sensitivity of T-2 should increase as well. However, like the ENNs

and BEA, T-2 can be detected much more sensitively as ammonium-adduct. In order to considerably increase the sensitivity of T-2, ammonium acetate could be added to the eluents, allowing for the detection of its ammonium-adduct. However, this will most likely negatively affect the sensitivities of other analytes. Moreover, since T-2 can be detected it should be rather unproblematic to add the structurally closely related HT-2 to the method as well.

While going through old quantitation files it was discovered that TeA could actually be detected previously and even on similar levels as reported by Puntscher *et al.* (2018). However, this was only after the method validation experiments were already finished. TeA was included in the first reference standard mixture, but because it was initially believed, that it cannot be detected, the compound was excluded from the final set of reference standard mixtures, which were used for the method validation. Its peaks were overlooked, while screening for the RTs of the analytes, in a broad MRM-chromatogram, due its very polar character, which lead to a low RT (3.4 min) and the compound's comparatively low sensitivity. Peak shape seemed sufficient, which does not confirm the requirements stated by Puntscher *et al.* (2018) that TeA needs a more alkaline pH or derivatization prior to analysis in order to achieve sufficient peak shapes. It appears like this analyte can be added to the method as well.

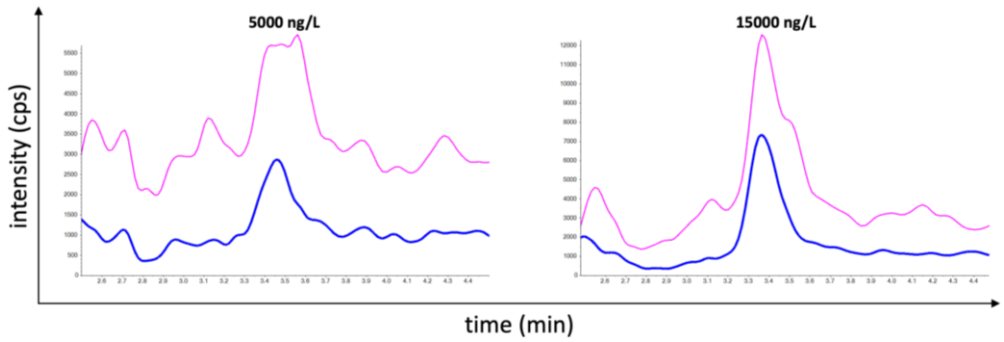


Figure 30: Tenuazonic acid peaks in matrix-matched calibration standards at two different concentration levels. The quantifying ion is given in blue (*m/z* 196.0 $[M-H]^-$ → 112.0), the qualifying ion is depicted in pink (*m/z* 196.0 $[M-H]^-$ → 138.9).
cps = ion counts per second, *m/z* = mass-to-charge-ratio

AOH and the OTs were significantly impacted by the sample cleanup for urine, which contradicts the results published by (Šarkanj *et al.*, 2018). However, this could be due to minor differences in the packing material of the SPE cartridges. With the sample cleanup for serum samples these mycotoxins and key metabolites were sufficiently recovered. Since OTA is an important regulated mycotoxin, which can be frequently detected in human bodily fluids (Braun *et al.*, 2020b; Šarkanj *et al.*, 2018), it might be reasonable to use a separate cleanup method, based on Preindl *et al.* (2019), for the determination of this and other mycotoxins in urine instead. It might also be sensible to spike the samples with isotopically labelled OTA prior to the sample cleanup procedure. This would account for the analyte loss, since the isotopically labelled ISs should

behave like the naturally occurring compound during sample cleanup. However, this would consume more resources and would therefore also be more expensive.

Moreover, possibilities to further enhance the sensitivities of the analytes include sample concentration, which is achieved after evaporation and reconstitution in a smaller volume, and direct analysis of the mycotoxin glucuronides instead of measuring the mycotoxins after enzymatic hydrolysis. However, this would require reference standards for all mycotoxin glucuronides, which are oftentimes not easily obtained.

5 Conclusion and outlook

Within this thesis, the LC-MS/MS method published by Šarkanj *et al.* (2018) for the quantification of multiple mycotoxins was modified and optimized. Several mycotoxins and key metabolites (n = 14) were added to the optimized method. However, the sensitivities as reported by Šarkanj *et al.* could not be achieved, due to rather high differences in individual matrix composition. Moreover, certain analytes (*e.g.* OTA) did not fulfill all in-house validation criteria. Although the optimized method did not reach the sensitivity of the earlier published method, it still enabled the detection of multiple mycotoxins in a proof-of-principle experiment. Furthermore, results of a screening attempt in serum showed that the method has excellent transferability.

Further research should be dedicated to the enhancement of the sensitivity of the optimized method and to the addition of important mycotoxins, which were not successfully validated in this thesis. Moreover, the optimized LC-MS/MS method should be validated in serum as well. Once validated, this method, as well as the method for urine, can be used for human mycotoxin exposure assessment. The acquired data can subsequently be related to data obtained through high-resolution mass spectrometry, revealing the link between mycotoxin exposure and its impact on the metabolome.

Abbreviations

$^{13}C_{17}$-AFM_1	$^{13}C_{17}$-aflatoxin M_1
$^{13}C_{13}$-CIT	$^{13}C_{13}$-citrinin
$^{13}C_{15}$-DON	$^{13}C_{15}$-deoxynivalenol
$^{13}C_{34}$-FB_1	$^{13}C_{34}$-fumonisin B_1
$^{13}C_{15}$-NIV	$^{13}C_{15}$-nivalenol
$^{13}C_{20}$-OTA	$^{13}C_{20}$-ochratoxin A
$^{13}C_{18}$-ZEN	$^{13}C_{18}$-zearalenone
$^{2}H_4$-AOH	$^{2}H_4$-alternariol
ACN	acetonitrile
AF	aflatoxin
AFB-N^7-gua	aflatoxin-N^7-guanine
$AFB_1/B_2/G_1/G_2/M_1/M_2/P_1/Q_1$	aflatoxin $B_1/B_2/G_1/G_2/M_1/M_2/P_1/Q_1$
AFL	aflatoxicol
AME	alternariol monomethyl ether
AOH	alternariol
BEA	beauvericin
bw	bodyweight
CAD	collision gas
CE	collision energy
CIT	citrinin
CRM	certified reference material
CUR	curtain gas
CXP	collision cell exit potential
DHC	dihydrocitrinone
DNA	deoxyribonucleic acid
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol
DP	declustering potential
E_2	17β-estradiol
ENN	enniatin
$ENNA/A_1/B/B_1$	enniatin $A/A_1/B/B_1$
EP	entrance potential
ESI	electrospray ionization
EU	European Union
$FB_1/B_2/B_3$	fumonisin $B_1/B_2/B_3$

FX	fusarenon-X
GOF	goodness-of-fit
GS1/GS2	ion source gas 1/ion source gas 2
GUS	β-glucuronidase
H_2O	water
HAc	acetic acid
HBM	human biomonitoring
HILIC	hydrophilic interaction chromatography
HLB	hydrophilic lipophilic balance
HPLC	high-performance liquid chromatography
HT-2	HT-2 toxin
IAC	immunoaffinity column
IARC	International Agency for Research on Cancer
IEC	ion-exchange chromatography
IPC	ion-pair chromatography
IS	internal standard
ISV	ionspray voltage
LC-MS/MS	liquid chromatography tandem mass spectrometry
m/z	mass-to-charge-ratio
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometer
MS/MS	tandem mass spectrometry
NARP	non-aqueous reversed-phase chromatography
NIV	nivalenol
NPC	normal-phase chromatography
OT	ochratoxin
OTA/B/C/α	ochratoxin A/B/C/α
PAT	patulin
PBS	phosphate-buffered saline
Q_1/Q_3	first quadrupole/third quadrupole
q_2	second quadrupole, collision cell
QqQ	triple-quadrupole mass spectrometer
R^2	correlation coefficient
RAL	resorcyclic acid lactone
R_E	extraction recovery

RPC	reversed-phase chromatography
RSD	relative standard deviation, coefficient of variation
RSD_r	repeatability
RSD_R	within-laboratory reproducibility
RT	retention time
S/N	signal-to-noise-ratio
SALLE	salting-out assisted liquid-liquid extraction
SEC	size-exclusion chromatography
sMRM	scheduled multiple reaction monitoring
SPE	solid phase extraction
SSE	signal suppression/enhancement
STC	sterigmatocystin
T-2	T-2 toxin
TDI	tolerable daily intake
TeA	tenuazonic acid
TEN	tentoxin
USA	United States of America
ZAN	zearalanone
ZEN	zearalenone
α-ZAL/β-ZAL	α-zearalanol/β-zearalanol
α-ZEL/β-ZEL	α-zearalenol/β-zearalenol

List of figures

List of tables

References

AB SCIEX. (2017). 6500/6500+ Series of Instruments - System User Guide. Retrieved from https://sciex.jp/Documents/manuals/6500-6500plus-system-user-guide-en.pdf (accessed: 18.05.2020)

Abdel-Wahhab, M.A., Hasan, A.M., Aly, S.E., and Mahrous, K.F. (2005). Adsorption of Sterigmatocystin by Montmorillonite and Inhibition of Its Genotoxicity in the Nile Tilapia Fish (Oreachromis Nilaticus). *Mutat Res, 582*(1-2), 20-27. doi:10.1016/j.mrgentox.2004.12.009

Adhikari, M., Negi, B., Kaushik, N., Adhikari, A., Al-Khedhairy, A.A., Kaushik, N.K., and Choi, E.H. (2017). T-2 Mycotoxin: Toxicological Effects and Decontamination Strategies. *Oncotarget, 8*(20), 33933-33952. doi:10.18632/oncotarget.15422

Ahamed, S., Foster, J.S., Bukovsky, A., and Wimalasena, J. (2001). Signal Transduction through the Ras/Erk Pathway Is Essential for the Mycoestrogen Zearalenone-Induced Cell-Cycle Progression in Mcf-7 Cells. *Mol Carcinog, 30*(2), 88-98.

