**Master Thesis**

# Optimisation and validation of an LC-MS/MS based multi-target method for the determination of mycotoxins in food and feed matrices

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# Acknowledgement

# Table of Content

# Zusammenfassung

Mykotoxine können ernsthafte toxikologische Effekte auf Menschen und Tieren haben, wenn kontaminierte Lebensmittel und Futtermittel aufgenommen werden. EU Verordnungen zu Grenzwerten von Mykotoxinen in spezifischen landwirtschaftlichen Rohstoffen erfordern die Entwicklung von zuverlässigen analytischen Methoden.

Ziel dieser Masterarbeit war die Optimierung und Validierung einer auf LC-MS/MS basierenden Methode für das Analytische Service Labor von Romer Labs Österreich. Die Methode soll die simultane Detektion von Mykotoxinen in vier unterschiedlichen Matrizes ermöglichen: Weizen, Mais, Schweinefutter und Silage. Sie basiert auf einer bereits existierenden Methode, die 2014 von Malachova *et al.* publiziert wurde und auf einem „dilute and shoot" Ansatz beruht. Die präsentierte Methode enthält jedoch eine geringere Anzahl an ausgewählten Mykotoxinen.

Die Probenaufarbeitung wurde aus der Literatur übernommen. Ein einzelner Extraktionsschritt mit einer Acetonitril/Wasser/Essigsäure Mischung wurde verwendet. Die daraus erhaltenen Extrakte wurden 1+1 verdünnt und direkt in das System injiziert. MS-Parameter wurden durch direkte Infusion der Standards mit einer integrierten Spritzenpumpe optimiert. Nach Experimenten mit unterschiedlichen Methodenvariationen, wurden zwei getrennte chromatographische Läufe für den positiven und negativen Ionisationsmodus gewählt. Der „scheduled multiple reaction monitoring" Modus wurde mit einem Retentionszeitfenster von 72 Sekunden eingesetzt.

56 nicht-markierte Mykotoxine und 22 interne Standards waren in der Methodenvalidierung inkludiert. Während dieser Prozedur wurden Blank-Matrixproben vor und nach der Extraktion mit Standards versetzt. Bestimmungsgrenzen unter den EU Grenzwerten wurden für Futtermittel-Matrizes erzielt, während für bestimme Lebensmittel Nachweisgrenzen unter, aber Bestimmungsgrenzen über den Grenzwerten erreicht wurden. Die höchsten Matrixeffekte wurden bei Silage beobachtet, gefolgt von Schweinefutter, Mais und Weizen. Es konnte außerdem gezeigt werden, dass $^{13}$C-markierte, interne Standards, welche mit einem Vorbehandlungsprogramm des Probenaufgebers injiziert wurden, Matrixeffekte kompensieren können. Die Wiederfindungen reichte von 30% bis 146% in Weizen, von 22 bis 114% in Mais, von 25 bis 114% in Schweinefutter und von 18 bis 65% in Silage. Nicht für alle Analyten konnten die EU Anforderungen bezüglich Wiederfindung erfüllt werden. Kompromisse müssen allerdings durch die hohe Anzahl an unterschiedlichen Analyten in einer Methode akzeptiert werden. Alle Analyten erreichen zufriedenstellende Wiederholbarkeit, die den EU Anforderungen entspricht. Die Methodenrichtigkeit wurde für vier Mykotoxine mit zertifizierten Referenzmaterialien und durch Teilnahme an einem Ringversuch für Mais, mit 8 unterschiedlichen Mykotoxinen gezeigt. Z-Faktoren zwischen -2 und +2 wurden erreicht, was zufriedenstellende Ergebnisse bedeutet.


Schlüsselwörter: Mykotoxine, Multi-Methode, Flüssigkeitschromatographie, Tandem-Massenspektrometrie, Validierung, interne Standards

# Abstract

Mycotoxins can cause severe toxicological effects on animals and humans when contaminated food or feed is ingested. EU regulations on maximum limits of mycotoxins in specific agricultural commodities make the development of reliable analytical methods necessary.

The aim of this master thesis was the optimisation and validation of an LC-MS/MS based method for the Analytical Service Lab of Romer Labs Austria. The method should enable the simultaneous detection of mycotoxins in four different matrices: wheat, maize, silage and pig feed. It is based on an already existing method, published by Malachova *et al.* in 2014 using a dilute and shoot approach. The presented method however consists of a smaller number of measured mycotoxins.

Sample preparation procedure was adopted from literature. A single extraction step with an acetonitrile/water/acetic acid mixture was used. The resulted extracts were diluted 1+1 and directly injected into the system without any further clean-up. MS parameters were optimised by direct infusion of analytical standards with an integrated syringe pump. After experiments on different method varieties, two separate chromatographic runs for the positive and the negative ionisation mode were chosen. The scheduled multiple reaction monitoring mode was used with a retention time window of 72 seconds.

56 unlabelled mycotoxins and 22 internal standards were included into the method validation. During this procedure, blank matrix samples were spiked before and after the extraction step to evaluate the method performance characteristics. LOQs below the maximum limits in EU regulations were achieved for feed matrices, while for some food matrices, LODs below but LOQs above the maximum limits were obtained. Most matrix effects were observed in silage, followed by pig feed, maize and wheat. $^{13}$C-labelled internal standards, injected with an autosampler pre-treatment program were proven to be feasible for the compensation of matrix effects. Apparent recoveries ranged from 30 to 146% in wheat, from 22 to 114% in maize, from 25 to 114% in pig feed and from 18 to 65% in silage. Not for all analytes, the EU requirements regarding recoveries could be fulfilled. However, some compromises have to be accepted due to the high number of different analytes in one method. All analytes showed acceptable repeatabilities, meeting the EU requirements. Method trueness was demonstrated for four mycotoxins with certified reference materials as well as by participating in a proficiency test for maize, containing 8 different mycotoxins. Z scores between -2 and +2 were achieved, meaning acceptable results.

Keywords: mycotoxins, multi-target method, liquid chromatography, tandem mass spectrometry, validation, internal standards

# List of Abbreviations

| | |
|---|---|
| ACN | acetonitrile |
| CE | collision energy |
| CRM | certified reference material |
| CXP | cell exit potential |
| DP | declustering potential |
| EFSA | European Food Safety Authority |
| ELISA | enzyme-linked immunosorbent assay |
| ESI | electrospray ionisation |
| GC | gas chromatography |
| HAc | acetic acid |
| HPLC | high-performance liquid chromatography |
| JECFA | FAO/WHO Joint Expert Committee on Food Additives of the United Nations |
| LC-MS/MS | liquid chromatography tandem mass spectrometry |
| LFD | lateral flow device |
| LOD | limit of detection |
| LOQ | limit of quantification |
| $m/z$ | mass to charge ratio |
| MIP | molecular imprinted polymers |
| MRM | multiple reaction monitoring |
| MS | mass spectrometer |
| $Q_1$ | first quadrupole |
| $q_2$ | collision cell |
| $Q_3$ | third quadrupole |
| QqQ | triple quadrupole mass spectrometer |
| $R^2$ | correlation coefficient |
| $R_A$ | apparent recovery |
| $R_E$ | recovery of extraction |
| RSD | relative standard deviation |
| S/N | signal to noise ratio |
| sMRM | scheduled multiple reaction monitoring |
| SSE | signal suppression or enhancement |
| TLC | thin layer chromatography |
| TOF | time of flight mass analyser |

**Mycotoxins**

| | |
|---|---|
| 15-AcDON | 15-acetyldeoxynivalenol |
| 3-AcDON | 3-acetyldeoxynivalenol |
| $AFB_1$ | aflatoxin $B_1$ |
| $AFB_2$ | aflatoxin $B_2$ |
| $AFG_1$ | aflatoxin $G_1$ |
| $AFG_2$ | aflatoxin $G_2$ |
| $AFM_1$ | aflatoxin $M_1$ |
| AOH | alternariol |
| D3G | deoxynivalenol-3-glucoside |
| DAS | diacetoscirpenol |
| DON | deoxynivalenol |
| $FA_1$ | fumonisin $A_1$ |
| $FA_2$ | fumonisin $A_2$ |
| $FB_1$ | fumonisin $B_1$ |
| $FB_2$ | fumonisin $B_2$ |
| $FB_3$ | fumonisin $B_3$ |
| FUM | fumonisin |
| FusX | fusarenone-X |
| HT-2 | HT-2 toxin |
| MON | moniliformin |
| NEO | neosolaniol |
| NIV | nivalenol |
| OTA | ochratoxin A |
| OTB | ochratoxin B |
| PAT | patulin |
| STG | sterigmatocystin |
| T-2 | T-2 toxin |
| ZAN | zearalanone |
| ZEN | zearalenone |
| $\alpha$-ZOL | $\alpha$-zearalenol |
| $\beta$-ZOL | $\beta$-zearalenol |

# 1   Introduction

## 1.1   Mycotoxins

Mycotoxins are secondary metabolites produced by filamentous fungi, with the ability causing a variety of acute and/or chronic toxic effects on animals and humans. Involvement of mycotoxins in human disease can occur in both, industrialised and developing countries. The negative effects range from immunosuppression, mutagenicity, teratogenicity, carcinogenicity to death. The extent of exposure, age as well as the nutritional status and synergistic effects of other mycotoxins and chemicals affect the toxic impact on the individual (Peraica et al., 1999). Disease caused by growth of fungi on animal or human hosts are called mycoses, while dietary, respiratory, dermal and other exposure to mycotoxins result in disease called mycotoxicoses. One common characteristic of both types is that they are generally not communicable from person to person. Inhalation of spores from the environment is a common cause of mycosis, while the majority of mycotoxicoses is caused by eating contaminated food. Moreover, contaminated feed can lead to carry-over into meat and milk products, which could contain toxic residues, and biotransformation products (Bennet & Klich, 2003).

Depending on the stage of growth, producing moulds found on agricultural commodities are generally divided into field and storage fungi. Toxin production depends on many factors such as composition of nutrients, moisture content, temperature, pH value, competition or presence of insects and so forth. Therefore, in some cases, a strict distinction into these two groups is not always possible (Atanda et al., 2001). Some of the most frequently contaminated products are cereals, nuts, spices and fruits. This is not only a health risk, but also has impact on the world trade and is connected to high financial loses (Marin et al., 2013).

More than 300 mycotoxins have been chemically characterised, but not all of them are of major concern in the food and feed industry. The ones with high importance are mainly produced by the genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps*. Some of the most important (groups of) mycotoxins produced by these fungi are aflatoxins, ochratoxins, patulin (PAT), fumonisins (FUMs), ergot alkaloids, trichothecenes and zearalenone (ZEN) (Steyn, 1995).

Aflatoxins are produced by *Aspergillus* specia, especially *Aspergillus flavus*. Warm temperatures and high humidity are potential risk factors for their production on different types

of grain. Out of all mycotoxins, aflatoxins are worldwide the most regulated ones. They can cause a variety of severe toxicological effects such as cancerogenity, immunosuppression and mutagenicity (Richard, 2012). Aflatoxin $B_1$ ($AFB_1$) for instance has been listed as the most potent natural carcinogen (Squire, 1981). Besides the common $AFB_1$, aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$) that occur on agricultural commodities, aflatoxin $M_1$ ($AFM_1$) has a substantial role due to the occurrence in milk. It is the hydroxylated metabolite of $AFB_1$ that is produced in dairy cattle after ingestion of contaminated feed. It is secreted with the milk and has severe carcinogenic potential. EU Commission regulation No 1881/2006 has set maximum limits for $AFM_1$ in milk and specific dairy products, making it the only regulated mycotoxin metabolite in those commodities (Prandini et al., 2009).

Ochratoxins are produced by different *Aspergillus* and *Penicillium* species. They occur on a high variety of commodities such as raisins, barley, soy beans, coffee and grains stored under warm temperature and high moisture content conditions. The level of contamination is often rather low, whereas the toxin may accumulate in the blood and tissues of either animals or humans. Concerning disease, ochratoxin A (OTA) is of higher importance than ochratoxin B (OTB) (Richard, 2012). Different studies showed carcinogenic, renal toxic, nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxin effects (Heussner & Bingle, 2015).

*Fusarium verticillioides* (formerly *F. moniliforme*) and related species are producers of fumonisins and infect mainly maize as the most common commodity. The toxicological effects are significant for fumonisin $B_1$ ($FB_1$) and fumonisin $B_2$ ($FB_2$), while fumonisin $B_3$ ($FB_3$), fumonisin $A_1$ ($FA_1$) and fumonisin $A_2$ ($FA_2$) occur in relatively low amounts and are less toxic (Peraica et al., 1999). The toxicity of FUMs often involves the liver and also cancer promoting activities have been shown (Geldberblom et al., 1988).

One of the first reported mycotoxicoses is ergotism, caused by ergot alkaloids with symptoms such as gangrene, central nervous and gastrointestinal effects. The two different types gangrenous and convulsive ergotism have been described extensively (Richard, 2012). Ergot alkaloids are most commonly produced by *Claviceps* species. A C9-C10 double bound in the tetracyclic ergoline ring system of ergot alkaloids allows epimerisation at the C atom at position 8 resulting in the occurrence of two different epimeric forms. In the group of ergopeptines the isomers with left-hand rotation are called ergopeptines, while the less toxic isomers with right-hand rotation are called ergopeptinines (Crews, 2015). This epimerisation is a special issue challenging the development and validation of an analytical method.

More than 140 trichothecenes have been chemically characterised, but only a few are of importance for the food and feed industries. These include deoxynivalenol (DON), nivalenol (NIV), diacetoscirpenol (DAS), T-2 toxin (T-2) and their derivatives such as 3-

acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), fusarenone-X (FusX) and HT-2 toxin (HT-2). They are mainly produced by moulds of the genus *Fusarium*, but also other genera have been found to produce trichothecenes (WHO, 1990).

Zearalenone is produced by *Fusarium graminearum*. It has powerful estrogenic activity and has been assessed as phytoestrogen, mycoestrogen and growth promotant (Bennet & Klich, 2003).

Some plants can protect themselves against mycotoxins by enzymatic conversion into less polar metabolites. Especially field fungi such as DON, ZEN, $FB_1$, T-2, HT-2 and NIV are targets of this process. Deoxynivalenol-3-glucoside (D3G) for example is the metabolic product of DON. This metabolites are often named as masked mycotoxins, since they are not detected by conventional, targeted analytical techniques. Modern mass spectrometric methods however made the identification of many of these metabolites possible. Masked mycotoxins can either be covalent derivatives of the initial mycotoxin or a non-covalent association with matrix components. Although it is assumed, that they are less toxic than their unmodified form, it could be that the native form are liberated through cleavage of the polar group during mammalian metabolism. Only a limited number of papers has been published on the toxic effects of masked mycotoxins and no regulations concerning maximum levels are available (Berthiller et al., 2015a).

Besides the toxicological effects of single mycotoxins, synergistic and additive effects have been observed in co-contaminated samples (Šegvić Klarić, 2012).

The mentioned negative effects caused by mycotoxins make implementation of regulatory limits necessary. More than 100 countries have established regulations and guidelines for specific mycotoxins. Maximum limits are based on scientific opinions of authoritative bodies such as the European Food Safety Authority (EFSA) or the FAO/WHO Joint Expert Committee on Food Additives of the United Nations (JECFA) (van Egmond et al., 2007).

The EU Commission regulation No 1881/2006 has set maximum limits for mycotoxins in different food products. There have been several amendments (EC No 1126/2007, EC No 105/2010, EC No 165/2010, EC No 165/2013, EC No 212/2014, EC No 1137/2015) resulting in maximum limits for aflatoxins ($AFB_1$ and total level), DON, FUMs ($FB_1$ and $FB_1$), OTA, PAT, T-2 & HT-2, ZEN and citrinin in cereals and various other foods. Furthermore, maximum levels in feed for $AFB_1$ and ergot alkaloids, as well as guidance levels for DON, $FB_1$ & $FB_2$, OTA, T-2 & HT-2 and ZEN have been established by several EU Directives and Commission Regulations (EC No 32/2002, EC No 576/2006, EC No 165/2013, EC No 637/2013). Table 1

and Table 2 summarise the most important maximum and guidance levels in food and feed commodities within the European Union with relevance for this thesis.

**Table 1: Maximum / guidance levels set by the European Union for different food commodities**

| Mycotoxin | Food commodity | Limit [µg/kg] |
|---|---|---|
| Aflatoxins | Processed maize | 5[a,c] / 10[a,d] |
| | Processed cereals | 2[a,c] / 4[a,d] |
| DON | Unprocessed maize | 1750[a] |
| | Unprocessed durum wheat and oats | 1750[a] |
| $FB_1$ & $FB_1$ | Maize and maize based foods intended for direct human | 1000[a] |
| OTA | Maize and maize based foods intended for direct human | 5[a] |
| T-2 & HT-2 | Maize for direct human consumption | 100[b] |
| | Other cereals for direct human consumption | 50[b] |
| ZEN | Maize intended for direct human consumption | 100[a] |
| | Cereals intended for direct human consumption | 75[a] |

[a] maximum level, [b] guidance level, [c] $AFB_1$, [d] total aflatoxin

**Table 2: Maximum / guidance levels set by the European Union for different feed commodities**

| Mycotoxin | Feed Commodity | Limit [µg/kg] |
|---|---|---|
| $AFB_1$ | All feed materials | 20[a] |
| | Complementary and complete feed | 10[a] |
| DON | Cereals and cereal products | 8000[b] |
| | Complementary and complete feed | 5000[b] |
| $FB_1$ & $FB_2$ | Maize and maize based products | 60000[b] |
| | Complementary and complete feed for pigs, horses, rabbits and pet animals | 5000[b] |
| OTA | Cereals and cereal products | 250[b] |
| | Complementary and complete feeding stuffs for pigs | 250[b] |
| T-2 & HT-2 | Cereal products | 500[b] |
| | Compound feed | 250[b] |
| ZEN | Cereals and cereal products | 2000[b] |

[a] maximum level, [b] guidance level

## 1.2   Analysis of mycotoxins

The previously mentioned maximum limits for mycotoxins, set by the EU require a regular control of different food and feed products by the use of reliable analytical methods.

The high chemical diversity of mycotoxins is a major challenge for the development of appropriate analytical methods for quantitative determination. Some of the main differences in the chemical properties include polarity, UV absorption, fluorescence and the ionic nature dependent on the pH value. Another challenge is the wide range of food and feed products where mycotoxins occur. Hence, many currently applied methods target single analytes and specific matrices (Krska & Molinelli, 2006).

On the one hand, different rapid testing techniques have been developed, which are often used to obtain a qualitative statement, if specific mycotoxins are present above a certain threshold or not. This statement can be achieved in a very short amount of time and with little to no lab equipment, ideally already on the field. Disadvantages are the often high matrix dependency and poor accuracy and precision. More complex methods have to be used if a confirmation or more accurate quantitative result is required. Many rapid screening methods are based on antigen-antibody interactions, such as the use of enzyme-linked immunosorbent assays (ELISA). They are however labour intensive, highly matrix dependent and sometimes show unacceptable precision. Other alternatives are lateral flow devices (LFD) and dipsticks, which can be used directly on the field. Different types of biosensors, spectroscopic techniques and molecularly imprinted polymers (MIP) are among the most important trends for rapid screening of mycotoxins. (Krska & Molinelli, 2006, Huybrechts, 2014). On the other hand chromatographic methods can target multiple analytes and are among the most common ones for the quantification of mycotoxins. These include gas chromatography (GC), thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Fluorescence and UV-absorption enable the chromatographic quantification of several mycotoxins (Contreras-Medina et al., 2013). Aflatoxins ($B_1$, $B_2$, $G_1$, $G_2$, $M_1$), ergot alkaloids, OTA, PAT and ZEN are naturally fluorescent, while derivatisation is necessary to enable UV or fluorescence detection of type A trichothecenes and FUMs (Coker et al., 1984, Gori & Troiano, 2012).

A trend that has become very popular during the last years is the simultaneous determination of multiple mycotoxins with liquid chromatography tandem mass spectrometry (LC-MS/MS). Since it is also the method of choice in this work, it is further explained in chapter 1.2.1

### 1.2.1  Liquid chromatography tandem mass spectrometry

HPLC is a chromatographic method for the separation of different analytes, depending on their physiochemical properties. An HPLC system usually covers the eluent(s), a pump, mixer,

injector, column, thermostat, detector and computer. The sample is injected into the liquid mobile phase and then pumped through the column, which is packed with the stationary phase. The analytes are retained and therefore separated through different interaction mechanisms with the stationary phase. The interactions can depend on polarity, electrical charge and molecular size. They can be divided into: normal-phase HPLC (apolar mobile phase + polar stationary phase), reversed-phase HPLC (polar mobile phase + apolar stationary phase), ion exchange HPLC (attraction or repulsion, dependent on electrical charge), size-exclusion chromatography (separation based on their size) and other types. Reversed-phase HPLC is the most common one, especially for LC-MS/MS systems (Rasmussen, 2001).

After the chromatographic separation, ionisation is required to enable detection with the mass spectrometer (MS). The ionisation techniques can be divided into hard and soft ionisation. During hard ionisation, a high number of fragments is formed besides the actual ion. This effect is reduced during soft ionisation. Moreover, soft ionisation techniques show good results for sensitive and thermally labile compounds. Electrospray ionisation (ESI) is a soft ionisation technique and usually preferred for LC-MS/MS instruments. In a first step, the mobile phase from the HPLC is nebulised to a fine spray of charged droplets. There is a counter flow of heated drying gas which leads to reduced droplet size by evaporation of the solvent. Finally, a critical point is reached, at which the ions are desorbed into the gas phase. The type of ion that is formed depends of the polarity of the analyte, property of the solvents and possible impurities that can be involved in the formation of specific ions (Ho et al., 2012, Gross, 2013).

Thereafter the ions are accelerated to the mass analyser. For this work, a special kind of a triple quadrupole mass spectrometer (QqQ) was used. In detail, within this thesis an Sciex QTrap 4500 was used, where additional scans, only attainable using this kind of system, can be performed. Other common types of mass analysers are time of flight mass analyser (TOF), sector field mass analyser and different types of ion traps.

A quadrupole mass analyser consists of four cylindrical, metal rods, parallel to each other. The opposite ones are connected and have the same potential. A combination of direct current and alternating current leads to an inhomogen, electric field. Ions that enter the quadrupole, oscillate through this electrical field and the ones with a specific mass to charge ratio ($m/z$) can pass (Gross, 2013).

Tandem mass spectrometry involves multiple steps with fragmentation of ions occurring in between. Using a tandem mass spectrometer, parent or precursor ions are formed in the ion source and separated according their $m/z$ in the first quadrupole ($Q_1$). Particular precursor ions can be fragmented in the collision cell ($q_2$). Those resulting fragments, also called product ions, can be separated in the third quadrupole ($Q_3$) and detected.

A Sciex QTrap 4500 instrument was used in the MRM (multiple reaction monitoring) and sMRM (scheduled multiple reaction monitoring). The declustering potential (DP) is used in $Q_1$ to control the solvent clusters that could remain on the ions, after entering the vacuum chamber. The second quadrupole acts as a collision cell: the ions are fragmented through collision with a gas such as $N_2$. Depending on the collision energy (CE), specific fragments are formed (product ions), which are further transferred to the $Q_3$. The voltage that is applied to remove the product ions from the $q_2$ is called cell exit potential (CXP). In the sMRM mode, the specific mass ranges are only scanned in a defined time window, depending on the observed retention time of the analyte, resulting in less time to scan one transition. Hence, more data points for a chromatographic peak can be achieved. During the software controlled compound optimisation, a precursor ion, two product ions, DP, CE and CXP are typically evaluated for each analyte (AB Sciex, 2013, Gross 2013).

