# Development, validation and application of an advanced LC-MS/MS based multiclass method for the analysis of animal feed with a special focus on globalisation and climate change

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# Contents

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# Aims and Structure of the Thesis

The presented thesis was performed within the Green Area of the Austrian Competence Centre for Feed and Food Quality Safety and Innovation (FFoQSI GmbH) within the work package on-field/plant quality assurance led by Univ.-Prof. Dr. Rudolf Krska. The focus of the Green Area is on topics from the cultivation of plants to plant-based feed and food. This research focus includes the identification of agro-contaminants with respect to global trade and climate change. This thesis is conducted as a cumulative work and consists of two major parts. The first part (**Introduction**) is divided into three chapters. Chapter 1 discusses the relevance of agro-contaminants in food and feed including definitions, classifications, toxicological profiles, and the legislative situation in the European Union. Chapter 2 focusses on the importance of (emerging) mycotoxins with respect to the influence of climate change on future contamination patterns and emerging hazards. Chapter 3 is providing background information for the most relevant publications 1 and 2 focusing on analytical method development emphasising state of the art concepts, as well as limitations and challenges in multiclass method development, validation and application. The second part (**Original Works**) lists four major Science Citation Index (SCI) ranked publications selected for this thesis. A brief description of the individual co-author's contribution is provided at the beginning of each publication. The overall goal of the presented thesis was to provide a liquid chromatography tandem mass spectrometry-based method for the quantitative determination of agro-contaminants including: secondary fungal metabolites (including all regulated mycotoxins), plant toxins, bacterial metabolites, pesticides and veterinary drugs in complex feed. More specifically, two sub-objectives are in the focus of this work. Objective one included a compatibility testing of multiple substance classes with a generic extraction protocol, as well as an investigation of technical limitations with respect to the number of substances, which could be measured within one analytical run (Publication #1). High compositional variances that are typically encountered in feed matrices were part of objective number two. The focus was set on the development of a strategy to circumvent the lack of compositional knowledge of complex feed matrices. A comprehensive characterisation and assignment of analytical performance data from single feed material to the final compound feed formula was conducted (Publication #2). The successful development and validation of this multiclass approach opens up new possibilities of application e.g. to monitor the influence of climate change on mycotoxin occurrence which was investigated within Publication #3 and Publication #4.

# Abstract

The impact of environmental chemical pollution has steadily increased in recent decades due to an excessive use of pesticides and pharmacologically active agents. In addition, alterations in contamination patterns of natural toxins such as mycotoxins are evident and triggered by changing climate conditions. This has led to an elevated consumer awareness in recent years and increased the legislative pressure especially within the European Union (EU) and the pressure on public health authorities to understand rapidly changing contamination patterns and to prevent the importation of potentially hazardous substances. Consequently, there is a growing need for accurate, reliable and comprehensive analytical methods, which allow the sensitive, selective and rapid determination of both natural and anthropogenic pollutants in the agro-food sector. Advances in the area of chromatography-mass spectrometry coupling techniques enabled the development of such multi-target approaches. However, the majority of published quantitative methods are not yet covering more than 400 analytes, and approaches exceeding this number are exclusively applicable for qualitative screening purposes.

This thesis describes the first quantitative multiclass approach enabling the accurate quantification of >1,400 agro-contaminants including 750 natural biotoxins (secondary fungal metabolites, plant- and bacterial toxins), 500 pesticides and 150 veterinary drugs which was developed and fully validated for the analysis of complex compound feed (Publication #1). Optimization of MS/MS (cycle and dwell time), as well as HPLC/UHPLC conditions (injection volume, flow rate and chromatographic column) were carried out in an unprecedented way. In order to reduce the overall measurement error, retention windows and cycle times were thoroughly optimized to ensure sufficient dwell times. We have shown that potential benefits of an UHPLC system with respect to matrix effects are reduced, because the prevention of overlapping events with co-eluting matrix components is diminished, with increasing number of target compounds. The method was successfully validated for 1,219 multiclass contaminants in two complex feed matrices according to EU SANTE validation guideline. Recovery of the extraction protocol complied with the 70-120% criterion for 91% of analytes in cattle and 95% of analytes in chicken feed. Limits of quantification were below 10 µg/kg for >1,000 analytes in both feed matrices. Matrix effects revealed as the major limitation of this approach especially in view of intra-matrix variations. These effects are in particular evident for matrices composed of different individual components as in case of compound feed.

Since current validation guidelines are neglecting this issue, most publications in feed analysis are based on the validation of single feed material. Within a comprehensive pre-validation study (Publication #2), we have shown that significant differences in apparent recoveries and especially matrix effects exist between single feed ingredients and complex compound feed as well as between individual compound feed samples. In order to circumvent the lack of a true blank sample material and to simulate compositional variances, model compound feed samples for chicken, pig and cattle feed were prepared in-house and compared and statistically tested with real compound feed samples. The results revealed compound feed modelling as a suitable solution to solve a neglected issue and ensure a more realistic estimation of the method's performance. Therefore, the final method validation was carried out using artificial in-house prepared sample material by combining the most relevant single feed ingredients for the respective compound feed formulas.

The method's applicability was tested two-fold, first within an investigation study (split in publications) on the occurrence of regulated (Publication #3) and non-regulated mycotoxins (Publication #4) in maize harvested in Serbia (between 2012-2015) with special focus on the influence of changing climate conditions. In 204 maize samples we have detected 109 different non-regulated secondary fungal metabolites, whereby every single maize sample was contaminated with 13 – 55 contaminants. In addition, we observed a significant influence of weather conditions on the occurrence of 20 regulated mycotoxins and its derivates. Aflatoxins were the most dominant contaminants in samples collected in very hot and dry years. Fumonisins in contrast, showed a very high prevalence (76-100%) in samples from each year. The second applicability testing was conducted within a pilot set of real complex chicken (n = 68) and cattle (n = 64) feed samples, providing first insights in exposure scenarios of co-occurrence of relevant agro-contaminants (Publication #1). The results revealed an average co-contamination of 56 compounds in chicken and 45 compounds in cattle feed including representatives of almost all investigated substance classes. High contamination levels of phytoestrogens, such as daidzein and genistein and mycoestrogens, like zearalenone and alternariol were observed in 58% of cattle and 91% of chicken feed samples.

In summary this work represents the first comprehensive quantitative multiclass approach allowing a monitoring of co-contaminations in the agro-food sector. Generated co-exposure data are of great interest for scientific advisory bodies which can serve as a basis of the assessment of potential additive, synergistic, or antagonistic effects. The transferability of this approach to other food commodities enables extensive total exposure screening to be conducted as an essential part of the dietary-exposome assessment.

# Zusammenfassung

Die Auswirkungen des Eintrags von Chemikalien haben in den letzten Jahrzehnten aufgrund des Einsatzes von Pestiziden und pharmakologisch wirksamen Substanzen zugenommen. Darüber hinaus nehmen Veränderungen in den Kontaminationsmustern natürlicher Toxine wie Mykotoxine aufgrund sich ändernder Klimabedingungen zu. Dies hat in den letzten Jahren zu einem erhöhten Konsumentenbewusstsein geführt und den Gesetzgebungsdruck insbesondere innerhalb der Europäischen Union (EU) sowie den Druck auf die Gesundheitsbehörden erhöht. Ziel ist es Kenntnisse der sich stetig ändernden Kontaminationsmuster zu generieren und die Einfuhr von potenziell gefährlichen Schadstoffen zu verhindern. Infolgedessen besteht ein wachsender Bedarf an präzisen, zuverlässigen und umfassenden Analysemethoden, mit denen sowohl natürliche als auch anthropogene Schadstoffe im Agrar- und Lebensmittelsektor empfindlich, selektiv und schnell bestimmt werden können. Fortschritte auf dem Gebiet der Chromatographie mit massenspektrometrischer Detektion ermöglichten die Entwicklung solcher Multi-Analyt-Ansätze. Die Mehrzahl der veröffentlichten quantitativen Methoden deckt jedoch nicht mehr als 400 Analyten ab, und Ansätze, die diese Anzahl überschreiten, sind ausschließlich für qualitative Screeningzwecke anwendbar.

Diese Arbeit beschreibt den ersten quantitativen Ansatz für mehrere Substanzklassen, der die Bestimmung von >1.400 Agrarkontaminanten ermöglicht. Die Methode beinhaltet 750 natürliche Biotoxine (sekundäre Pilzmetabolite, pflanzliche und bakterielle Toxine), 500 Pestizide und 150 Tierarzneimittel und wurde für die Analyse von Komplexfuttermitteln entwickelt und vollständig validiert (Publication #1). Die Optimierung von MS/MS (Scan und Dwell Zeiten) sowie HPLC/UHPLC-Bedingungen (Flussrate, Injektionsvolumen, Trennsäulen) wurde in umfangreicher Weise durchgeführt. Dies beinhaltete unter anderem eine präzise Anpassung der Akquisitionsparameter, mit dem Ziel die Gesamtmessunsicherheit zu reduzieren. Wir haben gezeigt, dass die potenziellen Vorteile eines UHPLC-Systems in Bezug auf Matrixeffekte abnehmen, da die Vermeidung überlappender Ereignisse mit Co-eluierenden Matrixkomponenten mit zunehmender Anzahl von Zielverbindungen verringert wird. Die Methode wurde erfolgreich für 1.219 Kontaminanten in zwei komplexen Futtermittelmatrizen gemäß der EU-SANTE-Validierungsrichtlinie validiert. Die Wiederfindung der Extraktion entsprach dem 70-120% Kriterium für 91% der Analyten in Rinderfutter und 95% der Analyten in Hühnerfutter. Die Bestimmungsgrenzen lagen für >1.000 Analyten in beiden Matrizen unter

10 µg/kg. Matrixeffekte erwiesen sich als Hauptlimitierung dieses Ansatzes, insbesondere im Hinblick auf Intra-Matrix-Variationen. Diese Effekte treten vorwiegend bei Matrizen auf, die aus verschiedenen Einzelkomponenten bestehen, wie im Fall von Mischfutter.

Da aktuelle Validierungsrichtlinien dieses Problem vernachlässigen, basieren die meisten wissenschaftlichen Publikationen aus dem Bereich der Futtermittelanalyse auf der Validierung von einzelnen Futtermittelbestandteilen. In einer umfassenden Vorvalidierungsstudie (Publication #2) haben wir gezeigt, dass zwischen einzelnen Futtermittelbestandteilen und komplexen Mischfuttermitteln sowie innerhalb komplexer Futtermittel signifikante Unterschiede, vor allem im Hinblick auf Matrixeffekte und scheinbarer Wiederfindungsraten, existieren. Um das Problem der Nicht-Verfügbarkeit eines passenden Probenmaterials, welches völlig frei von natürlicher Kontamination ist, zu umgehen und Variationen hinsichtlich der Zusammensetzung zu simulieren, wurden Mischfutterproben für Hühner- und Rinderfutter im Labor modelliert und mit realen Mischfutterproben verglichen und statistisch getestet. Die Ergebnisse zeigten, dass die Modellierung von Mischfuttermitteln einen geeigneten Ansatz darstellt, um einerseits ein vernachlässigtes Problem zu lösen und andererseits eine realistischere Abschätzung der Leistungsfähigkeit der Methode sicherzustellen. Die Methodenvalidierung wurde daher unter Verwendung von künstlich hergestelltem Probenmaterial durchgeführt, indem die relevantesten Einzelfuttermittelbestandteile für die jeweiligen Mischfutterproben kombiniert wurden.

Die Anwendbarkeit der Methode wurde in 2 Studien getestet, zunächst im Rahmen einer Untersuchungsstudie (auf 2 Publikationen aufgeteilt) zum Auftreten regulierter (Publication #3) und nicht regulierter Mykotoxine (Publication #4) in Mais, geerntet in Serbien (zwischen 2012 und 2015). Besonderer Fokus lag hierbei auf dem Einfluss sich ändernder Klimabedingungen auf das Kontaminationsmuster. In 204 Maisproben haben wir 109 verschiedene nicht regulierte sekundäre Pilzmetabolite nachgewiesen, wobei jede einzelne Maisprobe zwischen 13-55 Kontaminanten beinhaltete. Zusätzlich beobachteten wir einen signifikanten Einfluss der Wetterbedingungen auf das Auftreten von 20 regulierten Mykotoxinen und deren Derivaten. Aflatoxine waren die dominierenden Kontaminanten in Maisproben, die in sehr heißen und trockenen Jahren geerntet wurden. Im Gegensatz dazu zeigten Fumonisine eine sehr hohe Prävalenz (76-100%) in Proben aus jedem Jahr. Der zweite Anwendbarkeitstest wurde in einem Pilot-Set von realen Hühner- (n=68) und Rinderfuttermittelproben (n=64) durchgeführt, um erste Einblicke in Simultankontaminationen zu erhalten (Publication #1). Die Ergebnisse zeigten eine durchschnittliche Co-Kontamination von 56 Verbindungen in Hühner- und 45 Verbindungen in Rinderfutter, einschließlich Vertretern fast aller untersuchten

Substanzklassen. Bei 58% der Rinder- und 91% der Hühnerfuttermittelproben wurden hohe Kontaminationen von Phytoöstrogenen wie Daidzein und Genistein sowie Mykoöstrogenen wie Zearalenon und Alternariol beobachtet.