Al-Jaal, B.A., Jaganjac, M., Barcaru, A., Horvatovich, P., and Latiff, A. (2019). Aflatoxin, Fumonisin, Ochratoxin, Zearalenone and Deoxynivalenol Biomarkers in Human Biological Fluids: A Systematic Literature Review, 2001-2018. *Food Chem Toxicol, 129*, 211-228. doi:10.1016/j.fct.2019.04.047

Ali, N., Blaszkewicz, M., and Degen, G.H. (2015). Occurrence of the Mycotoxin Citrinin and Its Metabolite Dihydrocitrinone in Urines of German Adults. *Arch Toxicol, 89*(4), 573-578. doi:10.1007/s00204-014-1363-y

Ali, N., and Degen, G.H. (2019). Citrinin Biomarkers: A Review of Recent Data and Application to Human Exposure Assessment. *Arch Toxicol*. doi:10.1007/s00204-019-02570-y

Alshannaq, A., and Yu, J.H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *Int J Environ Res Public Health, 14*(6). doi:10.3390/ijerph14060632

Alves, I., Oliveira, N.G., Laires, A., Rodrigues, A.S., and Rueff, J. (2000). Induction of Micronuclei and Chromosomal Aberrations by the Mycotoxin Patulin in Mammalian Cells: Role of Ascorbic Acid as a Modulator of Patulin Clastogenicity. *Mutagenesis, 15*(3), 229-234. doi:10.1093/mutage/15.3.229

Andrade, J.E., Ju, Y.H., Baker, C., Doerge, D.R., and Helferich, W.G. (2015). Long-Term Exposure to Dietary Sources of Genistein Induces Estrogen-Independence in the Human Breast Cancer (Mcf-7) Xenograft Model. *Mol Nutr Food Res, 59*(3), 413-423. doi:10.1002/mnfr.201300780

Armorini, S., Altafini, A., Zaghini, A., and Roncada, P. (2016). Ochratoxin a in Artisan Salami Produced in Veneto (Italy). *Food Addit Contam Part B Surveill, 9*(1), 9-14. doi:10.1080/19393210.2015.1098735

Asam, S., Konitzer, K., Schieberle, P., and Rychlik, M. (2009). Stable Isotope Dilution Assays of Alternariol and Alternariol Monomethyl Ether in Beverages. *J Agric Food Chem, 57*(12), 5152-5160. doi:10.1021/jf900450w

Aupanun, S., Poapolathep, S., Giorgi, M., Imsilp, K., and Poapolathep, A. (2017). An Overview of the Toxicology and Toxicokinetics of Fusarenon-X, a Type B Trichothecene Mycotoxin. *J Vet Med Sci, 79*(1), 6-13. doi:10.1292/jvms.16-0008

Bailey, G.S., Loveland, P.M., Pereira, C., Pierce, D., Hendricks, J.D., and Groopman, J.D. (1994). Quantitative Carcinogenesis and Dosimetry in Rainbow Trout for Aflatoxin B1 and Aflatoxicol, Two Aflatoxins That Form the Same DNA Adduct. *Mutat Res, 313*(1), 25-38. doi:10.1016/0165-1161(94)90030-2

Becker-Algeri, T.A., Castagnaro, D., de Bortoli, K., de Souza, C., Drunkler, D.A., and Badiale-Furlong, E. (2016). Mycotoxins in Bovine Milk and Dairy Products: A Review. *J Food Sci, 81*(3), R544-552. doi:10.1111/1750-3841.13204

Belhassen, H., Jiménez-Díaz, I., Ghali, R., Ghorbel, H., Molina-Molina, J.M., Olea, N., and Hedili, A. (2014). Validation of a Uhplc-Ms/Ms Method for Quantification of Zearalenone, Alpha-Zearalenol, Beta-Zearalenol, Alpha-Zearalanol, Beta-Zearalanol and Zearalanone in Human Urine. *J Chromatogr B Analyt Technol Biomed Life Sci, 962*, 68-74. doi:10.1016/j.jchromb.2014.05.019

Bellio, A., Bianchi, D.M., Gramaglia, M., Loria, A., Nucera, D., Gallina, S., Gili, M., and Decastelli, L. (2016). Aflatoxin M1 in Cow's Milk: Method Validation for Milk Sampled in Northern Italy. *Toxins (Basel), 8*(3). doi:10.3390/toxins8030057

Bennett, J.W., and Klich, M. (2003). Mycotoxins. *Clin Microbiol Rev, 16*(3), 497-516. doi:10.1128/cmr.16.3.497-516.2003

Blasi, F., Rocchetti, G., Montesano, D., Lucini, L., Chiodelli, G., Ghisoni, S., Baccolo, G., Simonetti, M.S., and Cossignani, L. (2018). Changes in Extra-Virgin Olive Oil Added with Lycium Barbarum L. Carotenoids During Frying: Chemical Analyses and Metabolomic Approach. *Food Res Int, 105*, 507-516. doi:10.1016/j.foodres.2017.11.061

Bouslimi, A., Bouaziz, C., Ayed-Boussema, I., Hassen, W., and Bacha, H. (2008). Individual and Combined Effects of Ochratoxin a and Citrinin on Viability and DNA Fragmentation in Cultured Vero Cells and on Chromosome Aberrations in Mice Bone Marrow Cells. *Toxicology, 251*(1-3), 1-7. doi:10.1016/j.tox.2008.06.008

Braun, D., Ezekiel, C.N., Abia, W.A., Wisgrill, L., Degen, G.H., Turner, P.C., Marko, D., and Warth, B. (2018). Monitoring Early Life Mycotoxin Exposures Via Lc-Ms/Ms Breast Milk Analysis. *Anal Chem, 90*(24), 14569-14577. doi:10.1021/acs.analchem.8b04576

Braun, D., Ezekiel, C.N., Marko, D., and Warth, B. (2020a). Exposure to Mycotoxin-Mixtures Via Breast Milk: An Ultra-Sensitive Lc-Ms/Ms Biomonitoring Approach. *ChemRxiv preprint*. doi:10.26434/chemrxiv.11763555.v2

Braun, D., Schernhammer, E., Marko, D., and Warth, B. (2020b). Longitudinal Assessment of Mycotoxin Co-Exposures in Exclusively Breastfed Infants. *bioRxiv preprint*. doi:10.1101/2020.03.27.011072

Brousmiche, D.W., O'Gara, J.E., Walsh, D.P., Lee, P.J., Iraneta, P.C., Trammell, B.C., Xu, Y., and Mallet, C.R. (2008). Functionalization of Divinylbenzene/N-Vinylpyrrolidone Copolymer Particles: Ion Exchangers for Solid Phase Extraction. *J Chromatogr A, 1191*(1-2), 108-117. doi:10.1016/j.chroma.2008.01.076

Bryła, M., Ksieniewicz-Woźniak, E., Waśkiewicz, A., Szymczyk, K., and Jędrzejczak, R. (2018). Natural Occurrence of Nivalenol, Deoxynivalenol, and Deoxynivalenol-3-Glucoside in Polish Winter Wheat. *Toxins (Basel), 10*(2). doi:10.3390/toxins10020081

Carballo, D., Font, G., Ferrer, E., and Berrada, H. (2018). Evaluation of Mycotoxin Residues on Ready-to-Eat Food by Chromatographic Methods Coupled to Mass Spectrometry in Tandem. *Toxins (Basel), 10*(6). doi:10.3390/toxins10060243

Carvajal, M., Rojo, F., Méndez, I., and Bolaños, A. (2003). Aflatoxin B1 and Its Interconverting Metabolite Aflatoxicol in Milk: The Situation in Mexico. *Food Addit Contam, 20*(11), 1077-1086. doi:10.1080/02652030310001594478

Cavaliere, C., Foglia, P., Guarino, C., Nazzari, M., Samperi, R., and Laganà, A. (2007). Determination of Aflatoxins in Olive Oil by Liquid Chromatography-Tandem Mass Spectrometry. *Anal Chim Acta, 596*(1), 141-148. doi:10.1016/j.aca.2007.05.055

Chagas, G.M., Klüppel, M.L., Campello Ade, P., Buchi Dde, F., and de Oliveira, M.B. (1994). Alterations Induced by Citrinin in Cultured Kidney Cells. *Cell Struct Funct, 19*(2), 103-108. doi:10.1247/csf.19.103

Choi, C.Y., Nakajima-Adachi, H., Kaminogawa, S., and Sugita-Konishi, Y. (2000). Nivalenol Inhibits Total and Antigen-Specific Ige Production in Mice. *Toxicol Appl Pharmacol, 165*(1), 94-98. doi:10.1006/taap.2000.8930

Chulze, S.N. (2010). Strategies to Reduce Mycotoxin Levels in Maize During Storage: A Review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 27*(5), 651-657. doi:10.1080/19440040903573032

Ciegler, A., Beckwith, A.C., and Jackson, L.K. (1976). Teratogenicity of Patulin and Patulin Adducts Formed with Cysteine. *Appl Environ Microbiol, 31*(5), 664-667.

Coulombe, R.A., Shelton, D.W., Sinnhuber, R.O., and Nixon, J.E. (1982). Comparative Mutagenicity of Aflatoxins Using a Salmonella/Trout Hepatic Enzyme Activation System. *Carcinogenesis, 3*(11), 1261-1264. doi:10.1093/carcin/3.11.1261

Creppy, E.E. (2002). Update of Survey, Regulation and Toxic Effects of Mycotoxins in Europe. *Toxicol Lett, 127*(1-3), 19-28. doi:10.1016/s0378-4274(01)00479-9

Cummings, O.T., Strickland, E.C., Enders, J.R., and McIntire, G. (2018). Impact of Beta-Glucuronidase Mediated Hydrolysis on Haldol(R) Urinalysis. *J Anal Toxicol, 42*(4), 214-219. doi:10.1093/jat/bkx103

D'Mello, J.P.F., Placinta, C.M., and Macdonald, A.M.C. (1999). Fusarium Mycotoxins: A Review of Global Implications for Animal Health, Welfare and Productivity. *Animal Feed Science and Technology, 80*(3-4), 183-205. doi:10.1016/S0377-8401(99)00059-0

da Silva, L.P., Madureira, F., de Azevedo Vargas, E., Faria, A.F., and Augusti, R. (2019). Development and Validation of a Multianalyte Method for Quantification of Mycotoxins and Pesticides in Rice Using a Simple Dilute and Shoot Procedure and Uhplc-Ms/Ms. *Food Chem, 270*, 420-427. doi:10.1016/j.foodchem.2018.07.126

Dänicke, S., and Winkler, J. (2015). Invited Review: Diagnosis of Zearalenone (Zen) Exposure of Farm Animals and Transfer of Its Residues into Edible Tissues (Carry over). *Food Chem Toxicol, 84*, 225-249. doi:10.1016/j.fct.2015.08.009

Degen, G.H., Ali, N., and Gundert-Remy, U. (2018). Preliminary Data on Citrinin Kinetics in Humans and Their Use to Estimate Citrinin Exposure Based on Biomarkers. *Toxicol Lett, 282*, 43-48. doi:10.1016/j.toxlet.2017.10.006

Dietrich, D.R., Heussner, A.H., and O'Brien, E. (2005). Ochratoxin A: Comparative Pharmacokinetics and Toxicological Implications (Experimental and Domestic Animals and Humans). *Food Addit Contam, 22 Suppl 1*, 45-52. doi:10.1080/02652030500309327

Duś-Żuchowska, M., Bajerska, J., Krzyżanowska, P., Chmurzyńska, A., Miśkiewicz-Chotnicka, A., Muzsik, A., and Walkowiak, J. (2018). The Central European Diet as an Alternative to the Mediterranean Diet in Atherosclerosis Prevention in Postmenopausal Obese Women with a High Risk of Metabolic Syndrome - a Randomized Nutrition-Al Trial. *Acta Sci Pol Technol Aliment, 17*(4), 399-407. doi:10.17306/J.AFS.0593

Dutton, M.F., Ehrlich, K., and Bennett, J.W. (1985). Biosynthetic Relationship among Aflatoxins B1, B2, M1, and M2. *Appl Environ Microbiol, 49*(6), 1392-1395.