### 1.2.2 LC-MS/MS in mycotoxin analysis

For the analysis of mycotoxins, LC-MS/MS has become the state of art, due to its possibility to quantify almost all mycotoxins simultaneously (Pereira et al., 2014). It is the most sensitive and selective method to detect mycotoxins. It enables the detection of a high number of different analytes in complex matrices (Berthiller et al., 2015b). Several methods for the simultaneous determination of mycotoxins were developed in the last years. One of the first containing an high number of analytes, was first published by Berthiller et al. (2005), containing of 9 major *Fusarium* mycotoxins in maize. This method was modified and expanded by Sulyok et al. (2006, 2007, 2010), by changing from a sample clean-up procedure with Mycosep® colums, to a dilute and shoot approach. Continuous expansion to several other matrices and analytes lead to a method containing more than 300 fungal and bacterial metabolites (Malachova et al., 2014).

Several factors have to be taken into account when developing a multi-mycotoxin method. The toxins that should be included in the method have to be chosen first. Their occurrence differs a lot between different agricultural commodities. The working range depends on the specifications of the available instrument as well as specific requirements concerning limits of detection and expected contaminations in the samples of interest (Krska et al., 2017).

Sample preparation prior to the analysis is a critical step to receive reliable results. High heterogeneity of mycotoxins in food and feed leads to some challenges. It is of utmost importance that the analysed sample is representative for the bulk. The whole primary sample must be homogen, therefore mixing and grinding is necessary. Sometimes a high enough toxin

concentration in the sample is needed to ensure enough sensitivity (Ridgway, 2012). The EU has established Commission Regulation (EC) No 401/2006, which lays down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuff. The regulation has been amended several times (latest version: Commission Regulation (EU) No 519/2014).

It is not possible to measure solid samples with an LC-MS/MS system, hence several sample preparation steps are mandatory prior to analysis. Grinding reduces the particle size and facilitates the subsequent extraction step with an organic solvent. During this extraction step, the mycotoxins should be transferred from the solid matrix to the liquid solvent. A centrifugation or filtration step is necessary to separate the liquid extract and the solid components. This extract can be further diluted to decrease the amount of matrix components and the organic fraction in the final solution. The aim is to obtain a clean extract, enriched with the mycotoxins of interest that can be measured with the LC-MS/MS method (Ridgway, 2012).

When choosing an appropriate extraction solvent for an LC-MS/MS based multi-target methods that covers a large number of analytes, compromises have to be made. A large proportion of acetonitrile (ACN) was shown to be suitable for most mycotoxins with the exception of acidic ones (e.g. FUMs). 1% acetic acid (HAc) improves the extraction of these compounds, but negativity affects the results for basic mycotoxins. It also promotes the epimerisation of ergot alkaloids from the ergopeptine to the ergopeptinine form (Sulyok et al., 2006, 2007).

Another challenge are matrix effects. They are caused by compounds in the sample that-co elute with the analyte and distort the results. This can lead to either signal suppression or enhancement (SSE). Some strategies for reducing the matrix effects are clean-up of the sample, sample dilution and the use of matrix matched calibrations (Petal, 2011). Solid phase extraction columns for clean-up can remove matrix compounds for the most important mycotoxins. They are however not designed for such a high variety of analytes that are included in modern LC-MS/MS based multi-target methods. Another possible clean-up approach is the use of the so called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, which is often used for the analysis of pesticides. An extraction of mycotoxins with a mixture of ACN/$H_2O$ and the addition of salt should lead to an ACN phase, containing the analytes of interest and a water phase, containing polar matrix compounds. A second matrix removal step by primary secondary amines and/or C18 material is often applied for pesticide analysis. Primary secondary amines would however remove acidic mycotoxins, while C18 would bind the apolar ones. For LC-MS/MS methods that include more than 20 different mycotoxins, each clean-up step would cause discrimination of certain compounds (Krska et

al., 2017). Recoveries, obtained from matrix matched calibration during the validation, combined with a dilute and shoot approach, meaning that the sample is extracted, diluted and then directly injected into the LC-MS/MS system, has shown satisfactory results in several proficiency tests for a method, containing 331 secondary metabolites of fungi and bacteria in food matrices (Malachova et al., 2015). Matrix matched calibrations are calibration functions prepared in blank matrix extracts and can be used for the compensation of possible matrix effects. They however require blank matrix material, which can be hard to find for specific analytes. In addition, often the blank sample, which is used for the preparation of the matrix matched standards, is not completely comparable with the matrix analysed.

If isotopically labelled internal standards are spiked to the sample prior to extraction, they can compensate both, losses of the analyte during the extraction procedure as well as matrix effects during the MS analysis. This approach is however limited due to the high amount and associated high costs of internal standard that has to be added to the sample. In practice, the internal standard is usually added after the extraction procedure, resulting in compensation of matrix effects only. Isotopically-labelled standards have different molecular masses, but the same chromatographic properties as the unlabeled counterpart. Hence, they co-elute with the analyte of interest during the chromatographic run and can be separated in the mass spectrometer due to the differences in the *m/z* ratios. Generally, $^{13}$C- or $^{15}$N-labelled internal standards are preferred over $^{2}$H- or $^{18}$O-labelled ones, due to the higher stability of C-C and C-N bonds. Especially the number of commercially available $^{13}$C-labelled internal standards is growing. However, the high costs and remaining lack of availability for many analytes are the main drawbacks of this approach (Rychlik & Asam, 2008, Varga et al., 2012).

## 1.3 Validation

Validation is necessary to show that the analytical method is suitable and reliable for the intended use. Moreover, defined method acceptance criteria for specific method performance characteristics should ensure appropriate results. In this thesis, the method validation procedure included the parameters linear range and working range, limit of detection (LOD), limit of quantification (LOQ), different types of precision, stability of the analytes in matrix extract, selectivity, apparent recovery ($R_A$), SSE, recovery of extraction ($R_E$) and the method trueness. It is not possible to define acceptance criteria for each validation parameter, since the method includes such a high variety of mycotoxins, resulting in parameters that can be far from optimal for specific analytes. Hence, some compromises have to be accepted.

The linear range is the concentration range where the signal intensity is proportional to the analyte concentration. The acceptance criteria for the linearity is based on the correlation coefficient ($R^2$). In this thesis, $R^2$ values > 0.98 were accepted. The working range is the segment of the linear range that is used for the calibration function during the validation and in the routine analysis. The limit of detection is the lowest analyte concentration where a qualitative statement is possible, while the limit of quantification is the lowest analyte concentration where a quantitative statement is possible. In this thesis, the acceptance criteria was a signal to noise (S/N) ratio of 3:1 for the LOD and 10:1 for LOQ. LOD and LOQ were determined in all four matrices and in neat solvent in sevenfold repetition. Different types of precision can be distinguished. For the repeatability of the instrument itself, the same sample was measured 10 times in a row and the relative standard deviation (RSD) was calculated. For the reproducibility, the same experiment was performed with a different, but identical HPLC column, to prove that a change of the HPLC column does not have negative effects on the precision. Lab precision was tested by comparing the RSD of sample extracts, to show that comparable results are achieved, under the same lab conditions. The stability of the analytes in matrix extracts at room temperature is an important parameter for measuring of longer sequences (e.g. over the weekend), when the vials cannot be cooled. For this thesis, the stability was tested by measuring the same vials on different days (after thawing, after 1 day, 2 days, 4 days), after storing them at room temperature and calculating the RSD. Selectivity refers to the possibility of detecting analytes in the presence of other components. This means, that no interfering peaks should be present for both transitions in the retention time window of each analyte in each matrix. The selectivity should be tested close to the LOQ. The recovery is the amount of analyte that is detected, compared to its true concentration. SSE indicates the amount of matrix effects, while $R_E$ indicates the recovery of extraction. The $R_A$ is the product of both of them. The recovery does not have to be 100%, but it should be constant and reproducible. The method trueness is the closeness of the measured value to the actual value (FDA, 2013).

Commission Regulation (EU) No 519/2014 lays down requirements for the recovery range and repeatability of specific mycotoxins at defined concentrations. The recoveries and repeatabilities (in form of RSDs) are summarized in Table 3.

**Table 3: Requirements on the recovery and repeatability for specific mycotoxins according to Commission Regulation (EU) No 519/2014**

| Analyte | Concentration range [µg/kg] | Recovery [%] | RSD [%] |
|---|---|---|---|
| AFM$_1$ | 0.00001-0.00005 | 60 - 120 | May be calculated as 0.66 times RSD of the reproducibility |
| | >0.00005 | 70 - 110 | |
| AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$ | <0.001 | 50 - 120 | |
| | 0.001 – 0.01 | 70 - 110 | |
| | > 0.01 | 80 - 110 | |
| OTA | <1 | 50 - 120 | ≤ 40 |
| | >1 | 70 - 110 | ≤ 20 |
| PAT | <20 | 50 - 120 | ≤ 30 |
| | 20-50 | 70 - 105 | ≤ 20 |
| | > 50 | 75-105 | ≤ 15 |
| DON | 100-500 | 60-110 | ≤ 20 |
| | >500 | 70-120 | ≤ 20 |
| ZEN | ≤50 | 60-120 | ≤ 40 |
| | >50 | 70-120 | ≤ 25 |
| FB$_1$, FB$_2$ | ≤500 | 60-120 | ≤ 30 |
| | >500 | 70-120 | ≤ 20 |
| T-2, HT-2 | 15-250 | 60-130 | ≤ 30 |
| | >250 | 60 -130 | ≤ 25 |

# 2 Objectives of the work

The main objective of this thesis was the optimisation and validation of an LC-MS/MS based multitoxin screening method for the Analytical Service Lab, Romer Labs Austria. A list of analytes and agricultural commodities that should be included in the method was available. The analytes included regulated mycotoxins and toxins with guidance levels, emerging toxins, modified toxins as well as substances that were relevant due to their increased detection in the recent years. A total number of 77 analytes was on the list, while not for all of them analytical standards were in-house or commercially available. In-house-produced reference materials were produced under the trade name 'Biopure' by the production department of Romer Labs, Austria. Fermentation of fungi, followed by stepwise purification based on chromatographic methods and characterisation by appropriate independent methods, lead to mycotoxin reference materials with traceable concentration values. $^{13}$C-labelled internal standards were available for 22 analytes, an analytical standard was, but no internal standards were available for 37 analytes. The matrices of interest were wheat, maize, feed and silage. Representative samples of this matrices were used, but compromises had to be made especially for feed and silage due to lack of uniformity. While silage mainly varies in water and plant content, many different compositions and recipes for feed can be distinguished. Moreover, both matrices show high batch-to-batch variations. It was decided to use fresh maize silage and finished pig feed for the work of this thesis, due to their highest relevance in the routine analysis.

The method was based on an already existing LC-MS/MS multi-toxin method published in 2014 by Malachova *et al.* for more than 300 analytes, containing mycotoxins as well as other fungal and bacterial metabolites. Already existing LC-MS/MS methods in the Analytical Service, Romer Labs Austria and the R&D department of Romer Labs Austria further facilitated the progress.

Method optimisation included the evaluation of ideal MS parameters, while the HPLC parameters were mainly adopted from literature (Sulyok et al., 2006). During validation, the apparent recovery, consisting of matrix effects and the recovery of extraction, had to be evaluated. The main difference of this method to the one from Malachova *et al.* (2014) is that $^{13}$C-labeled internal standards in combination with liquid standards in neat solvents were preferred in this method. For all analytes with $^{13}$C-labeled internal standards, a quantification using the ratios between the unlabelled and labelled analytes will be performed for the correction for matrix effects, while the apparent recoveries evaluated during validation will be

used for all other analytes in the routine analysis. Other parameters such as LOD, LOQ, working range and precision also had to be determined.

It should be mentioned, that the same method was also optimised and validated in the Romer Labs Analytical Service Labs in Singapore and Union (MO, USA), to offer a unified service at all three locations. The methods running in Singapore and Union are however not part of this thesis.

# 3 Materials and methods

## 3.1 Experimental set-up

The experimental set-up of this work is summarised in Figure 1.

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**Preparation:**

- <u>Mycotoxin standards</u>: 80 mycotoxins (68 unlabelled, 22 internal standards)
- <u>Blank matrices</u>: wheat, maize, pig feed, silage

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**Method optimisation:**

- <u>MS parameters</u>: precursor ion, mass transitions for 2 product ions, DP, CE, CXP
- <u>LC-MS/MS parameters</u>: retention time, method variations
- <u>Working range</u>: linear range, respective LOD + LOQ, in neat solvent and all validated matrices
- <u>Internal standards</u>: concentration, solvent, injection program

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**Method validation:**

- <u>Contaminations in blank matrices</u>: standard addition approach
- <u>Ion ratio</u>: area of qualifier divided by area of quantifier
- <u>Recoveries</u>: $R_A$, SSE, $R_E$
- <u>LOD + LOQ</u>: signal to noise ratio: 3/1 for LOD, 10/1 for LOQ
- <u>Precision parameters</u>: repeatability, reproducibility, stability, lab precision
- <u>Trueness</u>: certified reference materials, proficiency testing

**Figure 1: Summary of the experimental set-up of this work**

## 3.2 Laboratory equipment

<u>Instruments:</u>

- Vortexer: Vortex1. IKA (Staufen, Germany)
- Analytical balance: Sartorius AY303, readability: 0.001 g. Sartorius (Göttingen, Germany)

- Precision balance: Sartorius CPA223D-OCE, readability: 0.00001 g. Sartorius (Göttingen, Germany)
- Rotary shaker: GFL 3015. GFL (Burgwedel, Germany)
- Evaporation unit: EVA-EC1-S-24. VLM (Bielefeld, Germany)
- Drying chamber: FD 260. Binder (Tuttlingen, Germany)
- Centrifuge: Thermo Fisher Multifuge, X3 FR. Thermo Fisher (Waltham, USA)
- HPLC column: Gemini C18 column, 150 x 4.6 mm i.d. 5 μm particle size. Phenomenex (Aschaffenburg, Germany)
- Security guard cartridge: Gemini C18 4x4 mm i.d., Phenomenex (Aschaffenburg, Germany)
- LC/MS System: Series 1260 HPLC (equipped with G1312B binary pump, G1329A autosampler and G1316A column thermostat). Agilent (Waldbronn, Germany) Connected to an QTrap 4500 and with an TurboV ESI source, Sciex (Foster City, USA)

General lab equipment:

- HPLC glass vials and vial caps: 1.5 mL. clear glass. Markus Bruckner Analysetechnik (Linz, Austria)
- HPLC glass vials with conical insert: 300 μL. clear 33. w/Patch. Verex (Steyr, Austria)
- Research pipettes: 2-20 μL, 20-200 μL, 100-1000 μL, 500-5000 μL. Eppendorf (Hamburg, Germany)
- Pipette tips: EpT.I.P.S.: 2-200 μL, 50-1000 μL, 100-5000 μL. Eppendorf (Hamburg, Germany)
- Centrifuge Tubes with screw caps: 15 and 50 mL. VWR (Leuven, Belgium)
- Measuring cylinders: 10 mL, 25 mL, 100 mL, 250 mL, 1000 mL. Schott (Mainz, Germany)
- Glass bottles and caps: 50 mL, 100 mL, 250 mL, 1000 mL, 2,5 L. Schott (Mainz, Germany)
- Beakers: different sizes. Schott (Mainz, Germany)
- Volumetric flasks. different sizes. DWK (Wertheim, Germany)
- Instrument Syringe: 1 mL, Model 1001, BFP Syringe. Hamilton (Bonaduz, Switzerland)
- Instrument Syringe: 100 μL, Model 1710 TLLX Syringe. Hamilton (Bonaduz, Switzerland)

## 3.3   Chemicals and reagents

<u>Chemicals</u>

- Acetic Acid (glacial): HiPerSolv Chromanoform, HPLC grade, VWR (Leuven, Belgium)
- Acetonitrile: Lichrosolv, Purity ≥ 99.9%, HPLC grade, Merck (Darmstadt, Germany)
- Ammonium Acetate: Purity ≥ 99.9%, trace metal basis, Sigma Aldrich (St. Louis, USA)
- Methanol: Lichrosolv, Purity ≥ 99.9%, HPLC grade, Merck (Darmstadt, Germany)
- Water: HiPerSolv Chromanoform, HPLC grade, VWR (Leuven, Belgium)

<u>Standard solutions:</u>

The department for reference materials of Romer Labs Austria has in-house produced all of the internal standards and most of the analytical standards. 3-AcDON (100 µg/mL), 15-AcDON (100 µg/ml), 15-acetoxyscirpenol (50 µg/mL), agroclavine (100 µg/ml), α-zearalenol (α-ZOL, 10 µg/mL), beauvericin (100 µg/mL), β-zearalenol (β-ZOL, 10 µg/mL), D3G (50 µg/mL), DAS (100 µg/mL), DON (100 µg/mL), FusX (100 µg/mL), gliotoxin (100µg/mL), HT-2 (100 µg/mL), mycophenolic acid (100µg/mL), neosolaniol (NEO, 100 µg/mL), NIV (100 µg/mL), OTA (10 µg/mL), OTB (10 µg/mL), PAT (100 µg/mL), penicillic acid (100µg/mL), sterigmatocystin (STG, 50 µg/mL), T-2 (100 µg/mL), T-2 triol (100 µg/mL), zearalanone (ZAN, 10 µg/mL), ZEN (100 µg/mL), $^{13}C$-3-AcDON (25 µg/mL), $^{13}C$-15-AcDON (10 µg/mL), $^{13}C$-AFM$_1$ (0.5 µg/mL), $^{13}C$-AFG$_1$ (0.5 µg/mL), $^{13}C$-AFG$_2$ (0.5 µg/mL), $^{13}C$-AFB$_1$ (0.5 µg/mL), $^{13}C$-D3G (10 µg/mL), $^{13}C$-DAS (25 µg/mL), $^{13}C$-DON (25 µg/mL), $^{13}C$-HT-2 (25 µg/mL), $^{13}C$-mycophenolic acid (100 µg/mL), $^{13}C$-NIV (25 µg/mL), $^{13}C$-OTA (10 µg/mL), $^{13}C$-PAT (25 µg/mL), $^{13}C$-roquefortine C (25 µg/mL), $^{13}C$-STG (25 µg/mL), $^{13}C$-T-2 (25 µg/mL) and $^{13}C$-ZEN (25 µg/mL) were commercial available under the trade name 'Biopure' and dissolved in pure acetonitrile. FB$_1$ (50 µg/mL), FB$_2$ (50 µg/mL), FB$_3$ (50 µg/mL), $^{13}C$-FB$_1$ (25 µg/mL), $^{13}C$-FB$_2$ (10 µg/mL) and $^{13}C$-FB$_3$ (10 µg/mL) were dissolved in ACN/H$_2$O 50:50 (v/v) and moniliformin (MON, 100 µg/mL) in ACN/H$_2$O 90:10. The following standards were received from the Romer Labs production department in dried down form and a defined volume of ACN was added: alternariol (AOH, 100 µg/mL), elymoclavine (100 µg/mL), ergine (100 µg/mL), ergocornine (100 µg/mL), ergocorninine (25 µg/mL), ergocristine (100 µg/mL), ergocristinine (25 µg/mL), ergocrpytine (100 µg/mL), ergocryptinine (25 µg/mL), ergometrine (100 µg/mL), ergometrinine (25 µg/mL), ergosine (100 µg/mL), ergosinine (25 µg/mL), ergotamine (100 µg/mL), ergotaminine (25 µg/mL), roquefortine C (100 µg/mL) and tenuazonic acid (100 µg/mL).

Solid standards from 'Biopure' were used to produce liquid standards with concentrations, higher than the ones that are commercially available. Appropriate amount of the standards were put into weighting pans, which were then transferred into volumetric flasks and filled up with ACN. Separate stock were prepared by putting 1.25 mg of solid standard in 25 mL weighting pans and filling up with ACN, for $AFB_1$ (purity: 98.8 %), $AFB_2$ (purity: 98.4 %), $AFG_1$ (purity: 98.0%) and $AFG_2$ (purity: 97.1 %). For T-2 tetraol, (purity 98%), 1 mg of solid standard was filled up with 5 mL ACN. The concentration of those self-prepared standards was quantified with the analytical method and standards used at 'Biopure' for the quality control of their standards.

An $AFM_1$ standard solution in ACN with a higher concentration (38.5 µg/mL) than the one that is commercially available was received from the Romer Labs production department.

Solid mycotoxin standards were purchased from Sigma Aldrich and filled up with ACN to receive liquid standards for the following analytes: enniatin A (1000 µg/mL), enniatin A1, (1000 µg/mL), enniatin B (500 µg/mL), enniatin B1 (1000 µg/mL), dihydrolysergol (100 µg/mL) and fusaric acid (100 µg/mL).

For the validation, multi-analyte stock solutions had to be prepared (see chapter 3.5.1). The concentration of the standard solutions used during the validation are also listed in Table 6-Table 9.

Samples

For the validation, representative blank matrix samples were required for spiking experiments, as well as for the preparation of matrix matched standards. In-house produced, blank quality control material by the brand Biopure was used for the validation of wheat. The maize sample was provided by the Analytical Service, Romer Labs Austria. Silage and pig feed samples were provided by Biomin. All samples were received grounded. It should be noticed, that the samples were not real blank samples for all analytes, since it is hardly impossible to get blank matrix material for some mycotoxins. Hence some contaminations in the used material could not be avoided and were detected according to chapter 3.5.2.2.

## 3.4 Method optimisation

Method optimisation was carried out to receive ideal LC-MS/MS parameters, which can further be used for the method validation and routine analysis. At first, MS/MS mass transitions were investigated (see chapter 3.4.1). HPLC parameters and sample preparation procedure were

not optimised for this thesis but adopted from literature (Sulyok et al., 2006), due to years of experimenting in these studies. For the sMRM mode, retention times are required and were detected (see chapter 3.4.2). Experiments on combining the positive and negative polarity mode in one chromatographic run and on the effects of different method parameters on the peak shape and reproducibility are described in chapter 3.4.3. In preparation to the validation, working range and respective LOD and LOQ for each analyte in each matrix were investigated (see chapter 3.4.5). Finally, the concentrations for the internal standards were chosen and an autosampler pre-treatment program was implemented to facilitate the injection of the internal standards (see chapter 3.4.6).

### 3.4.1 MS parameters

Some of the MS parameters have already been optimised beforehand. It was recognized, that several of these parameters were unfavourable. Hence, a re-optimisation was performed.

The following analytes were optimised/re-optimesed by the author of this thesis: $AFG_1$, agroclavine, beauvericin, enniatin A, enniatin B, enniatin B1, ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine, elymoclavine, ergine, fusaric acid, mycophenolic acid, penicillic acid, roquefortine C, tenuazonic acid, $^{13}C$-mycophenolic acid.

The optimisation of the MS parameters was performed by direct infusion of diluted standard solutions into the mass spectrometer with an integrated Harvard syringe pump, a flow rate of 7 µL/min, entrance potential of 10 V and curtain gas of 20 psi. The dilutions were prepared in 10 fold steps with mobile phase A which was composed of methanol/water/acetic acid 10:89:1 (v/v/v) and 5 mM ammonium acetate, to receive signal intensities of approximately $10^6$ for the base peak. In the Q1 MS (Q1) mode of Analyst 1.6.2, the ionised precursor molecules were investigated in positive and negative mode. The one showing the highest signal intensity was chosen for software controlled compound optimisation. As a result, mass transitions with optimal DP, CE and CXP were received. The two product ions with the highest signal intensity were chosen as quantifier and qualifier transitions and included into the LC-MS/MS method. A measurement of the single compounds by LC-MS/MS with this method was needed to receive the retention times of the different analytes (see chapter 3.4.2). For this purpose, a MRM method monitoring the different mass transitions over the whole chromatographic run was used. For analytes with fragmentations to non-analytes specific products (e.g. loss of water) other transitions had to be chosen.