Zusammenfassend stellt diese Arbeit den ersten umfassenden quantitativen Ansatz für die Bestimmung mehrerer Substanzklassen dar, welches eine umfangreiche Überwachung von Co-Kontaminationen im Agrar- und Lebensmittelsektor ermöglicht. Expositionsmuster sind für wissenschaftliche Beratungsgremien von großem Interesse und bieten erste Einblicke in mögliche synergistische, additive oder antagonistische Effekte. Die Übertragbarkeit dieses Ansatzes auf andere Lebensmittelgruppen ermöglicht darüber hinaus die Durchführung eines umfassenden Gesamtexpositionsscreenings als Grundlage für die Beurteilung des menschlichen Exposoms.

# Introduction

The introduction part is divided into three major sections. The first section provides relevant information on the major chemical contaminants and residues from the agricultural sector, as well as an overview about the current legislative situation. The second section describes the impact of climate change scenarios to alterations in mycotoxin production and related consequences. The third section introduces the reader to the field of multiclass contaminant analysis, including state of the art techniques and their limitations, as well as challenges in method development and validation of such approaches.

# 1 Agricultural Contaminants

## 1.1 Definitions and General Aspects

Most food commodities contain synthetic or natural chemicals that may pose a toxic hazard to the consumer. Within food toxicology, these chemicals are classified as natural and anthropogenic contaminants (Nasreddine & Parent-Massin, 2002). Natural contaminants are unintentionally present in food and feed and can be formed during storage and processing. Most relevant representatives are mycotoxins, which are secondary metabolites formed by fungi, bacterial toxins, and natural toxins of plant origin. These compound groups, collectively referred to as biotoxins, have already been shown to trigger toxic, teratogenic, genotoxic and carcinogenic effects in animal models and in humans. On the other hand, anthropogenic contaminants, also commonly referred to as residues, are intentionally introduced to food and feed during the production such as pesticides, veterinary drugs, or as additives (Müller & Steinhart, 2007; Nasreddine & Parent-Massin, 2002).

Both, natural and anthropogenic contaminants are ubiquitous in the food and feed chain. Due to several food scandals in recent years, consumer awareness not only for high-quality but in particular for safe food which is free of any risk, has increased significantly. This has also enhanced the requirements at the producer´s level to ensure the absence of potentially harmful compounds in their products. Thereby, every contractor involved in the food chain is affected, commencing with the production of feed for livestock husbandry (Gerssen et al., 2019). For global food security, animal derived food sources are highly significant since they contribute 25% of protein and 18% of calorie intake worldwide (FAOSTAT, 2016). The global demand for milk and meat between 2005 and 2050 has led to a projected increase of 48% and 57% due to rising incomes in developing countries and fast-growing population (Alexandratos & Bruinsma, 2012; Kim et al., 2019). With constant feed efficiency, an expansion estimate of 21% livestock production between 2010 and 2025 demands an increase of the world feed supply from 6.0 to 7.3 billion tons of dry matter (Kim et al., 2019; Mottet et al., 2017). Therefore, adequate risk management and practical measures are necessary in order to reduce absorption and carry-over of noxious substances from feed to food of animal origin (milk, eggs, meat, and organs). Especially animal food products, produced on a day-to-day basis such as milk and eggs, show increased residue levels e.g. of pesticides, veterinary drugs, or biotoxins after prolonged exposure (Kan & Meijer, 2007). The most frequent and potentially harmful classes of agro-contaminants will be discussed in the following chapter.

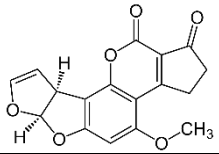
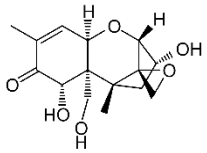
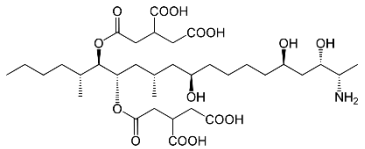
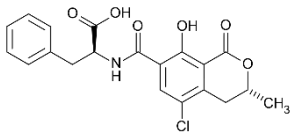
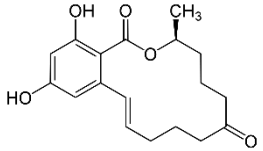
## 1.2 Major Classes of Contaminants

### 1.2.1 Mycotoxins

Mycotoxins are fungal secondary metabolites, which can lead to illness or even death in crops, animals, and humans (Pitt et al., 2012). All mycotoxins are natural products of low-molecular-weight, as their structural appearance ranges from single heterocyclic rings with ~50 Da to groups of multiple-rings with a molecular weight of >500 Da. Depending on several factors like age, gender, species, nutritional and health status, mycotoxins can pose acute toxicity (nausea, vomiting, death) or chronic toxicity (carcinogenic, mutagenic, nephrotoxic or cytotoxic effects) (Bennett & Klich, 2003; Pitt, 2000). Throughout history these toxic substances have caused severe epidemics in humans and animals. In 1960, aflatoxicosis was responsible for the death of 100,000 turkeys in the United Kingdom, causing further disease and death in other animals (Rodricks, J.V.; Hesseltine, C.W.; Mehlman, 1977); between 1942 and 1948, alimentary toxic aleukia (ATA) triggered by T-2 toxin, killed ~100,000 Russian people after they had eaten overwintered grain contaminated with *Fusarium sporotrichioides* (Joffe, 1978); and hundreds of thousands of people in Europe died in the last millennium due to ergotism caused by ergot alkaloids (Smith, J. E.; Moss, 1985).

Taking the global food commodities affected by mycotoxigenic fungi into account, three fungal genera stand out: *Fusarium, Penicillium,* and *Aspergillus*. *Fusarium* species are destructive pathogens mainly affecting cereal crops, producing their mycotoxins before or directly after harvest. In contrast, *Penicillium* and *Aspergillus* species are commonly associated with food commodities during storage and drying with exception of *Aspergillus flavus* which can produce mycotoxins under three conditions: as a pathogen, a commensal and as a storage fungus (Pitt, 2000; Pitt et al., 2012). Mycotoxin production depends on several factors such as climatic and storage conditions, including temperature, water activity and pH; agricultural practices like crop rotation and irrigation; to the crop varieties and to the fungi itself including factors like species, competing pressure and the degree of invasion (Magan & Aldred, 2007). Based on extensive analytical results and detailed information regarding the distribution of mycotoxigenic fungi in staple crops, there are only a few fungal toxins with agricultural importance: aflatoxins, trichothecenes, especially deoxynivalenol and nivalenol, fumonisins, as well as ochratoxin A and zearalenone (Miller, 1995). Table 1 provides an overview of selected mycotoxins with agricultural relevance including structural information, their main producing fungi and the toxicological profile.

**Table 1: Overview of mycotoxins with agricultural relevance**

| Mycotoxin | Fungal Origin | Toxic Effects | Opinions from EC* |
|---|---|---|---|
| **aflatoxin $B_1$**  | *Aspergillus* spp.<br><br>*A. flavus, A. parasiticus* | acute:<br>acute toxic effect in the liver<br><br>chronic:<br>carcinogenic and hepatotoxic | (EFSA Scientific Panel on Contaminants in the Food Chain, 2004, 2007; The Scientific Committee on Food, 1996) |
| **deoxynivalenol**  | *Fusarium* spp.<br><br>*F. graminearum, F. culmorum, F. cerealis* | acute:<br>vomiting, feed refusal<br><br>chronic:<br>immunotoxicity, anorexia | (EFSA Panel on Contaminants in the Food Chain, 2013, 2017b; European Food Safety Authority, 2013; The Scientific Committee on Food, 1999) |
| **fumonisin $B_1$**  | *Fusarium* spp.<br><br>*F. verticillioides, F. proliferatum, F. moniliforme, F. oxysporum* | porcine pulmonary oedema, equine encephalomalacia, nephrotoxicity, possibly oesophageal carcinoma | (EFSA Panel on Contaminants in the Food Chain, 2018b, 2018a; The Scientific Committee on Food, 2000b) |
| **ochratoxin A**  | *Aspergillus* spp., *Penicillium* spp.<br><br>*A. ochraceus, A. carbonarius, A. niger, P. verrucosum* | nephrotoxic, hepatotoxic, neurotoxic, teratogenic, immunotoxic, possibly carcinogenic | (EFSA Panel on Contaminants in the Food Chain, 2020; EFSA Scientific Panel on Contaminants in the Food Chain, 2006) |
| **zearalenone**  | *Fusarium* spp.<br><br>*F. graminearum, F. culmorum, F. cerealis, F. equiseti* | oestrogenic, haematotoxic, hepatotoxic, immunotoxic, genotoxic, possibly carcinogenic | (EFSA Panel on Contaminants in the Food Chain, 2011b, 2017a; The Scientific Committee on Food, 2000a) |

* EC = European Commission

## Aflatoxins

The most important aflatoxin producing fungi are *Aspergillus parasiticus* and *Aspergillus flavus*. With aflatoxin $B_1$, these fungi produce the most potent liver carcinogen known (Khlangwiset et al., 2011). Based on the carcinogenic potential of aflatoxin $B_1$, human exposure levels should be decreased as low as reasonably achievable. Various susceptible plant-derived food commodities (including milk for its hydroxylated metabolite aflatoxin $M_1$) are addressed by current EU legislation. Milk is of particular relevance, since a contamination with aflatoxin $M_1$ can occur following exposure of lactating cattle to aflatoxin $B_1$ present in compound feed. Aflatoxin carry-over model calculations from feed into milk revealed aflatoxin $M_1$ levels might exceed the current statutory limits under circumstantial maximum exposure. Imported feedstuff such as maize, sunflower cake, rice bran, or soy beans are among the most important carriers of aflatoxin $B_1$ (EFSA Scientific Panel on Contaminants in the Food Chain, 2004).

The formation of aflatoxins can occur before and after harvest and they are ubiquitous in a broad range of food and feed commodities, especially nuts (peanuts, brazil nuts and other tree nuts) and grains (maize, wheat, barley and rice) (Pitt et al., 2013). Figure 1 illustrates the pattern of development of aflatoxins in maize.

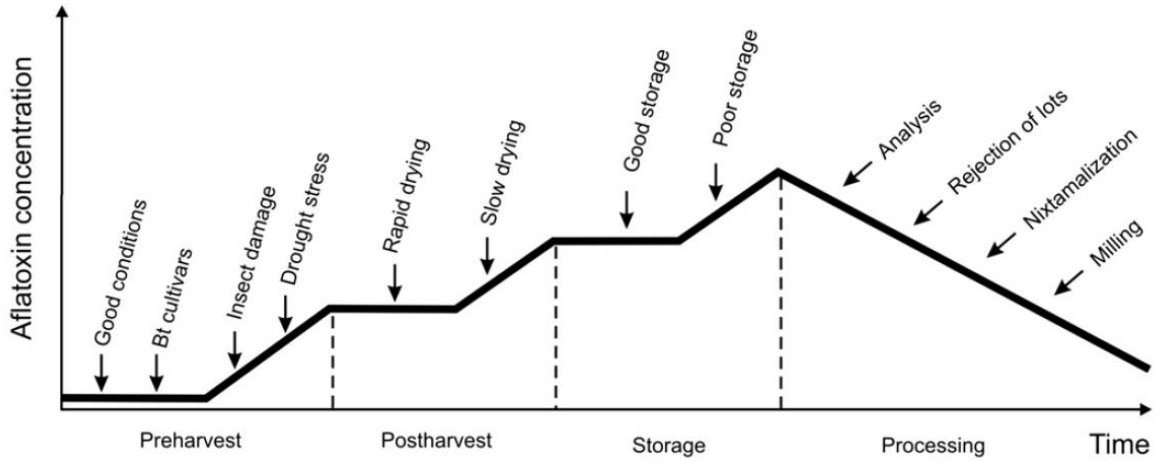



*Figure 1: Time course of aflatoxin production and reduction in maize. Nixtamalization is a process usually applied in Central America for the preparation of maize meals as part of the tortillas production. Aflatoxins are destroyed during this process. (Pitt et al., 2013)*

Within preharvest conditions, insect damage and drought stress are triggers of aflatoxin production. Preventing insect damage by using Bt maize cultivars decreases the formation of aflatoxins significantly (Dowd, 2009). To keep aflatoxin levels low during the postharvest period, rapid drying is essential. Furthermore, well-constructed silos are necessary to prevent moisture migration during storage. Maize lots with excessive aflatoxin contamination have to be rejected in order to control aflatoxin levels and meet food safety objectives (Pitt et al., 2013).