Ebrahem, M., Kersten, S., Valenta, H., Breves, G., and Dänicke, S. (2014). Residues of Deoxynivalenol (Don) and Its Metabolite De-Epoxy-Don in Eggs, Plasma and Bile of Laying Hens of Different Genetic Backgrounds. *Arch Anim Nutr, 68*(5), 412-422. doi:10.1080/1745039X.2014.949029

EC. (2002). Commission Decision of 12 August 2002 - Implementing Council Directive 96/23/Ec Concerning the Performance of Analytical Methods and the Interpretation of Results. Retrieved from https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF (accessed: 18.05.2020)

EC. (2006). Commission Regulation (Ec) No 1881/2006 of 19 December 2006 - Setting Maximum Levels for Certain Contaminants in Foodstuffs. Retrieved from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1881-20140701&from=EN (accessed: 18.05.2020)

EC. (2013). Commission Recommendation of 27 March 2013 on the Presence of T-2 and Ht-2 Toxin in Cereals and Cereal Products. Retrieved from https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:091:0012:0015:EN:PDF (accessed: 18.05.2020)

EFSA. (2005). Opinion of the Scientific Panel on Contaminants in the Food Chain [Contam] Related to Fumonisins as Undesirable Substances in Animal Feed. *The EFSA Journal, 3*(7), 1-32. doi:10.2903/j.efsa.2005.235

EFSA. (2011). Scientific Opinion on the Risks for Animal and Public Health Related to the Presence of Alternaria Toxins in Feed and Food. *The EFSA Journal, 9*(10), 2407. doi:doi.org/10.2903/j.efsa.2011.2407

EFSA. (2012). Scientific Opinion on the Risks for Public and Animal Health Related to the Presence of Citrinin in Food and Feed. *The EFSA Journal, 10*(3), 1-82. doi:10.2903/j.efsa.2012.2605

EFSA. (2013). Scientific Opinion on the Safety and Efficacy of Micro-Organism Dsm 11798 When Used as a Technological Feed Additive for Pigs. *11*(5), 3203. doi:10.2903/j.efsa.2013.3203

EFSA. (2014a). Evaluation of the Increase of Risk for Public Health Related to a Possible Temporary Derogation from the Maximum Level of Deoxynivalenol, Zearalenone, and Fumonisins for Maize and Maize Products. Retrieved from https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2014.3699 (accessed: 18.05.2020)

EFSA. (2014b). Scientific Opinion on the Risks to Human and Animal Health Related to the Presence of Beauvericin and Enniatins in Food and Feed. *EFSA Journal, 12*(8), 1-174. doi:doi.org/10.2903/j.efsa.2014.3802

EFSA. (2017). Risks to Human and Animal Health Related to the Presence of Deoxynivalenol and Its Acetylated and Modified Forms in Food and Feed. Retrieved from https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2017.4718 (accessed: 18.05.2020)

EFSA. (n.d.). Glossary. Retrieved from http://www.efsa.europa.eu/en/glossary-taxonomy-terms (accessed: 18.05.2020)

Escrivá, L., Manyes, L., Font, G., and Berrada, H. (2017). Mycotoxin Analysis of Human Urine by Lc-Ms/Ms: A Comparative Extraction Study. *Toxins (Basel), 9*(10). doi:10.3390/toxins9100330

Eskola, M., Kos, G., Elliott, C.T., Hajšlová, J., Mayar, S., and Krska, R. (2019). Worldwide Contamination of Food-Crops with Mycotoxins: Validity of the Widely Cited 'Fao Estimate' of 25%. *Crit Rev Food Sci Nutr*, 1-17. doi:10.1080/10408398.2019.1658570

Estiarte, N., Crespo-Sempere, A., Marín, S., Ramos, A.J., and Worobo, R.W. (2018). Stability of Alternariol and Alternariol Monomethyl Ether During Food Processing of Tomato Products. *Food Chem, 245*, 951-957. doi:10.1016/j.foodchem.2017.11.078

Eurachem. (2014). Eurachem Guide: The Fitness for Purpose of Analytical Methods - a Laboratory Guide to Method Validation and Related Topics. 2. Retrieved from https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf (accessed: 18.05.2020)

EURL. (2016). Guidance Document on the Estimation of Lod and Loq for Measurements in the Field of Contaminants in Feed and Food. Retrieved from https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-guidance_document_lod_en.pdf (accessed: 18.05.2020)

Fan, K., Xu, J., Jiang, K., Liu, X., Meng, J., Di Mavungu, J.D., Guo, W., Zhang, Z., Jing, J., Li, H.*, et al.* (2019). Determination of Multiple Mycotoxins in Paired Plasma and Urine Samples to Assess Human Exposure in Nanjing, China. *Environ Pollut, 248*, 865-873. doi:10.1016/j.envpol.2019.02.091

Ferri, F., Brera, C., De Santis, B., Fedrizzi, G., Bacci, T., Bedogni, L., Capanni, S., Collini, G., Crespi, E., Debegnach, F.*, et al.* (2017). Survey on Urinary Levels of Aflatoxins in Professionally Exposed Workers. *Toxins (Basel), 9*(4). doi:10.3390/toxins9040117

Föllmann, W., Behm, C., and Degen, G.H. (2014). Toxicity of the Mycotoxin Citrinin and Its Metabolite Dihydrocitrinone and of Mixtures of Citrinin and Ochratoxin a in Vitro. *Arch Toxicol, 88*(5), 1097-1107. doi:10.1007/s00204-014-1216-8

Franco, C.M., Fente, C.A., Vázquez, B.I., Cepeda, A., Mahuzier, G., and Prognon, P. (1998). Interaction between Cyclodextrins and Aflatoxins Q1, M1 and P1. Fluorescence and Chromatographic Studies. *J Chromatogr A, 815*(1), 21-29. doi:10.1016/s0021-9673(98)00509-3

Frizzell, C., Ndossi, D., Verhaegen, S., Dahl, E., Eriksen, G., Sørlie, M., Ropstad, E., Muller, M., Elliott, C.T., and Connolly, L. (2011). Endocrine Disrupting Effects of Zearalenone, Alpha- and Beta-Zearalenol at the Level of Nuclear Receptor Binding and Steroidogenesis. *Toxicol Lett, 206*(2), 210-217. doi:10.1016/j.toxlet.2011.07.015

Gao, W., Jiang, L., Ge, L., Chen, M., Geng, C., Yang, G., Li, Q., Ji, F., Yan, Q., Zou, Y.*, et al.* (2015). Sterigmatocystin-Induced Oxidative DNA Damage in Human Liver-Derived Cell Line through Lysosomal Damage. *Toxicol In Vitro, 29*(1), 1-7. doi:10.1016/j.tiv.2014.08.007

Gekle, M., Sauvant, C., Schwerdt, G., and Silbernagl, S. (1998). Tubulotoxic Mechanisms of Ochratoxin A. *Kidney Blood Press Res, 21*(2-4), 277-279. doi:10.1159/000025877

Gosetti, F., Mazzucco, E., Zampieri, D., and Gennaro, M.C. (2010). Signal Suppression/Enhancement in High-Performance Liquid Chromatography Tandem Mass Spectrometry. *J Chromatogr A, 1217*(25), 3929-3937. doi:10.1016/j.chroma.2009.11.060

Groopman, J.D., Wogan, G.N., Roebuck, B.D., and Kensler, T.W. (1994). Molecular Biomarkers for Aflatoxins and Their Application to Human Cancer Prevention. *Cancer Res, 54*(7 Suppl), 1907s-1911s.

Gross, J.H. (2011). *Mass Spectrometry: A Textbook* (2 ed.). Heidelberg, Germany: Springer.

Grover, S., and Lawrence, C.B. (2017). The Alternaria Alternata Mycotoxin Alternariol Suppresses Lipopolysaccharide-Induced Inflammation. *Int J Mol Sci, 18*(7). doi:10.3390/ijms18071577

Gruber-Dorninger, C., Novak, B., Nagl, V., and Berthiller, F. (2017). Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. *J Agric Food Chem, 65*(33), 7052-7070. doi:10.1021/acs.jafc.6b03413

Haighton, L.A., Lynch, B.S., Magnuson, B.A., and Nestmann, E.R. (2012). A Reassessment of Risk Associated with Dietary Intake of Ochratoxin a Based on a Lifetime Exposure Model. *Crit Rev Toxicol, 42*(2), 147-168. doi:10.3109/10408444.2011.636342

Hanai, T. (1991). *Liquid Chromatography in Biomedical Analysis* (Vol. 50). Amsterdam, Netherlands: Elsevier Science.

Hessel-Pras, S., Kieshauer, J., Roenn, G., Luckert, C., Braeuning, A., and Lampen, A. (2019). In Vitro Characterization of Hepatic Toxicity of Alternaria Toxins. *Mycotoxin Res, 35*(2), 157-168. doi:10.1007/s12550-018-0339-9

Heussner, A.H., and Bingle, L.E. (2015). Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins (Basel), 7*(10), 4253-4282. doi:10.3390/toxins7104253

Hidalgo-Ruiz, J.L., Romero-González, R., Martínez Vidal, J.L., and Garrido Frenich, A. (2019). A Rapid Method for the Determination of Mycotoxins in Edible Vegetable Oils by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry. *Food Chem, 288*, 22-28. doi:10.1016/j.foodchem.2019.03.003

Horvatovich, K., Hafner, D., Bodnár, Z., Berta, G., Hancz, C., Dutton, M., and Kovács, M. (2013). Dose-Related Genotoxic Effect of T-2 Toxin Measured by Comet Assay Using Peripheral Blood Mononuclear Cells of Healthy Pigs. *Acta Vet Hung, 61*(2), 175-186. doi:10.1556/AVet.2013.010

Huertas-Pérez, J.F., Arroyo-Manzanares, N., García-Campaña, A.M., and Gámiz-Gracia, L. (2017). Solid Phase Extraction as Sample Treatment for the Determination of Ochratoxin a in Foods: A Review. *Crit Rev Food Sci Nutr, 57*(16), 3405-3420. doi:10.1080/10408398.2015.1126548

IARC. (1987). Iarc Monographs on the Evaluation of the Carcinogenic Risks to Humans. *Overall Evaluations of Carcinogenity: An Updating of IARC Monographs Volumes 1 to 42.* Retrieved from https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-Supplements/Overall-Evaluations-Of-Carcinogenicity-An-Updating-Of-IARC-Monographs-Volumes-1–42-1987 (accessed: 18.05.2020)

IARC. (1993). Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. *Monograph on the Evaluation of Carcinogenic Risks to Humans Volume 56.* Retrieved from https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Naturally-Occurring-Substances-Food-Items-And-Constituents-Heterocyclic-Aromatic-Amines-And-Mycotoxins-1993 (accessed: 18.05.2020)

IARC. (2002). Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 82.* Retrieved from https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Traditional-Herbal-Medicines-Some-Mycotoxins-Naphthalene-And-Styrene-2002 (accessed: 18.05.2020)

IARC. (2012). Chemical Agents and Related Occupations. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100F.* Retrieved from https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Chemical-Agents-And-Related-Occupations-2012 (accessed: 18.05.2020)

IUPAC. (1997). Compendium of Chemical Terminology, 2nd Ed. (the “Gold Book“). Retrieved from https://goldbook.iupac.org/terms/view/L03540 (accessed: 18.05.2020)

Jain, R.B. (2016). Ratio-Based Vs. Model-Based Methods to Correct for Urinary Creatinine Concentrations. *Environ Sci Pollut Res Int, 23*(16), 16417-16431. doi:10.1007/s11356-016-6818-7

Jeong, Y., Schäffer, A., and Smith, K. (2017). Equilibrium Partitioning of Organic Compounds to Oasis Hlb((R)) as a Function of Compound Concentration, Ph, Temperature and Salinity. *Chemosphere, 174*, 297-305. doi:10.1016/j.chemosphere.2017.01.116

Kabak, B., and Dobson, A.D. (2017). Mycotoxins in Spices and Herbs-an Update. *Crit Rev Food Sci Nutr, 57*(1), 18-34. doi:10.1080/10408398.2013.772891

Karabulut, S., Paytakov, G., and Leszczynski, J. (2014). Reduction of Aflatoxin B1 to Aflatoxicol: A Comprehensive Dft Study Provides Clues to Its Toxicity. *J Sci Food Agric, 94*(15), 3134-3140. doi:10.1002/jsfa.6663

Kiseleva, M., Chalyy, Z., Sedova, I., and Aksenov, I. (2020). Stability of Mycotoxins in Individual Stock and Multi-Analyte Standard Solutions. *Toxins (Basel), 12*(2). doi:10.3390/toxins12020094

Knasmüller, S., Cavin, C., Chakraborty, A., Darroudi, F., Majer, B.J., Huber, W.W., and Ehrlich, V.A. (2004). Structurally Related Mycotoxins Ochratoxin a, Ochratoxin B, and Citrinin Differ in Their Genotoxic Activities and in Their Mode of Action in Human-Derived Liver (Hepg2) Cells: Implications for Risk Assessment. *Nutr Cancer, 50*(2), 190-197. doi:10.1207/s15327914nc5002_9