### 3.4.2 LC-MS/MS parameters

Chromatographic separation was performed at 25 °C on a Gemini C18 column, 150 x 4.6 mm i.d. 5 µm particle size, equipped with a C18 security guard cartridge, 4 x 4 mm i.d. (all from Phenomenex). An injection volume of 10 µL was used. Eluent A was composed of methanol/water/acetic acid 10:89:1 (v/v/v) and eluent B of methanol/water/acetic acid 97:2:1 (v/v/v). Additionally, both eluents contained 5 mM ammonium acetate. Elution was carried out with the following gradient: until 2 min: 100% A, linear increase of B to 50% within 3 minutes, further linear increase of B to 100% within 9 min, 100% B for 4 minutes, 2.5 min column re-equilibration at 100% A.

Figure 2 shows the gradient graphically. A flow rate of 1 mL/min was used and a six-port valve was responsible to transfer the column effluent either to the mass spectrometer (between 2.8 and 20.5 min; no flow splitting was used) or to the waste.
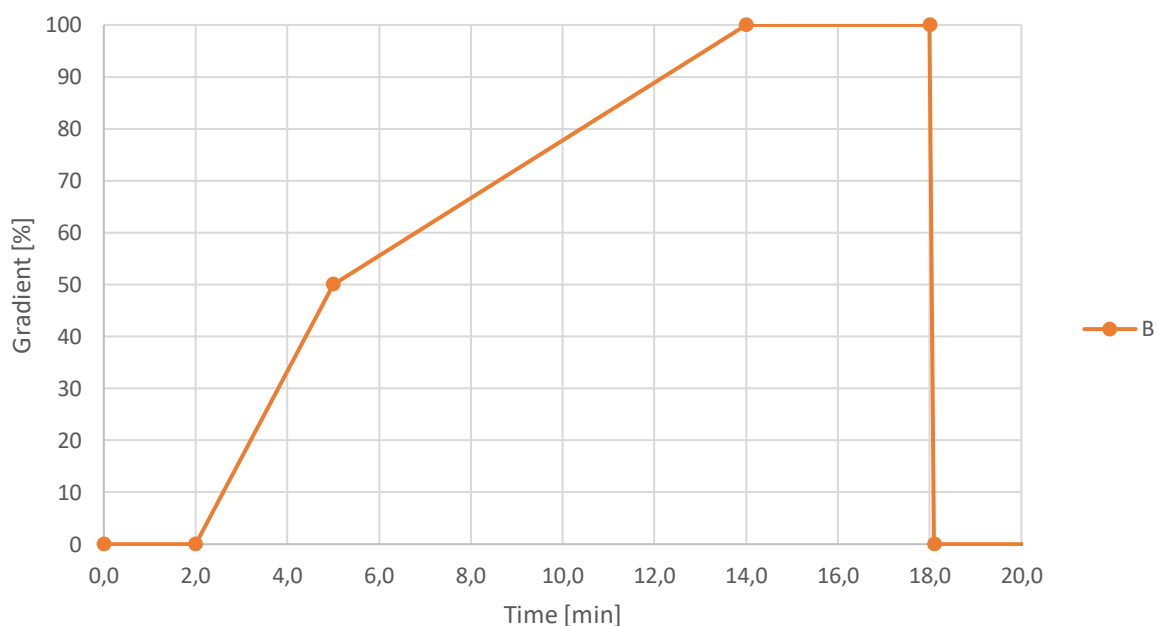


**Figure 2: Gradient of eluent A and B throughout the chromatographic run**

ESI-MS/MS was performed in MRM mode and sMRM mode. Several experiments were performed on separate chromatographic runs for the positive and negative mode and on combining both modes in one run with fast polarity switching (see chapter 3.4.3).

The following settings were used: source temperature: 550 °C, curtain gas: 30 psi, ion source gas 1 (sheath gas): 60 psi, ion source gas 2 (drying gas): 60 psi, ion spray voltage +4500 V and -4500 V, collision gas (nitrogen): high/medium.

Two mixed standard solutions, one for the positive and one for the negative mode, containing all analytes in the concentrations that were ideal for compound optimisation, were prepared in eluent A. For the analytes, were the compound optimisation was not carried out by the author, the concentrations were assumed. The standard solutions were measured with an MRM method: dwell times of 10 ms for positive mode and 50 ms in negative mode. The obtained retention time, peak shape, signal intensity of quantifier and qualifier as well as noise intensity were evaluated. The parameters were adopted for the investigation of the linear range and for the validation. In case that one of the parameters was unsatisfactory or the qualifier showed better results than the quantifier transition, MS compound optimisation was repeated or the two product ions had to be switched.

The received retention time for each analyte was used for the sMRM method.

### 3.4.3   Method variations

13 different varieties of the LC-MS/MS methods, described in chapter 3.4.2 were tested. These included MRM in positive / negative mode and both combined with dwell times of 10, 25 and 50 ms. Then, sMRM methods with the obtained retention times and retention time windows of 60, 72 and 90 s were created and compared for the positive / negative mode and both combined. On the combined method, a scan time of 0.5 s instead of 1.0 s was also tested. The Analyst software set the dwell times in the sMRM methods automatically.

A mixed standard, containing representative, 6 analytes for the positive and 6 analytes for the negative mode was prepared in eluent A. The concentrations in µg/L were $AFM_1$: 50, AOH: 100, D3G: 500, DAS: 500, DON; 1000, enniatin A: 100, ergocornine: 100, NEO: 500, NIV: 1000, OTB: 500, ZAN: 100, ZEN: 100. The same vial was measured in fivefold repetition for each method. The signal intensity of the product ions, as well as relative standard deviations and the amount of data points per peak were evaluated.

### 3.4.4   Sample and solvent preparation

Eluents and solvents

For the preparation of eluent A and B, ammonium acetate was weighed into centrifuge tubes, pre-dissolved in water and added into the glass bottle that should contain the eluents. The tube was rinsed with water, which was then also added into the glass bottles, to ensure transfer of

the salt into the eluent. The remaining volume of water, methanol and finally acetic acid was then added and the bottle thoroughly shaken.

Extraction solvent was composed of ACN/H$_2$O/HAc 79:20:1 *(v/v/v)* and dilution solvent of ACN/H$_2$O/HAc 20:79:1 *(v/v/v)*. During the preparation, water and acetonitrile were added first, followed by acetic acid and thorough shaking.

<u>Sample preparation</u>

5.0 ± 0.1 g of ground matrix sample were weighed into 50 mL centrifuge tubes and 20 mL of extraction solvent were added. The samples were extracted for 90 min at room temperature by using a rotary shaker. After a centrifugation step of 5 min at 5000 rpm, 500 µL of the supernatant was transferred into glass vials, diluted with the same amount of dilution solvent followed by thorough mixing. 10 µL of this mix were directly injected into the LC-MS/MS system without any further pre-treatment steps. This procedure was used for the extraction of blank matrix (for matrix matched calibration) (see chapter 3.5.1) and will be used after implementation of this method into the routine analysis. For the spiked samples during the validation, this procedure was miniaturised by a factor of 10 (500 mg of matrix + 2 mL of extraction solvent, 15 mL centrifuge tubes), to keep the amount of standard and matrix material as low as possible.

### 3.4.5   Linear range, respective LODs and LOQs

Prior to validation, the working range of each analyte had to be defined. The working range for analytical methods is usually smaller than the linear range.

Several dilutions of all standards, prepared in eluent A and matrix blank extract/dilution solvent 50:50 (v/v) were injected 5 times into the LC-MS/MS system. The peak areas were then plotted against the concentration in Microsoft Excel 2016. The respective linear range was estimated by visual observation. The respective LOD and LOQ were calculated for quantifier and qualifier transition. Signal to noise (S/N) ratios were calculated: 3:1 corresponds to the LOD and 10:1 to the LOQ. An exact calculation of the LODs and LOQs was performed during the validation (see chapter 3.5.2.3).

### 3.4.6 Internal standards

$^{13}$C-labeled internal standards were included in the validation, to prove the compensation of signal suppression or enhancement effects. For the calculation of this compensation, see chapter 3.5.2.4.

Two separate mixes, one for the positive and one for the negative mode, containing all internal standards were prepared in brown glass vials. Respectively, the concentration of the internal standard should be in the middle of the working range of the unlabeled mycotoxin. The concentrations in µg/mL of the internal standards in the mixes are shown in Table 4 for the positive mode and in Table 5 for the negative mode. This concentrations were used for all further experiments that included internal standards.

A pre-treatment program of the integrated Agilent G1329A autosampler was created to inject the mixes into the LC-MS/MS system: 1 µL was drawn out of the vial containing the internal standards, before drawing the 10 µL from the vial to be analysed, followed by subsequent injection. Small volumes can be used with this approach, so that the amount of internal standards can be kept as low as possible. The 1:10 dilution of the internal standards during this step has to be considered during calculation steps.

The mix for the negative mode was diluted in pure ACN, while for the positive mode pure ACN and ACN/$H_2O$ 50:50 (v/v) were tested before the validation, due to poor solubility of FUMs in 100% ACN. Peak shape and RSDs of each internal standard were compared for the 2 different solvents.

**Table 4: List of available internal standards for the positiv mode**

| Positive mode | |
|---|---|
| Internal standard | c [µg/L] |
| STG | 100 |
| Roquefortine C | 200 |
| DAS | 200 |
| T-2 | 400 |
| OTA | 400 |
| FB$_3$ | 500 |
| HT-2 | 1250 |
| FB$_1$ | 450 |
| FB$_2$ | 450 |
| Mycophenolic acid | 2250 |

**Table 5: List of available internal standards for the negative mode**

| Negative mode | |
|---|---|
| Internal standard | c [µg/L] |
| ZEN | 120 |
| D3G | 800 |
| 3-AcDON | 1500 |
| DON | 1500 |
| NIV | 1500 |
| PAT | 3000 |

| | |
|---|---|
| AFB$_1$ | 70 |
| AFB$_2$ | 70 |
| AFG$_1$ | 70 |
| AFG$_2$ | 70 |
| AFM$_1$ | 70 |
| 15-AcDON | 2000 |

## 3.5 Method validation

Method validation was performed for four different matrices: wheat, maize, finished pig feed and silage. At first, four multi-analyte stock solutions were prepared (see chapter 3.5.1) to facilitate the preparation of calibration functions and to keep the workload as low as possible. Then three different types of calibration curves were prepared in sevenfold repetition (see chapter 3.5.1). These functions were used for the data evaluation. The ion ratio of the qualifier/quantifier transition was calculated for each analyte (see chapter 3.5.2.1) and the quantification of possible contaminations in the used matrix material was performed via standard addition approach (see chapter 3.5.2.2). Recovery of the extraction step and matrix effects were calculated for each analyte in the four different commodities (see chapter 3.5.2.4), as well as their limits of detection and limits of quantification (see chapter 3.5.2.3). Experiments on several types of precision were carried out for wheat and maize (see chapter 3.5.3). Finally, the trueness was tested for four mycotoxins (DON, ZEN, NIV and OTA) with certified reference materials (CRMs) in neat solvent and one proficiency test in maize was attended (see chapter 3.6).

### 3.5.1 Preparation of the calibration functions

The four individual multi-analyte stock solutions were prepared by mixing mycotoxin standards with solvent: stock A + B for the positive mode, stock C + D for the negative mode. ACN was preferred as solvent and used for stock A + C. Due to poor solubility of some analytes in pure ACN, other solvent combinations had to be used: ACN/H$_2$O 50:50 (v/v) for stock B, ACN/H$_2$O 90:10 (v/v) for stock D. Table 6 - Table 9 show the preparation of the four stock solutions with the concentration and the added volume of the initial mycotoxin standard and the final concentration of the stock solution, as well as the amount of additionally added solvent (last row in each table).

Stock solutions were freshly prepared for each of the model matrices and used for all further validation experiments. The preparation should be done as fast as possible and exposure to light and heat should be avoided. The solutions were stored at -20°C and brought to room temperature before use.

**Table 6: Multi-analyte stock solution A**

| Stock A | | | |
|---|---|---|---|
| Analyte/solvent | $c$ sta. [µg/L] | $V$ sta. [µL] | $c$ sto. [µg/L] |
| Enniatin A | 10000 | 2.1* | 100 |
| Enniatin A1 | 10000 | 2.1* | 100 |
| Enniatin B1 | 10000 | 2.1* | 100 |
| Enniatin B | 50000 | 4.1* | 100 |
| Agroclavine | 10000 | 21 | 100 |
| Beauvericin | 10000 | 21 | 100 |
| Elymoclavine | 10000 | 21 | 100 |
| Ergine | 10000 | 21 | 100 |
| Dihydrolysergol | 10000 | 105 | 500 |
| Ergocornine | 10000 | 105 | 500 |
| Ergocristine | 10000 | 105 | 500 |
| Ergocryptine | 10000 | 105 | 500 |
| Ergometrine | 10000 | 105 | 500 |
| Ergosine | 10000 | 105 | 500 |
| Ergotamine | 10000 | 105 | 500 |
| $AFB_1$ | 40000 | 175 | 333.3 |
| $AFB_2$ | 40000 | 175 | 333.3 |
| $AFG_1$ | 40000 | 175 | 333.3 |
| $AFG_2$ | 40000 | 175 | 333.3 |
| $AFM_1$ | 40000 | 175 | 333.3 |
| DAS | 10000 | 210 | 1000 |
| NEO | 10000 | 210 | 1000 |
| Roquefortine C | 10000 | 210 | 1000 |
| STG | 50000 | 210 | 500 |
| Ergocorninine | 25000 | 420 | 500 |
| Ergocristinine | 25000 | 420 | 500 |
| Ergocryptinine | 25000 | 420 | 500 |
| Ergometrinine | 25000 | 420 | 500 |
| Gliotoxin | 10000 | 420 | 2000 |

**Table 7: Multi-analyte stock solution B**

| Stock B | | | |
|---|---|---|---|
| Analyte/solvent | $c$ sta. [µg/L] | $V$ sta. [µL] | $c$ sto. [µg/L] |
| $FB_1$ | 50000 | 840 | 13333 |
| $FB_2$ | 50000 | 840 | 13333 |
| $FB_3$ | 50000 | 840 | 13333 |
| $ACN/H_2O$ 50:50 (v/v) | | 630 | |

**Table 8: Multi-analyte stock solution C**

| Stock C | | | |
|---|---|---|---|
| Analyte/solvent | $c$ sta. [µg/L] | $V$ sta. [µL] | $c$ sto. [µg/L] |
| ZEN | 10000 | 42 | 266.6 |
| AOH | 10000 | 210 | 1333 |
| ZAN | 10000 | 210 | 133.3 |
| 3-AcDON | 10000 | 630 | 4000 |
| NIV | 10000 | 630 | 4000 |
| FusX | 10000 | 700 | 4444 |
| α-ZOL | 10000 | 700 | 444.4 |
| β-ZOL | 10000 | 700 | 444.4 |
| D3G | 50000 | 1400 | 4444 |
| DON | 10000 | 1400 | 8888 |
| PAT | 10000 | 4200 | 26666 |
| ACN | | 4928 | |

| | | | |
|---|---|---|---|
| HT-2 | 10000 | 420 | 2000 |
| T-2 | 10000 | 420 | 2000 |
| Fusaric acid | 10000 | 630 | 3000 |
| Penicillic acid | 10000 | 630 | 3000 |
| OTA | 10000 | 700 | 333.3 |
| OTB | 10000 | 700 | 333.3 |
| 15-Acetoxyscirpenol | 50000 | 840 | 2000 |
| T-2 triol | 50000 | 1260 | 3000 |
| 15-AcDON | 10000 | 2100 | 10000 |
| Mycophenolic acid | 10000 | 2100 | 10000 |
| T-2 tetraol | 20000 | 2100 | 20000 |
| ACN | | 4445 | |

**Table 9: Multi-analyte stock solution D**

| Stock D | | | |
|---|---|---|---|
| Analyte/solvent | $c$ sta. [µg/L] | $V$ sta. [µL] | $c$ sto. [µg/L] |
| MON | 10000 | 210 | 2000 |
| ACN/H$_2$O 90:10 (v/v) | | 10290 | |

*c* sta.: concentration in the initially used mycotoxin standard in [µg/L]

*V* sta.: volume of the initial mycotoxin standard, added to the multi-analyte stock solution in [µL]

*c* sto.: final concentration of the mycotoxin in the multi-analyte stock solution in [µg/L]

Three different types of calibration functions had to be prepared for the validation, which are in this work referred as liquid standards, matrix matched standards and spiked samples. Matrix matched standards were spiked with the stock solutions after the extraction step, spiked samples where spiked before the extraction step, liquid standards contained no matrix and were prepared in neat solvents. The in chapter 3.3 described blank model matrix samples were used.

The whole procedure was performed in sevenfold repetition and 7 different levels with the following relative concentrations:

**Table 10: Relative concentration levels, which were used during the validation**

| Level | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Relative concentration | 300 | 100 | 30 | 10 | 3 | 1 | 0.3 |

The levels were chosen individually for each analyte based on the linearity, occurrence data provided by Biomin (within their Spectrum 380® survey), regulations and respective LOD + LOQ. Level 6 should be close to the respective LOQ, level 7 close the respective LOD. Levels 1-6 were used for the calibration functions of most analytes. For some mycotoxins with high respective LOQs, only levels 1-5 were used, meaning that level 6 corresponds to the LOD and level 5 to the LOQ, to keep the amount of standard as low as possible. Biomin provided

mycotoxin occurrence data of their samples for the past several months, measured by other existing LC-MS/MS multimethods. These data were reviewed in order to select the appropriate working range, which will be needed in future after implementation of the method into routine analysis.

For the liquid standard calibration and matrix matched calibration, the highest concentration levels (level 1) were prepared by mixing 300 µL of stock A with 45 µL of stock B for the positive mode and 225 µL of stock C with 150 µL of stock D for the negative mode. The solvents were then evaporated to dryness with $N_2$ using an evaporation unit and the remaining mycotoxins were re-suspended in 1000 µL of the required solvent. In this step and for the further dilutions (level 2-6), dilution solvent/extraction solvent 50:50 (v/v) was used for the liquid standards. The blank samples were extracted according to 3.4.4. A mix of this extract and dilution solvent 50:50 (v/v) was used as the solvent for the matrix matched calibration. The concentration levels 2-7 were prepared as follows:

- Level 2:       330 µL level 1       + 660 µL solvent
- Level 3:       100 µL level 1       + 900 µL solvent
- Level 4:        33 µL level 1       + 957 µL solvent
- Level 5:       300 µL level 4       + 700 µL solvent
- Level 6:       100 µL level 4       + 900 µL solvent
- Level 7:        30 µL level 4       + 970 µL solvent


For the preparation of spiked samples, 500 mg of ground blank matrix samples (see chapter 3.4.4) were weighed into 15 mL centrifuge tubes. Before spiking, pre-dilutions had to be prepared for the higher levels. Table 11 shows the pre-dilution steps prior to the spiking experiments, including the volumes of the multi-analyte stock solution and the volumes of the solvent that were mixed.

**Table 11: Pre-dilutions of the multi-analyte stocks for spiking of blank samples**

| Pre-dilution | V [µL] | V solvent [µL] | Solvent |
|---|---|---|---|
| Stock A 1:20 | 300 (stock A) | 5700 | ACN |
| Stock B 1:15 | 270 (stock B) | 3780 | ACN/$H_2O$ 50:50 (v/v) |
| Stock B 1:150 | 200 (stock B1:15) | 1800 | ACN/$H_2O$ 50:50 (v/v) |
| Stock C 1:30 | 200 (stock C) | 5800 | ACN |
| Stock D 1:40 | 125 (stock D) | 4875 | ACN/$H_2O$ 90:10 (v/v) |

Appropriate amounts of the stock solutions/pre-dilutions were then added to the blank matrix:

Positive mode:

Level 1: 1200 µL stock A          + 180 µL stock B

Level 2: 400 µL stock A           +  60 µL stock B

Level 3: 120 µL stock A           + 270 µL stock1 B 1:15

Level 4: 40 µL stock A            +  90 µL stock B 1:15

Level 5: 240 µL stock A 1:20      +  27µL stock B 1:15

Level 6: 80 µL stock A 1:20       +  90 µL stock B 1:150

Level 7: 24 µL stock A 1:20       +  27 µL stock B 1:150


Negative mode:

Level 1: 900 µL stock C           + 600 µL stock D

Level 2: 300 µL stock C           + 200 µL stock D

Level 3: 90 µL stock C            +  60 µL stock D

Level 4: 30 µL stock C            +  20 µL stock D

Level 5: 270 µL stock C 1:30      + 240 µL stock D 1:40

Level 6: 90 µL stock C 1:30       +  80 µL stock D 1:40

Level 7: 27 µL stock C 1:40       +  24 µL stock D 1:40


The centrifuge tubes were stored uncapped in an incubator at 37°C overnight, to allow solvent evaporation and establishment of equilibrium between the analytes and matrix. Extraction was performed as described in chapter 3.4.4 (miniaturised version for the validation), so that the same concentration levels as for the liquid standards and matrix matched standards were received.

### 3.5.2 Data evaluation

The data evaluation was performed in Analyst 1.6.2 and Microsoft Excel 2016. The peak areas were automatically calculated with the quantitative wizard function of the Analyst Software and the integration of the peaks was manually corrected if necessary. A smoothing width of 3 and a peak splitting factor of 2 were chosen. This is especially important for the calculation of the LOD and LOQ, because the smoothing width reduces the amount of noise and therefore leads to a seemingly lower LOD and LOQ. For the construction of the calibration functions and their slopes, Microsoft Excel 2016 was used. It has to be noticed, that a 1/x weighting of the calibration function is not possible in Microsoft Excel. A comparison of the calculated recoveries for representative analytes between a 1/x weighted linear calibration function, created by the Analyst Software with the non-weighted in Microsoft Excel, showed only minimal differences in the results. Microsoft Excel was therefore preferred, to facilitate the handling of such a large amount of data. However, for the routine analysis and tests on the trueness of the method, 1/x weighted calibration curves from the Analyst Software are used.

#### 3.5.2.1 Ion ratio

The 7 replicates of the liquid standard at concentration level 3 were used to calculate the average ion ratio. The peak area of the qualifier transition was divided by the peak area of the quantifier transition. Besides the retention time, quantifier and qualifier transitions, the ion ratio can be used as an additional confirmation criteria for the specific analyte during routine analysis. It should however be taken into account, that slight changes at different concentrations may be possible.

#### 3.5.2.2 Quantification of blank material

The analysis of the used matrices prior to validation showed, that not all of the used matrices were real blank samples for every mycotoxin included in the method. For some analytes, clear peaks in the blank extract showed contamination in the samples. In this case, the peak area in the blank extract was subtracted from the peak area in the matrix matched calibration and spiked sample calibration, to eliminate errors during the calculation of recoveries. For the calculation of LOD + LOQ, the contaminations had to be quantified, wherefore the standard addition approach was used. Figure 3 shows an example of calculating the contamination of a specific mycotoxin in the matrix material. In this example, a 5-point calibration function was created by plotting the peak area of the matrix matched standard vs. the analyte concentration.