**Trichothecenes**

*Fusarium graminearum* and *Fusarium culmorum* are the main sources of the major type B trichothecenes deoxynivalenol (DON) and nivalenol (NIV). In contrast to aflatoxins, these toxins do not appear to be carcinogenic but cause a variety of immunological effects. Additionally, DON causes feed refusal in pigs at levels near 8 mg/kg of feed and anorexia, nausea, vomiting, abdominal pain, and diarrhoea in humans (Pitt, 2000). In farm and companion animals, forage maize and cereal grains are the main contributor for exposure. Humans in contrast are mainly exposed by grains and grain-based products. Absorption, distribution and excretion of DON is rapid. Similar toxic effects to DON can be expected by its precursors 3-acetyldeoxynivalenol (3-Ac-DON) and 15-acetyldeoxynivalenol (15-Ac-DON) since they are largely deacetylated, as well as by deoxynivalenol-3-glucoside (DON-3-glucoside) which is cleaved in the intestines. Therefore, a group tolerable daily intake (TDI) of 1 µg/kg for the sum of DON, 3-Ac-DON, 15-Ac-DON and DON-3-glucoside was established (EFSA Panel on Contaminants in the Food Chain, 2017b).

**Fumonisins**

Fumonisins are produced by several *Fusarium* species, mainly from *F. verticillioides and F. proliferatum*. In the U.S.A., China, Europe, South America and southern Africa, fumonisins are very common corn contaminants with three main naturally occurring forms fumonisins $B_1$, $B_2$ and $B_3$ (Miller, 1995). The toxic mode of action of fumonisins is directed to the sphingolipid metabolism based on a competition with sphingosine. In horses and other equine species, fumonisins cause leukoencephalomalacia (LEM) leading to liquefactive necrotic lesions in the white matter of the cerebral hemispheres (Pitt, 2000). In humans, several clinical effects, such as oesophageal cancer, liver cancer or growth impairment have been discussed, but a causal connection with fumonisin exposure has never been proven. Hence, a dose-response analysis was conducted in order to establish a TDI which is set at 1 µg $FB_1$/kg bw per day (EFSA Panel on Contaminants in the Food Chain, 2018a).

**Ochratoxin A**

The toxicological profile of ochratoxin A (OTA) includes immunosuppressive, embryonic, and potential carcinogenic effects. Furthermore, ochratoxin A is an acute nephrotoxin causing necrosis of renal tubules and periportal liver cells (Pitt, 2000). Ochratoxin A can be produced

by *Aspergillus* and *Penicillium* species, including *A. ochraceus*, *A. carbonarius*, *A. niger*, *P. verrucosum* and is often found together with other toxins such as citrinin (Miller, 1995). Human exposure to ochratoxin A occurs from cereals and cereal products, beer, wine, grape juice, cocoa and cocoa products, brewed coffee and pork meat. In 2006 a TDI of 17 ng/kg bw per day was established, but is no longer valid due to neoplastic effects of OTA. A margin of exposure approach will therefore be conducted for risk characterisation (EFSA Panel on Contaminants in the Food Chain, 2020).

**Zearalenone**

Zearalenone (ZON) is a potent oestrogenic toxin due to its binding affinity to estrogen receptors. It is primary produced by *F. graminearum* and *F. culmorum* and mainly occurs in maize and in addition in wheat grains and barley. Pigs show a susceptibility to ZON leading to genital problems such as oedematous swelling of the vulva, or prolapse of the vagina and rectum. In humans, precocious pubertal changes in children have been described (Miller, 1995; Pitt, 2000). A TDI of 0.25 µg ZON/kg bw per day was derived from a 10 µg/kg bw no observed effect level for oestrogenic effects in immature piglets (EFSA Panel on Contaminants in the Food Chain, 2017a).

**Mycotoxins covered in the developed multiclass method**

In total 739 secondary fungal metabolites were implemented in the final multiclass approach including all mycotoxins (aflatoxin $B_1$, $B_2$, $G_1$, $G_2$, deoxynivalenol, fumonisins $B_1$, $B_2$, HT-2 toxin, T2 toxin, ochratoxin A, zearalenone, citrinin and patulin) with an existing or recommended regulatory limit based on Commission Regulation (EC) 1881/2006 (European Commission, 2006a), as well as its masked forms such as deoxynivalenol-3-glucoside or zearalenone-14-glucoside and ergot alkaloids. To the best of our knowledge, this high number of secondary fungal metabolites within one method represents the most comprehensive analytical scope with respect to this substance class and was originally developed by Sulyok et al. in 2006 (Sulyok et al., 2006) for the determination of all regulated mycotoxins in cereal based materials. This method has been applied successfully in routine operations ever since and served as the basis for the further development of the multiclass approach.

### 1.2.2 Natural Toxins of Plant Origin

Phytotoxins, or plant toxins, are secondary plant metabolites which protect the plant from bacteria, fungi and herbivores as a chemical defence. These substances can lead to anti-nutritional effects and may exhibit chronic or acute toxicity. Plant toxins which are present in edible crops are so called inherent plant toxins, in addition phytotoxins may enter the food and feed chain by contamination of crops by non-edible plants (Mol et al., 2011). Inherent plant toxins include e.g. cyanogenic glycosides such as linamarin and lotaustralin present in cassava, or glycoalkaloids such as solanine and chaconin in potatoes (Speijers et al., 2010). Pyrrolizidine alkaloids are one of the most comprehensively described representatives of phytotoxins, because they exert teratogenic, hepatotoxic, genotoxic and carcinogenic effects. They occur as inherent plant toxins in herbal medicine and show a high global abundance of food and feed contamination (Wiedenfeld & Edgar, 2011).

**Pyrrolizidine alkaloids**

Pyrrolizidine alkaloids (PA) belong to the families of Asteraceae with its most important members *Jacobeae vulgaris* (ragwort) and *Senecio vulgaris* (common groundsel), as well as Boraginaceae and Fabaceae. About 3% of all flowering plants worldwide may contain PAs and their content in plant material depends on several factors, such as harvest, storage, species and plant organ. The structure of PAs is characterised by a necine base in form of an amino alcohol and an acid part which is called a necic acid (EFSA Panel on Contaminants in the Food Chain, 2011a). Exposure in humans occurs by the use of herbal products, like comfrey, as they are applied in traditional medicines and honey where the contamination occurs via transfer of contaminated plant pollen by bees. An additional source of PAs exposure is milk as it has already been shown to contain these toxic substances in samples from milk-producing animals fed by PA-containing plant material (Wiedenfeld & Edgar, 2011). Overall carry-over rates for PAs from feed to milk are at 0.1%, but for specific PAs such as jacoline this figure can be up to 7%. Although PAs have been recognised as noxious substances for livestock and eventual concentrations e.g. of jacoline in milk may present a considerable risk for consumer, the European Commission has not yet established maximum levels for PAs in animal feedstuff (Hoogenboom et al., 2011).

**Phytoestrogens**

Another widely distributed class of substances with plant origin are the polyphenolic non-steroidal phytoestrogens (PE) which do not pose a direct toxic threat, but also play an important role in public health due to their estrogenic effectiveness. The definition of PEs refers to substances that trigger estrogenic effects on the central nervous system, stimulate growth of the genital tract of female animals and induce estrus (Kurzer & Xu, 1997). Phytoestrogens are compounds that exert long-term effects on human and animal health, the most important representatives are the chickpea isoflavone biochanin A, the soy isoflavones daidzein and genistein, the isoflavonoid-derived coumestan coumestrol, as well as the clover isoflavone formononetin and the flaxseed lignans (Dixon, 2004). PEs are of particular interest since combinations of the phytoestrogen genistein with mycoestrogens, such as zearalenone and alternariol showed interactive effects *in vitro*. How far interactions influence consumer's health is not yet fully discovered. However, it is certain that co-exposure between phyto- and mycoestrogens as native food constituents is a realistic scenario and these interactions should be incorporated into current risk assessment procedures (Vejdovszky et al., 2017).

**Natural toxins covered in the developed multiclass method**

Natural toxins covered by the multiclass approach included 47 plant toxins and 15 unspecific bacterial metabolites. The group of plant toxins is subdivided into pyrrolizidine- and tropane alkaloids, cyanogenic glycosides, phenolic phytohormones and unspecific plant toxins.

### 1.2.3 Pesticides

In addition to naturally occurring toxic substances, chemical compounds which are deliberately applied to agricultural crops, such as pesticides, cause increasing concern among consumers. To maintain high crop yields, agricultural industry is in favour of using chemicals as an integral part of agricultural production and modern farming practices. In the conventional sense, pesticides are chemical compounds used to control, mitigate, repel, or kill any pest. Pesticides are a co-formulation of one or more active ingredients with other materials (Margni et al., 2002). The classification of pesticides can either be made based on the way these substances enter the target, including contact, systemic, fumigants, stomach poisons, and repellents, or based on the

pesticide function and the pest organism they kill (Yadav & Devi, 2017). An overview of pesticide classification based on target pests is provided in Table 2.

**Table 2: Target based classification of pesticides (modified after Leong et al., 2020)**

| Pesticide class | Target pests/functions | Example |
|---|---|---|
| Acaricides | kill mites that feed on plants and animals | bifenazate |
| Algaecides | inhibit algae growth | diuron |
| Avicides | used against birds | avitrol |
| Bactericides | kill bacteria or acts against bacteria | copper complexes |
| Desiccants | remove water from plants | silica |
| Fungicides | kill fungi (including blights, mildews and molds) | azoxystrobin |
| Herbicides | kill weeds and other plants | atrazine |
| Insecticides | kill insects and other arthropods | acephate |
| Larvicides | inhibits growth of larvae | methoprene |
| Molluscicides | inhibit or kill molluscs (snails, and slugs) | methiocarb |
| Moth balls | prevention against moth larvae | p-dichlorobenzene |
| Nematicides | kill nematodes that act as parasites of plants | aldicarb |
| Ovicides | inhibits the growth of eggs of insects and mites | benzoxazin |
| Piscicides | used against fishes | rotenone |
| Repellents | repel pests by its taste or smell | permethrin |
| Rodenticides | used against rodents (rats, and mice) | flocoumafen |
| Termiticides | kill termites | fipronil |
| Virucides | used against viruses | scytovirin |

About 500 active substances are approved within the European Union for use as agricultural pesticides, with fungicides, herbicides and insecticides as the most common (Silva et al., 2019). Although pesticide use has a benefit to the agricultural output, these chemical substances also exert negative impacts on the environment and on humans. In particular long-term effects of pesticides through transfer to the natural environment or ingestion of contaminated foodstuff raise major concerns within the general public. These concerns are not unfounded, as the use of pesticides has increased dramatically in recent decades and continues to increase in most countries (Steingrímsdóttir et al., 2018). The world average yields of maize, wheat and rice more than doubled since 1960, as the use of pesticides increased by 15 to 20-fold. Annually, about 3 million tons of pesticides are applied worldwide, representing a corresponding market value of USD 40 billion (Silva et al., 2019). An overview about the agri-environmental indicator on the use of pesticides per area of cropland at national level for the period of 1990 and 2017 is shown in Table 3.

**Table 3: Agri-environmental indicator on the use of pesticides per area of cropland at national level for the period 1990 to 2017 for selected countries (FAO, 2020)**

| Country | 1990 | 2017 | Absolute Change | Relative Change |
|---|---|---|---|---|
| | *in kg/hectare (ha)* | | *in kg* | *in %* |
| Africa | 0.31 | 0.29 | - 0.02 | - 6 |
| Argentina | 0.95 | 4.88 | + 3.93 | +414 |
| Asia | 2.12 | 3.67 | + 1.55 | + 73 |
| Australia | 1.04 | 2.04 | +1.00 | + 96 |
| Austria | 2.82 | 3.34 | + 0.52 | + 18 |
| Brazil | 0.88 | 5.95 | + 5.07 | + 576 |
| Canada | 0.71 | 2.37 | + 1.66 | + 234 |
| China | 5.87 | 13.07 | + 7.20 | + 123 |
| East Asia | 6.14 | 12.74 | + 6.60 | + 107 |
| Europe | 1.34 | 1.65 | + 0.31 | + 23 |
| European Union | 3.24 | 3.09 | - 0.15 | - 5 |
| France | 5.14 | 3.63 | - 1.51 | - 29 |
| Germany | 2.52 | 4.03 | + 1.51 | + 60 |
| Ireland | 1.93 | 6.47 | + 4.54 | + 235 |
| Israel | 5.78 | 12.61 | + 6.83 | + 118 |
| Italy | 8.40 | 6.14 | - 2.26 | - 27 |
| Japan | 15.22 | 11.76 | - 3.46 | - 23 |
| Netherlands | 10.70 | 7.90 | - 2.80 | - 26 |
| New Zealand | 1.30 | 7.89 | + 6.59 | + 507 |
| Northern America | 1.88 | 2.51 | + 0.63 | + 34 |
| Oceania | 1.03 | 2.09 | + 1.06 | + 103 |
| Portugal | 2.99 | 5.44 | + 2.45 | + 82 |
| South America | 1.13 | 5.42 | + 4.29 | + 380 |
| South Korea | 11.89 | 12.37 | + 0.48 | + 4 |
| Spain | 1.96 | 3.59 | + 1.63 | + 83 |
| Taiwan | 11.18 | 13.30 | + 2.12 | + 19 |
| Turkey | 1.08 | 2.31 | + 1.23 | + 114 |
| United Kingdom | 4.41 | 3.24 | - 1.17 | - 27 |
| United States | 2.14 | 2.54 | + 0.40 | + 19 |
| World | 1.54 | 2.63 | + 1.09 | + 71 |

The use of pesticides (in total 1,357 applied substances) per area of cropland increased worldwide by 71% between 1990 and 2017 (FAO, 2020). Countries with the highest amounts of pesticides applied per unit of cropland in 2017, measured in kilogram per hectare, are located in East Asia including Taiwan (13.30 kg/ha), China (13.07 kg/ha), South Korea (12.37 kg/ha) and Japan (11.76 kg/ha). Between 1990 and 2017 the amounts of pesticides used within the agricultural sector in Eastern Asia increased by 107%. In contrast, the use of pesticides per unit cropland was reduced by 5% within the European Union within in the same time period. A graphical illustration about the applied pesticides per unit cropland between 1990 and 2017 is illustrated in Figure 2.