Kőszegi, T., and Poór, M. (2016). Ochratoxin A: Molecular Interactions, Mechanisms of Toxicity and Prevention at the Molecular Level. *Toxins (Basel), 8*(4), 111. doi:10.3390/toxins8040111

Kovalenko, A.V., Soldatenko, N.A., Fetisov, L.N., and Strel'tsov, N.V. (2011). More Accurate Determination of the Minimum Allowable Level of Sterigmatocystin in Piglet Feed. *Russian Agricultural Sciences, 37*(6), 504-507. doi:doi.org/10.3103/S1068367411060097

Kovalsky, P., Kos, G., Nährer, K., Schwab, C., Jenkins, T., Schatzmayr, G., Sulyok, M., and Krska, R. (2016). Co-Occurrence of Regulated, Masked and Emerging Mycotoxins and Secondary Metabolites in Finished Feed and Maize-an Extensive Survey. *Toxins (Basel), 8*(12). doi:10.3390/toxins8120363

Kowalska, K., Habrowska-Górczyńska, D.E., Urbanek, K.A., Domińska, K., and Piastowska-Ciesielska, A.W. (2018). Estrogen Receptor Alpha Is Crucial in Zearalenone-Induced Invasion and Migration of Prostate Cancer Cells. *Toxins (Basel), 10*(3). doi:10.3390/toxins10030098

Krejci, M.E., Bretz, N.S., and Koechel, D.A. (1996). Citrinin Produces Acute Adverse Changes in Renal Function and Ultrastructure in Pentobarbital-Anesthetized Dogs without Concomitant Reductions in [Potassium]Plasma. *Toxicology, 106*(1-3), 167-177. doi:10.1016/0300-483x(95)03183-g

Kupski, L., Freitas, M., Ribeiro, D., Furlong, E.B., and Fernandes, E. (2016). Ochratoxin a Activates Neutrophils and Kills These Cells through Necrosis, an Effect Eliminated through Its Conversion into Ochratoxin Alpha. *Toxicology, 368-369*, 91-102. doi:10.1016/j.tox.2016.09.001

Lega, F., Angeletti, R., Stella, R., Rigoni, L., Biancotto, G., Giusepponi, D., Moretti, S., Saluti, G., and Galarini, R. (2017). Abuse of Anabolic Agents in Beef Cattle: Could Bile Be a Possible Alternative Matrix? *Food Chem, 229*, 188-197. doi:10.1016/j.foodchem.2017.02.069

Li, T., Jiang, G., Qu, H., Wang, Y., Xiong, Y., Jian, Q., Wu, Y., Duan, X., Zhu, X., Hu, W.*, et al.* (2017). Comparative Transcriptome Analysis of Penicillium Citrinum Cultured with Different Carbon Sources Identifies Genes Involved in Citrinin Biosynthesis. *Toxins (Basel), 9*(2). doi:10.3390/toxins9020069

Liang, B., Du, X., Li, P., Guo, H., Sun, C., Gao, J., and Wang, S. (2017). Orf6 Gene Encoded Glyoxalase Involved in Mycotoxin Citrinin Biosynthesis in Monascus Purpureus Yy-1. *Appl Microbiol Biotechnol, 101*(19), 7281-7292. doi:10.1007/s00253-017-8462-7

Lin, S.Y., Hsu, W.H., Lin, C.C., and Chen, C.J. (2014). Mass Spectrometry-Based Proteomics in Chest Medicine, Gerontology, and Nephrology: Subgroups Omics for Personalized Medicine. *Biomedicine (Taipei), 4*, 25. doi:10.7603/s40681-014-0025-y

Liu, B.H., Yu, F.Y., Wu, T.S., Li, S.Y., Su, M.C., Wang, M.C., and Shih, S.M. (2003). Evaluation of Genotoxic Risk and Oxidative DNA Damage in Mammalian Cells Exposed to Mycotoxins, Patulin and Citrinin. *Toxicol Appl Pharmacol, 191*(3), 255-263. doi:10.1016/s0041-008x(03)00254-0

Liu, Y., Du, M., and Zhang, G. (2014). Proapoptotic Activity of Aflatoxin B1 and Sterigmatocystin in Hepg2 Cells. *Toxicol Rep, 1*, 1076-1086. doi:10.1016/j.toxrep.2014.10.016

Liu, Z., Zhao, X., Wu, L., Zhou, S., Gong, Z., Zhao, Y., and Wu, Y. (2020). Development of a Sensitive and Reliable Uhplc-Ms/Ms Method for the Determination of Multiple Urinary Biomarkers of Mycotoxin Exposure. *Toxins (Basel), 12*(3). doi:10.3390/toxins12030193

Llewellyn, G.C., McCay, J.A., Brown, R.D., Musgrove, D.L., Butterworth, L.F., Munson, A.E., and White, K.L., Jr. (1998). Immunological Evaluation of the Mycotoxin Patulin in Female B6c3f1 Mice. *Food Chem Toxicol, 36*(12), 1107-1115. doi:10.1016/s0278-6915(98)00084-2

Lutsky, II, and Mor, N. (1981). Alimentary Toxic Aleukia (Septic Angina, Endemic Panmyelotoxicosis, Alimentary Hemorrhagic Aleukia): T-2 Toxin-Induced Intoxication of Cats. *Am J Pathol, 104*(2), 189-191.

Malachová, A., Dzuman, Z., Veprikova, Z., Vaclaviková, M., Zachariasová, M., and Hajslová, J. (2011). Deoxynivalenol, Deoxynivalenol-3-Glucoside, and Enniatins: The Major Mycotoxins Found in Cereal-Based Products on the Czech Market. *J Agric Food Chem, 59*(24), 12990-12997. doi:10.1021/jf203391x

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., and Toman, J. (2016). Ochratoxin A: 50 Years of Research. *Toxins (Basel), 8*(7). doi:10.3390/toxins8070191

Mally, A. (2012). Ochratoxin a and Mitotic Disruption: Mode of Action Analysis of Renal Tumor Formation by Ochratoxin A. *Toxicol Sci, 127*(2), 315-330. doi:10.1093/toxsci/kfs105

Mally, A., and Dekant, W. (2009). Mycotoxins and the Kidney: Modes of Action for Renal Tumor Formation by Ochratoxin a in Rodents. *Mol Nutr Food Res, 53*(4), 467-478. doi:10.1002/mnfr.200800149

Marasas, W.F.O. (2001). Discovery and Occurrence of the Fumonisins: A Historical Perspective. *Environmental Health Perspectives, 109*, 239-243. doi:DOI 10.1289/ehp.01109s2239

Marin, S., Ramos, A.J., Cano-Sancho, G., and Sanchis, V. (2013). Mycotoxins: Occurrence, Toxicology, and Exposure Assessment. *Food Chem Toxicol, 60*, 218-237. doi:10.1016/j.fct.2013.07.047

Mariño-Repizo, L., Goicoechea, H., Raba, J., and Cerutti, S. (2018). A Simple, Rapid and Novel Method Based on Salting-out Assisted Liquid-Liquid Extraction for Ochratoxin a Determination in Beer Samples Prior to Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 35*(8), 1622-1632. doi:10.1080/19440049.2018.1486045

Massart, F., Meucci, V., Saggese, G., and Soldani, G. (2008). High Growth Rate of Girls with Precocious Puberty Exposed to Estrogenic Mycotoxins. *J Pediatr, 152*(5), 690-695, 695 e691. doi:10.1016/j.jpeds.2007.10.020

McMillan, A., Renaud, J.B., Burgess, K.M.N., Orimadegun, A.E., Akinyinka, O.O., Allen, S.J., Miller, J.D., Reid, G., and Sumarah, M.W. (2018). Aflatoxin Exposure in Nigerian Children with Severe Acute Malnutrition. *Food Chem Toxicol, 111*, 356-362. doi:10.1016/j.fct.2017.11.030

Merrill, A.H., Jr., Sullards, M.C., Wang, E., Voss, K.A., and Riley, R.T. (2001). Sphingolipid Metabolism: Roles in Signal Transduction and Disruption by Fumonisins. *Environ Health Perspect, 109 Suppl 2*, 283-289. doi:10.1289/ehp.01109s2283

Moake, M.M., Padilla-Zakour, O.I., and Worobo, R.W. (2006). Comprehensive Review of Patulin Control Methods in Foods. *Comprehensive Reviews in Food Science and Food Safety, 4*(1), 8-29. doi:10.1111/j.1541-4337.2005.tb00068.x

Moukas, A., Panagiotopoulou, V., and Markaki, P. (2008). Determination of Patulin in Fruit Juices Using Hplc-Dad and Gc-Msd Techniques. *Food Chem, 109*(4), 860-867. doi:10.1016/j.foodchem.2008.01.015

Muñoz, K., Cramer, B., Dopstadt, J., Humpf, H.U., and Degen, G.H. (2017). Evidence of Ochratoxin a Conjugates in Urine Samples from Infants and Adults. *Mycotoxin Res, 33*(1), 39-47. doi:10.1007/s12550-016-0261-y

Ok, H.E., Kang, Y.W., Kim, M., and Chun, H.S. (2013). T-2 and Ht-2 Toxins in Cereals and Cereal-Based Products in South Korea. *Food Additives & Contaminants Part B-Surveillance, 6*(2), 103-109. doi:10.1080/19393210.2012.735703

Osteresch, B., Viegas, S., Cramer, B., and Humpf, H.U. (2017). Multi-Mycotoxin Analysis Using Dried Blood Spots and Dried Serum Spots. *Anal Bioanal Chem, 409*(13), 3369-3382. doi:10.1007/s00216-017-0279-9

Ostry, V., Malir, F., Toman, J., and Grosse, Y. (2017). Mycotoxins as Human Carcinogens-the Iarc Monographs Classification. *Mycotoxin Res, 33*(1), 65-73. doi:10.1007/s12550-016-0265-7

Pahlke, G., Tiessen, C., Domnanich, K., Kahle, N., Groh, I.A., Schreck, I., Weiss, C., and Marko, D. (2016). Impact of Alternaria Toxins on Cyp1a1 Expression in Different Human Tumor Cells and Relevance for Genotoxicity. *Toxicol Lett, 240*(1), 93-104. doi:10.1016/j.toxlet.2015.10.003

Palumbo, R., Crisci, A., Venâncio, A., Cortiñas Abrahantes, J., Dorne, J.L., Battilani, P., and Toscano, P. (2020). Occurrence and Co-Occurrence of Mycotoxins in Cereal-Based Feed and Food. *Microorganisms, 8*(1). doi:10.3390/microorganisms8010074

Partanen, H.A., El-Nezami, H.S., Leppänen, J.M., Myllynen, P.K., Woodhouse, H.J., and Vähäkangas, K.H. (2010). Aflatoxin B1 Transfer and Metabolism in Human Placenta. *Toxicol Sci, 113*(1), 216-225. doi:10.1093/toxsci/kfp257

Pierron, A., Bracarense, A.P.F.L., Cossalter, A.M., Laffitte, J., Schwartz-Zimmermann, H.E., Schatzmayr, G., Pinton, P., Moll, W.D., and Oswald, I.P. (2018). Deepoxy-Deoxynivalenol Retains Some Immune-Modulatory Properties of the Parent Molecule Deoxynivalenol in Piglets. *Arch Toxicol, 92*(11), 3381-3389. doi:10.1007/s00204-018-2293-x

Pietri, A., Bertuzzi, T., Fortunati, P., and Gualla, A. (2003). Excretion Pattern of Aflatoxins in Buffalo Milk and Carry-over in Mozzarella Cheese. *Italian Journal of Animal Science, 2*, 302-304. doi:doi.org/10.4081/ijas.2003.11675993