The concentration in the blank sample corresponds to the absolute value of the x-intercept and is marked by the blue arrow.
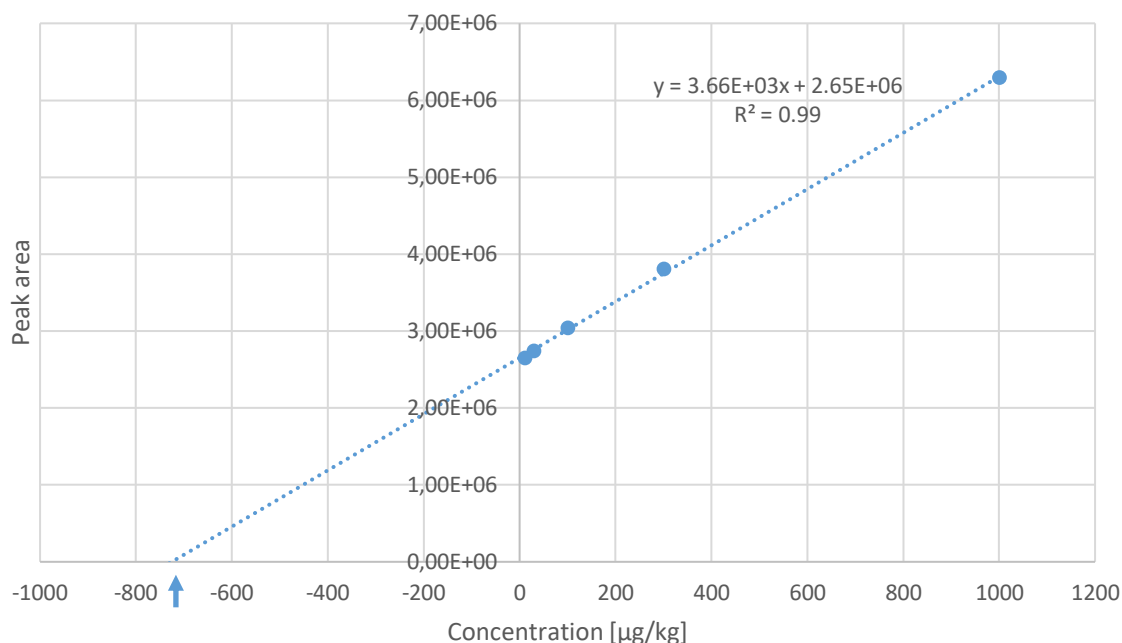


**Figure 3: Example for calculation the contamination in the matrix material of interest with the standard addition approach**

### 3.5.2.3 LODs + LOQs

The limits of detection and limits of quantification were calculated for all mycotoxins in the different commodities and in neat solvent (extraction solvent/dilution solvent 50:50 (v/v)) by using the signal to noise ratio approach. The retention time window of the sMRM method was too small, to get valuable results by using the noise before and after the analyte peak, so that the noise of blank matrix extract/dilution solvent 50:50 (v/v) was used for the calculations. The 7 replicates of the matrix matched standard calibration levels that were closest to a S/N ratio of 3/1 for the LOD and 10/1 for the LOQ were used for the calculations.

Figure 4 shows an example of how the highest and lowest point of the noise were evaluated, which were then used for the calculation of the noise with F1. The calculation of the signal was performed according to Figure 5 and F2. Both together enabled the calculation of the S/N ratio with F3.
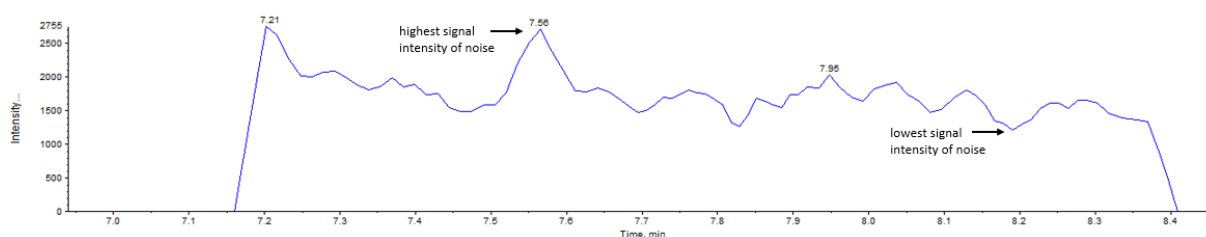
**Figure 4: Example for the calculation of noise**
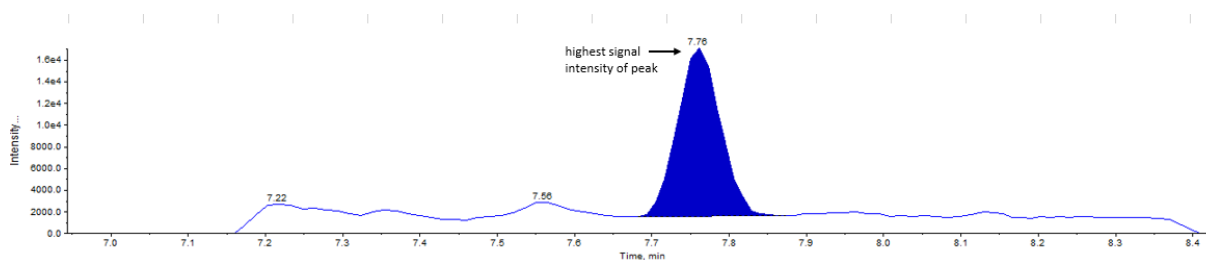


**Figure 5: Example for the calculation of the signal intensity**

F1: noise $=$ highest signal intensity of noise $-$ lowest signal intensity of noise

F2: signal $=$ highest signal intensity of peak $- \left( \frac{\text{noise}}{2} + \text{lowest signal intensity of noise} \right)$

F3: signal to noise $= \frac{\text{signal}}{\text{noise}}$

### 3.5.2.4 Recovery

Linear calibration curves were constructed for each analyte by plotting the peak area versus the analyte concentration, using Microsoft Excel. Separate calibration curves were gained for the liquid standards, matrix matched standards and spiked samples. The slopes of the functions were used to calculate $R_A$, SSE and $R_E$, by using F4-6.

A SSE above 100% means, that the signals are enhanced by matrix effects, SSE below 100% means a signal suppression due to matrix effects.

The formulas F4-6 were used by Matuszewski (2003) and modified by Sulyok et al. (2006) and adopted for this thesis.

For analytes with internal standards, additionally the peak area of the analyte divided by the peak area of the internal standard vs. analyte concentration divided by concentration of the internal standard was plotted in Microsoft Excel. To calculate the recoveries with internal standards, the slopes were inserted into F 4-6.

$$\text{F4: } R_A\ (\%) = \frac{\text{slope}_{\text{spiked sample}}}{\text{slope}_{\text{liquid standard}}} * 100$$

$$\text{F5: } SSE\ (\%) = \frac{\text{slope}_{\text{spiked extract}}}{\text{slope}_{\text{liquid standard}}} * 100$$

$$\text{F6: } R_E(\%) = \frac{R_A}{SSE} * 100 = \frac{\text{slope}_{\text{spiked sample}}}{\text{slope}_{\text{spiked extract}}} * 100$$

### 3.5.3 Precision

For wheat and maize, additional tests on different types of precision were carried out:

- The <u>repeatability</u> was calculated by successively measuring the same vial (level 3 from matrix matched calibration) 10 times. The RSD values were calculated from the peak area for each analyte.
- To test the <u>stability</u> of the analytes at room temperature, three replicates of level 3 from the matrix matched calibration were measured on different days: directly after thawing, after 1 day, 2 days and 4 days. RSDs of the peak areas between the days and the average RSD of the three replicates were calculated for each analyte.
- <u>Ruggedness</u>: An identical HPLC-column was available in-house. The same experiment as for the repeatability was performed with this column and the difference in RSDs was analysed. The LC-MS/MS method was slightly adjusted, due to some retention time shifts during this experiment.
- For the <u>lab precision,</u> the 7 replicates of level 3, spiked samples were measured one after another and the RSDs of the peak area were calculated for each analyte.

## 3.6 Trueness

### 3.6.1 Certified reference materials

In-house produced certified reference materials of the brand Biopure were available for the following analytes: DON, NIV, OTA and ZEN. They were diluted in extraction solvent/dilution solvent 50:50 (v/v) and measured with the LC-MS/MS method during the validation to quantify them with the liquid standard calibration function. The results were then compared with the certified values. The following formulas, published by Linsinger (2010) were used in this thesis:

F7: $\Delta_m = |c_m - c_{CRM}|$

$\Delta_m$.....absolute difference between mean measured value and certified value

$c_m$.....mean measured value

$c_{CRM}$.....certified value

F8: $u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$

$u_\Delta$.....combined uncertainty of results and certified value

$u_m$..... uncertainty of the measurement result

$u_{CRM}$..... uncertainty of the certified value

F9: $U_\Delta = 2 * u$

$U_\Delta$.....expanded uncertainty of difference between result and certified value

If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measured results and the certified value.

### 3.6.2 Proficiency test

Method trueness was tested by participating in food chemistry proficiency test 04319 from FAPAS. A maize sample that contained the analytes $AFB_1$, DON, ZEN, OTA, $FB_1$, $FB_2$, T-2 and HT-2 was received. The sample preparation procedure was carried out according to 3.4.4 and the extracts were measured with the existing LC-MS/MS methods. Quantification was performed by using a liquid standard calibration function with the same concentrations that were used during the validation. The whole procedure was carried out in fivefold repetition and

each replicate was measured 3 times. Internal standards and the $R_E$ values from the validation were used for the correction of the measured values. The results were then statistically analysed by FAPAS by calculating z scores and reporting them for each analyte. Z scores between -2 and +2 mean acceptable results.

# 4 Results and Discussion

## 4.1 Optimised LC-MS/MS parameters

Software controlled compound optimisation was accomplished to receive the most abundant MRM transitions by choosing ideal MS parameters such as DP, CE and CXP. The chosen ESI polarity mode (positive or negative) was based on the signal intensity of the precursor ions. 62 analytes showed higher signals in the positive mode, while 18 showed higher signals in the negative mode. Most mycotoxins that were ionised in the positive mode formed $[M+H]^+$ ions, but for some analytes $[M+NH_4]^+$ adducts gave higher signal intensities. The ion with the highest signal intensity was used as a precursor ion, except for $^{13}C$-mycophenolic acid, where the $[M+NH_4]^+$ adduct showed the highest intensity, but the $[M+H]^+$ adduct was used as precursor ion, to adjust it with the unlabelled mycophenolic acid. In the negative ionisation mode, $[M-H]^-$ ions were used for the majority of analytes, while $[M+CH_3COO]^-$ adducts were used for a smaller number of mycotoxins. The formation of stable sodium adducts, which do not yield detectable product ions for some analytes according to Sulyok et al. (2006) was minimised by adding 5 mM ammonium acetate to eluent A + B.

The two product ions with the highest signal intensities were chosen as quantifier and qualifier transition. Precurser ions, product ions and CE did not show significant differences, compared to the ones published by Malachova et al. (2014), while the DP is more instrument dependent and showed more differences. All analytes showed at least two product ions, except for MON which showed only one product ion (see Sulyok et al., 2006).

After successful optimisation of the MS parameters, two mixes, containing all analytes for the positive and negative mode were prepared and injected into the LC-MS/MS system with the MRM method described in chapter 3.4.2. Retention times, necessary for the sMRM method were received and the peak shape, signal intensity and intensity of the noise were evaluated. Internal standards were not included in this experiment, because of the same retention time as the unlabelled analyte. Analytes that have the same MRM transitions were additionally injected in separate chromatographic runs to make annotation of the peaks possible.

Some toxins caused problems during these runs. Fusaric acid showed a very broad peak and strong tailing. It was difficult to get the whole peak into a retention time window of 72 or 90 seconds for the sMRM mode, since slight retention time shifts would cause an incomplete peak. This was the only analyte that showed such a broad peak, so that it was decided that the retention time window is not extended. T-2 tetraol showed a very low peak intensity and

suboptimal peak shape. The automatic compound optimisation, was amended by a manual compound optimisation with the Analyst Software. Nevertheless, the signal intensity could not be improved. It was further tried, to dilute the standard with a combination of eluent A/B 50:50 (v/v) instead of eluent A only, to exclude solubility problems. After unsatisfactory results, it was decided to accept the suboptimal peak shape and to continue without further improvement attempts. Tenuazonic acid showed very low signal intensity and unsatisfactory peak shape, which could not be used for linear calibration functions. The analyte had to be excluded from the final method.

For the group of the ergot alkaloids, it was not always possible to fully separate the ergopeptine and the ergopeptinine epimers, which show the same MRM transitions. Moreover, an epimerisation of a part of the ergopeptine form to the ergopeptinine form was expected due to the acidic pH of the eluents. This makes a precise quantification of the analytes with the present method impossible. Ergocornine/ergocorninine, ergocryptine/ergocryptinine, ergometrine/ergometrinine and ergocristine/ergocristinine showed separate peaks for the two epimers, while ergotamine/ergotaminine and ergosine/ergosinine showed co-elution and only one peak. Hence, in the routine analysis it will not be possible to report separate results for ergotamine/ergotaminine and ergosine/ergosinine, but only for the sum of the two epimers together. The same effect was observed by Sulyok et al. (2007). It was decided to only include ergotamine and ergosine in the validation procedure and to exclude ergotaminine and ergosinine.

Concerning the high number of analytes with different chemical characteristics in one LC-MS/MS method, compromises had to be accepted. Some parameters were far from optimal for some analytes and ideal peak shape or peak separation could not be expected. Especially for analytes that elute before minute 8, suboptimal peak shape was observed. Nevertheless, it was possible to receive peaks that are suitable for quantification, except for tenuazonic acid and separate quantification of ergotamine/ergotaminine and ergosine/ergosinine. It was also not possible to avoid co-elution for some of the other analytes, but this is of minor importance due to different MRM transitions. For all other analytes with the same MRM transitions, the peaks could be fully separated.

15-acetoxyscirpenol, $AFB_1$, enniatin A1, ergocryptine, $FB_1$, $FB_2$, $FB_3$, OTA and STG, showed higher signal intensities or higher S/N ratios for the initial qualifier transition than for the quantifier transitions. For this analytes, the product ions were switched.

The received retention times ranged from 3.14 to 15.99 min. Figure 6 shows the retention time windows (72 s) in the sMRM mode for all analytes, positive and negative polarity mode combined. Especially the period of time between 8 and 10 minutes is critical, due to the high

number of eluting analytes. When the repeatability is tested, special attention should be paid to these analytes. It was tested, if acceptable peak shape and repeatability can be achieved when combining positive and negative polarity mode in one chromatographic run with fast polarity switching or if two separate chromatographic runs have to be used for the validation and routine analysis (see chapter 4.1.1).

Table 12 shows the optimised MS parameters, as well as the retention times and ion ratio for all analytes with internal standards, included in the method optimisation.

The ion ratio of the two product ions was calculated during the validation, by dividing the peak area of the qualifier transition by the one from the quantifier transition. For some analytes, the primary transition showed higher signal intensities than the secondary transition, resulting in ion ratios > 1. In this case the noise of the qualifier transition was clearly higher, so that better LODs and LOQs were achieved with the chosen quantifier transition. For the internal standards and the analytes that were not included in the validation, the ion ratio was not calculated.

The two MRM transitions, expected retention time and the ion ratio can be used as confirmation criteria for a specific analyte.
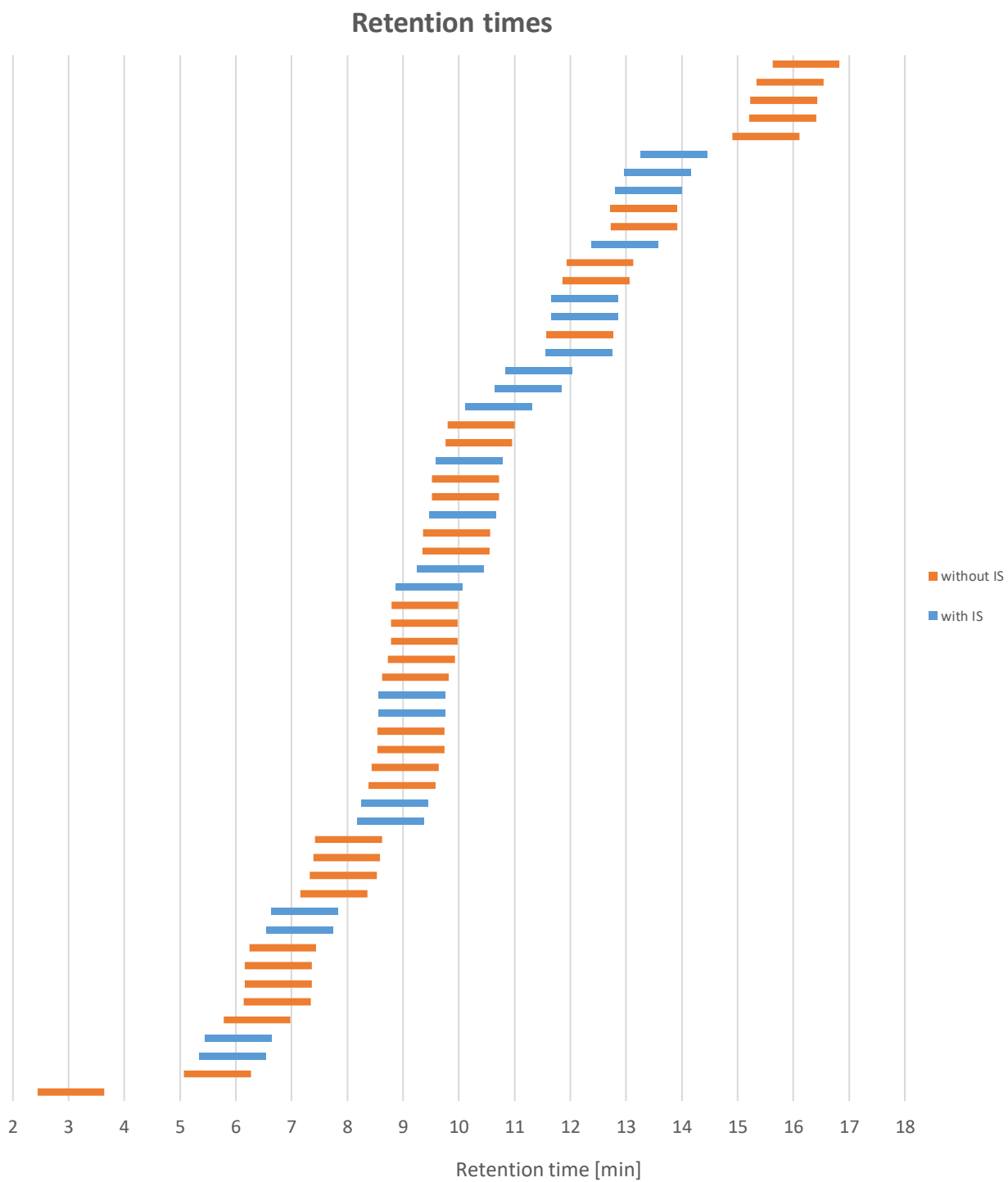
**Figure 6: Retention time window of all analytes included in the method**

**Table 12: Optimised MS parameters, retention times and ion ratios**

| Analyte | Ionisation mode | Molecular mass [g/mol] | Precursor ion | Q1 *m/z* | DP [V] | $r_t$ [min] | Q3 *m/z* | CE [eV] | CXP [V] | Ion ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| 15-AcDON | positive | 338.4 | [M+H]+ | 339.0 | 71 | 8.53 | 137.1 / 321.1 | 15 / 13 | 10 / 14 | 0.48 |
| 15-Acetoxyscirpenol | positive | 324.4 | [M+NH4]+ | 342.1 | 36 | 8.98 | 107.0 / 265.2 | 21 / 13 | 8 / 8 | 0.79 |
| 3-AcDON | negative | 338.4 | [M+CH3COO]- | 397.1 | -55 | 8.61 | 307.2 / 337.1 | -22 / -14 | -9 / -9 | 3.42 |
| AFB$_1$ | positive | 312.3 | [M+H]+ | 313.1 | 86 | 9.95 | 241.0 / 285.0 | 51 / 33 | 12 / 6 | 1.16 |
| AFB$_2$ | positive | 314.3 | [M+H]+ | 315.0 | 106 | 9.61 | 287.0 / 259.1 | 37 / 41 | 6 / 12 | 0.84 |
| AFG$_1$ | positive | 328.3 | [M+H]+ | 328.9 | 81 | 9.22 | 243.0 / 200.1 | 37 / 51 | 10 / 8 | 0.60 |
| AFG$_2$ | positive | 330.3 | [M+H]+ | 331.0 | 76 | 8.91 | 313.0 / 245.0 | 35 / 43 | 8 / 8 | 0.53 |
| AFM$_1$ | positive | 328.3 | [M+H]+ | 329.0 | 76 | 8.92 | 273.0 / 259.0 | 35 / 35 | 14 / 14 | 0.38 |
| Agroclavine | positive | 238.3 | [M+H]+ | 239.0 | 71 | 7.52 | 208.2 / 183.2 | 25 / 25 | 8 / 8 | 0.98 |
| α -ZOL | negative | 320.4 | [M-H]- | 319.2 | -110 | 13.08 | 274.9 / 159.9 | -28 / -40 | -11 / -7 | 0.30 |
| AOH | negative | 258.2 | [M-H]- | 257.0 | -105 | 12.29 | 215.0 / 213.0 | -36 / -32 | -7 / -9 | 1.43 |
| Beauvericin | positive | 783.9 | [M+NH4]+ | 801.3 | 101 | 15.57 | 784.4 / 134.1 | 29 / 93 | 16 / 8 | 0.52 |
| β-ZOL | negative | 320.4 | [M-H]- | 319.2 | -110 | 12.22 | 274.9 / 159.9 | -28 / -40 | -11 / -7 | 0.44 |
| D3G | negative | 458.5 | [M+CH3COO]- | 517.1 | -15 | 6.81 | 427.1 / 457.1 | -28 / -20 | -11 / -13 | 1.30 |
| DAS | positive | 366.4 | [M+NH4]+ | 384.2 | 36 | 9.73 | 307.1 / 104.9 | 17 / 55 | 10 / 8 | 0.85 |
| Dihydrolysergol | positive | 256.2 | [M+H]+ | 257.1 | 86 | 6.40 | 208.1 / 167.0 | 33 / 53 | 6 / 12 | 0.74 |
| DON | negative | 296.3 | [M+CH3COO]- | 355.1 | -40 | 6.89 | 59.2 / 295.1 | -40 / -16 | -13 / -7 | 0.91 |
| Elymoclavine | positive | 254.1 | [M+H]+ | 255.1 | 76 | 6.30 | 224.1 / 180.1 | 21 / 53 | 8 / 12 | 0.41 |
| Enniatin A | positive | 681.5 | [M+NH4]+ | 699.2 | 110 | 15.99 | 210.3 / 555.4 | 39 / 41 | 16 / 6 | 0.22 |
| Enniatin A1 | positive | 667.4 | [M+NH4]+ | 685.0 | 76 | 15.70 | 668.2 /210.2 | 25 /45 | 20 / 8 | 0.41 |
| Enniatin B | positive | 639.4 | [M+NH4]+ | 657.2 | 96 | 15.27 | 640.2 / 196.2 | 25 / 37 | 16 / 10 | 0.56 |