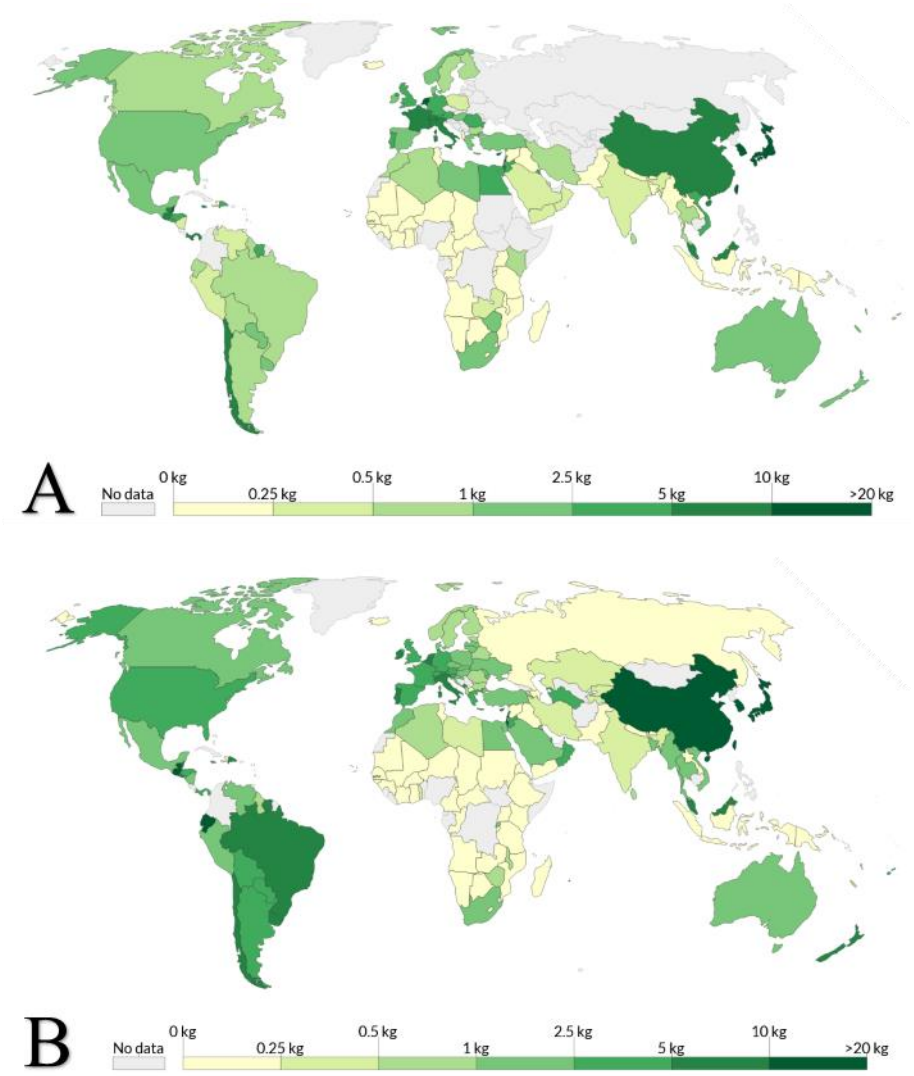



Figure 2: Average pesticide application per unit of cropland, measured in kilograms per hectare between 1990 (A) and 2017 (B) *(FAO, 2020)*

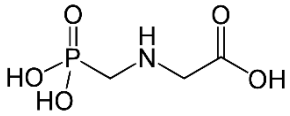
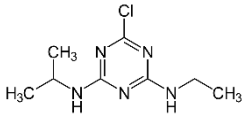
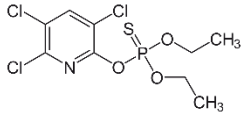
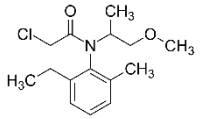
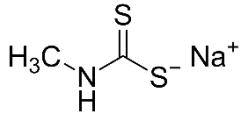
Pesticide exposure can take place in several ways, e.g. when pesticides are used within our environment, when people work with pesticides and when pesticides are applied for food preservation (Bonner & Alavanja, 2017). Based on the estimates from the World Health Organization (WHO), about 3 million cases of pesticide poisoning occur every year and of these, 220,000 fatalities are recorded (Kumar et al., 2012). Acute pesticide poisoning includes typical symptoms like nausea, vomiting, headache, tremor, panic attacks, circulatory problems etc. and an explicit diagnosis requires a scientific analysis of urine and blood pesticide residues, since these symptoms are also imputed to other illnesses. Beside acute poisoning, pesticides also trigger chronic illnesses and weaken the immune system after exposure over a prolonged period, even under comparatively low concentrations. Agricultural workers are at especially high risk, but the general population is also affected, since pesticides drift from the field via pollution routes, resulting in contaminated foodstuff (Laumann, 2012).

Currently, many commonly used pesticides are classified as probably or possibly carcinogenic for humans (Leong et al., 2020). Some pesticides such as organochlorines, sulfallate and creosote are labelled as carcinogenic based on the outcome of several animal studies, while other pesticides such as lindane, chlordane and DDT are identified as tumour promoters (Dich et al., 1997). Results of epidemiological studies revealed positive correlation of pesticide exposure with malignancies like non-Hodgkin lymphomas and leukaemia towards agricultural workers, breast cancer for women with elevated blood levels of DDE, as well as multiple myelomas, sarcomas, cancer of testicles, pancreas, prostate, lungs, kidneys, ovaries, intestines, liver and brain tumours, while brain and prostate cancer showed the most consistent correlation amongst those (Leong et al., 2020). Additionally, clear evidence exists between the exposure of pesticides and the formation of neurological damage. This is particularly true for the connection between pesticide exposure and the occurrence of disruptions in psychomotoric and cognitive functions, as well as depression and anxiety (Beseler & Stallones, 2008; Laumann, 2012; Leong et al., 2020). Table 4 provides an overview of the most common pesticides used in the US and their health effects.

**Pesticides covered in the developed multiclass method**

The final approach covered 504 EU regulated pesticides from various groups e.g. carbamates, organophosphates, neonicotinoids, or pyrethroids and 3 (atrazine, chlorpyrifos, metolachlor) of the 5 mainly applied pesticides in the US (Table 4). The implementation of relevant persistent organic pollutants (POP) was not feasible, since these substances show low compatibility to liquid chromatography based analytical techniques. Therefore, 12 priority substances (aldrin, chlordane, dieldrin, dichlorodiphenyltrichloroethane, endrin, heptachlor, hexachlorobenzene, mirex, polychlorinated biphenyl, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, toxaphene), also known as the “dirty dozen”, which are banned by the POP convention of 2004 (United Nations Environment Programme, 2001) are not included in the final scope. In addition, very polar compounds such as glyphosate and its metabolites (glufosinate, AMPA) were excluded as well. In order to ensure an accurate and reliable determination of these substances, a derivatisation step prior extraction would be necessary. This step would hamper the applicability of a generic extraction protocol.

**Table 4: Most commonly used pesticides in the US and their potential negative effects (modified after Leong et al., 2020)**

| Pesticides | Application | Health Effects |
|---|---|---|
| **glyphosate** (organophosphate)  | used on maize, soy grain, canola, barley, wheat, cotton, home gardens and parks | ▪ mitochondrial metabolism disruptions<br>▪ cellular oxidative stress<br>▪ chronic kidney and liver diseases<br>▪ endocrine system disruption leading to reproductive system impairment, cancer and birth defects |
| **atrazine** (triazine)  | most commonly used weed killer to protect maize, sorghum, pines, sugarcane | ▪ reproductive system toxicity<br>▪ birth defects and cancer, especially breast and prostate cancers<br>▪ miscarriages<br>▪ endocrine disruption |
| **chlorpyrifos** (organophosphate)  | applied in home and garden settings, also used in almonds, apples, cotton, oranges, and corn crops | ▪ low-dose exposure can cause cholinesterase inhibition leading to respiratory system intoxication<br>▪ severe conditions affect central nervous system and can cause arrhythmias, muscle paralysis and coma<br>▪ death can occur due to cardiac arrest or respiratory failure |
| **metolachlor** (chloroacetanilide)  | used on sorghum, corn, soybean, potatoes, peanuts, lawns, stone fruits, safflower | ▪ symptoms of intoxication include nausea, diarrhoea, short breath, convulsions, dark urine, and jaundice<br>▪ cause liver damage, eyes, skin and mucous membranes irritation<br>▪ possibly carcinogenic |
| **metam sodium** (dithiocarbamate)  | used as fumigant on potatoes | ▪ allergic dermatitis<br>▪ respiratory allergy like asthma<br>▪ birth defects<br>▪ mutagenic |

**Reduction in Pesticide Efficacy**

The pesticide level on the plant, which depends on the product's formulation, dose, concentration and application procedure determines the plant protection. As the pesticide interaction mainly occurs with the plant surface, pesticides are exposed to rainfall, sun radiation, wind and other environmental factors. The effectiveness and availability of pesticides are affected by absorption, penetration, transport (volatilization, wash and runoff processes) and degradation (photolysis, microbial breakdown and detoxification) processes. (Figure 3) These factors subsequently depend on environmental conditions including fluctuations in precipitation and temperature variations (Delcour et al., 2015; Matzrafi, 2019). Furthermore, an enrichment of atmospheric $CO_2$ increases the pest related damage, as the herbicide sensitivity of some weed species is reduced and the competitiveness level increases (Varanasi et al., 2016).

Beside pesticide dissipation, especially the ecotoxic potential of pesticides will determine the future application since the efficacy will be diminished due to climate change scenarios (Delcour et al., 2015).

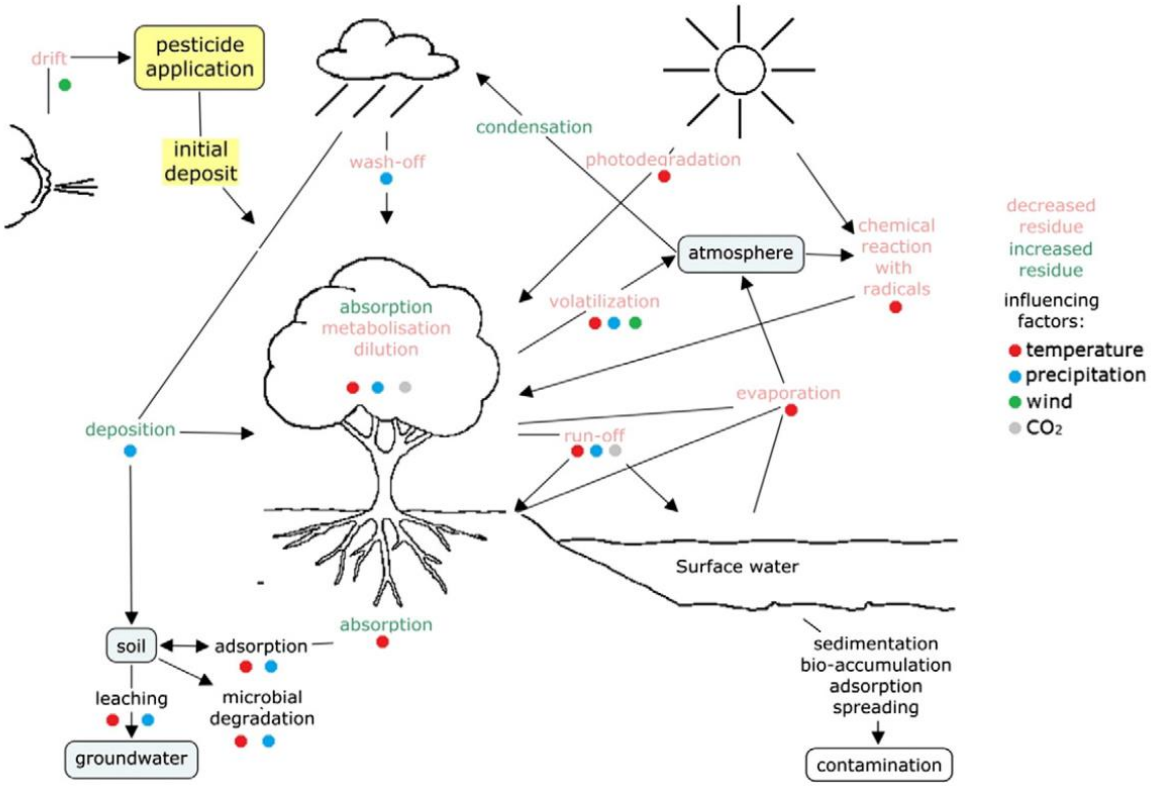



*Figure 3: Environmental factors influencing pesticide activity (Delcour et al., 2015)*