Ponce-García, N., Serna-Saldivar, S.O., and Garcia-Lara, S. (2018). Fumonisins and Their Analogues in Contaminated Corn and Its Processed Foods - a Review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 35*(11), 2183-2203. doi:10.1080/19440049.2018.1502476

Preindl, K., Braun, D., Aichinger, G., Sieri, S., Fang, M., Marko, D., and Warth, B. (2019). A Generic Liquid Chromatography-Tandem Mass Spectrometry Exposome Method for the Determination of Xenoestrogens in Biological Matrices. *Anal Chem, 91*(17), 11334-11342. doi:10.1021/acs.analchem.9b02446

Puntscher, H., Aichinger, G., Grabher, S., Attakpah, E., Kruger, F., Tillmann, K., Motschnig, T., Hohenbichler, J., Braun, D., Plasenzotti, R*., et al.* (2019a). Bioavailability, Metabolism, and Excretion of a Complex Alternaria Culture Extract Versus Altertoxin Ii: A Comparative Study in Rats. *Arch Toxicol, 93*(11), 3153-3167. doi:10.1007/s00204-019-02575-7

Puntscher, H., Hankele, S., Tillmann, K., Attakpah, E., Braun, D., Kütt, M.L., Del Favero, G., Aichinger, G., Pahlke, G., Höger, H*., et al.* (2019b). First Insights into Alternaria Multi-Toxin in Vivo Metabolism. *Toxicol Lett, 301*, 168-178. doi:10.1016/j.toxlet.2018.10.006

Puntscher, H., Kütt, M.L., Skrinjar, P., Mikula, H., Podlech, J., Fröhlich, J., Marko, D., and Warth, B. (2018). Tracking Emerging Mycotoxins in Food: Development of an Lc-Ms/Ms Method for Free and Modified Alternaria Toxins. *Anal Bioanal Chem, 410*(18), 4481-4494. doi:10.1007/s00216-018-1105-8

Qian, G.-S., Ross, R.K., Yu, M.C., Yuan, J.-M., Gao, Y.-T., Henderson, B.E., Wogan, G.N., and Groopman, J.D. (1994). A Follow-up Study of Urinary Markers of Aflatoxin Exposure and Liver Cancer Risk in Shanghai, People's Republic of China. *Cancer Epidemiology, Biomarkers & Prevention, 3*(1), 3-10.

Qiao, X., Zhang, J., Yang, Y., Yin, J., Li, H., Xing, Y., and Shao, B. (2020). Development of a Simple and Rapid Lc-Ms/Ms Method for the Simultaneous Quantification of Five Alternaria Mycotoxins in Human Urine. *J Chromatogr B Analyt Technol Biomed Life Sci, 1144*, 122096. doi:10.1016/j.jchromb.2020.122096

Rašić, D., Mladinić, M., Želježić, D., Pizent, A., Stefanović, S., Milićević, D., Konjevoda, P., and Peraica, M. (2018). Effects of Combined Treatment with Ochratoxin a and Citrinin on Oxidative Damage in Kidneys and Liver of Rats. *Toxicon, 146*, 99-105. doi:10.1016/j.toxicon.2018.03.002

Rheeder, J.P., Marasas, W.F., and Vismer, H.F. (2002). Production of Fumonisin Analogs by Fusarium Species. *Appl Environ Microbiol, 68*(5), 2101-2105. doi:10.1128/aem.68.5.2101-2105.2002

Richard, J.L. (2007). Some Major Mycotoxins and Their Mycotoxicoses--an Overview. *Int J Food Microbiol, 119*(1-2), 3-10. doi:10.1016/j.ijfoodmicro.2007.07.019

Riley, R.T., and Voss, K.A. (2006). Differential Sensitivity of Rat Kidney and Liver to Fumonisin Toxicity: Organ-Specific Differences in Toxin Accumulation and Sphingoid Base Metabolism. *Toxicol Sci, 92*(1), 335-345. doi:10.1093/toxsci/kfj198

Sakai, K., Kinoshita, H., Shimizu, T., and Nihira, T. (2008). Construction of a Citrinin Gene Cluster Expression System in Heterologous Aspergillus Oryzae. *J Biosci Bioeng, 106*(5), 466-472. doi:10.1263/jbb.106.466

Šarkanj, B., Ezekiel, C.N., Turner, P.C., Abia, W.A., Rychlik, M., Krska, R., Sulyok, M., and Warth, B. (2018). Ultra-Sensitive, Stable Isotope Assisted Quantification of Multiple Urinary Mycotoxin Exposure Biomarkers. *Anal Chim Acta, 1019*, 84-92. doi:10.1016/j.aca.2018.02.036

Šarkanj, B., Warth, B., Uhlig, S., Abia, W.A., Sulyok, M., Klapec, T., Krska, R., and Banjari, I. (2013). Urinary Analysis Reveals High Deoxynivalenol Exposure in Pregnant Women from Croatia. *Food Chem Toxicol, 62*, 231-237. doi:10.1016/j.fct.2013.08.043

Schellinger, A.P., and Carr, P.W. (2006). Isocratic and Gradient Elution Chromatography: A Comparison in Terms of Speed, Retention Reproducibility and Quantitation. *J Chromatogr A, 1109*(2), 253-266. doi:10.1016/j.chroma.2006.01.047

Schlittenbauer, L., Seiwert, B., and Reemtsma, T. (2015). Matrix Effects in Human Urine Analysis Using Multi-Targeted Liquid Chromatography-Tandem Mass Spectrometry. *J Chromatogr A, 1415*, 91-99. doi:10.1016/j.chroma.2015.08.069

Selmanoglu, G., and Koçkaya, E.A. (2004). Investigation of the Effects of Patulin on Thyroid and Testis, and Hormone Levels in Growing Male Rats. *Food Chem Toxicol, 42*(5), 721-727. doi:10.1016/j.fct.2003.12.007

Shier, W.T., Shier, A.C., Xie, W., and Mirocha, C.J. (2001). Structure-Activity Relationships for Human Estrogenic Activity in Zearalenone Mycotoxins. *Toxicon, 39*(9), 1435-1438. doi:10.1016/s0041-0101(00)00259-2

Shinozuka, J., Suzuki, M., Noguchi, N., Sugimoto, T., Uetsuka, K., Nakayama, H., and Doi, K. (1998). T-2 Toxin-Induced Apoptosis in Hematopoietic Tissues of Mice. *Toxicol Pathol, 26*(5), 674-681. doi:10.1177/019262339802600512

Singh, N., Bansal, M., Pal, S., Alam, S., Jagdale, P., Ayanur, A., and Ansari, K.M. (2018). Cox-2/Ep2-Ep4/Beta-Catenin Signaling Regulates Patulin-Induced Intestinal Cell Proliferation and Inflammation. *Toxicol Appl Pharmacol, 356*, 224-234. doi:10.1016/j.taap.2018.08.009

Slobodchikova, I., Sivakumar, R., Rahman, M.S., and Vuckovic, D. (2019). Characterization of Phase I and Glucuronide Phase Ii Metabolites of 17 Mycotoxins Using Liquid Chromatography-High-Resolution Mass Spectrometry. *Toxins (Basel), 11*(8). doi:10.3390/toxins11080433

Snyder, L.R., Kirkland, J.J., and Dolan, J.W. (2010). *Introduction to Modern Liquid Chromatography* (3 ed.). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., and Kizek, R. (2010). Deoxynivalenol and Its Toxicity. *Interdiscip Toxicol, 3*(3), 94-99. doi:10.2478/v10102-010-0019-x

Souci, S.W., Fachmann, W., and Kraut, H. (2008). *Food Composition and Nutrition Tables* (Vol. 7). Stuttgart: Medpharm Scientific Publishers.

Spadaro, D., Ciavorella, A., Frati, S., Garibaldi, A., and Gullino, M.L. (2007). Incidence and Level of Patulin Contamination in Pure and Mixed Apple Juices Marketed in Italy. *Food Control, 18*(9), 1098-1102. doi:10.1016/j.foodcont.2006.07.007

Sreemannarayana, O., Marquardt, R.R., Frohlich, A.A., and Juck, F.A. (1986). Some Acute Biochemical and Pathological Changes in Chicks after Oral Administration of Sterigmatocystin. *Journal of the American College of Toxicology, 5*, 275-287. doi:doi.org/10.3109/10915818609140752

Streit, E., Naehrer, K., Rodrigues, I., and Schatzmayr, G. (2013). Mycotoxin Occurrence in Feed and Feed Raw Materials Worldwide: Long-Term Analysis with Special Focus on Europe and Asia. *J Sci Food Agric, 93*(12), 2892-2899. doi:10.1002/jsfa.6225

Sun, D., Qiu, N., Zhou, S., Lyu, B., Zhang, S., Li, J., Zhao, Y., and Wu, Y. (2019). Development of Sensitive and Reliable Uplc-Ms/Ms Methods for Food Analysis of Emerging Mycotoxins in China Total Diet Study. *Toxins (Basel), 11*(3). doi:10.3390/toxins11030166

Sundstøl Eriksen, G., Pettersson, H., and Lundh, T. (2004). Comparative Cytotoxicity of Deoxynivalenol, Nivalenol, Their Acetylated Derivatives and De-Epoxy Metabolites. *Food Chem Toxicol, 42*(4), 619-624. doi:10.1016/j.fct.2003.11.006

Sweeney, M.J., and Dobson, A.D. (1998). Mycotoxin Production by Aspergillus, Fusarium and Penicillium Species. *Int J Food Microbiol, 43*(3), 141-158. doi:10.1016/s0168-1605(98)00112-3

Tangni, E.K., Waegeneers, N., Van Overmeire, I., Goeyens, L., and Pussemier, L. (2009). Mycotoxin Analyses in Some Home Produced Eggs in Belgium Reveal Small Contribution to the Total Daily Intake. *Sci Total Environ, 407*(15), 4411-4418. doi:10.1016/j.scitotenv.2008.10.060

Theumer, M.G., Henneb, Y., Khoury, L., Snini, S.P., Tadrist, S., Canlet, C., Puel, O., Oswald, I.P., and Audebert, M. (2018). Genotoxicity of Aflatoxins and Their Precursors in Human Cells. *Toxicol Lett, 287*, 100-107. doi:10.1016/j.toxlet.2018.02.007

Tiessen, C., Ellmer, D., Mikula, H., Pahlke, G., Warth, B., Gehrke, H., Zimmermann, K., Heiss, E., Fröhlich, J., and Marko, D. (2017). Impact of Phase I Metabolism on Uptake, Oxidative Stress and Genotoxicity of the Emerging Mycotoxin Alternariol and Its Monomethyl Ether in Esophageal Cells. *Arch Toxicol, 91*(3), 1213-1226. doi:10.1007/s00204-016-1801-0

Tolosa, J., Rodríguez-Carrasco, Y., Ferrer, E., and Mañes, J. (2019). Identification and Quantification of Enniatins and Beauvericin in Animal Feeds and Their Ingredients by Lc-Qtrap/Ms/Ms. *Metabolites, 9*(2). doi:10.3390/metabo9020033

Torović, L., Dimitrov, N., Assunção, R., and Alvito, P. (2017). Risk Assessment of Patulin Intake through Apple-Based Food by Infants and Preschool Children in Serbia. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 34*(11), 2023-2032. doi:10.1080/19440049.2017.1364434

Uchigashima, M., Yamaguchi Murakami, Y., Narita, H., Nakajima, M., and Miyake, S. (2012). Development of an Immuno-Affinity Column for Ochratoxin Analysis Using an Organic Solvent-Tolerant Monoclonal Antibody. *Methods, 56*(2), 180-185. doi:10.1016/j.ymeth.2011.09.006