38

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Enniatin B1 | positive | 653.4 | [M+NH4]+ | 671.2 | 71 | 15.59 | 654.3 / 196.1 | 25 / 39 | 14 / 10 | 0.36 |
| Ergine | positive | 267.1 | [M+H]+ | 268.1 | 11 | 6.04 | 223.2 / 208.1 | 27 / 31 | 8 / 8 | 0.42 |
| Ergocornine | positive | 561.7 | [M+H]+ | 562.1 | 36 | 9.09 | 223.2 / 268.1 | 43 / 33 | 6 / 8 | 0.40 |
| Ergocorninine | positive | 561.7 | [M+H]+ | 562.1 | 36 | 10.18 | 223.2 / 268.1 | 43 / 33 | 6 / 8 | 0.36 |
| Ergocristine | positive | 609.7 | [M+H]+ | 610.2 | 96 | 9.88 | 223.1 / 268.1 | 43 / 37 | 8 / 10 | 0.18 |
| Ergocristinine | positive | 609.7 | [M+H]+ | 610.2 | 96 | 11.13 | 223.1 / 268.1 | 43 / 37 | 8 / 10 | 0.23 |
| Ergocryptine | positive | 575.7 | [M+H]+ | 576.1 | 88 | 9.15 | 223.0 / 267.9 | 43 / 35 | 12 / 8 | 0.24 |
| Ergocryptinine | positive | 575.7 | [M+H]+ | 576.1 | 88 | 10.72 | 223.0 / 267.9 | 43 / 35 | 12 / 8 | 0.22 |
| Ergometrine | positive | 325.4 | [M+H]+ | 325.8 | 51 | 6.42 | 223.1 / 208.0 | 31 / 37 | 10 / 8 | 0.46 |
| Ergometrinine | positive | 325.4 | [M+H]+ | 325.8 | 51 | 7.06 | 208.0 / 223.1 | 37 / 31 | 8 / 10 | 0.37 |
| Ergosine | positive | 547.6 | [M+H]+ | 548.1 | 46 | 8.90 | 223.2 / 208.0 | 41 / 45 | 8 / 6 | 0.41 |
| Ergosinine | positive | 547.6 | [M+H]+ | 548.1 | 46 | 8.90 | 223.2 / 208.0 | 41 / 45 | 8 / 6 | * |
| Ergotamine | positive | 581.7 | [M+H]+ | 582.1 | 91 | 9.14 | 223.0 / 268.2 | 39 / 35 | 8 / 10 | 0.14 |
| Ergotaminine | positive | 581.7 | [M+H]+ | 582.1 | 91 | 9.14 | 223.0 / 268.2 | 39 / 35 | 8 / 10 | * |
| FB$_1$ | positive | 721.8 | [M+H]+ | 722.4 | 121 | 10.81 | 352.3 / 334.3 | 51 / 59 | 12 / 10 | 0.86 |
| FB$_2$ | positive | 705.8 | [M+H]+ | 706.3 | 96 | 12.73 | 336.4 / 354.3 | 51 / 45 | 10 / 8 | 0.34 |
| FB$_3$ | positive | 705.8 | [M+H]+ | 706.3 | 96 | 11.92 | 336.4 / 354.3 | 51 / 45 | 10 / 8 | 0.43 |
| Fusaric acid | positive | 179.1 | [M+H]+ | 179.9 | 26 | 8.70 | 134.1 / 91.9 | 21 / 33 | 10 / 14 | 0.29 |
| FusX | negative | 354.4 | [M+CH3COO]- | 413.3 | -40 | 7.69 | 59.1 / 262.9 | -44 / -22 | -13 / -13 | 0.40 |
| Gliotoxin | positive | 326.4 | [M+H]+ | 327.0 | 41 | 10.12 | 263.1 / 245.1 | 13 / 23 | 10 / 10 | 0.74 |
| HT-2 | positive | 424.5 | [M+NH4]+ | 442.1 | 26 | 11.19 | 263.2 / 215.2 | 17 / 17 | 10 / 6 | 1.06 |
| MON | negative | 98.1 | [M-H]- | 96.9 | -10 | 3.14 | 41.0 | -18 | -11 | ** |
| Mycophenolic acid | positive | 320.3 | [M+H]+ | 321.0 | 111 | 12.01 | 206.9 / 159.0 | 33 / 45 | 12 / 8 | 0.55 |
| NEO | positive | 382.4 | [M+NH4]+ | 400.2 | 36 | 7.75 | 305.1 / 185.2 | 17 / 29 | 8 / 10 | 0.69 |
| NIV | negative | 312.3 | [M+CH3COO]- | 371.1 | -35 | 5.50 | 281.1 / 59.0 | -22 / -46 | -7 / -5 | 1.16 |
| OTA | positive | 403.8 | [M+H]+ | 404.0 | 56 | 13.2 | 239.0 / 358.0 | 33 / 21 | 14 / 10 | 0.76 |
| OTB | positive | 369.4 | [M+H]+ | 370.1 | 56 | 11.9 | 205.0 / 103.2 | 33 / 77 | 12 / 6 | 0.31 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PAT | negative | 154.1 | [M-H]- | 153.0 | -45 | 5.40 | 80.9 / 108.8 | -14 / -10 | -21 / -9 | 0.40 |
| Penicillic acid | positive | 170.2 | [M+H]+ | 171.0 | 56 | 7.78 | 125.0 / 97.1 | 17 / 21 | 10 / 8 | 0.19 |
| Roquefortine C | positive | 389.5 | [M+H]+ | 390.0 | 51 | 10.17 | 193.1 / 321.9 | 37 / 29 | 18 / 10 | 0.77 |
| STG | positive | 324.3 | [M+H]+ | 324.9 | 86 | 13.62 | 310.1 / 280.9 | 35 / 51 | 10 / 10 | 0.92 |
| T-2 | positive | 466.5 | [M+NH4]+ | 484.2 | 51 | 12.02 | 215.0 / 305.1 | 29 / 19 | 14 / 6 | 0.74 |
| T-2 tetraol | positive | 298.3 | [M+NH4]+ | 316.1 | 31 | 5.20 | 215.1 / 281.1 | 12 / 11 | 10 / 11 | 0.81 |
| T-2 triol | positive | 382.5 | [M+NH4]+ | 400.1 | 21 | 10.26 | 215.2 / 105.1 | 19 / 61 | 8 / 8 | 0.20 |
| Tenuazonic acid | negative | 197.2 | [M-H]- | 196.1 | -150 | 8.74 | 139.0 /111.9 | -26 / -32 | -7 / -9 | * |
| ZAN | negative | 320.4 | [M-H]- | 319.1 | -110 | 13.08 | 275.2 / 161.0 | -30 / -38 | -7 / -5 | 0.15 |
| ZEN | negative | 318.4 | [M-H]- | 317.1 | -75 | 13.32 | 174.9 / 131.0 | -34 / -42 | -13 / -13 | 0.60 |
| $^{13}$C-3-AcDON | negative | 355.4 | [M+CH3COO]- | 414.1 | -50 | 8.50 | 332.2 / 354.1 | -22 / -14 | -9 / -9 | *** |
| $^{13}$C-15-AcDON | positive | 355.4 | [M+CH3COO]- | 356.1 | 71 | 8.53 | 145.2 / 338.2 | 15 / 13 | 6 / 10 | *** |
| $^{13}$C-AFB$_1$ | positive | 329.3 | [M+H]+ | 330.1 | 91 | 9.95 | 301.1 / 255.1 | 33 / 53 | 8 / 14 | *** |
| $^{13}$C-AFB$_2$ | positive | 331.3 | [M+H]+ | 332.1 | 81 | 9.61 | 303.1 / 273.2 | 39 / 43 | 8 / 16 | *** |
| $^{13}$C-AFG$_1$ | positive | 345.3 | [M+H]+ | 346.1 | 76 | 9.22 | 257.1 / 212.1 | 39 / 59 | 8 / 12 | *** |
| $^{13}$C-AFG$_2$ | positive | 347.3 | [M+H]+ | 348.0 | 56 | 8.91 | 330.1 / 259.1 | 37 / 43 | 8 / 8 | *** |
| $^{13}$C-AFM$_1$ | positive | 345.3 | [M+H]+ | 346.1 | 76 | 8.92 | 288.1 / 273.0 | 35 / 35 | 16 / 6 | *** |
| $^{13}$C-D3G | negative | 479.5 | [M+CH3COO]- | 538.2 | - 20 | 6.81 | 478.1 / 447.3 | -20 / -30 | -13 / -7 | *** |
| $^{13}$C-DAS | positive | 385.5 | [M+NH4]+ | 403.2 | 41 | 9.73 | 324.2 / 244.1 | 17 / 23 | 10 / 6 | *** |
| $^{13}$C-DON | negative | 311.4 | [M+CH3COO]- | 370.1 | - 45 | 6.89 | 59.1 / 310.1 | - 36/ -16 | - 7 / -7 | *** |
| $^{13}$C-FB$_1$ | positive | 755.9 | [M+H]+ | 756.4 | 86 | 10.81 | 374.3 / 356.4 | 53 / 59 | 10 / 10 | *** |
| $^{13}$C-FB$_2$ | positive | 739.9 | [M+H]+ | 740.5 | 111 | 12.73 | 358.2 / 340.3 | 53 / 55 | 10 / 10 | *** |
| $^{13}$C-FB$_3$ | positive | 739.9 | [M+H]+ | 740.5 | 111 | 11.92 | 358.2 / 340.3 | 53 / 53 | 10 / 10 | *** |
| $^{13}$C-HT-2 | positive | 446.6 | [M+NH4]+ | 464.3 | 46 | 11.19 | 278.0 / 113.0 | 19 / 65 | 6 / 8 | *** |
| $^{13}$C-Mycophenolic acid | positive | 337.4 | [M+H]+ | 338.1 | 106 | 12.01 | 169.2 / 218.1 | 49 / 31 | 12 / 12 | *** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| [13C]-NIV | negative | 327.4 | [M+CH3COO]- | 386.1 | - 40 | 5.50 | 58.9 / 295.1 | -52 / -22 | -5 / -9 | *** |
| [13C]-OTA | positive | 423.9 | [M+H]+ | 424.1 | 66 | 13.17 | 250.0 / 377.1 | 33 / 21 | 10 / 6 | *** |
| [13C]-PAT | negative | 161.1 | [M-H]- | 159.9 | - 50 | 5.40 | 115.0 / 86.0 | -12 / -16 | -9 / -7 | *** |
| [13C]-Roquefortine C | positive | 411.5 | [M+H]+ | 412.1 | 21 | 10.17 | 201.1 / 339.2 | 39 / 29 | 8 /10 | *** |
| [13C]-STG | positive | 342.3 | [M+H]+ | 343.0 | 81 | 13.62 | 297.0 / 327.0 | 55 / 37 | 12 / 10 | *** |
| [13C]-T-2 | positive | 490.6 | [M+NH4]+ | 508.2 | 41 | 12.02 | 229.1 / 198.1 | 29 / 31 | 12 / 12 | *** |
| [13C]-ZEN | negative | 336.4 | [M-H]- | 335.1 | - 85 | 13.32 | 185.0 / 139.9 | -36 / -44 | -15 / -5 | *** |

$r_t$: retention time

*analyte was not included in the validation, hence ion ratio was not calculated

**ion ratio could not be calculated since only one product ion was available

***ion ratio was not calculated for internal standards

### 4.1.1 Method varieties

By testing different method variations, it was tried to combine positive and negative polarity mode into one chromatographic run to save considerable amounts of time during the routine analysis. The mix, containing 12 representative analytes (see chapter 3.4.3) was measured to obtain the method with the best performance parameters. The mix contained analytes with high and low signal intensities. The critical time frame was between minute 8 and 10 due to the high number of analytes eluting in this period of time (see Figure 6). With $AFM_1$, ergocornine and DAS, the mix contained 3 analytes that elute during this critical period. Moreover, dwell times of 10, 25 and 50 ms were tested for the MRM method in positive and negative mode as well as both of them combined. In the sMRM mode, the dwell times were automatically set by the software.

The results of the 13 method varieties are summarised in Table 13. A higher dwell time leaded to less data points. Especially in the positive mode it was difficult to receive enough data points, which should not be less than 8. The signal intensities for the different methods, were heavily analyte dependent, so that it was difficult to find a real trend. Therefore further variations of the method were tested.

**Table 13: Tested methods with the set dwell times and the received data points in the positive and negative mode**

| Method | Dwell time [ms] | DB (+) | DB (-) |
|---|---|---|---|
| (+) MRM | 10 | 7-10 | - |
| (+) MRM | 25 | 5-7 | - |
| (+) MRM | 50 | 3 | - |
| (-) MRM | 10 | - | 20-35 |
| (-) MRM | 25 | - | 12-20 |
| (-) MRM | 50 | - | 7-10 |
| (+)(-) MRM | 10 | 6-8 | approx. 6 |
| (+)(-) MRM | 25 | 3 | 3 |
| (+)(-) MRM | 50 | 3 | 3 |
| (+) sMRM | Software | 12-20 | |
| (-) sMRM | Software | | 25-35 |
| (+)(-) sMRM | Software | 8-10 | 7-9 |
| (+)(-) sMRM scan time 0.5 | Software | 14-20 | 13-23 |

(+): positive mode

(-): negative mode

DB: data points

The repeatability and data points of the additionally tested methods were evaluated, by measuring the same vial in fivefold repetition with different sMRM methods.

For the first variation of the method with a scan time of 1 s in positive and negative mode combined, the number of data points per peak was between 7 and 17 in the positive and between 8 and 20 in the negative mode, but too often below 10. The RSD values were in most cases below 5%, but the used concentrations were rather high and more fluctuations can be expected close to the LOQ. Due to the low number of data points it was decided, that this method is not suitable for further experiments.

The second variation with a scan time of 1 s, with two separated runs in positive and negative mode resulted in more data points (11-20 for the positive mode, 14-22 in the negative mode).

For the third variation with a scan time of 0.5 s and two separate runs, between 21 and 38 data points for the positive and between 26 and 45 for the negative mode were received, but the RSD values were higher than for the variation with a scan time of 1 s.

Signal intensities and RSD values were for most analytes better in the separated methods. However, the advantage of the combined method is that for each sample, only one chromatographic run of about 20 minutes is needed. Measuring time and organic solvents are saved, but on the other hand the wear of the instrument is highly increased due to fast polarity switching.

A retention time window of 72 s was chosen. The RSD values were for 7 out of 12 analytes better than at 90 s. This is due to less overlapping of the time windows and therefore more available time for each analyte. The sMRM method with a scan time of 1 s and two separate chromatographic runs for positive and negative mode was chosen, due to good repeatability and satisfactory amount of data points for all of the tested analytes. This method was used for the validation and all further experiments.

### 4.1.2  Internal standards

An autosampler pre-treatment program was implemented to include the internal standards automatically with every injection (see chapter 3.4.6). Without this approach, the $^{13}$C-labelled analytes have to be added to each vial manually, which is a clear disadvantage regarding workload and the use of larger amounts of expensive internal standards. Moreover, the amount of standards can be kept as low as possible. Internal standards were available for $FB_1$, $FB_2$

and $FB_3$, which show poor solubility in pure ACN. Hence it was tested if the signal intensities and RSDs are acceptable with this solvent, or if a mixture had to be used. $ACN/H_2O$ 50:50 (v/v) was tested as an alternative to see if this solvent has adverse effects on the repeatability of other analytes for the positive mode, since one vial contained all internal standards. Table 14 shows the RSDs for the positive mode in pure ACN and $ACN/H_2O$ 50:50 (v/v). The RSD in pure ACN was comparable or slightly better for most analytes than in $ACN/H_2O$ 50:50 (v/v). The FUMs however, showed RSD values between 34.6 and 39.2% in pure ACN, which is clearly unacceptable, while values between 3.1 and 5.6% were observed in the $ACN/H_2O$ mixture. The signal intensity was slightly lower for most of the analytes in the mixture, but remarkable higher for the FUMs. It was therefore decided to use the $ACN/H_2O$ mixture for the internal standards in the positive mode.

**Table 14: Signal intensity and repeatability of internal standards in the positive mode in $ACN/H_2O$ 50:50 (v/v) and pure ACN**

| Internal standard | Quotient of peak area ACN/H₂O 50:50 (v/v) and pure ACN | RSD ACN/H₂O 50:50 (v/v) [%] | RSD pure ACN [%] |
|---|---|---|---|
| AFB₁ | 0.9 | 11.5 | 8.8 |
| AFB₂ | 0.9 | 9.4 | 8.8 |
| AFG₁ | 0.8 | 9.9 | 5.4 |
| AFG₂ | 0.9 | 8.6 | 8.8 |
| AFM₁ | 0.9 | 6.5 | 5.8 |
| DAS | 0.9 | 5.4 | 5.2 |
| FB₁ | 20.0 | 3.7 | 41.1 |
| FB₂ | 17.3 | 3.1 | 39.2 |
| FB₃ | 18.4 | 5.6 | 34.6 |
| HT2 | 1.0 | 10.6 | 5.9 |
| OTA | 1.1 | 6.3 | 2.2 |
| Roquefortine C | 0.9 | 8.3 | 5.8 |
| STG | 0.9 | 4.4 | 2.8 |
| 15-AcDON | 0.9 | 13.5 | 10.8 |
| Mycophenolic acid | 0.9 | 8.3 | 8.2 |

## 4.2 Chromatograms

The following figures show chromatograms for the positive and negative mode with all analytes, included in the final method and internal standards. The extracted ion chromatograms (XIC) display the peaks in different colours for each mass transitions (two

colour lines per analyte due to the quantifier and qualifier transitions). The signal intensity of the peaks is plotted against the retention time. The chromatograms also show that co-elution could not be avoided for all analytes. ^{13}C-labeled internal standards have the same retention time as the unlabelled mycotoxins, so that in some cases 4 transitions can be observed. All analytes exhibit reasonable peaks shapes, although not all of them are perfectly symmetric. Figure 7 (pos mode) and Figure 8 (neg mode) show the XIC chromatograms of level 3 matrix matched standards for wheat.
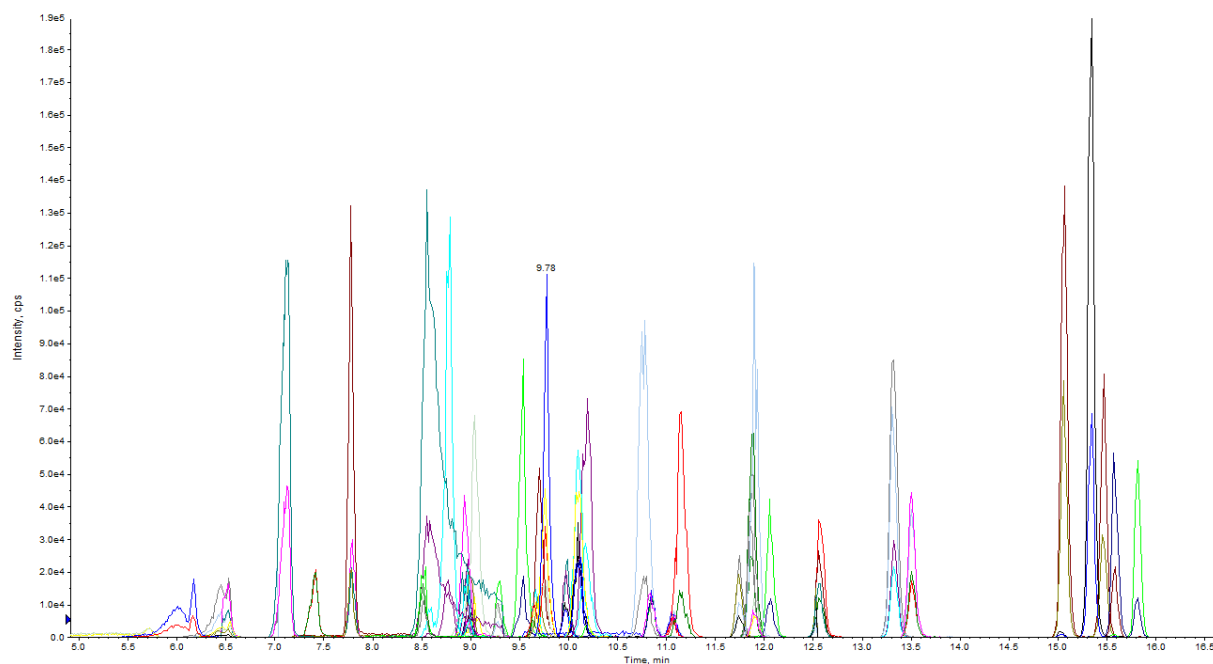


**Figure 7: Chromatogram in the positive ionisation mode**
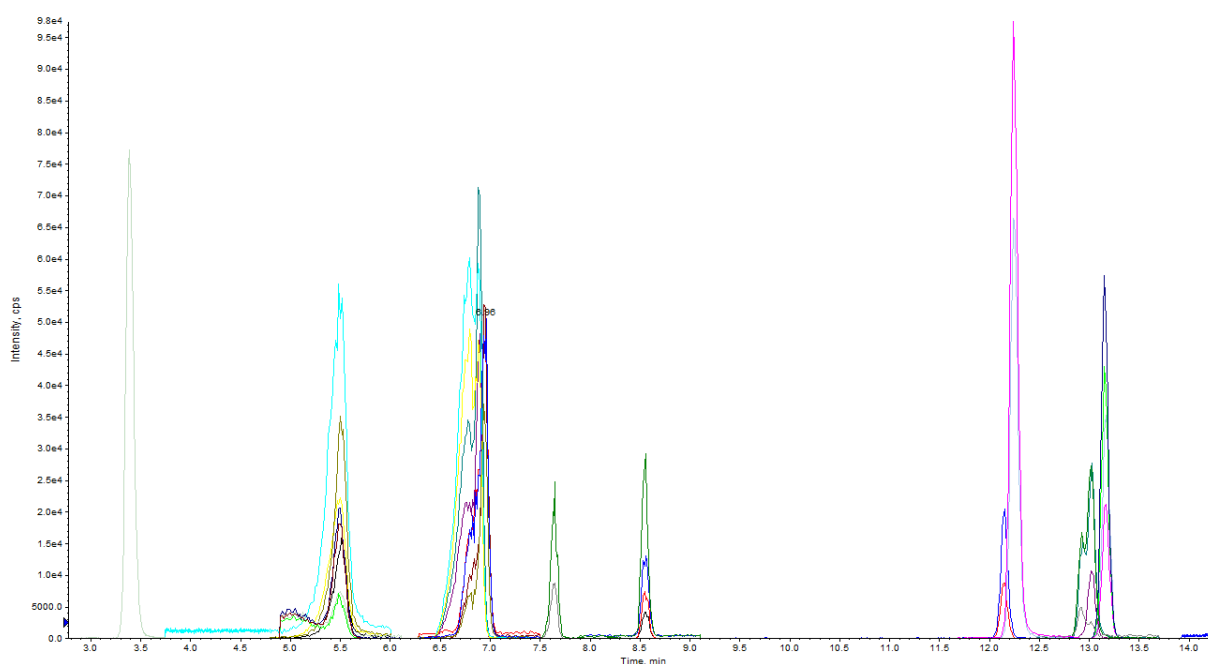
**Figure 8: Chromatogram in the negative ionisation mode**

## 4.3 Quantification of blank material

The available matrix samples were no real blank material for all of the mycotoxins, included in the final method. They showed peaks for some analytes in the unspiked extracts and hence contamination. The quantification was performed with the standard addition approach (see chapter 3.5.2.2), since not for all of the analytes other analytical methods were in-house available. The standard addition approach allows quantification, without any additional methods. Table 15 summarises the results for the calculated contaminations in the used samples. Especially pig feed and silage showed contamination for a high number of analytes. The wheat blank quality control material only showed contamination for the enniatins (A, A1, B, B1). The peaks in the contaminated matrix material were used for the calculation of the LOD and LOQ during the validation. Moreover, the peak area in the contaminated material was subtracted from the peak area in the matrix matched calibration and in the spiked sample calibration functions.

**Table 15: Contamination in the matrix material that was used for the validation**

| Analyte | c in wheat [µg/kg] | c in maize [µg/kg] | c in pig feed [µg/kg] | c in silage [µg/kg] |
|---|---|---|---|---|
| 15-AcDON | - | - | 518.7 | - |
| AOH | - | - | 15.3 | - |
| Beauvericin | - | 1.2 | 4.9 | 158.7 |
| D3G | - | - | 99.4 | 127.8 |
| DON | - | - | 343.2 | 1356.8 |
| Ennatin A | 1.0 | - | 2.0 | 1.2 |
| Enniatin A1 | 3.7 | - | 10.2 | 4.7 |
| Enniatin B | 18.8 | - | 166.9 | 8.6 |
| Enniatin B1 | 7.7 | - | 43.9 | 12.7 |
| $FB_1$ | - | 14.8 | - | 170.1 |
| $FB_2$ | - | 4.0 | - | 56.3 |
| $FB_3$ | - | 7.2 | - | 27.1 |
| Fusaric Acid | - | - | - | 2428.2 |
| MON | - | 2.4 | 12.3 | 31.2 |
| NIV | - | - | - | 816.8 |
| ZEN | - | - | 22.7 | 261.1 |

## 4.4 Method validation

Some of method performance characteristics that were evaluated during the validation are summarised in Table 16 (wheat), Table 17 (maize), Table 18 (pig feed) and Table 19 (silage). The tables include LOD, LOQ, the linear working range, $R_A$, SSE and $R_E$. The recoveries were first calculated separately for each of the 7 replicates that were used during the validation and further used to calculate the average values and the RSDs.