Dry conditions in particular hamper the activity of many pesticides, indicating higher dose or concentration levels of applied pesticides are needed, or the frequency of application has to be increased. It is more than likely, that these practices pose a higher risk for humans to be exposed

to pesticidal residues in their food. This is compounded by the fact that soil organic matter will decrease and evaporation rates increase with elevated temperatures, resulting in an increased root uptake of pesticides, which would otherwise remain bound to organic matter (Miraglia et al., 2009). Varying levels of $CO_2$ and temperature are both associated with several cases of conditional resistance. This is based on a reduction in the sensitivity of the pest to the pesticide due to changing environmental conditions. (Table 5)

**Table 5: Influence of climate change on pesticide sensitivity (modified after Matzrafi, 2019)**

| Pest species | Pesticide | Environmental factors |
|---|---|---|
| *Anopheles arabiensis* | deltamethrin, $\lambda$-cyhalothrin | Reduced efficacy under elevated temperatures 30/35° C |
| *Frankliniella occidentalis* | avermectin | Reduced efficacy under elevated temperatures (21 < 26 < 33° C) |
| *Nilaparvata lugens* | triazophos | Reduced efficacy under enriched $CO_2$ levels, from 375 to 750 ppm |
| *Alopecurus myosuroides* | diclofop-methyl, pinoxaden | Reduced efficacy under elevated temperatures, 28/34° C |
| *Amaranthus palmeri* | mesotrione | Reduced efficacy under elevated temperatures (25 < 32.5 < 40° C) |
| *Conyza canadensis* | glyphosate | Reduced efficacy under elevated temperatures (16 < 22 < 28 < 34° C) |
| *Kochia scoparia* | glyphosate, dicamba | Reduced efficacy under elevated temperatures (17.5 < 25 < 32.5° C) |
| *Chenopodium album* | glyphosate | Reduced efficacy at enriched $CO_2$ levels from 360 to 720 ppm |

Currently, two resistance mechanisms are known. The target-site mode of action includes gene amplifications, which increase the binding niches and thus decreases pesticide efficacy, as well as gene mutations resulting in structural modifications in the pest organism binding site leading to reduced pesticide affinity. Changing environmental conditions may lead to response alterations influencing upstream related genes like transcription factors and signal transductors. An even more complex mode of action is associated with the non-target site resistance including detoxification processes such as conjugation, oxygenation and reduction of pesticides. Pesticide detoxification, for instance, can be increased due to higher enzymatic efficacy, triggered by elevated temperatures (Matzrafi, 2019). Future pesticide use will be adapted on the presence and magnitude of pests, weeds and crop diseases, which are inevitably influenced by environmental changes. In order to provide a transparent insight to actual and future use levels of pesticides at the international level, a harmonized monitoring program, based on state-of-the-art analytical methods, is mandatory (Miraglia et al., 2009).

### 1.2.4 Veterinary Drugs

The quality and nature of food is directly related to human health. In particular the quality of animal derived food products is increasingly concerning public health agencies, as the use of veterinary drugs (VD) in the field of agro-industry and animal husbandry has started to play an important role. In this context, food contamination with veterinary drug residues and especially antibiotic resistance have become the key issues. In order to provide adequate amounts of food for a growing world population, the use of veterinary medicinal products is essential, as drugs improve feed efficiency, weight gain and prevent diseases in food producing animals (Beyene, 2015). The most widely applied class of veterinary drug on a global level are antimicrobials, which are licensed in many jurisdictions and currently used for therapeutic treatment in veterinary medicine. These includes e.g. penicillins, tetracyclines, aminoglycosides, sulfonamides, amphenicols, arsenicals and nitroimidazoles. Beside antimicrobials, non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of acute and chronic inflammatory pain; beta agonists as partitioning agents in food animals to cause growth modifications; and antiprotozoals such as coccidiostat feed additives (Baynes et al., 2016; Danaher et al., 2016). If residues of veterinary drugs or their metabolites remain in animal foodstuff, they can cause adverse and even toxic effects on consumers' health. Intoxications after the consumption of meat containing residues of e.g. clenbuterol triggered symptoms like nausea, headache, dizziness, tachycardia and gross tremors of the extremities. Furthermore, the presence of mainly antibiotic residues may cause allergic reactions and negatively impacts on the human intestinal microflora.

Beside misuse and abuse of pharmacological active agents, a variety of routes exist for violative veterinary drugs to enter the food chain. The major problems are cross-contamination scenarios occurring in feed mills, where medical meal residuals maybe retained at different points along the production line, resulting in contamination of meal batches prior processing. Cross-contamination of feedstuff can also occur in trucks used to transport both non-medicated and medicated feed, as well as through insufficient purging of feeding system when medicated feedstuff is replaced by withdrawal feeds. Even this low contamination of medical residuals is sufficient to accumulate violative veterinary drugs in milk, eggs or tissues from food producing animals fed with contaminated feeds (Glenn Kennedy et al., 2000). In a study carried out in Northern Ireland, the extent of the animal feed contamination with undeclared antimicrobial additives was investigated. In 71 (44.1%) of 161 feed materials which were declared to be free of medication by the manufacturers, antimicrobials were detected, 26.1% of those in quantifiable concentrations. The most common detected antimicrobials were chlortetracycline

(15.2%), sulfonamides (6.9%), penicillin (3.4%) and ionophores (3.4%). While the detected concentrations for chlortetracycline were unlikely to cause residues in animal meat, the amounts of sulfonamides were sufficient to cause violative tissue residues (>100 µg/kg) if the animals are fed directly prior to slaughter (Lynas et al., 1998).

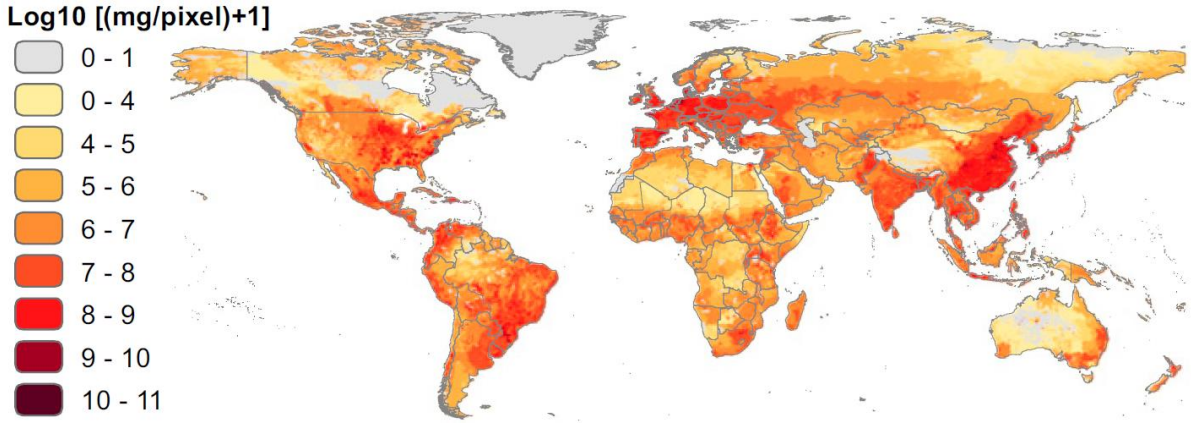



*Figure 4: Worldwide antimicrobial consumption in livestock in mg per 10 km$^2$ pixels (modified after Van Boeckel et al., 2015)*

Overall antimicrobial consumption trends in food producing animals are projected to rise by 67% between 2010 (63,151 ± 1,560 tons) and 2030 (105,596 ± 3,605 tons). The expected ranking will be China (30%), the United States (10%), Brazil (8%), India (4%) and Mexico (2%). The highest projected increase in antimicrobial agent consumption will occur in Myanmar (205%), Indonesia (202%), Nigeria (163%), Peru (160%) and Vietnam (157%). Estimated antimicrobial agent consumption growth for animals in BRICs countries (Brazil, Russia, India, China and South Africa) is at 99% (Ibrahim et al., 2020). Major reasons for this increasing trend are the growing number of animals raised for food production (66%) and a shift in farming practises (34%) towards intensive farming systems by 2030. Furthermore, significant differences in antimicrobial consumption patterns are observed and reveals important geographical heterogeneity across continents (Figure 4). Thus, antimicrobial hotspots appear in South and Southeast Asia in the southeast coast of China's Sichuan and Guangdong regions, in the Red River delta in Vietnam, in Mumbai and Delhi, as well as the south coast of India and in Thailand including the northern suburbs of Bangkok. The highest consumption of antimicrobials within America occurs in the suburbs of Mexico City, in the south of Brazil and midwestern and southern parts of the United States. Within the European Union, antimicrobial consumption is basically associated with chicken production. Hotspots are observed in the Netherlands, in Flanders (Belgium), the Po Valley in Italy and in Brittany (France). Notable hotspots in Africa were observed in Johannesburg (South Africa) including its surrounding townships and the Nile delta. Without regulatory actions, the compound annual growth rate

(2.60%) will exceed the projected annual growth rate of human population (0.98%) by almost threefold (Van Boeckel et al., 2015).

## Antimicrobial Resistance

As described above, in terrestrial animal feeding the use of antibiotics, growth promoters and other pharmacological active agents is necessary to maintain animal health and productivity and ensure food and feed safety, as well as public health. However, these benefits might reverse as the growing resistance of antimicrobials is rising as a global concern (Morley et al., 2005), since resistant bacteria could be transferred to humans through the food chain (Reig & Toldrá, 2008). For 70 years the use of veterinary drugs in animal feed has been widely spread, not only for disease treatment, but also to improve feed utilization and boost growth. However, veterinary drug residues may enter the feed chain by using feed ingredients of animal origin like in aquacultures, or by carry-over of veterinary drugs in feed during production. The compliance with the Code recommendations including flushing, sequencing and cleaning during feed manufacture after the production of medicated feed is therefore important. Additionally, the illegal use of banned substances such as chloramphenicol or nitrofurans, as well as the disease preventive application of high quantities of veterinary drugs may result in critical and unsafe amounts of residues in animal products such as milk, eggs or meat (FAO, 2010). Since farming animals are treated with medication based on the same active ingredients as those we rely on for humans, the partially nonessential use of veterinary drugs is accelerating the "use up" of effectiveness of these agents (CDDEP, 2016).

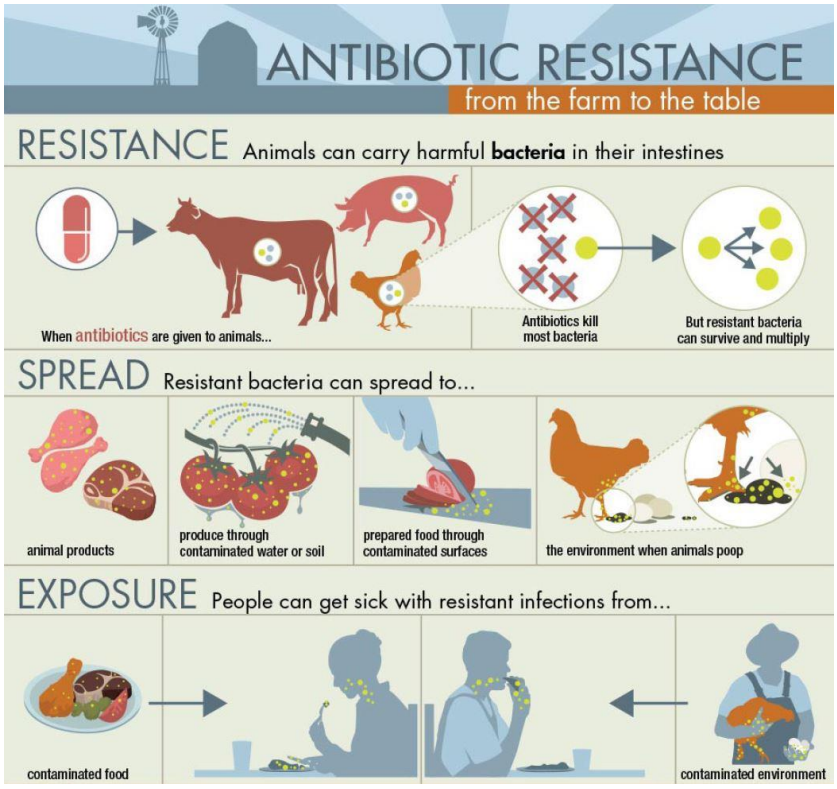



*Figure 5: Antibiotic resistance from farm to table (CDDEP, 2016)*

Livestock feeding with feed additives like growth promoters or antibiotics cause the development of resistant bacteria. (Figure 5)

The resistance to the action of antimicrobial drugs can be based on the bacterial physiology or its inherent structure (constitutive resistance), or can be acquired through developing mechanisms to circumvent the drug action by genetic mutations (acquired resistance). Resistant bacteria can be transferred by the movement of carrier animals between herds, through environmental exposure such as contaminated soils and facilities, through the exposure of feed and water, or the transmission through vectors like insects, birds and wildlife. Finally, the consumption of exposed animal products by humans lead to infections of resistant bacterial organisms and pose a serious threat to public health (CDDEP, 2016; Morley et al., 2005).