Ueno, Y., Hosoya, M., Morita, Y., Ueno, I., and Tatsuno, T. (1968). Inhibition of the Protein Synthesis in Rabbit Reticulocyte by Nivalenol, a Toxic Principle Isolated from Fusarium Nivale-Growing Rice. *J Biochem, 64*(4), 479-485. doi:10.1093/oxfordjournals.jbchem.a128919

Vejdovszky, K., Hahn, K., Braun, D., Warth, B., and Marko, D. (2017a). Synergistic Estrogenic Effects of Fusarium and Alternaria Mycotoxins in Vitro. *Arch Toxicol, 91*(3), 1447-1460. doi:10.1007/s00204-016-1795-7

Vejdovszky, K., Schmidt, V., Warth, B., and Marko, D. (2017b). Combinatory Estrogenic Effects between the Isoflavone Genistein and the Mycotoxins Zearalenone and Alternariol in Vitro. *Mol Nutr Food Res, 61*(3). doi:10.1002/mnfr.201600526

Vettorazzi, A., van Delft, J., and López de Cerain, A. (2013). A Review on Ochratoxin a Transcriptomic Studies. *Food Chem Toxicol, 59*, 766-783. doi:10.1016/j.fct.2013.05.043

Vidal, A., Ouhibi, S., Ghali, R., Hedhili, A., De Saeger, S., and De Boevre, M. (2019). The Mycotoxin Patulin: An Updated Short Review on Occurrence, Toxicity and Analytical Challenges. *Food Chem Toxicol, 129*, 249-256. doi:10.1016/j.fct.2019.04.048

Viegas, S., Veiga, L., Figueredo, P., Almeida, A., Carolino, E., Sabino, R., Veríssimo, C., and Viegas, C. (2013). Occupational Exposure to Aflatoxin B1 in Swine Production and Possible Contamination Sources. *J Toxicol Environ Health A, 76*(15), 944-951. doi:10.1080/15287394.2013.826569

Viegas, S., Veiga, L., Malta-Vacas, J., Sabino, R., Figueredo, P., Almeida, A., Viegas, C., and Carolino, E. (2012). Occupational Exposure to Aflatoxin (Afb(1)) in Poultry Production. *J Toxicol Environ Health A, 75*(22-23), 1330-1340. doi:10.1080/15287394.2012.721164

Voss, K.A., Smith, G.W., and Haschek, W.M. (2007). Fumonisins: Toxicokinetics, Mechanism of Action and Toxicity. *Animal Feed Science and Technology, 137*(3-4), 299-325. doi:10.1016/j.anifeedsci.2007.06.007

Warth, B., Preindl, K., Manser, P., Wick, P., Marko, D., and Buerki-Thurnherr, T. (2019). Transfer and Metabolism of the Xenoestrogen Zearalenone in Human Perfused Placenta. *Environ Health Perspect, 127*(10), 107004. doi:10.1289/EHP4860

Warth, B., Sulyok, M., Berthiller, F., Schuhmacher, R., and Krska, R. (2013). New Insights into the Human Metabolism of the Fusarium Mycotoxins Deoxynivalenol and Zearalenone. *Toxicol Lett, 220*(1), 88-94. doi:10.1016/j.toxlet.2013.04.012

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Fröhlich, J.*, et al.* (2012). Development and Validation of a Rapid Multi-Biomarker Liquid Chromatography/Tandem Mass Spectrometry Method to Assess Human Exposure to Mycotoxins. *Rapid Commun Mass Spectrom, 26*(13), 1533-1540. doi:10.1002/rcm.6255

Waters. (2019). Spe Method Development. Retrieved from https://www.waters.com/waters/en_US/SPE-Method-Development/nav.htm?cid=10083845&locale=en_US (accessed: 18.05.2020)

Wu, T.S., Yang, J.J., Yu, F.Y., and Liu, B.H. (2013). Cardiotoxicity of Mycotoxin Citrinin and Involvement of Microrna-138 in Zebrafish Embryos. *Toxicol Sci, 136*(2), 402-412. doi:10.1093/toxsci/kft206

Zhao, H., Chen, X., Shen, C., and Qu, B. (2017). Determination of 16 Mycotoxins in Vegetable Oils Using a Quechers Method Combined with High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 34*(2), 255-264. doi:10.1080/19440049.2016.1266096

Zheng, X., Zhang, X., Zhao, L., Apaliya, M.T., Yang, Q., Sun, W., Zhang, X., and Zhang, H. (2017). Screening of Deoxynivalenol Producing Strains and Elucidation of Possible Toxigenic Molecular Mechanism. *Toxins (Basel), 9*(6). doi:10.3390/toxins9060184

Zhou, W., Yang, S., and Wang, P.G. (2017). Matrix Effects and Application of Matrix Effect Factor. *Bioanalysis, 9*(23), 1839-1844. doi:10.4155/bio-2017-0214

Zinedine, A., Soriano, J.M., Moltó, J.C., and Mañes, J. (2007). Review on the Toxicity, Occurrence, Metabolism, Detoxification, Regulations and Intake of Zearalenone: An Oestrogenic Mycotoxin. *Food Chem Toxicol, 45*(1), 1-18. doi:10.1016/j.fct.2006.07.030

Zouaoui, N., Mallebrera, B., Berrada, H., Abid-Essefi, S., Bacha, H., and Ruiz, M.J. (2016). Cytotoxic Effects Induced by Patulin, Sterigmatocystin and Beauvericin on Cho-K1 Cells. *Food Chem Toxicol, 89*, 92-103. doi:10.1016/j.fct.2016.01.010

Appendix

Table A1: Performance characteristics for the first validation run including retention time (RT), correlation coefficient (R^2), extraction recovery (R_E), relative standard deviation (RSD), intraday precision (RSD_r), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	RT	R^2	Ion ratio[a]		R_E + RSD		RSD_r		SSE[c]	LOD	LOQ
				spiking levels[b]	level 1	level 2	level 1	level 2			
	(min)			(ng/L)	(%)	(%)	(%)	(%)	(%)	(ng/L)	(ng/L)
Aflatoxicol	18.1	0.9991	0.78	800/13333	78 ± 4	104 ± 5	-	-	65	225	450
Aflatoxin B_1	17.6	0.9998	0.65	120/2000	77 ± 3	106 ± 8	-	-	85	30	60
Aflatoxin B_2	16.7	0.9993	0.52	200/3333	73 ± 8	103 ± 6	-	-	91	60	121
Aflatoxin G_1	16.7	0.9998	1.66	140/2333	74 ± 4	104 ± 7	-	-	98	34	68
Aflatoxin G_2	-	-	-	200/3333	-	-	-	-	-	-	-
Aflatoxin M_1	14.7	0.9994	0.55	140/2333	76 ± 2	101 ± 4	-	-	94	27	55
Aflatoxin M_2	13.8	0.9999	0.59	600/10000	75 ± 6	105 ± 4	-	-	75	99	198
Aflatoxin P_1	14.6	0.9999	0.32	600/10000	62 ± 4	97 ± 4	-	-	63	105	210
Aflatoxin Q_1	14.9	1.0000	0.65	600/10000	-	80 ± 11	-	-	77	2243	4487
Aflatoxin-N^7-guanine	11.0	0.9999	0.15	40/667	-	68 ± 22	-	-	43	29	58
Alternariol	18.4	0.9988	1.68	1000/16667	-	-	-	-	28%	250	500
Alternariol monomethyl ether	20.7	0.9992	0.17	60/1000	70 ± 9	52 ± 6	-	-	20	15	30
Citrinin	19.0	0.9972	0.11	3000/50000	43 ± 21	41 ± 17	-	-	64	1076	2152
Dihydrocitrinone	16.2	0.9989	0.71	1200/20000	82 ± 1	98 ± 1	-	-	106	333	666
Deoxynivalenol	8.5	1.0000	2.94	4000/66667	65 ± 7	87 ± 2	-	-	94	998	1997
Deepoxy-deoxynivalenol	10.2	0.9997	0.94	2000/30000	69 ± 9	82 ± 7	-	-	28	584	1167
Nivalenol	5.7	1.0000	0.88	1000/16667	99 ± -	92 ± 4	-	-	86	411	821
Fumonisin B_1	14.9	0.9998	0.97	400/6667	24 ± 41	19 ± 37	-	-	96	92	184
Ochratoxin A	20.6	0.9997	0.33	40/667	61 ± 44	76 ± 26	-	-	98	24	48
Ochratoxin B	19.5	0.9989	0.31	40/667	54 ± 7	92 ± 15	-	-	88	38	75
Ochratoxin α	15.1	0.9979	0.16	200/3333	-	128 ± 4	-	-	81	108	216
Sterigmatocystin	21.1	1.0000	0.86	10/167	-	77 ± 8	-	-	99	13	26
T-2 toxin	20.3	0.9993	0.77	6000/100000	-	84 ± 5	-	-	66	8054	16108
Tentoxin	18.2	0.9995	0.57	200/3333	81 ± 6	93 ± 4	-	-	42	46	91
Zearalanone	20.7	0.9996	1.32	600/10000	74 ± 5	105 ± 3	-	-	18	268	537
α-Zearalanol	19.6	0.9996	0.082	1200/20000	93 ± 12	106 ± 2	-	-	53	406	811
β-Zearalanol	18.8	0.9995	0.26	1200/20000	84 ± 0	112 ± 4	-	-	45	190	380
Zearalenone	20.8	0.9999	1.22	400/6667	80 ± 6	89 ± 3	-	-	99	75	150
α-Zearalenol	19.8	0.9996	0.61	200/3333	93 ± 14	91 ± 7	15	8	43	100	201
β-Zearalenol	19.0	0.9996	0.65	200/3333	75 ± 14	109 ± 4	-	-	59	83	166