**Table 16: Method performance characteristics in wheat**

| Wheat | | | Working range | | $R_A$ | | SSE | | $R_E$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analyte | LOD [µg/kg] | LOQ [µg/kg] | From [µg/kg] | To [µg/kg] | Average [%] | RSD [%] | Average [%] | RSD [%] | Average [%] | RSD [%] |
| 15-AcDON | 87.0 | 277.7 | 240 | 24000 | 88 | 9.7 | 103 | 5.3 | 85 | 7.2 |
| 15-Acetoxyscirpenol | 14.1 | 50.4 | 48 | 4800 | 89 | 26.6 | 97 | 24.3 | 93 | 21.1 |
| 3-AcDON | 27.2 | 74.9 | 72 | 7200 | 84 | 10.6 | 103 | 7.5 | 82 | 4.5 |
| AFB1 | 0.9 | 2.9 | 2.6 | 800 | 70 | 6.8 | 88 | 3.8 | 80 | 7.1 |
| AFB2 | 1.9 | 7.2 | 8 | 800 | 71 | 12.1 | 89 | 4.2 | 80 | 10.1 |
| AFG$_1$ | 1.6 | 6.1 | 8 | 800 | 68 | 19.6 | 87 | 8.3 | 78 | 13.1 |
| AFG$_2$ | 3.5 | 11.8 | 8 | 800 | 72 | 12.5 | 89 | 8.2 | 80 | 7.8 |
| AFM$_1$ | 1.0 | 3.1 | 2 | 800 | 71 | 10.6 | 88 | 6.4 | 80 | 4.2 |
| Agroclavine | 0.1 | 0.4 | 0.8 | 240 | 67 | 9.0 | 90 | 5.1 | 75 | 5.9 |
| α -zearalenol | 2.2 | 7.2 | 8 | 800 | 79 | 5.2 | 96 | 7.4 | 82 | 6.1 |
| Alternariol | 0.9 | 2.5 | 2.4 | 800 | 56 | 3.4 | 79 | 4.9 | 72 | 4.0 |
| Beauvericin | 0.1 | 0.3 | 0.8 | 240 | 84 | 15.7 | 102 | 8.6 | 83 | 12.6 |
| β-zearalenol | 2.5 | 8.4 | 8 | 800 | 87 | 2.4 | 100 | 4.3 | 88 | 4.6 |
| D3G | 3.3 | 13.7 | 8 | 2664 | 29 | 2.5 | 47 | 8.5 | 62 | 7.6 |
| DAS | 1.8 | 5.1 | 8 | 2400 | 68 | 10.5 | 86 | 5.3 | 79 | 8.1 |
| Dihydrolysergol | 1.3 | 5.3 | 4 | 1200 | 73 | 7.6 | 106 | 3.1 | 69 | 5.7 |
| DON | 10.9 | 48.8 | 53.3 | 16000 | 68 | 4.8 | 94 | 2.4 | 73 | 4.3 |
| Elymoclavine | 0.5 | 1.6 | 0.8 | 240 | 61 | 7.2 | 97 | 5.4 | 63 | 5.8 |
| Enniatin A | 0.1 | 0.3 | 0.8 | 240 | 84 | 14.2 | 94 | 5.4 | 89 | 15.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Enniatin A1 | 0.1 | 0.2 | 0.8 | 240 | 114 | 19.8 | 126 | 11.8 | 92 | 27.9 |
| Enniatin B | 0.1 | 0.2 | 0.8 | 240 | 90 | 12.3 | 111 | 5.6 | 81 | 12.4 |
| Enniatin B1 | 0.1 | 0.2 | 0.8 | 240 | 73 | 13.1 | 90 | 6.0 | 81 | 12.9 |
| Ergine | 0.4 | 1.5 | 0.8 | 240 | 57 | 7.6 | 92 | 4.9 | 61 | 5.6 |
| Ergocornine | 0.4 | 1.3 | 1.2 | 400 | 83 | 3.1 | 99 | 7.9 | 84 | 7.3 |
| Ergocorninine | 0.4 | 1.1 | 1.2 | 1200 | 115 | 12.0 | 106 | 3.1 | 109 | 11.2 |
| Ergocristine | 0.8 | 2.3 | 1.2 | 400 | 91 | 7.0 | 100 | 6.1 | 92 | 11.0 |
| Ergocristinine | 0.2 | 0.9 | 1.2 | 1200 | 97 | 7.8 | 86 | 2.8 | 114 | 8.6 |
| Ergocryptine | 0.5 | 1.2 | 1.2 | 400 | 85 | 11.1 | 90 | 5.2 | 95 | 11.8 |
| Ergocryptinine | 0.3 | 0.8 | 1.2 | 1200 | 111 | 13.5 | 102 | 4.0 | 110 | 16.1 |
| Ergometrine | 1.3 | 4.1 | 4 | 1200 | 73 | 15.1 | 92 | 4.6 | 79 | 11.9 |
| Ergometrinine | 0.3 | 0.8 | 1.2 | 1200 | 89 | 13.9 | 107 | 5.1 | 83 | 10.6 |
| Ergosine | 0.4 | 1.3 | 1.2 | 1200 | 112 | 11.1 | 106 | 5.6 | 106 | 15.5 |
| Ergotamine | 1.0 | 1.9 | 1.2 | 400 | 123 | 11.7 | 103 | 10.4 | 119 | 11.1 |
| FB$_1$ | 3.9 | 12.2 | 16 | 4800 | 40 | 8.7 | 98 | 3.7 | 41 | 9.0 |
| FB$_2$ | 1.3 | 5.1 | 4.8 | 4800 | 55 | 8.7 | 106 | 4.6 | 52 | 8.3 |
| FB$_3$ | 3.6 | 12.5 | 16 | 4800 | 55 | 6.4 | 101 | 6.9 | 55 | 9.9 |
| Fusaric acid | 12.7 | 43.9 | 24 | 7200 | 59 | 12.6 | 99 | 9.7 | 60 | 5.1 |
| FusX | 17.0 | 80.3 | 80 | 8000 | 66 | 5.5 | 89 | 5.5 | 74 | 2.1 |
| Gliotoxin | 18.0 | 72.3 | 48 | 4800 | 84 | 14.3 | 110 | 8.6 | 76 | 9.2 |
| HT-2 | 17.6 | 65.6 | 48 | 4800 | 87 | 15.7 | 103 | 14.6 | 85 | 9.0 |
| MON | 1.2 | 3.4 | * | * | * | * | * | * | * | * |
| Mycophenolic acid | 38.5 | 140.6 | 80 | 24000 | 145 | 7.6 | 129 | 9.8 | 113 | 6.5 |
| NEO | 1.4 | 5.3 | 8 | 2400 | 72 | 7.4 | 90 | 5.5 | 80 | 7.3 |
| NIV | 23.1 | 89.4 | 72 | 7200 | 49 | 9.9 | 82 | 9.8 | 60 | 1.9 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| OTA | 1.0 | 3.5 | 2.6 | 800 | 73 | 6.5 | 101 | 3.4 | 72 | 3.9 |
| OTB | 1.0 | 3.2 | 2.6 | 800 | 72 | 8.2 | 98 | 5.3 | 73 | 4.9 |
| PAT | 164.0 | 547.7 | 480 | 48000 | 65 | 6.0 | 103 | 6.1 | 63 | 3.2 |
| Penicillic acid | 20.8 | 70.4 | 72 | 7200 | 75 | 8.0 | 97 | 5.9 | 77 | 3.2 |
| Roquefortine C | 1.6 | 4.9 | 8 | 2400 | 70 | 10.3 | 89 | 3.9 | 79 | 8.4 |
| STG | 0.5 | 1.7 | 1.2 | 1200 | 80 | 8.8 | 101 | 3.9 | 79 | 6.5 |
| T-2 | 2.6 | 9.8 | 4.8 | 4800 | 82 | 11.8 | 99 | 7.2 | 83 | 7.1 |
| T-2 tetraol | 155.9 | 483.8 | 432 | 43200 | 75 | 20.2 | 113 | 7.6 | 67 | 20.5 |
| T-2 triol | 32.9 | 99.3 | 72 | 7200 | 77 | 16.2 | 98 | 14.5 | 79 | 16.4 |
| ZAN | 0.3 | 1.2 | 0.8 | 240 | 77 | 3.8 | 96 | 9.4 | 81 | 7.7 |
| ZEN | 0.8 | 1.5 | 1.6 | 480 | 74 | 3.9 | 93 | 7.3 | 81 | 5.1 |

**Table 17: Method performance characteristics in maize**

| Maize Analyte | LOD [µg/kg] | LOQ [µg/kg] | Working range From [µg/kg] | To [µg/kg] | R_A Average [%] | RSD [%] | SSE Average [%] | RSD [%] | R_E Average [%] | RSD [%] |
|---|---|---|---|---|---|---|---|---|---|---|
| 15-AcDON | 53.9 | 185.6 | 240 | 24000 | 84 | 18.4 | 94 | 11.3 | 89 | 11.8 |
| 15-Acetoxyscirpenol | 5.6 | 17.2 | 16 | 4800 | 81 | 16.8 | 92 | 14.6 | 91 | 24.6 |
| 3-AcDON | 19.7 | 66.8 | 72 | 7200 | 49 | 8.9 | 62 | 3.6 | 79 | 11.7 |
| AFB1 | 2.1 | 6.5 | 8 | 800 | 53 | 13.3 | 62 | 14.0 | 86 | 5.3 |
| AFB2 | 2.3 | 10.5 | 8 | 800 | 68 | 13.8 | 76 | 23.6 | 91 | 8.8 |
| AFG1 | 1.9 | 6.3 | 8 | 800 | 60 | 13.3 | 73 | 18.4 | 84 | 10.8 |
| AFG2 | 5.2 | 12.7 | 8 | 800 | 71 | 13.7 | 76 | 6.7 | 93 | 8.3 |
| AFM1 | 1.2 | 3.6 | 2.4 | 800 | 75 | 13.4 | 85 | 13.9 | 88 | 5.9 |
| Agroclavine | 0.2 | 0.6 | 0.8 | 240 | 66 | 13.1 | 90 | 10.8 | 73 | 4.1 |
| α -zearalenol | 2.5 | 7.8 | 8 | 800 | 67 | 4.4 | 82 | 2.3 | 82 | 5.3 |
| Alternariol | 0.8 | 2.8 | 2.4 | 800 | 28 | 7.3 | 41 | 4.6 | 68 | 4.4 |
| Beauvericin | 0.1 | 0.16 | 0.8 | 240 | 85 | 13.1 | 91 | 16.4 | 94 | 4.3 |
| β-zearalenol | 3.4 | 10.9 | 8 | 800 | 66 | 2.7 | 84 | 3.6 | 80 | 4.2 |
| D3G | 3.7 | 11.5 | 8 | 2664 | 22 | 3.5 | 44 | 4.3 | 50 | 5.4 |
| DAS | 2.3 | 5.1 | 8 | 2400 | 65 | 10.4 | 69 | 14.6 | 94 | 11.0 |
| Dihydrolysergol | 1.2 | 4.6 | 4 | 1200 | 73 | 17.5 | 91 | 16.0 | 81 | 3.3 |
| DON | 37.9 | 153.3 | 160 | 16000 | 57 | 2.7 | 79 | 2.0 | 72 | 3.8 |
| Elymoclavine | 0.4 | 1.3 | 0.8 | 240 | 59 | 17.4 | 87 | 13.9 | 67 | 4.9 |
| Enniatin A | 0.1 | 0.3 | 0.8 | 240 | 71 | 13.0 | 77 | 16.2 | 92 | 6.3 |
| Enniatin A1 | 0.2 | 0.5 | 0.8 | 240 | 96 | 23.0 | 92 | 31.2 | 108 | 13.1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Enniatin B | 0.04 | 0.1 | 0.8 | 240 | 90 | 13.9 | 90 | 16.4 | 101 | 5.1 |
| Enniatin B1 | 0.1 | 0.4 | 0.8 | 240 | 75 | 14.1 | 82 | 14.8 | 92 | 6.3 |
| Ergine | 0.4 | 1.2 | 0.8 | 240 | 78 | 30.1 | 126 | 28.0 | 62 | 3.5 |
| Ergocornine | 0.8 | 2.2 | 1.2 | 400 | 48 | 12.1 | 62 | 26.1 | 81 | 20.7 |
| Ergocorninine | 0.5 | 1.7 | 1.2 | 1200 | 77 | 11.1 | 68 | 11.8 | 113 | 7.6 |
| Ergocristine | 0.5 | 1.7 | 1.2 | 400 | 32 | 16.8 | 38 | 29.6 | 89 | 24.0 |
| Ergocristinine | 0.5 | 1.3 | 1.2 | 1200 | 54 | 11.1 | 48 | 11.2 | 112 | 11.7 |
| Ergocryptine | 0.6 | 1.5 | 1.2 | 400 | 41 | 15.3 | 45 | 32.3 | 97 | 22.7 |
| Ergocryptinine | 0.4 | 1.3 | 1.2 | 1200 | 65 | 11.4 | 56 | 14.5 | 116 | 8.9 |
| Ergometrine | 1.3 | 3.5 | 4 | 1200 | 68 | 21.2 | 82 | 21.4 | 83 | 2.2 |
| Ergometrinine | 0.3 | 0.8 | 1.2 | 1200 | 33 | 15.3 | 34 | 18.3 | 99 | 5.8 |
| Ergosine | 0.8 | 2.5 | 1.2 | 1200 | 86 | 13.0 | 86 | 17.4 | 101 | 5.8 |
| Ergotamine | 0.9 | 3.4 | 1.2 | 400 | 60 | 12.1 | 75 | 29.2 | 84 | 21.2 |
| FB$_1$ | 4.2 | 13.3 | 16 | 4800 | 60 | 8.3 | 95 | 12.6 | 63 | 6.3 |
| FB$_2$ | 1.2 | 5.3 | 4.8 | 4800 | 72 | 12.8 | 105 | 11.5 | 68 | 3.3 |
| FB$_3$ | 2.8 | 11.0 | 16 | 4800 | 69 | 11.1 | 100 | 13.8 | 70 | 4.0 |
| Fusaric acid | 25.8 | 91.4 | 72 | 7200 | 75 | 12.6 | 95 | 11.8 | 79 | 2.4 |
| FusX | 24.5 | 81.3 | 80 | 8000 | 46 | 3.6 | 66 | 4.5 | 70 | 5.1 |
| Gliotoxin | 7.4 | 29.3 | 16 | 4800 | 58 | 7.8 | 87 | 9.8 | 66 | 8.3 |
| HT-2 | 10.3 | 42.0 | 48 | 4800 | 85 | 8.0 | 93 | 9.6 | 92 | 13.3 |
| MON | 1.2 | 2.9 | * | * | * | * | * | * | * | * |
| Mycophenolic acid | 87.6 | 317.2 | 240 | 24000 | 81 | 16.0 | 90 | 17.8 | 91 | 12.0 |
| NEO | 2.0 | 5.8 | 8 | 2400 | 78 | 12.5 | 83 | 12.7 | 93 | 2.4 |
| NIV | 19.2 | 76.6 | 72 | 7200 | 57 | 10.9 | 97 | 7.3 | 58 | 6.6 |
| OTA | 1.1 | 3.7 | 2.6 | 800 | 78 | 10.9 | 96 | 11.3 | 81 | 1.7 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| OTB | 1.2 | 4.9 | 2.6 | 800 | 80 | 9.6 | 94 | 10.4 | 85 | 2.1 |
| PAT | 167.5 | 513.0 | 480 | 48000 | 39 | 3.4 | 100 | 2.9 | 39 | 2.9 |
| Penicillic acid | 17.3 | 62.4 | 72 | 7200 | 73 | 9.9 | 85 | 8.7 | 86 | 6.2 |
| Roquefortine C | 2.2 | 6.1 | 8 | 2400 | 63 | 8.9 | 73 | 12.0 | 87 | 3.7 |
| STG | 0.3 | 1.0 | 1.2 | 1200 | 89 | 12.6 | 98 | 13.5 | 90 | 2.0 |
| T-2 | 2.9 | 9.8 | 4.8 | 4800 | 90 | 12.8 | 97 | 12.7 | 93 | 2.7 |
| T-2 tetraol | 143.2 | 461.9 | 432 | 43200 | 114 | 16.2 | 140 | 13.1 | 82 | 10.5 |
| T-2 triol | 32.0 | 72.7 | 72 | 7200 | 79 | 13.1 | 88 | 17.5 | 91 | 12.7 |
| ZAN | 0.20 | 0.80 | 0.8 | 240 | 62 | 5.1 | 77 | 3.0 | 81 | 5.9 |
| ZEN | 0.60 | 1.70 | 1.6 | 480 | 72 | 3.9 | 87 | 2.7 | 83 | 5.2 |

**Table 18: Method performance characteristics in pig feed**

| Pig feed | | | Working range | | R$_A$ | | SSE | | R$_E$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analyte | LOD [µg/kg] | LOQ [µg/kg] | From [µg/kg] | To [µg/kg] | Average [%] | RSD [%] | Average [%] | RSD [%] | Average [%] | RSD [%] |
| 15-AcDON | 51.0 | 170.0 | 240 | 24000 | 62 | 3.9 | 79 | 6.8 | 80 | 5.0 |
| 15-Acetoxyscirpenol | 5.3 | 20.3 | 16 | 4800 | 60 | 4.5 | 76 | 6.9 | 79 | 4.1 |
| 3-AcDON | 33.0 | 133.1 | 72 | 7200 | 42 | 10.6 | 43 | 5.9 | 98 | 10.7 |
| AFB1 | 2.3 | 7.0 | 8 | 800 | 55 | 3.8 | 69 | 9.5 | 81 | 10.2 |
| AFB2 | 2.3 | 9.6 | 8 | 800 | 51 | 4.9 | 64 | 13.8 | 81 | 8.2 |
| AFG$_1$ | 4.6 | 15.2 | 8 | 800 | 51 | 4.0 | 65 | 12.1 | 78 | 7.9 |
| AFG$_2$ | 4.8 | 18.1 | 8 | 800 | 54 | 3.4 | 69 | 11.7 | 79 | 8.5 |
| AFM$_1$ | 2.4 | 7.1 | 8 | 800 | 54 | 5.2 | 70 | 10.2 | 78 | 7.6 |
| Agroclavine | 0.1 | 0.4 | 0.8 | 240 | 114 | 22.6 | 171 | 21.4 | 67 | 5.2 |
| α-zearalenol | 2.4 | 10.6 | 8 | 800 | 60 | 4.6 | 66 | 1.9 | 92 | 4.2 |
| Alternariol | 2.9 | 9.5 | 8 | 800 | 54 | 4.5 | 58 | 2.8 | 92 | 2.0 |
| Beauvericin | 0.1 | 0.2 | 0.8 | 240 | 61 | 6.5 | 74 | 3.0 | 82 | 5.8 |
| β-zearalenol | 7.9 | 32.2 | 8 | 800 | 70 | 4.0 | 74 | 2.3 | 94 | 3.4 |
| D3G | 24.5 | 82.9 | 80 | 2664 | 30 | 6.9 | 37 | 5.3 | 82 | 7.2 |
| DAS | 1.5 | 5.2 | 8 | 2400 | 63 | 4.2 | 75 | 4.1 | 84 | 2.7 |
| Dihydrolysergol | 2.6 | 8.0 | 4 | 1200 | 48 | 8.9 | 71 | 5.8 | 67 | 8.2 |
| DON | 63.3 | 211.0 | 160 | 16000 | 69 | 7.9 | 78 | 7.5 | 88 | 6.7 |
| Elymoclavine | 0.8 | 3.1 | 2.4 | 240 | 42 | 9.3 | 65 | 6.5 | 65 | 7.9 |
| Enniatin A | 0.1 | 0.2 | 0.8 | 240 | 54 | 5.2 | 69 | 4.5 | 78 | 3.6 |
| Enniatin A1 | 0.1 | 0.3 | 0.8 | 240 | 58 | 13.5 | 78 | 9.0 | 75 | 8.7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Enniatin B | 0.1 | 0.3 | 0.8 | 240 | 36 | 20.7 | 67 | 4.2 | 55 | 21.1 |
| Enniatin B1 | 0.1 | 0.3 | 0.8 | 240 | 52 | 13.9 | 72 | 5.0 | 72 | 11.9 |
| Ergine | 0.8 | 3.1 | 2.4 | 240 | 25 | 7.8 | 49 | 10.3 | 50 | 6.9 |
| Ergocornine | 1.1 | 3.4 | 1.2 | 400 | 65 | 7.4 | 68 | 4.9 | 95 | 4.5 |
| Ergocorninine | 0.6 | 2.1 | 1.2 | 1200 | 62 | 6.5 | 60 | 2.7 | 104 | 6.3 |
| Ergocristine | 0.6 | 2.9 | 1.2 | 400 | 46 | 7.5 | 43 | 6.5 | 97 | 5.6 |
| Ergocristinine | 0.3 | 1.5 | 1.2 | 1200 | 49 | 7.3 | 45 | 2.5 | 110 | 6.4 |
| Ergocryptine | 0.4 | 1.6 | 1.2 | 400 | 53 | 12.1 | 53 | 12.0 | 100 | 3.2 |
| Ergocryptinine | 0.3 | 1.1 | 1.2 | 1200 | 63 | 4.7 | 57 | 2.2 | 110 | 4.3 |
| Ergometrine | 1.4 | 8.3 | 4 | 1200 | 36 | 14.9 | 47 | 9.7 | 77 | 12.2 |
| Ergometrinine | 0.4 | 1.3 | 1.2 | 1200 | 57 | 6.2 | 91 | 4.2 | 63 | 5.0 |
| Ergosine | 1.0 | 2.9 | 1.2 | 1200 | 58 | 4.9 | 68 | 5.4 | 85 | 6.5 |
| Ergotamine | 0.7 | 2.7 | 1.2 | 400 | 60 | 6.6 | 60 | 3.3 | 100 | 3.7 |
| $FB_1$ | 3.1 | 11.3 | 16 | 4800 | 41 | 3.1 | 88 | 3.4 | 46 | 4.0 |
| $FB_2$ | 3.7 | 9.8 | 16 | 4800 | 50 | 1.9 | 90 | 1.9 | 56 | 3.2 |
| $FB_3$ | 3.8 | 14.5 | 16 | 4800 | 50 | 3.6 | 90 | 2.0 | 56 | 3.1 |
| Fusaric acid | 18.7 | 48.9 | 24 | 7200 | 54 | 11.4 | 86 | 2.3 | 63 | 11.3 |
| FusX | 244.4 | 864.7 | 266.6 | 8000 | 43 | 10.7 | 43 | 3.8 | 98 | 9.9 |
| Gliotoxin | 6.0 | 26.1 | 16 | 4800 | 57 | 3.2 | 80 | 4.5 | 71 | 5.1 |
| HT-2 | 23.4 | 66.8 | 48 | 4800 | 68 | 3.4 | 88 | 7.9 | 78 | 7.7 |
| MON | 0.7 | 2.3 | * | * | * | * | * | * | * | * |
| Mycophenolic acid | 73.0 | 276.0 | 240 | 24000 | 71 | 7.2 | 87 | 7.2 | 82 | 5.8 |
| NEO | 2.2 | 7.6 | 8 | 2400 | 62 | 2.6 | 76 | 2.4 | 82 | 3.0 |
| NIV | 33.5 | 131.5 | 72 | 7200 | 54 | 7.2 | 66 | 11.2 | 82 | 11.5 |
| OTA | 0.9 | 3.3 | 2.6 | 800 | 66 | 2.5 | 84 | 2.3 | 79 | 2.3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| OTB | 0.9 | 3.4 | 2.6 | 800 | 66 | 2.5 | 85 | 3.0 | 78 | 2.9 |
| PAT | 52.4 | 184.1 | 160 | 48000 | 65 | 5.2 | 81 | 2.7 | 80 | 3.3 |
| Penicillic acid | 15.0 | 56.8 | 72 | 7200 | 65 | 2.7 | 83 | 4.9 | 79 | 4.1 |
| Roquefortine C | 2.7 | 9.7 | 8 | 2400 | 54 | 3.5 | 72 | 5.3 | 75 | 3.9 |
| STG | 0.4 | 1.5 | 1.2 | 1200 | 67 | 2.0 | 84 | 1.7 | 80 | 2.6 |
| T-2 | 2.2 | 9.7 | 4.8 | 4800 | 71 | 4.1 | 86 | 3.7 | 83 | 3.3 |
| T-2 tetraol | 85.6 | 231.5 | 188 | 56400 | 57 | 7.5 | 80 | 3.2 | 71 | 8.6 |
| T-2 triol | 23.9 | 127.7 | 72 | 7200 | 66 | 19.0 | 77 | 17.2 | 87 | 16.2 |
| ZAN | 0.7 | 3.2 | 2.4 | 240 | 58 | 4.9 | 62 | 2.6 | 94 | 3.8 |
| ZEN | 0.4 | 1.3 | 1.6 | 480 | 70 | 6.4 | 77 | 7.4 | 91 | 5.2 |