Between 2000 and 2018, the fraction of antimicrobials with resistance >50% (P50) increased from 0.12 to 0.23 in cattle, from 0.13 to 0.34 in pigs and from 0.15 to 0.41 in chickens in low- and middle-income countries (LMICs). Across LMICs it is estimated that about 9% of cattle, 18% of pigs, and 21% of chickens were raised in antimicrobial resistance hotspots in 2013. (Van Boeckel et al., 2019). Due to this threatening scenario, the FAO is working together with several stakeholders on programs to minimize antimicrobial use and implement their plans in the feed sector. Beside Good Husbandry and Good Hygiene Practice, disease control measures are very important. Based on the Codex Alimentarius code of practice to minimize and contain antimicrobial resistance (CAC/RCP 61-2005), future surveillance programs should be harmonised at an international level, including laboratory techniques to detect and quantify these compounds, sampling procedures and the careful choice of relevant microorganisms and veterinary drugs. An important role is assigned to officially recognised control laboratories as they are responsible for monitoring maximum residue limits of regulated veterinary drugs and control their approved application (FAO, 2005).

**Veterinary drugs covered in the developed multiclass method**

The multiclass method included 162 veterinary drugs from 12 major sub-substance classes (antiparasitics, antimicrobials, sulfonamides, coccidiostats, non-steroidal anti-inflammatory drugs, cephalosporines, ß-lactams, corticosteroids, tetracyclines, macrocyclic lactones, amphenicols and penicillins).

A comprehensive overview about the final scope (in total 1,467 analytes) of the multiclass method can be found in the online supplementary table S1 of Publication #1, at: https://ars.els-cdn.com/content/image/1-s2.0-S0021967320307779-mmc1.pdf

## 1.3 Legislation and Notification System

Due to several food affairs in recent years, European citizens have developed an increased awareness about food safety, the food production and the protection measures of Member States. Basically, three major food scandals in the late twentieth century have raised food safety concerns among European consumers, including the bovine spongiform encephalopathy (BSE) in British beef, the *E. coli* O157 outbreak in Scottish beef and the dioxin contamination of agri-foods in Belgium. As a result, a fundamental rethink within the European Union about food integrity took place. This was manifested in form of a re-organisation of the Directorates General in the Commission and the formation of the directorate DG SANCO in order to ensure health and consumer protection. Furthermore, food safety agencies in several Member States were created and the European Food Safety Authority (EFSA) was founded. Driven by the rational of an integrated "farm to fork" approach, the control of microbial contamination in food and the presence of potentially toxic substances, such as mycotoxins, pesticides and other agricultural contaminants was affirmed by legislative measures (McEvoy, 2002). Within the European Union, Commission Regulations (EC) 396/2005, 1881/2006, 37/2010 and their amendments are setting maximum levels for pesticides, mycotoxins and veterinary drugs in different food commodities including animal tissues (European Commission, 2005, 2006a, 2010). For animal feeding, maximum levels for undesirable substances such as aflatoxin $B_1$ or dioxins are regulated within the EC Directive 32/2002 (European Commission, 2002b).

The regulation for pesticides is set with a default value of 10 µg/kg valid for almost all food commodities. In contrast, very different regulatory limits exist for veterinary drugs and especially mycotoxins depending on the food category/compound combination. For example, maximum limits can range between 0.1 µg/kg for aflatoxin $B_1$ in baby food and 60,000 µg/kg for fumonisin $B_1$ in compound feed. In order to ensure that non-consumable products are not entering the European market and to allow an informational exchange between the Member States of the EU plus Switzerland, Norway, Iceland and Liechtenstein, the Rapid Alert System for Food and Feed (RASFF) was established. The category based RASFF notifications between 2012 and 2018 are depicted in Figure 6. Based on the annual RASFF report from 2018 including a total of 3,699 original notifications, 1,401 were classified as border rejection, 1,118 as alert, 675 as information for attention, 493 as information for follow-up and 12 as news notification. Official controls on the internal market are the largest category of notifications. Typically, these controls are conducted at a business operator such as manufacturer, wholesaler or retailer involving an inspection and a sampling for analytical purposes. Additionally, notifications

occur based on company own-checks, consumer complaints or when a food poisoning was at the origin (European Commission, 2019).

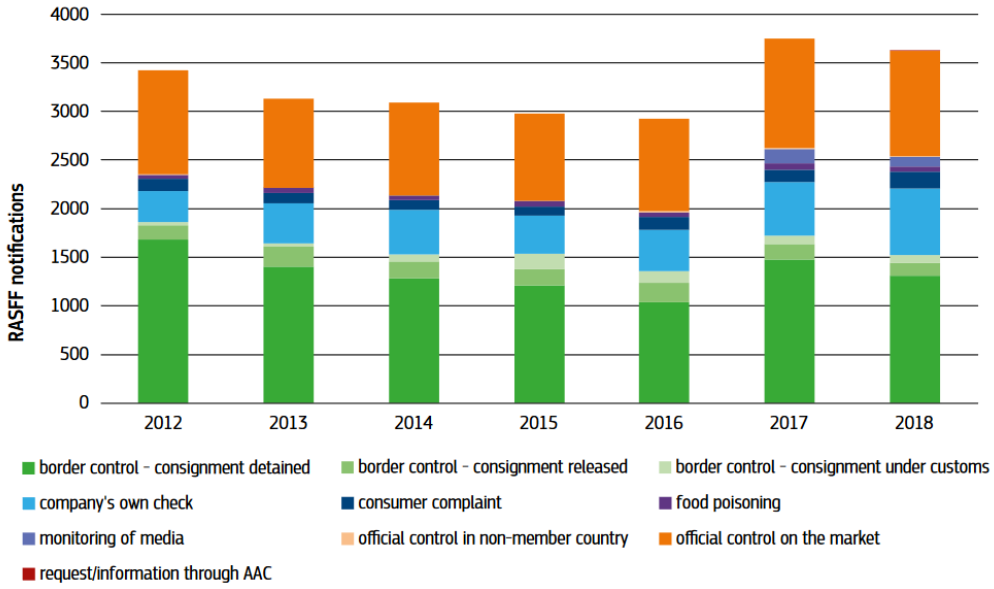



*Figure 6: Category based RASFF notifications between 2012 and 2018 (European Commission, 2019)*

Controls at the outer European Economic Area borders at points of border posts or entry amounted 42% of RASFF notifications in 2018. Considering notifications in feed, about 9% of the total RASFF notifications accounted for this category, with a considerable increase (+3%) compared to 2017. The top 10 hazard and product categories included, inter alia, mycotoxins with recurrent notifications for aflatoxins, ochratoxin A, deoxynivalenol, fumonisins and patulin, as well as pesticide residues with chlorpyrifos, carbofuran, methomyl, formetanate, tricyclazole, dinotefuran, dimethoate, omethoate, tolfenpyrad, acetamiprid, carbendazim, imazalil, triazophos, acephate and propargit. The largest fraction of the 313 notifications regarding feed was based on pathogenic micro-organisms, but also included agricultural-contaminants such as mycotoxins, pesticide residues and feed additives as non-compliance reason (European Commission, 2019).

# 2 Occurrence of Mycotoxins in View of Climate Change

## 2.1 Influence of Climate Change on the Agricultural Supply

Within the context of food production, environmental sustainability and food security are the key challenges. To ensure future food and nutrient supply, animal derived products are essential protein sources and therefore of major importance. About 100 g of protein per capita per day are consumed by the European population, approximately two thirds of which are derived from animal products, the rest from cereals, vegetables and pulses. With 96% of meat and 99% of fresh dairy products produced within the EU, the self-sufficiency for animal derived protein sources is very high. However, for the EU-based plant protein input an entirely different picture appears (De Visser et al., 2014). In 2017, the self-sufficiency rates for rapeseed, sunflower and soy amounted 79%, 42% and 5%, respectively. Consequently, about 17 million tonnes of crude protein (soy-based material) are imported annually, basically from Argentina, Brazil and the USA. Additionally, around 1.5 million tonnes of sunflower and 1 million tonnes of rapeseed-based crude protein are imported, mostly from the Ukraine. These protein sources are important ingredients for the production of compound animal feed, with soy meal as privileged ingredient. However, the growth prospects for plant proteins cultivated in the EU are limited in the conventional compound feed market, which will lead to a further increase of imports of plant-based protein sources by the EU (European Commission, 2018). These international trades are critical for two major reasons, first: a potential import of feed containing various agricultural contaminants, such as soy isoflavonoids (Durazzo et al., 2019) or carcinogenic mycotoxins ($AFB_1$) and mycoestrogens (ZON) (Calori-Domingues et al., 2018) can occur, and second: the increasing feed production and flows may lead to more greenhouse gas emissions, which negatively impacts on climate change (World Trade Organisation, 2009). A complex cause-effect relationship exists between climate change and agriculture. In addition to transport and industry, the agricultural sector is substantially responsible for generating large amounts of gas emissions, namely nitrous oxide ($N_2O$) and especially methane ($CH_4$) from livestock. In total, 10 – 12% of global greenhouse gas emissions (GHG) are related to agriculture (Fellmann et al., 2018). This has led to a significant rise in temperatures over the past century that has been accompanied by side effects such as droughts and heat waves, storms and floods, increased precipitation and higher levels of atmospheric carbon dioxide (Agovino et al., 2019). These mostly human-induced effects are challenging the maintenance of food supply and food safety, since agriculture itself is one of the most vulnerable sectors as yields are strongly depending on

favourable climate conditions. Based on models from the Intergovernmental Panel on Climate Change (IPCC), agricultural yields will significantly decline worldwide and most severely in lower latitude countries. Especially food insecure regions, such as Africa and south Asia will be threatened by a decline in crop production. For these regions, mean yield losses of 8% by 2050 for wheat, sorghum and maize are projected (Agovino et al., 2019; Parker et al., 2019).

In addition to yield losses, alterations in mycotoxin patterns and reduction of pesticidal activity (described in chapter 1.2.3) due to the changing climate conditions are serious challenges that must be faced across the agricultural sector in the future. Beside monitoring regulated mycotoxins, such as aflatoxins and fumonisins, a particular focus should be on "emerging mycotoxins" as evidence for their incidence is rapidly increasing (Gruber-Dorninger et al., 2017). The term of this chemically diverse group of mycotoxins in not yet clearly defined, but is nowadays often used for secondary fungal metabolites for which to date no regulation exist (Kovalsky et al., 2016). Commonly mentioned compounds in this group are precursors of aflatoxins, as well as alternariol, alternariol monomethyl ether, beauvericin, butenolide, culmorin, emodin, enniatins, ergot alkaloids, fusaproliferin, fusaric acid, moniliformin, mycophenolic acid, sterigmatocystin and tenuazonic acid (Gruber-Dorninger et al., 2017; Kovalsky et al., 2016). Multiclass methods covering a vast majority of agro-contaminants might reveal some surprising and unexpected findings, e.g. mycotoxins in unusual matrices or in untypical geographical regions (like aflatoxins in Europe) indicating the influence of global warming to the presence of these compounds (Gruber-Dorninger et al., 2017). In addition, focusing on a multitude of fungal metabolites including regulated, as well as emerging toxins is of particular relevance especially in view of an overall biological context as it might provide insights to the plant condition and interactions between plants and fungi.

## 2.2 Alterations in Fungal Metabolite Production

The production of secondary metabolites by mycotoxigenic fungi is influenced by environmental factors including relative humidity, temperature, drought, stress conditions of the plant and insect attacks (Miraglia et al., 2009). Climate change (CC) will have significant effects on these factors which will lead to changes in mycotoxin profile and infection patterns. Precise predictions regarding structural changes of the fungal community and related functional alterations are not yet feasible. However, these changes may lead to a discontinuous fungal response which moves the reaction to a different path. Furthermore, different types and amounts

of mutagenic mycotoxins on crops may occur e.g. due to elevated UV radiation from CC related temperature increase, resulting in the formation of mutated fungal strains capable to produce additional mutagenic mycotoxins in a cyclic manner (Paterson & Lima, 2011). Regions that are currently more used to a temperate climate will have to deal with serious changes with regards to the supply of natural resources as well as managing the changing mycotoxin infestation and related diseases. This is in particular true for the Mediterranean zones, which are identified as climate change hot spots. For these regions, extreme changes in $CO_2$, rainfall and temperature patterns are predicted which increases the risk of migration of pathogens as a result of shifts in response to significantly warmer and drought-like climatic conditions (Magan et al., 2011). Magan and Baxter (1996) highlighted that increased temperature (4°C) and $CO_2$ levels (600/700 ppm) might change the phyllosphere mycoflora of cereals during ripening leading to changes in plant physiology. Especially the photosynthetic capacity and transpiration will be affected by changes in stomatal patterns on leaf surfaces (Magan & Baxter, 1996). As a consequence, the colonisation of mycotoxigenic fungi might increase, leading to an elevated mycotoxin contamination of agricultural raw materials.