[a] calculated as the ratio of qualifying ion and quantifying ion

[b] spiking levels reported in following order: level 1/level 2

[c] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent

Table A2: Performance characteristics for the second validation run including retention time (RT), correlation coefficient (R^2), extraction recovery (R_E), relative standard deviation (RSD), intraday precision (RSD_r), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	RT	R^2	Ion ratio[a]		R_E + RSD		RSD_r		SSE[c]	LOD	LOQ
				spiking levels[b]	level 1	level 2	level 1	level 2			
	(min)			(ng/L)	(%)	(%)	(%)	(%)	(%)	(ng/L)	(ng/L)
Aflatoxicol	18.2	0.9998	0.82	800/13333	87 ± 2	94 ± 3	14	6	78	168	336
Aflatoxin B_1	17.6	0.9991	0.66	120/2000	112 ± 5	91 ± 2	5	5	82	36	72
Aflatoxin B_2	16.7	0.9994	0.51	200/3333	79 ± 5	94 ± 3	10	6	103	50	100
Aflatoxin G_1	16.8	0.9999	1.59	140/2333	91 ± 7	95 ± 3	9	6	109	36	72
Aflatoxin G_2	-	-	-	200/3333	-	-	-	-	-	-	-
Aflatoxin M_1	14.7	0.9993	0.54	140/2333	113 ± 1	95 ± 2	2	4	102	29	58
Aflatoxin M_2	13.8	0.9992	0.60	600/10000	113 ± 4	94 ± 6	3	5	89	118	235
Aflatoxin P_1	14.7	0.9998	0.30	600/10000	85 ± 5	89 ± 6	4	5	73	96	193
Aflatoxin Q_1	14.9	0.9995	0.75	600/10000	-	75 ± 6	-	5	138	1881	3762
Aflatoxin-N^7-guanine	11.0	0.9995	0.15	40/667	-	42 ± 29	-	23	54	40	81
Alternariol	18.4	0.9972	1.66	1000/16667	-	-	-	-	108	232	464
Alternariol monomethyl ether	20.7	0.9987	0.17	60/1000	64 ± 2	74 ± 12	8	18	30	12	24
Citrinin	19.0	0.9957	0.11	3000/50000	37 ± 11	37 ± 42	8	34	71	359	719
Dihydrocitrinone	16.0	0.9994	0.69	1200/20000	72 ± 19	85 ± 14	16	12	99	286	572
Deoxynivalenol	8.5	1.0000	2.79	4000/66667	94 ± 3	111 ± 8	6	7	90	1046	2093
Deepoxy-deoxynivalenol	10.2	0.9989	0.96	2000/30000	64 ± 8	97 ± 6	20	9	50	549	1099
Nivalenol	5.7	0.9999	0.85	1000/16667	115 ± 5	96 ± 10	5	7	84	255	510
Fumonisin B_1	14.9	0.9993	0.99	400/6667	55 ± 5	14 ± 23	8	24	80	56	112
Ochratoxin A	20.6	0.9992	0.34	40/667	46 ± 13	47 ± 51	20	52	89	15	30
Ochratoxin B	19.6	0.9997	0.33	40/667	-	60 ± 48	-	39	95	21	42
Ochratoxin α	15.0	0.9999	0.16	200/3333	-	91 ± 28	-	22	86	152	303
Sterigmatocystin	21.1	0.9980	0.84	10/167	53 ± 10	66 ± 1	14	14	111	2	4
T-2 toxin	20.3	0.9999	0.81	6000/100000	93 ± 11	96 ± 9	9	7	63	2477	4954
Tentoxin	18.2	0.9970	0.55	200/3333	94 ± 4	99 ± 5	3	4	41	51	101
Zearalanone	20.7	1.0000	1.30	600/10000	100 ± 4	104 ± 10	4	9	29	134	268
α-Zearalanol	19.6	0.9996	0.08	1200/20000	77 ± 20	69 ± 3	20	14	29	127	253
β-Zearalanol	18.8	0.9999	0.27	1200/20000	67 ± 7	88 ± 2	15	19	46	140	281
Zearalenone	20.8	0.9998	1.20	400/6667	97 ± 3	100 ± 11	8	12	100	67	134
α-Zearalenol	19.8	0.9994	0.62	200/3333	92 ± 2	73 ± 17	-	6	21	143	286
β-Zearalenol	19.0	0.9997	0.66	200/3333	88 ± 7	94 ± 2	18	20	51	56	111

[a] calculated as the ratio of qualifying ion and quantifying ion
[b] spiking levels reported in following order: level 1/level 2
[c] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent

Table A3: Performance characteristics for the third validation run including retention time (RT), correlation coefficient (R^2), extraction recovery (R_E), relative standard deviation (RSD), intraday precision (RSD_r), signal suppression/enhancement (SSE), limit of detection (LOD), and limit of quantification (LOQ)

Analyte	RT	R^2	Ion ratio[a]		R_E + RSD		RSD_r		SSE[c]	LOD	LOQ
				spiking levels[b]	level 1	level 2	level 1	level 2			
	(min)			(ng/L)	(%)	(%)	(%)	(%)	(%)	(ng/L)	(ng/L)
Aflatoxicol	18.1	0.9976	0.84	800/13333	71 ± 6	81 ± 4	-	-	64	112	224
Aflatoxin B_1	17.6	0.9997	0.69	120/2000	93 ± 6	94 ± 4	-	-	77	29	58
Aflatoxin B_2	16.7	0.9977	0.53	200/3333	72 ± 3	89 ± 4	-	-	82	21	42
Aflatoxin G_1	16.7	0.9981	1.59	140/2333	82 ± 3	84 ± 7	-	-	88	52	104
Aflatoxin G_2	-	-	-	200/3333	-	-	-	-	-	-	-
Aflatoxin M_1	14.7	0.9995	0.57	140/2333	83 ± 5	93 ± 8	-	-	95	28	55
Aflatoxin M_2	13.8	0.9993	0.57	600/10000	93 ± 3	86 ± 10	-	-	78	132	265
Aflatoxin P_1	14.6	0.9981	0.36	600/10000	69 ± 7	84 ± 10	-	-	62	135	270
Aflatoxin Q_1	14.9	0.9989	0.67	600/10000	-	82 ± 3	-	-	85	1216	2432
Aflatoxin-N^7-guanine	11.0	0.9986	0.14	40/667	-	50 ± 25	-	-	50	28	56
Alternariol	18.4	0.9970	1.64	1000/16667	-	-	-	-	101	83	166
Alternariol monomethyl ether	20.7	0.9988	0.17	60/1000	51 ± 3	62 ± 13	-	-	31	9	19
Citrinin	19.0	0.9995	0.11	3000/50000	25 ± 22	10 ± 68	-	-	68	574	1148
Dihydrocitrinone	15.8	0.9999	0.69	1200/20000	80 ± 12	96 ± -	-	-	93	267	534
Deoxynivalenol	8.5	0.9994	3.10	4000/66667	85 ± 5	102 ± 7	-	-	91	800	1600
Deepoxy-deoxynivalenol	10.2	0.9993	0.99	2000/30000	72 ± 14	101 ± 10	-	-	32	271	542
Nivalenol	5.7	0.9998	0.85	1000/16667	84 ± 2	90 ± 11	-	-	85	260	520
Fumonisin B_1	14.9	0.9997	1.00	400/6667	27 ± 4	12 ± 49	-	-	88	55	109
Ochratoxin A	20.6	0.9951	0.33	40/667	67 ± 6	80 ± 38	-	-	103	21	42
Ochratoxin B	19.5	0.9944	0.33	40/667	-	78 ± 29	-	-	78	30	60
Ochratoxin α	14.9	0.9946	0.15	200/3333	-	53 ± 75	-	-	78	188	377
Sterigmatocystin	21.1	0.9980	0.85	10/167	63 ± 2	79 ± 5	-	-	76	1	3
T-2 toxin	20.2	0.9982	0.76	6000/100000	-	93 ± 7	-	-	46	3240	6481
Tentoxin	18.2	0.9999	0.56	200/3333	87 ± 4	85 ± 7	-	-	43	32	65
Zearalanone	20.7	0.9991	1.33	600/10000	96 ± 10	96 ± 18	-	-	32	180	360
α-Zearalanol	19.6	0.9991	0.08	1200/20000	86 ± 6	95 ± 8	-	-	29	261	523
β-Zearalanol	18.8	0.9998	0.27	1200/20000	77 ± 2	97 ± 13	-	-	54	235	470
Zearalenone	20.8	0.9994	1.18	400/6667	59 ± 5	100 ± 7	-	-	105	53	106
α-Zearalenol	19.8	0.9990	0.66	200/3333	65 ± 11	98 ± 8	-	-	29	52	104
β-Zearalenol	19.0	0.9998	0.65	200/3333	74 ± 5	111 ± 10	-	-	57	54	109

[a] calculated as the ratio of qualifying ion and quantifying ion

[b] spiking levels reported in following order: level 1/level 2

[c] calculated as the ratio of matrix-matched calibration slope and solvent calibration slope and expressed as percent

Table A4: Calibration ranges (excluding zero) and calibration curves for analytes during in-house validation experiments in urine

Analyte	Internal standard[a]	Calibration range (ng/L)	Calibration curves[b]
Aflatoxicol	$^{13}C_{17}$-Aflatoxin M_1	400 - 40000	y = 0.0029x + 0.2743 / y = 0.0030x + 0.2496 / y = 0.0032x + 0.5741
Aflatoxin B_1	$^{13}C_{17}$-Aflatoxin M_1	30 - 6000	y = 0.0023x + 0.0036 / y = 0.0022x - 0.0906 / y = 0.0022x - 0.0098
Aflatoxin B_2	$^{13}C_{17}$-Aflatoxin M_1	100 - 10000	y = 0.0009x + 0.0077 / y = 0.0009x + 0.0238 / y = 0.0009x + 0.0651
Aflatoxin G_1	$^{13}C_{17}$-Aflatoxin M_1	35 - 7000	y = 0.0020x + 0.0020 / y = 0.0020x + 0.0001 / y = 0.0020x + 0.0004
Aflatoxin G_2	-	-	-
Aflatoxin M_1	$^{13}C_{17}$-Aflatoxin M_1	35 - 7000	y = 0.0036x + 0.0196 / y = 0.0034x - 0.1069 / y = 0.0035x + 0.0577
Aflatoxin M_2	$^{13}C_{17}$-Aflatoxin M_1	150 - 30000	y = 0.0013x + 0.0302 / y = 0.0013x - 0.2033 / y = 0.0014x + 0.0986
Aflatoxin P_1	$^{13}C_{17}$-Aflatoxin M_1	150 - 30000	y = 0.0024x + 0.0894 / y = 0.0020x + 0.0432 / y = 0.0020x + 0.5988
Aflatoxin Q_1	$^{13}C_{17}$-Aflatoxin M_1	1000 - 30000	y = 0.00003x - 0.0009 / y = 0.00003x - 0.0039 / y = 0.00003x + 0.0129
Aflatoxin-N^7-guanine	^{13}C-Aflatoxin M_1	67 - 2000	y = 0.0015x + 0.0035 / y = 0.0015x - 0.0176 / y = 0.0014x + 0.0432
Alternariol	$^{2}H_4$-Alternariol	500 - 50000	y = 131.5x + 1706 / y = 127.5x + 6169 / y = 0.0205x + 18.5540
Alternariol monomethyl ether	$^{2}H_4$-Alternariol	15 - 3000	y = 0.2020x - 5.3252 / y = 0.1515x + 0.0005 / y = 0.2186x + 0.0009
Citrinin	-	1500 - 150000	y = 3213x - 0.000002 / y = 4843x - 0.0001 / y = 3739x - 0.000004
Dihydrocitrinone	-	600 - 60000	y = 458.6x - 24111 / y = 407.0x - 4.742 / y = 462.1x - 28854
Deoxynivalenol	$^{13}C_{15}$-Deoxynivalenol	2000 - 200000	y = 0.0005x + 0.4342 / y = 0.0004x + 0.4607 / y = 0.0005x + 0.4561
Deepoxy-deoxynivalenol	$^{13}C_{15}$-Deoxynivalenol	1000 - 100000	y = 0.0012x - 0.0721 / y = 0.0009x + 0.6789 / y = 0.0011x + 0.3100
Nivalenol	$^{13}C_{15}$-Nivalenol	500 - 50000	y = 0.0029x - 0.3470 / y = 0.0027x - 0.1839 / y = 0.0029x + 0.7776
Fumonisin B_1	$^{13}C_{34}$-Fumonisin B_1	200 - 2000	y = 0.0068x - 0.3402 / y = 0.0080x - 1.3703 / y = 0.0087x - 0.6684
Ochratoxin A	$^{13}C_{20}$-Ochratoxin A	67 - 2000	y = 0.0035x - 0.0003 / y = 0.0034x - 0.0001 / y = 0.0032x + 0.1143
Ochratoxin B	$^{13}C_{20}$-Ochratoxin A	67 - 2000	y = 0.0177x + 0.5644 / y = 0.0121x - 0.0018 / y = 0.0121x + 0.5577
Ochratoxin α	$^{13}C_{20}$-Ochratoxin A	333 - 10000	y = 0.0016x + 0.2510 / y = 0.0008x - 0.0287 / y = 0.0008x + 0.1643
Sterigmatocystin	$^{13}C_{17}$-Aflatoxin M_1	5 - 500	y = 0.0009x + 0.0015 / y = 0.0076x - 0.0524 / y = 0.0065x + 0.0063
T-2 toxin	-	10000 - 300000	y = 4.856x + 14453 / y = 5.4x + 272.3 / y = 4.928x + 26145
Tentoxin	-	100 - 10000	y = 215.6x - 1232 / y = 220.7x + 2812 / y = 295.9x + 5867
Zearalanone	$^{13}C_{18}$-Zearalenone	300 - 30000	y = 0.0003x + 0.0033 / y = 0.0003x + 0.0165 / y = 0.0003x + 0.0194
α-Zearalanol	$^{13}C_{18}$-Zearalenone	300 - 60000	y = 0.0021x - 0.0821 / y = 0.0014x + 0.0279 / y = 0.0012x + 0.0106
β-Zearalanol	$^{13}C_{18}$-Zearalenone	300 - 60000	y = 0.0022x - 0.0044 / y = 0.0020x + 0.0055 / y = 0.0017x + 0.0128
Zearalenone	$^{13}C_{18}$-Zearalenone	100 - 20000	y = 0.0007x - 0.0299 / y = 0.0007x - 0.0018 / y = 0.0006x + 0.0738
α-Zearalenol	$^{13}C_{18}$-Zearalenone	100 - 10000	y = 0.0006x - 0.0258 / y = 0.0003x - 0.0164 / y = 0.0003x + 0.0121
β-Zearalenol	$^{13}C_{18}$-Zearalenone	100 - 10000	y = 0.0005x + 0.0049 / y = 0.0005x - 0.0098 / y = 0.0004x + 0.0246