**Table 19: Method performance characteristics in silage**

| Silage | | | Working range | | $R_A$ | | SSE | | $R_E$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analyte | LOD [µg/kg] | LOQ [µg/kg] | From [µg/kg] | To [µg/kg] | Average [%] | RSD [%] | Average [%] | RSD [%] | Average [%] | RSD [%] |
| 15-AcDON | 58.8 | 344.9 | 240 | 24000 | 55 | 2.7 | 75 | 7.7 | 74 | 5.7 |
| 15-Acetoxyscirpenol | 4.8 | 22.6 | 16 | 4800 | 49 | 8.3 | 66 | 6.8 | 74 | 8.9 |
| 3-AcDON | 92.1 | 423.5 | 240 | 7200 | 18 | 8.6 | 25 | 70.5 | 98 | 5.7 |
| AFB1 | 4.4 | 14.5 | 8 | 800 | 35 | 4.1 | 53 | 16.1 | 67 | 14.6 |
| AFB2 | 5.3 | 19.0 | 8 | 800 | 39 | 6.2 | 58 | 15.0 | 69 | 11.6 |
| AFG$_1$ | 4.6 | 21.0 | 8 | 800 | 43 | 5.2 | 62 | 12.7 | 69 | 11.7 |
| AFG$_2$ | 16.2 | 51.7 | 26.6 | 800 | 46 | 2.7 | 64 | 12.7 | 72 | 10.7 |
| AFM$_1$ | 3.6 | 11.5 | 8 | 800 | 47 | 5.5 | 69 | 10.6 | 69 | 8.0 |
| Agroclavine | 0.2 | 0.8 | 0.8 | 240 | 47 | 8.8 | 71 | 3.5 | 65 | 8.7 |
| α -zearalenol | 7.2 | 30.4 | 26.6 | 800 | 57 | 4.5 | 83 | 4.6 | 69 | 2.1 |
| Alternariol | 1.3 | 3.5 | 2.6 | 800 | 61 | 2.9 | 95 | 3.7 | 64 | 2.2 |
| Beauvericin | 0.1 | 0.4 | 0.8 | 240 | 41 | 18.2 | 69 | 5.8 | 59 | 12.5 |
| β-zearalenol | 8.3 | 37.5 | 26.6 | 800 | 65 | 2.0 | 93 | 3.9 | 70 | 3.5 |
| D3G | 32.8 | 109.5 | 80 | 2664 | 22 | 5.9 | 26 | 4.8 | 86 | 5.6 |
| DAS | 4.2 | 21.8 | 8 | 2400 | 47 | 2.5 | 61 | 4.1 | 78 | 3.5 |
| Dihydrolysergol | 1.8 | 5.4 | 4 | 1200 | 47 | 8.8 | 81 | 1.1 | 58 | 8.4 |
| DON | 38.9 | 129.8 | 160 | 16000 | 38 | 6.7 | 52 | 21.9 | 76 | 14.0 |
| Elymoclavine | 0.5 | 2.4 | 2.4 | 240 | 46 | 8.0 | 77 | 2.1 | 59 | 6.4 |
| Enniatin A | 0.04 | 0.1 | 0.8 | 240 | 56 | 5.2 | 79 | 3.7 | 72 | 3.0 |
| Enniatin A1 | 0.1 | 0.2 | 0.8 | 240 | 57 | 8.8 | 79 | 6.0 | 72 | 3.3 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Enniatin B | 0.1 | 0.2 | 0.8 | 240 | 54 | 4.7 | 74 | 3.2 | 73 | 2.4 |
| Enniatin B1 | 0.1 | 0.2 | 0.8 | 240 | 57 | 4.7 | 79 | 2.7 | 73 | 2.6 |
| Ergine | 0.6 | 2.5 | 2.4 | 240 | 32 | 13.9 | 42 | 15.1 | 77 | 11.3 |
| Ergocornine | 2.3 | 6.5 | 4 | 400 | 23 | 13.8 | 23 | 11.5 | 101 | 4.8 |
| Ergocorninine | 1.6 | 5.2 | 4 | 1200 | 28 | 8.6 | 34 | 5.3 | 80 | 4.2 |
| Ergocristine | 1.8 | 5.7 | 4 | 400 | 46 | 7.5 | 43 | 6.5 | 97 | 5.6 |
| Ergocristinine | 1.7 | 8.3 | 4 | 1200 | 20 | 10.0 | 25 | 6.3 | 80 | 5.9 |
| Ergocryptine** | 0.7 | 3.0 | 4 | 400 | 49 | 22.5 | 41 | 29.0 | 119 | 6.7 |
| Ergocryptinine | 1.2 | 3.9 | 4 | 1200 | 26 | 11.0 | 32 | 8.8 | 83 | 4.8 |
| Ergometrine | 1.4 | 4.9 | 4 | 1200 | 43 | 8.1 | 68 | 3.5 | 63 | 5.3 |
| Ergometrinine | 0.3 | 1.2 | 1.2 | 1200 | 58 | 18.7 | 88 | 5.5 | 66 | 18.0 |
| Ergosine | 1.3 | 4.8 | 4 | 1200 | 51 | 7.5 | 55 | 6.9 | 93 | 10.8 |
| Ergotamine | 2.3 | 8.2 | 4 | 400 | 38 | 7.6 | 36 | 5.2 | 106 | 6.7 |
| FB$_1$ | 7.8 | 26.1 | 16 | 4800 | 53 | 7.0 | 73 | 10.6 | 74 | 7.5 |
| FB$_2$ | 4.6 | 15.2 | 16 | 4800 | 53 | 3.7 | 72 | 9.0 | 74 | 8.7 |
| FB$_3$ | 4.0 | 13.5 | 16 | 4800 | 52 | 5.6 | 73 | 9.3 | 73 | 10.8 |
| Fusaric acid | 19.2 | 63.9 | 24 | 7200 | 50 | 9.4 | 71 | 11.5 | 70 | 7.2 |
| FusX | 52.3 | 196.6 | 266.6 | 8000 | 32 | 8.5 | 43 | 8.3 | 76 | 8.6 |
| Gliotoxin | 9.7 | 39.9 | 48 | 4800 | 51 | 3.4 | 74 | 4.9 | 69 | 3.8 |
| HT-2 | 11.0 | 39.6 | 48 | 4800 | 55 | 7.0 | 77 | 7.7 | 72 | 9.4 |
| MON | 1.2 | 3.9 | * | * | * | * | * | * | * | * |
| Mycophenolic acid | 94.8 | 344.5 | 240 | 24000 | 45 | 9.4 | 65 | 11.4 | 70 | 10.7 |
| NEO | 2.3 | 8.9 | 8 | 2400 | 52 | 3.2 | 67 | 4.0 | 77 | 3.4 |
| NIV | 37.7 | 125.7 | 72 | 7200 | 38 | 7.2 | 60 | 12.9 | 64 | 11.0 |
| OTA | 1.2 | 5.3 | 2.6 | 800 | 47 | 5.6 | 65 | 7.7 | 74 | 3.5 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| OTB | 1.8 | 6.4 | 2.6 | 800 | 46 | 7.2 | 61 | 5.6 | 76 | 3.0 |
| PAT | 51.7 | 218.1 | 160 | 48000 | 56 | 6.2 | 73 | 4.8 | 76 | 2.7 |
| Penicillic acid | 15.8 | 98.9 | 72 | 7200 | 55 | 3.0 | 71 | 8.9 | 78 | 8.9 |
| Roquefortine C | 6.2 | 19.0 | 8 | 2400 | 37 | 10.7 | 57 | 8.9 | 64 | 4.9 |
| STG | 1.1 | 4.2 | 4 | 1200 | 39 | 5.0 | 56 | 5.9 | 70 | 2.8 |
| T-2 | 4.1 | 14.6 | 16 | 4800 | 46 | 6.0 | 60 | 3.3 | 76 | 3.9 |
| T-2 tetraol | 102.3 | 387.4 | 188 | 56400 | 60 | 10.3 | 98 | 22.8 | 63 | 19.3 |
| T-2 triol | 22.3 | 99.0 | 72 | 7200 | 49 | 9.9 | 67 | 19.6 | 76 | 16.3 |
| ZAN | 0.9 | 4.7 | 2.4 | 240 | 55 | 4.0 | 80 | 5.2 | 69 | 3.0 |
| ZEN | 0.5 | 1.8 | 1.6 | 480 | 41 | 24.4 | 82 | 10.4 | 51 | 24.1 |

* MON did not show linear response according to its concentration the recoveries were hence calculated according to 4.4.3.4

** only two replicates were evaluated instead of seven

### 4.4.1 Limit of detection and limit of quantification

LODs and LOQs were calculated with the S/N ratio approach in the four different matrices and in neat solvent (see chapter 4.4.1). The LODs and LOQs for wheat, maize, pig feed and silage are shown in Table 16-Table 19, while Table 20 shows the LODs and LOQs in neat solvent. The calculated results were multiplied by a factor of 8 to convert the concentration from µg/L in the diluted extract to µg/kg in the solid sample prior to extraction, based on the sample preparation procedure. It has to be mentioned, that the LOD and LOQ values are highly dependent on the instrument sensitivity which can show significant day-to-day variations for MS instruments. The reported LODs and LOQs only reflect the sensitivity of the instrument during the validation procedure. During the routine analysis, the instrument performance can be ensured by measuring quality control samples with each sequence.

**Table 20: LOD and LOQ in neat solvent**

| Analyte | LOD [µg/kg] | LOQ [µg/kg] | Analyte | LOD [µg/kg] | LOQ [µg/kg] |
|---|---|---|---|---|---|
| 15-AcDON | 9.0 | 31.4 | Ergocryptinine | 0.1 | 0.4 |
| 15-Acetoscirpenol | 1.8 | 5.5 | Ergometrine | 0.3 | 1.0 |
| 3-AcDON | 5.2 | 16.6 | Ergometrinine | 0.1 | 0.3 |
| $AFB_1$ | 0.9 | 2.9 | Ergosine | 0.4 | 1.4 |
| $AFB_2$ | 0.4 | 1.8 | Ergotamine | 0.4 | 1.3 |
| $AFG_1$ | 0.6 | 1.9 | $FB_1$ | 5.4 | 16.3 |
| $AFG_2$ | 1.4 | 5.5 | $FB_2$ | 1.3 | 4.9 |
| $AFM_2$ | 0.5 | 1.6 | $FB_3$ | 2.8 | 10.0 |
| Agroclavine | 0.1 | 0.2 | Fusaric acid | 5.1 | 18.2 |
| α-ZOL | 0.6 | 2.6 | FusX | 3.7 | 35.3 |
| AOH | 0.8 | 2.6 | Gliotoxin | 6.3 | 20.5 |
| Beauvericin | 0.1 | 0.1 | HT-2 | 5.4 | 20.6 |
| β-ZOL | 0.9 | 3.2 | MON | 0.5 | 1.8 |
| D3G | 1.3 | 4.4 | Mycophenolic acid | 37.9 | 120.0 |
| DAS | 0.9 | 3.1 | NEO | 0.9 | 2.7 |
| Dihydrolysergol | 0.6 | 2.2 | NIV | 4.2 | 15.1 |
| DON | 7.3 | 27.9 | OTA | 0.8 | 2.6 |
| Elymoclavine | 0.2 | 0.9 | OTB | 1.1 | 4.2 |
| Enniatin A | 0.02 | 0.06 | PAT | 20.7 | 81.8 |
| Enniatin A1 | 0.04 | 0.1 | Penicillic acid | 5.4 | 26.8 |
| Enniatin B | 0.1 | 0.2 | Roquefortine C | 1.1 | 3.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Enniatin B1 | 0.1 | 0.2 | | STG | 0.4 | 1.2 |
| Ergine | 0.2 | 0.8 | | T-2 | 1.4 | 4.8 |
| Ergocornine | 0.4 | 1.4 | | T-2 tetraol | 56.2 | 176.5 |
| Ergocorninine | 0.2 | 0.7 | | T-2 triol | 9.7 | 27.2 |
| Ergocristine | 0.3 | 0.9 | | ZAN | 0.2 | 0.6 |
| Ergocristinine | 0.2 | 0.7 | | ZEN | 0.3 | 1.1 |
| Ergocryptine | 0.3 | 0.8 | | | | |

The results varied a lot between the different analyte-matrix combinations. The LOD and LOQ values were generally higher in the matrix samples than in neat solvent due to matrix effects and often higher noise in the matrix extracts occurred. Pig feed and silage showed higher LODs and LOQs for most analytes compared to wheat and maize, which was expected due to the higher amount of matrix effects (see chapter 4.4.3.2). The lowest LODs and LOQs were achieved for the group of enniatins (A, A1, B, B1) and beauvericin which showed LOQs below 0.5 µg/kg for all matrices and neat solvent. Mycophenolic acid, and T-2 tetraol exceeded an LOQ of 100 µg/kg in all matrices and neat solvent, while 15-AcDON and PAT exceeded an LOQ of 100 µg/kg in all matrices, but not in neat solvent. The highest LOQ was reported for FusX in pig feed (864.7 µg/kg).

Regarding the maximum levels set by the EU (see Table 1 and Table 2), it was possible to achieve LODs that were below the limits for all regulated analyte-matrix combinations. All LOQs were below the maximum level for feed matrices, which means that a quantitative statement, close to the regulated limit will be possible. In food matrices however, it was not always possible to obtain LOQs below the maximum level. For aflatoxins in processed cereals and processed maize and for HT-2 in cereals for direct human consumption other than maize, the LOQs during the validation were above those mentioned limits. Since the LOD was below the maximum level for all analyte-matrix combinations, at least a qualitative statement will be possible for the analysis of food samples. Moreover, the method was primarily designed for the analysis of feed samples, since food samples will only be of minor importance for the routine analysis.

Most of the LODs and LOQs were similar or higher than the ones published by Malachova et al. (2014). This was expected, since they used a QTrap 5500 from AB Sciex, which is a more sensitive instrument. Moreover, the results are not completely comparable with the mentioned publication, because a different approach for the calculations of LODs and LOQs was used.

### 4.4.2 Working range

The working range was based on the linear response of the analyte, according to its concentration, respective LOD and LOQ, occurrence data provided by Biomin and available regulations and guidance values. For most analytes, a working range with a factor of 300 between the lowest level and the highest level was chosen. For some analytes a factor of 100 was used, to keep the amount of needed standard during validation as low as possible. Moreover, occurrence data showed, that higher concentrations were not necessary for most samples. However, there can always be higher contaminated samples, which have to be diluted then. For some analytes that showed strong signal suppression effects, the calibration function was still linear ($R^2 > 0.98$) at the highest concentration level in the matrix matched standard and spiked sample, but not for the liquid standard. In the routine analysis, calibration functions from the liquid standards will be used. Hence, the linearity from the liquid standards is the most important one, so that in this case the highest levels were removed from all 3 types of calibration functions.

MON eluted close to the column dead time and did not show linear response according to its concentration (previously reported by Sulyok et al., 2006). Therefore, the slopes of a linear calibration curve could not be used for the calculation of the recoveries. It was decided to use the peak areas instead of the slope at three different concentration levels (low level, medium level, high level) and report the recoveries at this three levels separately (see chapter 4.4.3.4).

### 4.4.3 Recoveries

#### 4.4.3.1 Recoveries of extraction

Recoveries of extraction were calculated according to F4. They indicate the amount of analyte which can be extracted with the present conditions. Theoretically, values above 100% cannot be achieved if the used matrix is really a blank sample. However, $R_E$ values above 100% percent were especially observed for the ergopeptinine form in the group ergot alkaloids. This is not due to blank contamination in the matrix samples, but rather due to epimerisation of the ergopeptine form during the evaporation process at 37°C. Additionally, average values above 100% percent were observed for mycophenolic acid in wheat (113%), enniatin A1 (108%) and enniatin B (101%) in wheat. The enniatins in wheat were already quantified earlier (see chapter 4.3) and the peak area in the blank extract was subtracted from the peak area of the levels from the calibration functions. Lack of homogenous distribution of mycotoxins in the matrix

might be the reason for receiving values above 100% anyway. The same effect could cause the high $R_E$ of mycophenolic acid, which was not detected in the analysed blank samples.

The $R_E$ values were only an estimation based on spiking experiments and evaporation of the used solvent. The average values were therefore comparable for all matrices. Such approaches are merely an estimation and cannot fully represent the extraction of naturally contaminated samples during routine analysis afterwards. However, the results varied a lot between the different analytes. Values below 60% were received for $FB_1$, $FB_2$ and $FB_3$ in wheat and pig feed, for MON in all matrices, for NIV in wheat and maize, for fusaric acid in maize, for PAT in maize and for beauvericin, dihydrolysergol, elymoclavine and ZEN in silage. In contrast to the SSE, the $R_E$ values are not instrument dependent and can be compared to scientific literature using different MS systems. In general, analytes that showed low $R_E$ values in this work, also showed low $R_E$ values, while analytes with high $R_E$ values, also showed high $R_E$ values in the method from Malachova et al. (2014).

### 4.4.3.2  Signal suppression or enhancement

The signal suppression or enhancement refers to the amount of matrix effects, due to compounds that co-elute with the analyte and distort the result. A value above 100% percent indicates enhancement and was only observed for a small number of analytes. By setting a range of 100 ± 15%, only enniatin A1 (126) and mycophenolic acid (129%) in wheat, ergine (126%) and T-2 tetraol (140%) in maize and agroclavine in pig feed (171%) clearly exceeded this value.

Less signal suppression effects were observed in wheat (average SSE of 97%) than in maize (average SSE of 81%), pig feed (average SSE of 72%) and silage (average SSE of 64%). The higher matrix effects in silage and pig feed probably occurred due to the more complex composition of this agricultural commodities. Sulyok et al. (2006) also observed higher matrix effects in maize than in wheat. While in wheat only 3 analytes showed values below 85% (AOH: 79%, D3G: 47%, NIV: 82%), a majority of mycotoxins showed clear signal suppression in maize, pig feed and silage. 7 analytes in maize, 7 analytes in pig feed and 10 analytes in silage even showed SSE below 50%.

Available internal standards were used for the evaluation of possible compensations of the matrix effects. During the validation, they were representatively tested for the two matrices pig feed and silage, which showed more matrix effects than wheat and maize. Figure 9 and Figure 10 show a comparison between the SSE with and without internal standards for 22 analytes.

The SSE with internal standard is always higher than without internal standard and close to 100%. It was only below 90% for D3G in pig feed (89%) and for $AFG_1$ in silage (88%), but still significantly higher than without internal standard. $^{13}C$-labelled standards can therefore be used for the compensation of the matrix effects. In the routine analysis, the $R_A$ from the validation will be used for the correction of the results for all analytes without available internal standard, while the $R_E$ values will be used for the analytes with internal standards.
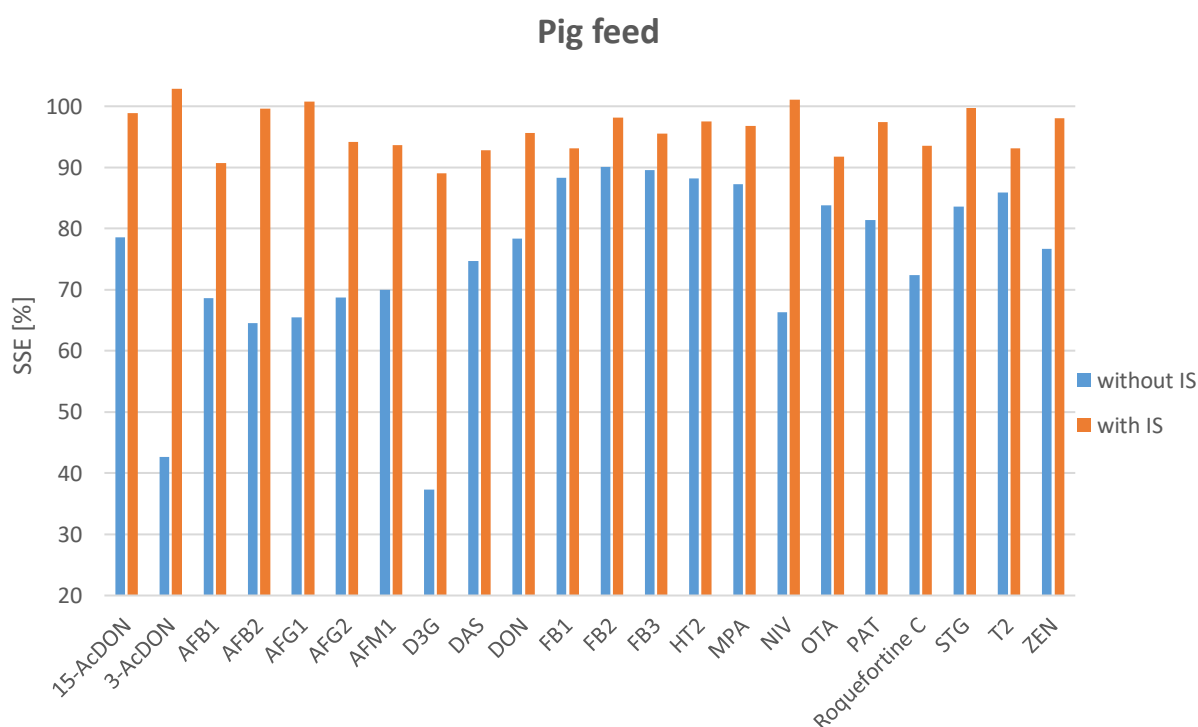


**Figure 9: Comparison of SSE with and without internal standards in pig feed**
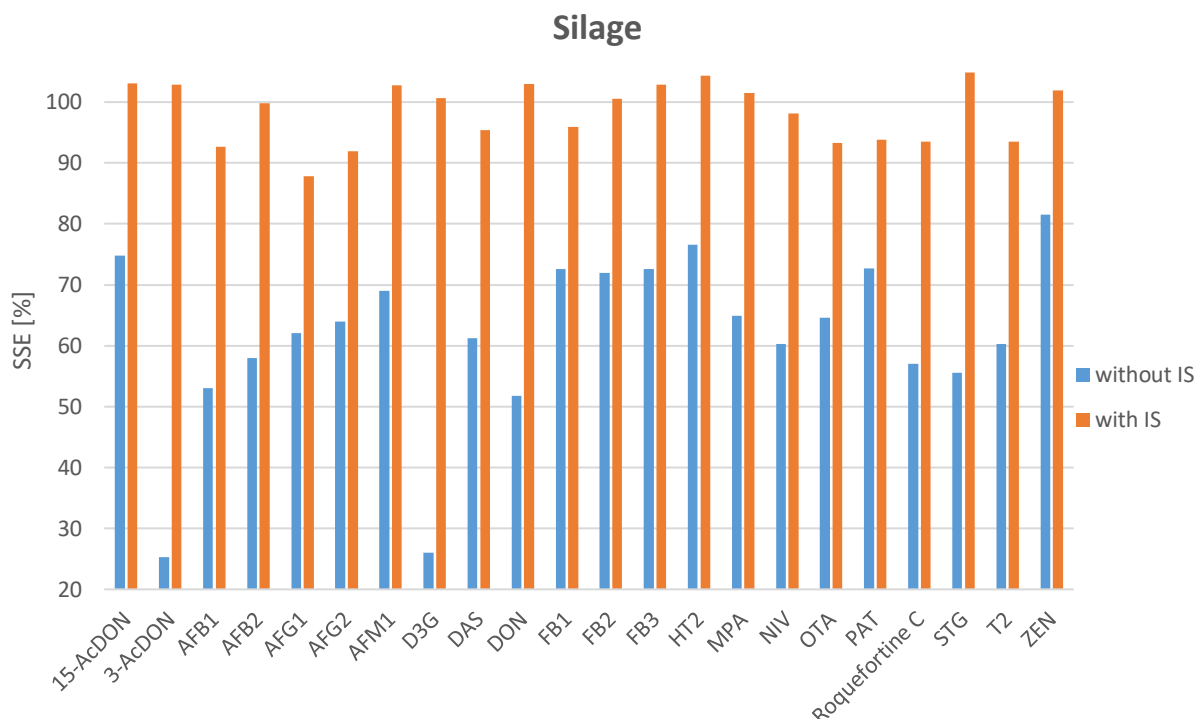
**Figure 10: Comparison of matrix effects with and without internal standards in silage**

### 4.4.3.3 Apparent recovery

The apparent recovery includes both, the recovery of extraction and signal suppression and enhancement effects. It should be used in the routine analysis as a correction factor for the matrix samples that are quantified with liquid standards. Hence, the evaluation has special importance during the validation. A low $R_E$ leads to low $R_A$, if the SSE is not above 100% and vice versa.