### 2.2.1 Impact of Climate Change on *Fusarium* species

In Europe, the most prevalent fungal genus in grains is the trichothecene-producing *Fusarium*. Most of the *Fusarium* species (in total 90 described phylogenetically distinct species) act as plant pathogens and produce a wide range of mycotoxins such as fumonisins in maize or trichothecenes in wheat. These compounds are able to contribute to the pathogenesis of *Fusarium* on crops and can also show toxic effects on the plant. The life cycle of this fungal species starts with the colonisation of the host, followed by sporulation dispersing the spores via rain splashes or wind (Perincherry et al., 2019). It is obvious, that optimum environmental conditions and climatic distribution influences on the life cycle and the presence of *Fusarium* species. The main deoxynivalenol producer *F. graminearum* is dominant in central and southern Europe, while *F. culmorum* is common on stem base, cereal roots and heads in Nordic areas including all Scandinavian countries (Moretti et al., 2019). Their temperature optimum for growth ranges from 20°C to 32°C, while a growth reduction was reported at 15°C. Toxin production e.g. of deoxynivalenol (with a temperature optimum of 28°C) is substantially higher in temperate regions, such as the Mediterranean where pathogens receive optimum environmental conditions, like extreme temperature, rainfall and lasting drought periods (Perincherry et al., 2019). One of the clearest indications of climate change influencing on

mycotoxin infection is given with the fusarium head blight disease (FHB) in wheat (Magan et al., 2011).

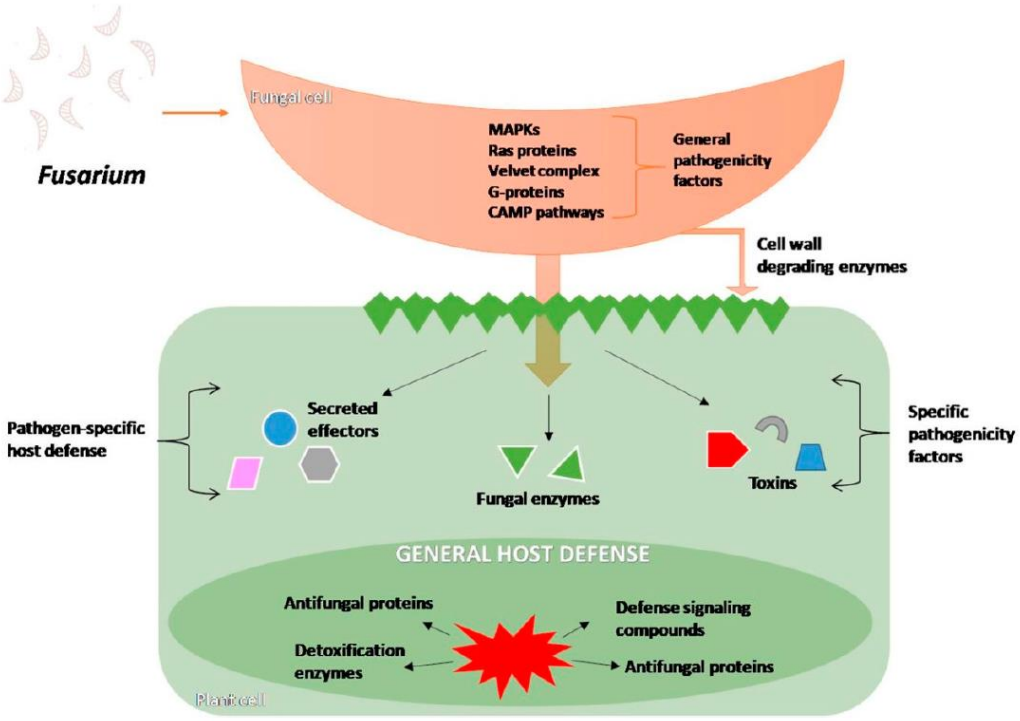



*Figure 7: Overview of Fusarium pathogenicity effectors, defense reaction of the host and signal transduction pathways (Perincherry et al., 2019)*

Typically, this infection occurs under humid and warm conditions and lead to a diverse and complex interaction between the fungi and the plant. Initial colonisation steps include adhesion and plant penetration along the pericarp of the seeds (e.g. *F. graminearum*) or by wounds through stomatal pores. After the successful colonisation with *Fusarium*, the fungi start to destroy plant tissue by producing host-specific toxins and override plant defense mechanisms (Figure 7). Secondary metabolites are produced by the fungi triggering a plant defense response. Specific cell wall degrading enzymes, such as lipases, pectinases, cellulases and xylanases enable the penetration into the plant cell wall (Perincherry et al., 2019). The most common species involved in FHB in cereal grains in cooler and maritime regions of Netherlands and Britain in the early 1990s was *F. culmorum*. An increase in the presence of *F. graminearum* and a decline in *F. culmorum* has changed the dominant species in these regions. At the beginning of 2000, *F. graminearum* has been reported as the most abundant *Fusarium* species (Moretti et al., 2019). In addition, alterations in environmental phenomena due to CC in different geographical regions lead to high location-to-location and year-to-year variability in incidents of FHB (Kriss et al., 2012; Scala et al., 2016). FHB causes severe yield losses up to 50% for small grain cereals and additionally decreases the cereal grain quality which in turn

leads to a further vulnerability for storage deterioration and mycotoxin production (Perincherry et al., 2019). In order to control the presence of *Fusarium* species triggering FHB, more effective fungicide treatments and new wheat cultivars with a higher tolerance level should be considered. However, it will require many years of development and adaption in order to implement these strategies. Therefore, it will be important to start adaption processes soon to mitigate the impact of climate change on crop disease and to ensure a sustainable wheat production as well as food safety and security (Moretti et al., 2019).

Furthermore, dry weather conditions during grain fill and late season rains are associated with an increased fumonisin production by *F. verticillioides* in maize. This species from the section *Liseola* has a growth optimum between 25 and 30°C resulting in a higher fumonisin production (Magan et al., 2011). In addition, the yield of fumonisins is maximised by *F. verticillioides* at $a_w$ (activity of water) values around 0.95, which will certainly be affected by warmer seasons. Moreover, the systematic transmission of *F. verticillioides* from plant to kernels increases under warmer conditions, which will further impact the fumonisin contamination in maize (Dall'Asta & Battilani, 2016).

### 2.2.2 Climate Change and the Risk of *Aspergillus* Contamination in Europe

Hot and dry weather conditions are also preferred by *Aspergillus flavus*, the main producer of aflatoxins. Hence, foreseen CC would provide favourable conditions for these toxins and lead to higher aflatoxin contamination of maize in traditionally temperate climates (Miraglia et al., 2009). For European policy makers especially, the presence of aflatoxins in feed and food is a "hot topic". Based on the realistic scenario of +2°C temperature increase in Europe, the plant-pathogen interactions might change in the whole continent (Medina et al., 2014; Tollefson, 2015). Current predictive models based on climate change data reveal that south Europe will become a high-risk area for aflatoxin contamination in maize. As depicted in Figure 8, even in the least conducive year with a low aflatoxin risk index, high yields of aflatoxin content in maize in south Europe will occur following a +2°C temperature increase. It is expected that aflatoxin contamination of maize and other food and feed commodities above the legal limits will increase and become more frequent in regions previously "free" of aflatoxins. This reduced availability of safe maize for the food, but in particular for the feed market will further impair the economic value, as contaminated maize batches will have to be diverted to non-agricultural uses, like biofuel production (Battilani et al., 2016).

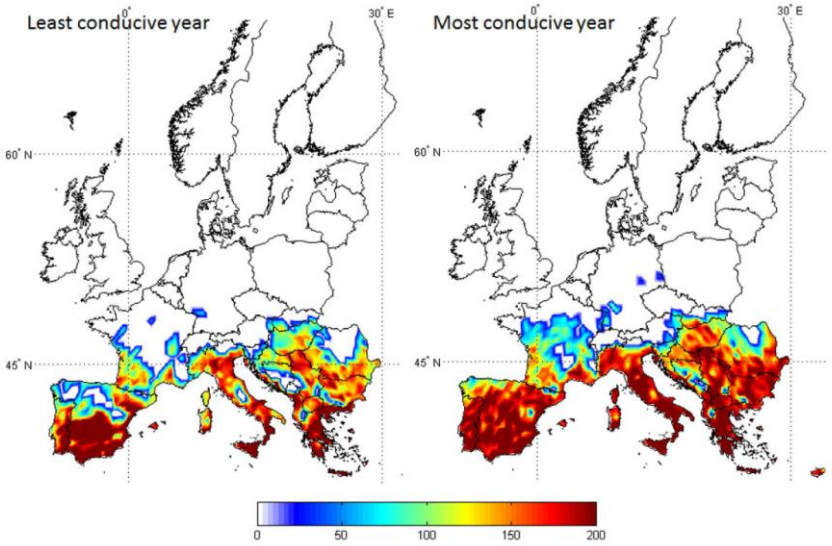



*Figure 8: European risk maps for aflatoxin contamination in maize based on +2°C climate scenario. The least (left) and the most (right) conducive years are depicted. The color scale (0-200) represents the aflatoxin risk index from low risk (white-blue) to high risk (yellow-red) (Battilani et al., 2016)*

Within a case study conducted by Van der Fels-Klerx et al. (2019), the authors investigated the influence of CC on the production of aflatoxin $B_1$ in maize and consequently aflatoxin $M_1$ in dairy cow's milk based on a full chain modelling approach. The focus was set on maize from Eastern Europe (Ukraine) intended for importation to the Netherlands as part of dairy cow's compound feed ingredient. In total, the authors applied five different carry-over models, three different climate models and one aflatoxin $B_1$ prediction model. An overview about the different climate model scenarios is depicted in Table 6, which are based on global circulation models reflecting maximum diversity with respect to the output of weather variables.

**Table 6: Climate baseline data and projected changes for 2030 based on three different models for Ukraine (modified by Van der Fels-Klerx et al., 2019)**

| Statistic | Baseline | 2030 | | |
|---|---|---|---|---|
| | | DMI | ETHZ | METO |
| mean $T_{min}$ (°C) | 10.4 | 10.6 | 10.9 | 11.4 |
| SD $T_{min}$ | 6.5 | 6.2 | 6.5 | 6.9 |
| mean $T_{max}$ (°C) | 20.8 | 20.3 | 20.6 | 21.3 |
| SD $T_{max}$ | 8.8 | 8.2 | 8.6 | 9.0 |
| Mean sum of precipitation (mm/year) | 360 | 333 | 244 | 250 |
| SD sum of precipitation | 120 | 127 | 120 | 99 |
| * SD = standard deviation | | | | |

With respect to the surface air temperate METO represents the warmest and DMI the coldest prediction model, while ETHZ falls in between. The aflatoxin forecast model is based on the aflatoxin-maize model previously described by Battilani et al., 2016. Aflatoxin carry-over model simulates bulk milk production at a dairy farm in Netherland with a herd size of 69 dairy cows taking three different composition scenarios (low-protein, high-protein and feed

ingredient with minimal and maximal ranges), as well as two different milk yield scenarios (normal and extreme lactation) into account. Predicted mean aflatoxin $B_1$ concentrations in Ukrainian maize are depicted in Figure 9. Compared to the baseline scenario, an expected decrease (-25% DMI) or increase (+52% ETHZ, +93% METO) in aflatoxin $B_1$ concentrations can be observed.

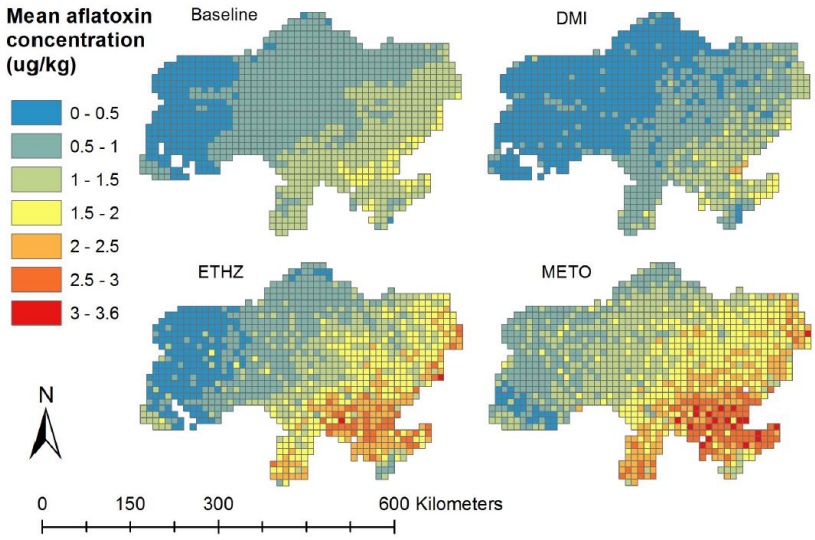



*Figure 9: Map overview of mean aflatoxin $B_1$ concentration in µg/kg in Ukrainian maize for baseline conditions and three different climate models for 2030 (DMI, ETHZ and METO)* (Van der Fels-Klerx et al., 2019)

Although the results strongly depend on the carry-over and the climate model used for the calculation, most of the calculations predict an increase up to 50% of maximum mean aflatoxin $M_1$ in milk by 2030 with exception for DMI. In addition, the authors suggest a stable (increase up to 0.6%) probability of aflatoxin $M_1$ findings exceeding the EU limit of 0.05 µg/kg by 2030 (Van der Fels-Klerx et al., 2019).