[a] isotopically labelled internal standard used for area correction

[b] calibration curves reported in following order: first validation run / second validation run / third validation run

Table A5: Calibration ranges (excluding zero) and calibration curves for analytes during screening attempt in serum

Analyte	Internal standard[a]	Calibration range (ng/L)	Calibration curve
Aflatoxicol	-	500 - 15000	y = 2219x - 103860
Aflatoxin B_1	-	10 - 3000	-
Aflatoxin B_2	-	10 - 3000	-
Aflatoxin G_1	-	10 - 3000	-
Aflatoxin G_2	-	10 - 3000	-
Aflatoxin M_1	-	10 - 3000	-
Aflatoxin M_2	-	10 - 3000	-
Aflatoxin P_1	-	10 - 3000	-
Aflatoxin Q_1	-	10 - 3000	-
Aflatoxin-N^7-guanine	-	50 - 15000	y = 405.3x + 4995
Alternariol	$^2H_4$-Alternariol	60 - 6000	y = 0.0068x - 0.1484
Alternariol monomethyl ether	$^2H_4$-Alternariol	20 - 6000	y = 0.0179x + 0.4031
Citrinin	-	10 - 3000	-
Dihydrocitrinone	-	20 - 6000	-
Deoxynivalenol	-	150 - 45000	-
Nivalenol	-	267 - 80000	-
Fumonisin B_1	-	250 - 75000	y = 163.5x - 114533
Ochratoxin A	-	20 - 6000	y = 609.7x - 0.2910
Ochratoxin B	-	60 - 6000	y = 2571x - 50051
Ochratoxin α	-	333 - 10000	y = 328.0x - 18726
Sterigmatocystin	-	15 - 1500	y = 411.4x + 9914
T-2 toxin	-	20 - 6000	-
Tentoxin	-	20 - 6000	y = 569.2x + 5055
Zearalanone	$^{13}C_{18}$-Zearalenone	200 - 6000	y = 0.0010x - 0.0038
α-Zearalanol	$^{13}C_{18}$-Zearalenone	267 - 8000	y = 0.0048x - 0.2694
β-Zearalanol	$^{13}C_{18}$-Zearalenone	267 - 8000	y = 414.0x - 0.3207
Zearalenone	$^{13}C_{18}$-Zearalenone	200 - 6000	y = 0.0029x - 0.0554
α-Zearalenol	$^{13}C_{18}$-Zearalenone	80 - 8000	y = 0.0015x - 0.0245
β-Zearalenol	$^{13}C_{18}$-Zearalenone	80 - 8000	y = 0.0015x - 0.0648

[a] isotopically labelled internal standard used for area correction

Table A6: Isotopically labelled internal standard spiking levels in urine

Internal Standard	Spiking concentration (ng/L)	Detected
$^{13}C_{17}$-Aflatoxin M_1	250	yes
$^{2}H_{4}$-Alternariol	200	yes
$^{13}C_{13}$-Citrinin	250	no
$^{13}C_{15}$-Deoxynivalenol	2000	yes
$^{13}C_{15}$-Nivalenol	400	yes
$^{13}C_{34}$-Fumonisin B_1	1000	yes
$^{13}C_{20}$-Ochratoxin A	100	yes
$^{13}C_{18}$-Zearalenone	662	yes

Table A7: Isotopically labelled internal standard spiking levels in serum

Internal Standard	Spiking concentration (ng/L)	Detected
$^{13}C_{17}$-Aflatoxin M_1	100	yes[a]
$^{2}H_{4}$-Alternariol	700	yes
$^{13}C_{13}$-Citrinin	50	no
$^{13}C_{15}$-Deoxynivalenol	1500	yes[a]
$^{13}C_{15}$-Nivalenol	500	yes[a]
$^{13}C_{34}$-Fumonisin B_1	1500	no
$^{13}C_{20}$-Ochratoxin A	100	yes[b]
$^{13}C_{18}$-Zearalenone	200	yes

[a] insufficient peak shapes
[b] only detected at the first half of the sequence

Table A8: Nursing mother urine samples analyzed during second validation run blinded and unblinded

Blinded	Unblinded (in-thesis classification)
MaU_1	Mother #2 day 5
MaU_2	Mother #1 day 1
MaU_3	Mother #3 day 2
MaU_4	Mother #1 day 4
MaU_5	Mother #3 day 3
MaU_6	Mother #2 day 1
MaU_7	Mother #3 day 4
MaU_8	Mother #1 day 5
MaU_9	Mother #2 day 3
MaU_10	Mother #3 day 5
MaU_11	Mother #1 day 3
MaU_12	Mother #2 day 4
MaU_13	Mother #3 day 1
MaU_14	Mother #1 day 2
MaU_15	Mother #2 day 2

Table A9: Sequences submitted during method optimization and validation

Sequence	File name	Description	File path
1	190917_MycoU_BS_Test_Seq1	test with standards provided by Šarkanj *et al.*	\\131.130.23.237\Analytik\06. Masterarbeiten\03.Patrick Windisch – MycoU\SCIEX OS\MycoU\Data
2	191007_MycoU_Seq2	first analysis with own set of matrix-matched and solvent calibrants with 5 µL injection volume, source temperatures at 450 °C and 550 °C various analytes added (*e.g.* aflatoxin B_1, beauvericin, ochratoxin α, …)	
3	191010_MycoU_Seq3	injection volume adjusted to 10 µL, source temperature adjusted to 450 °C various recently added analytes removed (*e.g.* beauvericin, tenuazonic acid, …)	
4	191014_MycoU_Seq4	analysis with eluents according to Braun *et al.* (2018)	
5	191015_MycoU_Seq5	screening for citrinin with regular eluents	
6	191028_MycoU_Seq6	new column: - test run with eluent B: ACN + 0.1 % HAc - test run with eluent B: MeOH + 0.1 % HAc Needle Flush Port adjusted to 20 sec	
7	191030_MycoU_Seq7	collision energies for various analytes adjusted Needle Flush Port still at 20 sec	
8	191031_MycoU_Seq8	Needle Flush Port changed back to default	
9	191031_MycoU_Seq9	determination of unknown retention times	
10	191104_MycoU_Seq10	test sequence in order to figure out retention times of analytes when MeOH + 0.1 % HAc is used as eluent B at flow rate of 0.1 mL/min and 0.2 mL/min	
11	191105_MycoU_Seq11	comparison of different eluents: - B: ACN + 0.1 % HAc - B: MeOH + 0.1 % HAc	
12	191107_MycoU_Seq12	matrix-matched standard 5 + 6 spiked with high concentrations of beauvericin and enniatins in order to figure out if these analytes can be detected at all	
13	191113_MycoU_Seq13	various analytes added (*e.g.* aflatoxin G_1, ochratoxin B, tentoxin, …) different eluent B used: ACN + 1 % MeOH + 0.1 % HAc short sequence in order to figure out the retention times of the newly added analytes	
14	191114_MycoU_Seq14	new set of matrix-matched and solvent calibrants prepared analysis at source temperatures of 450 °C and 550 °C	
15	191120_MycoU_Seq15	short sequence in order to figure out retention times for analytes with eluent B MeOH + 0.1 % HAc at flow rate of 0.2 mL/min	
16	191120_MycoU_Seq16	new analysis with ACN + 0.1 % HAc (eluent B) at two different flow rates (0.3 mL/min and 0.6 mL/min) and new gradient (number 2)	

Sequence	File name	Description	File path
17	191121_MycoU_Seq17	new gradient (number 3); short analysis in order to figure out retention times of analytes at two different flow rates (0.3 mL/min and 0.6 mL/min)	\\131.130.23.237\Analytik\06. Masterarbeiten\03.Patrick Windisch – MycoU\SCIEX OS\MycoU\Data
18	191121_MycoU_Seq18	analysis with eluent B ACN + 0.1 % HAc at two different flow rates (0.3 mL/min and 0.6 mL/min) and with gradient number 3 at source temperature of 550 °C	
19	191122_MycoU_Seq19	analysis with ACN + 0.1 % HAc (eluent B) at flow rate of 0.1 mL/min and source temperature of 550 °C	
20	191127_MycoU_Seq20	comparison analysis of different parameters: - all analytes + source temperature 450 °C - all analytes, source temperature 450 °C + cycle time adjusted - selected analytes, source temperature 450 °C + cycle time adjusted - all analytes, source temperature 450 °C, cycle time + ion source gas 1 and 2 adjusted	
21	191129_MycoU_Seq21	analysis with needle height adjusted to 10 mm	
22	191202_MycoU_Seq22	analysis with needle height adjusted to 0 mm at source temperature of 450 °C and 550 °C	
23	191205_MycoU_Seq23	quality control run after cleaning and calibration of the mass spectrometer	
24	191205_MycoU_Seq24	short sequence with solvent calibrants and quality control samples with source temperature at 450 °C, Gas 1: 60 psi, Gas 2: 40 psi + cycle time: 0.5 sec	
25	191206_MycoU_Seq25	comparison analysis of self-produced set of matrix-matched and solvent calibrants and matrix-matched calibrants provided by Šarkanj *et al.*	
26	191211_MycoU_Seq26	analysis of matrix-matched calibrants, spiked with isotopically labelled internal standard-mixture, in order to figure out concentration levels at which matrix-matched calibrants have to be spiked	
27	191217_MycoU_Seq27_VALIDATION_1	first validation run	
28	200116_MycoU_Seq28_VALIDATION_2	second validation run + proof-of-principle experiment	
29	200220_MycoU_Seq29_VALIDATION_3_Serum_MV1	third validation run + screening in serum	

Table A10: Sciex OS quantitation results files

File name	Description	File path
200110_Results_Seq27_Urine_VALIDATION_1_FINAL	first validation run	\\131.130.23.237\Analytik\06. Masterarbeiten\03.Patrick Windisch – MycoU\SCIEX OS \MycoU\Quantitation Results
200205_Results_Seq28_Urine_VALIDATION_2_FINAL	second validation run + proof-of-principle experiment	
200304_Results_Seq29_Urine_VALIDATION_3_FINAL	third validation run	
200309_Results_Seq29_Serum_SCREENING_FINAL	screening in serum	

Table A11: Excel files containing raw data from method validation and method validation performance characteristics

File name	Description	File path
191217_MycoU_Seq27_VALIDATION_1	first validation run	\\131.130.23.237\Analytik\06. Masterarbeiten\03.Patrick Windisch – MycoU\Excel
200116_MycoU_Seq28_VALIDATION_2	second validation run + proof-of-principle experiment	
200220_MycoU_Seq29_VALIDATION_3	third validation run + screening in serum	
200311_VALIDATION_FINAL	calculation of validation parameters across all three sequences	