The range of $R_A$ values varied a lot between the different analytes: values between 30 and 146% were observed in wheat, between 22 and 114% in maize, between 25 and 114% in pig feed and between 18 and 65% in silage. The average $R_A$ was the lowest in silage, and the highest in wheat, which is due to the SSE, due to the fact that the $R_E$ are similar in all matrices. Not for all of the 13 analytes with available EU requirements (see Table 3), high enough apparent recoveries were achieved. Most of the criteria were met for maize, where 6 analytes showed $R_A$ values above the EU requirements (AFM$_1$, FB$_2$, HT-2, OTA, T-2, ZEN), while in wheat 5 analytes (AFM$_1$, HT-2, OTA, T-2, ZEN) completely fulfilled the criteria and DON fulfilled the criteria only up to a concentration level of 500 µg/kg. In pig feed, only HT-2 and T-2 could completely meet the EU requirements, while DON could fulfil them until a concentration of 500

µg/kg and ZEN until a concentration of 50 µg/kg. In silage, for none of the analytes, $R_A$ values that fulfil the EU requirements were achieved. However, for a method that includes such a high variety of different analytes and agricultural commodities, some compromises have to be accepted. Therefore also the $R_A$ values that do not meet the EU criteria will be used for the correction of the measured values in routine analysis.

### 4.4.3.4  Moniliformin

Moniliformin did not show linear response of the signal intensity, according to its concentration (see chapter 4.4.2). For the calculation of the recoveries, the peak areas were compared instead of the slopes. Moreover it was observed, that the recovery varies between different concentration levels, so that the average recovery of all concentration levels could not be used. Hence, the recoveries were calculated at three separate levels (low, medium and high level). Table 21 shows the $R_A$, SSE and $R_E$ for MON in wheat, maize, pig feed and silage at 8, 240 and 2400 µg/kg. It is noticeable, that in all four matrices, signal suppression is observed in the lowest level, while signal enhancement occurs in the highest level. In silage, a SSE of 44% was observed in the low level, while a SSE of 157 was observed in the highest level. Matrix effects of MON are therefore highly concentration dependent.

**Table 21: Recoveries for moniliformin in wheat, maize, pig feed and silage at 8 µg/kg, 240 µg/kg and 2400 µg/kg**

| Wheat | | | | | | |
|---|---|---|---|---|---|---|
| | $R_A$ [%] | RSD [%] | SSE [%] | RSD [%] | $R_E$ [%] | RSD [%] |
| Low level | 40 | 6.6 | 83 | 4.0 | 48 | 7.1 |
| Medium level | 64 | 3.2 | 115 | 4.3 | 56 | 2.4 |
| High level | 81 | 2.5 | 150 | 1.8 | 54 | 1.4 |
| **Maize** | | | | | | |
| | $R_A$ [%] | RSD [%] | SSE [%] | RSD [%] | $R_E$ [%] | RSD [%] |
| Low level | 43 | 7.1 | 81 | 14.0 | 53 | 9.5 |
| Medium level | 56 | 4.0 | 99 | 2.8 | 57 | 1.4 |
| High level | 72 | 3.4 | 115 | 1.7 | 63 | 2.9 |
| **Pig feed** | | | | | | |
| | $R_A$ [%] | RSD [%] | SSE [%] | RSD [%] | $R_E$ [%] | RSD [%] |
| Low level | 59 | 12.9 | 97 | 8.0 | 62 | 13.5 |
| Medium level | 84 | 5.0 | 123 | 4.0 | 68 | 3.2 |
| High level | 95 | 4.0 | 138 | 2.9 | 69 | 2.1 |
| **Silage** | | | | | | |

| | $R_A$ [%] | RSD [%] | SSE [%] | RSD [%] | $R_E$ [%] | RSD [%] |
|---|---|---|---|---|---|---|
| Low level | 35 | 17.6 | 44 | 17.2 | 81 | 16.3 |
| Medium level | 56 | 8.8 | 65 | 5.5 | 86 | 8.0 |
| High level | 104 | 5.0 | 157 | 5.0 | 66 | 2.2 |

## 4.4.4 Selectivity

Selectivity was investigated by visual observation of the peaks from the quantifier and qualifier transition, at a concentration level close to the LOQ. If no interfering peaks were visible in the retention time window, selectivity was given. Sometimes, the period of time between the peaks with the same MRM transitions was small, so that more than one peak was visible in the chosen time window. This is of minor importance, for analytes with separated chromatographic peaks. Except for ergotamine/ergotaminine and ergosine/ergosinine (see chapter 4.1), all of this analytes could be completely separated.

For some analytes, additional peaks were observed in the chosen matrix samples for either the quantifier or qualifier transition. This matrix component peaks however did not co-elute with the actual analyte and therefore did not negatively affect the quantification. Selectivity was given for 54 unlabelled mycotoxins and 22 internal standards.

## 4.4.5 Precision

The repeatability, reproducibility, stability and lab precision were evaluated in wheat and maize. All values were received by calculating the RSDs from the peak areas. The results are summarised in Table 22. It should be noticed, that the evaluated RSDs are not the same over the complete linear range and were only tested in the middle of the working range. The values increase with decreasing concentration and vice versa.

**Table 22: Results of the precision experiments**

| | Maize | | | | Wheat | | | |
|---|---|---|---|---|---|---|---|---|
| Analyte | Repeat. | Rugg. | Stability | Lab Pr. | Repeat. | Rugg. | Stability | Lab Pr. |
| 15-AcDON | 6.9 | 5.4 | 12.8 | 6.7 | 7.6 | 4.0 | 9.5 | 11.4 |
| 15-Acetoxyscirpenol | 9.0 | 11.6 | 30.0 | 13.6 | 14.5 | 8.5 | 30.2 | 9.6 |
| 3-AcDON | 4.8 | 4.7 | 12.1 | 3.8 | 3.3 | 4.1 | 8.8 | 4.7 |
| $AFB_1$ | 4.7 | 3.4 | 5.1 | 4.9 | 6.7 | 3.0 | 3.5 | 11.1 |
| $AFB_2$ | 6.4 | 5.5 | 7.1 | 4.6 | 4.5 | 5.1 | 6.3 | 5.3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AFG$_1$ | 7.3 | 4.8 | 4.6 | 5.3 | 6.1 | 6.0 | 6.1 | 12.1 |
| AFG$_2$ | 6.3 | 9.0 | 5.0 | 6.3 | 7.4 | 6.4 | 6.8 | 10.2 |
| AFM$_1$ | 4.1 | 4.9 | 4.2 | 8.0 | 6.1 | 4.3 | 5.0 | 8.2 |
| Agroclavine | 2.8 | 5.8 | 6.1 | 5.8 | 3.2 | 4.1 | 1.5 | 4.9 |
| A-zearalenol | 4.6 | 4.7 | 3.9 | 5.6 | 2.4 | 1.3 | 3.6 | 4.5 |
| Alternariol | 5.8 | 5.5 | 4.4 | 7.5 | 1.4 | 0.8 | 3.6 | 3.7 |
| Beauvericin | 2.9 | 4.3 | 9.4 | 5.1 | 3.1 | 2.9 | 7.3 | 9.4 |
| B-zearalenol | 6.3 | 6.3 | 3.4 | 6.5 | 2.1 | 1.3 | 3.0 | 2.2 |
| D3G | 3.2 | 5.0 | 4.5 | 7.4 | 1.4 | 1.5 | 6.0 | 2.9 |
| DAS | 4.5 | 3.7 | 8.3 | 6.1 | 6.1 | 5.1 | 8.6 | 9.0 |
| Dihydrolysergol | 2.3 | 3.4 | 2.1 | 4.1 | 4.3 | 3.6 | 3.1 | 4.5 |
| DON | 3.4 | 2.5 | 10.4 | 3.5 | 3.7 | 1.9 | 7.3 | 5.5 |
| Elymoclavine | 3.2 | 3.6 | 3.1 | 3.6 | 4.2 | 5.3 | 2.8 | 3.8 |
| Enniatin A | 3.4 | 4.7 | 8.4 | 2.4 | 5.6 | 3.8 | 5.6 | 8.8 |
| Enniatin A1 | 3.7 | 8.2 | 5.6 | 7.8 | 3.7 | 6.2 | 5.5 | 9.1 |
| Enniatin B | 2.0 | 4.3 | 6.7 | 3.7 | 2.5 | 2.0 | 6.2 | 8.9 |
| Enniatin B1 | 2.8 | 4.4 | 6.0 | 5.5 | 3.8 | 3.7 | 4.0 | 7.7 |
| Ergine | 2.1 | 2.0 | 3.8 | 3.3 | 3.4 | 1.8 | 3.4 | 3.3 |
| Ergocornine | 6.7 | 5.5 | 7.9 | 6.5 | 10.0 | 7.0 | 7.6 | 10.7 |
| Ergocorninine | 3.4 | 4.9 | 5.0 | 6.4 | 3.9 | 5.3 | 6.9 | 8.0 |
| Ergocristine | 5.5 | 11.7 | 13.0 | 7.8 | 6.1 | 9.3 | 8.2 | 11.5 |
| Ergocristinine | 2.3 | 2.3 | 8.1 | 9.5 | 1.3 | 2.5 | 8.5 | 4.7 |
| Ergocryptine | 5.9 | 4.4 | 6.5 | 6.7 | 4.9 | 6.7 | 5.0 | 8.2 |
| Ergocryptinine | 4.1 | 3.6 | 6.1 | 5.3 | 4.0 | 3.7 | 3.2 | 8.2 |
| Ergometrine | 2.4 | 5.0 | 2.5 | 7.1 | 6.2 | 5.6 | 4.0 | 5.9 |
| Ergometrinine | 2.7 | 4.6 | 2.8 | 4.6 | 5.0 | 3.4 | 2.7 | 3.8 |
| Ergosine | 3.6 | 4.9 | 5.2 | 2.3 | 5.0 | 5.3 | 3.2 | 8.8 |
| Ergotamine | 4.2 | 3.8 | 8.2 | 5.3 | 4.5 | 7.9 | 5.2 | 9.3 |
| FB$_1$ | 3.8 | 3.3 | 4.9 | 7.6 | 4.6 | 3.2 | 4.9 | 6.6 |
| FB$_2$ | 2.6 | 3.0 | 4.2 | 4.4 | 4.4 | 2.1 | 5.0 | 7.6 |
| FB$_3$ | 3.8 | 4.6 | 5.5 | 7.8 | 3.3 | 3.4 | 5.0 | 5.5 |
| Fusaric acid | 3.9 | 4.1 | 3.7 | 2.4 | 5.4 | 3.2 | 1.9 | 5.5 |
| FusX | 3.8 | 3.1 | 11.6 | 3.1 | 2.5 | 2.2 | 8.9 | 1.7 |
| Gliotoxin | 7.3 | 8.4 | 18.6 | 30.3 | 6.7 | 9.4 | 18.8 | 11.6 |
| HT-2 | 9.8 | 7.2 | 28.5 | 11.9 | 7.7 | 7.7 | 25.2 | 11.7 |
| MON | 8.2 | 2.9 | 6.5 | 14.7 | 2.4 | 4.0 | 3.5 | 3.1 |
| Mycophenolic acid | 3.7 | 4.1 | 9.9 | 2.9 | 2.6 | 3.1 | 5.7 | 8.7 |
| NEO | 2.4 | 2.9 | 11.2 | 4.9 | 3.5 | 2.2 | 11.1 | 5.5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| NIV | 9.1 | 12.2 | 10.2 | 8.3 | 6.1 | 5.1 | 10.6 | 3.4 |
| OTA | 3.0 | 1.6 | 5.1 | 4.0 | 2.5 | 2.5 | 5.7 | 5.9 |
| OTB | 4.7 | 3.3 | 5.8 | 2.7 | 2.5 | 2.7 | 6.4 | 4.6 |
| PAT | 2.5 | 3.1 | 7.8 | 17.7 | 2.0 | 1.3 | 8.5 | 1.8 |
| Penicillic acid | 2.6 | 1.8 | 7.4 | 11.0 | 2.7 | 2.5 | 3.3 | 10.6 |
| Roquefortine C | 3.1 | 3.2 | 4.0 | 6.3 | 3.3 | 4.0 | 4.4 | 5.9 |
| STG | 1.8 | 1.8 | 2.7 | 4.4 | 1.7 | 2.3 | 3.0 | 7.2 |
| T-2 | 3.6 | 2.3 | 15.0 | 6.3 | 6.3 | 5.7 | 15.3 | 7.7 |
| T-2 tetraol | 8.0 | 5.4 | 16.6 | 9.4 | 5.8 | 5.9 | 17.3 | 7.6 |
| T-2 triol | 14.5 | 12.7 | 14.2 | 16.7 | 14.2 | 10.9 | 27.1 | 8.3 |
| ZAN | 5.2 | 6.7 | 4.0 | 8.1 | 2.5 | 2.0 | 4.0 | 4.9 |
| ZEN | 6.3 | 7.1 | 4.0 | 7.2 | 1.3 | 1.6 | 4.3 | 2.2 |
| **Average** | **4.7** | **5.0** | **7.9** | **7.0** | **4.6** | **4.2** | **7.3** | **6.9** |

Repeat.: Repeatability

Rugg.: Ruggedness

Lab Pr.: Lab Precision

The <u>repeatability</u> was calculated by measuring the same vial 10 times in a row. It shows the precision of the instrument itself, since the same vial, instrument and a short time frame are used. There was no great difference between the two matrices: maize showed an average RSD of 4.7, wheat of 4.6. They ranged from 1.8 to 14.5 for maize and 1.3 to 14.5 for wheat. No analyte showed values above 25 and only two analytes showed values above 10 (15-acetoscirpenol in wheat: 15.5, T-2 triol in maize: 14.5 and wheat: 14.2). Moreover, none of the analytes exceeded the EU requirements (see Table 3).

The <u>ruggedness</u> was evaluated by repeating the same experiment with a different HPLC column, but identical properties. The RSDs were comparable to the repeatability and showed an average value of 5.0 for maize and 4.2 for wheat. There was only one exception that showed values higher than 10, which was again T-2 triol (12.7 in wheat and 10.9 in maize), while no values above 15 were received. It was proven, that the method shows similar repeatability on an identical column, which is important because the lifespan of a HPLC column is limited and has to be changed from time to time.

<u>Stability</u> was tested, to prove the stability of the mycotoxins at room temperature, over a longer period of time (e.g. over the weekend). This has to be considered in the routine analysis, because the used autosampler does not include a cooling unit. It was expected, that the values would be higher than for the repeatability due to changes in sensitivity of the instrument over time as well as due to possible instabilities of the analytes itself. Most analytes showed results

below 10, while gliotoxin, T-2 and T-2 tetraol showed results above 15 for both matrices, HT-2 showed values above 25 for both matrices and T-2 triol for wheat only. 15-Acetoscirpenol even showed results above 30 for both matrices. Nevertheless, the signal intensities did not decrease dramatically between the first and last day of measuring for any of the analytes. The high RSDs rather result from fluctuations in the instrument sensitivity over time. Hence the values do not disprove the stability of the analytes, but rather show high intra-day repeatability.

The lab precision was investigated by including fluctuations of the extraction. The average value was 7.0 for maize and 6.9 for wheat. Some exceptions showed RSDs of higher than 15: gliotoxin, PAT and T-2 triol in maize. In wheat no values above 15 were observed.

### 4.4.6 Trueness

#### 4.4.6.1 Certified reference materials

Commission Regulation (EU) No 519/2014 recommends to include CRMs during the validation. The availability is however limited, so that in this work, CRMs were included only for DON, NIV, OTA and ZEN. The CRM standards were diluted and quantified during the validation. Table 23 shows the measured values and the certified values in µg/mL. For all 4 analytes, $m_\Delta$ is smaller than $U_\Delta$, meaning that no significant difference was detected.

**Table 23: Results for the quantification of certified reference materials**

| Analyte | Measured value [µg/mL] | Certified value [µg/mL] | $m_\Delta$ | $U_\Delta$ |
|---------|------------------------|-------------------------|------------|------------|
| DON | 25.03 | 24.74 | 0.29 | 1.11 |
| NIV | 25.30 | 25.04 | 0.26 | 3.54 |
| OTA | 10.68 | 10.01 | 0.67 | 6.68 |
| ZEN | 9.66 | 10.03 | 0.37 | 11.58 |

With the application of the CRMs in neat solvent the trueness for those four analytes in neat solvent was proven. Nevertheless, this approach does not prove trueness of the quantification in matrix. This was additionally started by the participation in proficiency tests.

#### 4.4.6.2 Proficiency test for maize

Method trueness in matrix was tested for maize by participating in a proficiency with the highest number of different mycotoxins from FAPAS. FAPAS was chosen, due to good experience

with their proficiency tests in the past. A total number of 101 participants submitted their results before the dead line. The methods that were used by the participants did not only include LC-MS/MS, but also ELISA, fluorometry and others. For each analyte z-scores between -2 and +2 were reached from at least 80% of the participating labs.

Table 24 shows the results for the method validated in the work of this thesis. For all of the 8 analytes that were included in the proficiency test, the measured values and the assigned values are given in µg/kg. Z-scores between -2 and +2, meaning acceptable results were achieved for all of the included analytes.

**Table 24: Results of the FAPAS proficiency test for maize**

| Analyte | Measured value [µg/kg] | Assigned value [µg/kg] | Z-score |
|---|---|---|---|
| DON | 610.4 | 489 | 1.4 |
| ZEN | 133.8 | 107 | 1.2 |
| T2 | 69.2 | 62.7 | 0.5 |
| HT2 | 82.2 | 78.3 | 0.2 |
| Sum (T2+HT2) | 151.5 | 142 | 0.3 |
| $AFB_1$ | 4.6 | 4.69 | -0.1 |
| $FB_1$ | 604.3 | 507 | 1.1 |
| $FB_2$ | 131 | 111 | 0.8 |
| Sum ($FB_1$+$FB_2$) | 735.2 | 599 | 1.3 |
| OTA | 6.2 | 4.73 | 1.4 |

# 5 Conclusion and outlook

During this master thesis, an LC-MS/MS multi target method for the determination of mycotoxins was optimised and validated on an 1260 HPLC from Agilent, coupled to a 4500 QTrap from AB Sciex. MS parameters were received by direct infusion of the standards into the system. The precursor ions showed higher signal intensities in the positive mode for 60 analytes, while 18 analytes showed higher signal intensities in the negative mode. Quantifier and qualifier transitions were received for all analytes except for moniliformin, which showed only one product ion.

Most of the HPLC parameters were adopted from literature, but some experiments on combining positive and negative polarity mode into one chromatographic run and on the length of the retention time window were performed. Some analytes did not show optimal peak shape during the chromatographic runs, but for 56 unlabelled mycotoxins and 22 internal standards, quantification was possible and they could be included in the validation procedure. Two separate chromatographic runs had to be used, due to unsatisfactory peak reproducibility and more wear of the instrument in the combined method. A retention time window of 72 s and a target scan time of 1 s were chosen for the sMRM method.

The validation was performed for the 4 matrices wheat, maize, pig feed and silage. LOQs below the maximum limits in the regulations could be achieved for feed matrices, while for some analyte-food commodity combinations, LODs below but LOQs above the maximum level were obtained. Hence, it will not always be possible to give quantitative statements close to the regulated limit for food samples.

The recoveries of extraction were only an estimation from spiking experiments and comparable for all of the four matrices. Only a small number of analytes showed signal enhancement, while more analytes showed signal suppression. The most matrix effects were observed in silage and pig feed, due to the more complex composition of this two commodities. Regarding the apparent recoveries, the EU requirements could not be met for all analyte-matrix combinations. This however has to be accepted, since a method that contains such a high number of analytes always includes conditions that are far from optimal for some analytes. It was further proven that matrix effects can be compensated with ^{13}C-labelled internal standards that are injected with an autosampler pre-treatment program.

All of the analytes showed acceptable repeatabilities, meeting the EU requirements. The use of a different HPLC column with the same properties, did not negatively affect the repeatability.

Moreover, the stability of the analytes in matrix extract at room temperature was tested. High RSD values were obtained for some analytes, which were not caused by analyte degradation but rather due to sensitivity fluctuations of the instrument.

Certified reference materials in neat solvent were available for DON, NIV, OTA and ZEN and quantified with the validated LC-MS/MS method. No significant differences between the measured values and the certified values were detected. Furthermore the method trueness in matrix was proven for maize by participating in a proficiency test from FAPAS which included 8 mycotoxins. The measured values were corrected with the recoveries, obtained during the validation. Z scores between -2 and +2 were achieved for all of the included analytes, meaning acceptable results.

It is planned to prove the trueness of the other three matrices by participating in further proficiency tests. The method should then be implemented in the Analytical Service Lab of Romer Labs Austria for routine analysis purposes. One of the main objectives of this method should be the decrease of amount of time necessary for the analysis, compared to already existing LC-MS/MS multi-target methods.

Finished pig feed was chosen as a representative feed matrix for the validation, since it has the highest relevance in the routine analysis. Other feed matrices however can have a high variety of different compositions and therefore also different performance characteristics. It is planned to expand the method to other feed matrices in the future, by carrying out further validation experiments.

# 6 Directories

## 6.1 References

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## 6.2  List of Tables

## 6.3  List of Figures

Figure 10: Comparison of matrix effects with and without internal standards in silage