Beside grain-based commodities, CC will also impact the other food commodities such as wine or nuts. Wine production will suffer enormously from CC, since the mycotoxin formation on grapes will change significantly. This is especially true for the production of ochratoxin A, as higher temperatures between 30-35°C favour the growth of the producing fungi *Aspergillus carbonarius* and *Aspergillus niger*. It is further expected that the prevalence of more dangerous mycotoxins, which are better adapted to higher temperatures, will occur in wine. Large wine producing areas especially in south Europe will experience massive challenges in maintaining uncontaminated wine production (Paterson et al., 2018). Nut production will especially be affected by drought stress which could develop cracking in pods or hull splitting. This might lead to an increased formation of *A. flavus* and aflatoxin contamination in peanuts and pistachios (Magan et al., 2011).

## 2.3 Aflatoxin associated Public Health Risk in Serbia

Typically, the infection rate of cereal crops by *Aspergillus* species and related aflatoxin contamination are rare in most of the European countries, as the climate conditions are still rather moderate. Aflatoxin contaminated foodstuff is therefore considered as an "import problem" within Europe and requires a strict control system of critical foods such as nuts and maize (Levic et al., 2013). Although the Serbian climate is traditionally considered as a humid subtropical or warm-humid continental climate, extreme weather conditions in this region of southern Europe are one of the greatest risks for contamination of cereals (including maize, barley, wheat etc.) by toxigenic fungi. In recent decades, extreme weather events in Serbia increased which was especially pronounced in the year 2012. In this year, the spring crops generation phase coincided with one of the hottest and driest periods ever reported in this country. As a result, this extreme climate event caused a significant damage in agricultural crop production and caused a severe "aflatoxin outbreak" (Milićević et al., 2020).

This outbreak was triggered by an increased production of *Aspergillus spp.*, basically *A. flavus* and *A. niger*. The incidence of these two *Aspergillus* species was between 3-16% before 2008 (Milićević et al., 2020) which increased due to extreme weather events and reached its peak in 2012 (Figure 10). About 95% of analysed grain contained high amounts of *A. flavus* and *A. niger* leading to highly contaminated aflatoxin maize batches intended for the use as animal feed (Levic et al., 2013).

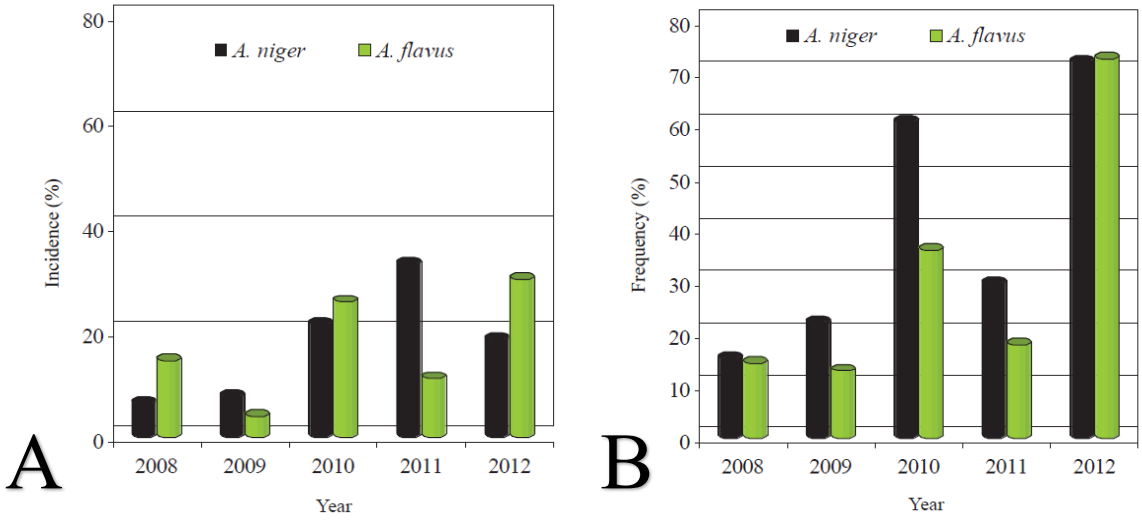



*Figure 10: Maximum incidence (A) and average frequency (B) of Aspergillus flavus and Aspergillus niger for grains, 2008-2012 (Levic et al., 2013)*

This unfolded a big milk crisis in Serbia in terms of exceeding EU regulatory limits (0.05 µg/kg) of aflatoxin $M_1$ in raw milk and dairy products. As a consequence, the Serbian Government

increased the EU maximum limit for aflatoxin $M_1$ in milk by a factor of 10 from 0.05 to 0.5 µg/kg in order to avoid massive financial losses. The value was restored to 0.05 µg/kg in 2014 as the situation in milk and dairy products improved again (Miocinovic et al., 2017). However, due to confused and scared consumer reactions, the milk supply chain was heavily shaken. Although milk represents a major food group in the Serbian diet, average consumption of milk and milk products significantly declined. Compared to 2012, the consumption of sterilised and pasteurised milk decreased by 11.4% in 2013, in Belgrade even by 26.6%. In addition, countries with higher food standards stopped the import of Serbian milk. An accumulation of stocks occurred at dairy processing facilities as a result of the decreased international and domestic demand. The prices of dairy products were cut monthly by 15 to 20% from the regular price throughout the period of the crisis (Popovic et al., 2017).

The Serbian "milk scandal" is a tragic example for the influence of extreme climate events on shift of fungal infestation and subsequently the presence of potentially harmful substances such as aflatoxins. A lack of reliable and comprehensive monitoring and control systems has led to an increased public health risk within the Serbian population. Although the Serbian Government and in particular the Ministry of Agriculture Forestry and Water Management has started to establish food safety systems following the principles of European Union legislation, there are still major deficiencies including its development, implementation and control. Serbian regulatory authorities e.g. have not yet established maximum limits for fumonisins, despite their health hazards and widespread occurrence (Milićević et al., 2020).

With respect to the Serbian "milk scandal", we have tested 204 maize samples from the northern part of the Republic of Serbia, collected in the time period of 2012 and 2015. The major objective was an investigation of the influence of climatic conditions on the presence of regulated mycotoxins (Publication #3). In addition, for the first time, samples of this region were tested for a broad spectrum of non-regulated secondary fungal metabolites. This included emerging, masked and modified forms of mycotoxins and should provide first insights to relevant mixtures of contaminants prevalent in this region (Publication #4).

# 3 Determination of Multiclass Contaminants

Due to the enormous variety of previously described potentially harmful substances which may enter different chains of food production, analytical chemistry has become more and more important. Food and feed analysis provide relevant information about contamination patterns, the chemical composition, quality control and ensures compliance with food and feed and trade laws. One of the biggest challenges in food safety is currently based on the risk assessment of continuously changing contaminant mixtures. The trend in analytical chemistry is therefore moving towards the development of fast, reliable and efficient procedures for the quantitative trace analysis of multiple target and non-target contaminants in food and feed (Farré & Barceló, 2013).

Detection and quantification of multi-contaminants at ultra-trace concentration levels has become routine for official control laboratories and the respective scientific community (Farré et al., 2012). This progress was mainly possible due to the coupling of chromatographic and mass spectrometric (MS) techniques. In the field of residue analysis in food, gas chromatography (GC) is playing a crucial role especially for the investigation of contaminants in certain food matrices with high fat content, or for compounds which are less sensitive to atmospheric pressure ionisation (API) or electrospray ionisation (ESI) (Farré & Barceló, 2013; Hernández et al., 2011; Plaza-Bolaños et al., 2010). Nevertheless, the greatest progress in food and feed analysis in recent years was based on liquid chromatography (LC) mass spectrometric analytical techniques. This coupling approach was born decades ago, but technological improvements in recent years have led to its increased applicability and broad use (Farré & Barceló, 2013). For the analysis of polar organic molecules in food, a combination of LC or ultra-performance LC (UPLC) with atmospheric pressure ionisation (API)-MS/MS is most frequently used. With regards to mass analysers, triple quadrupole (TQMS or QQQ) instruments are the most common, but high-resolution mass analyser such as quadrupole time-of-flight (Q-TOF) and quadrupole ion trap (Orbitrap) are gaining more popularity, as they provide accurate-mass measurements of product ions together with fragmentation patterns (Farré & Barceló, 2013; Reemtsma, 2003). The electrospray ionisation technique in MS is the most common for the analysis of moderate to high polar organic compounds (<1 kDa) (Smyth, 2003). The biggest advantage of other API interfaces such as atmospheric chemical ionisation (APCI) or atmospheric pressure photo-ionisation (APPI) compared to ESI is based on reduced matrix effects but the limits of detection are significantly higher compared to LC-ESI-MS/MS instruments (Farré & Barceló, 2013; Takino et al., 2003).

Due to the strong developments and advances in the field of LC-MS/MS coupling technology, the number of published "multiclass" analytical papers in peer-reviewed journals increased in recent years (Figure 11). Between 2009 and 2019 the number of publications dealing with multiple-compound analysis either based on triple quadrupole, or high-resolution mass spectrometry have almost doubled. Most of the publications in this area come from our institute with Prof. Rudolf Krska as the most frequently named author (32 publications), followed by Dr. Michael Sulyok (29 publications) also BOKU from IFA-Tulln and Prof. Sarah De Saeger (23 publications) from Ghent university.

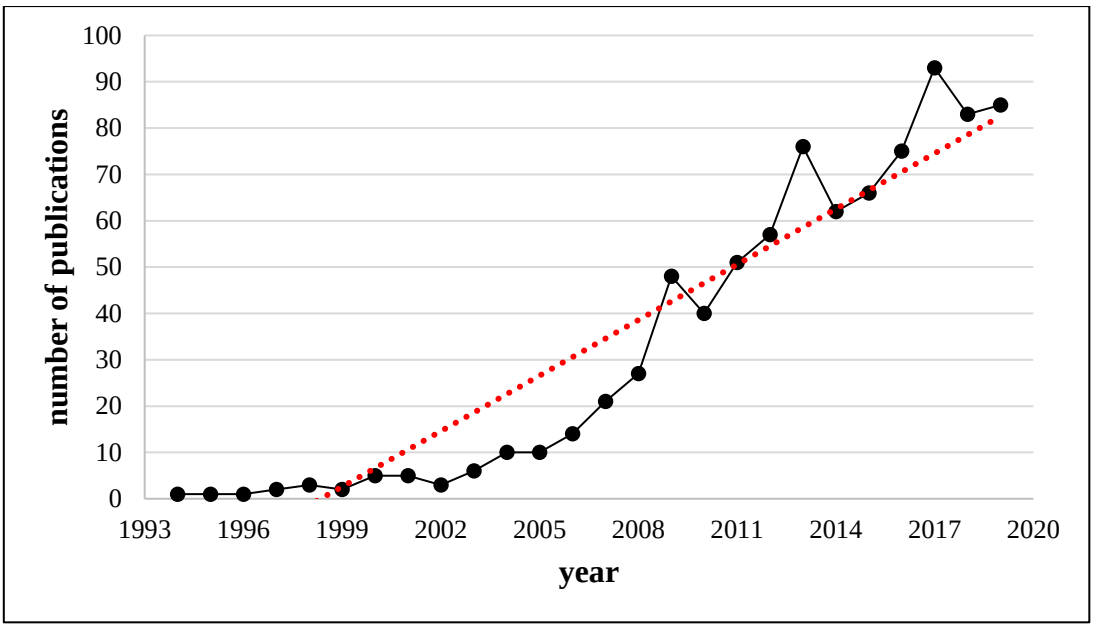



*Figure 11: Number of publications listed on Scopus (accessed 20 September 2020, search terms: "LC-MS/MS", "HRMS", "orbitrap", "multi", "contaminant", "residue", "veterinary drug", "pesticide", "mycotoxin")*

However, the number of "real" multiclass methods is still comparatively scarce, since most of the published approaches contain a maximum of two substance categories. Many of the publications only include one single substance class e.g. pesticides or veterinary drugs. The term multiclass, or multi-target in this case refers to the subcategory of the specific substance class e.g. tetracyclines, sulfonamides or penicillins in case of veterinary drugs. Table 7 provides an overview of multiclass analytical methods developed for the analysis of ≥ 2 different substance classes in food and feed matrices. Table 8 reports a list of multi-target approaches developed for the analysis of contaminants or residues in different food commodities.

## 3.1 Multi-compound Analytical Methods

**Table 7: Examples of multiclass analytical methods (≥2 substance classes) in food and feed analysis**

| Compounds | Matrix | Extraction protocol | Separation | Detection | Reference |
|---|---|---|---|---|---|
| | | *Multiclass approaches* | | | |
| mycotoxins, pesticides, veterinary drugs, plant toxins, bacterial metabolites (1,467) | compound feed (cattle, chicken) | Dilute & shoot acetonitrile/water/formic acid 79:20:1, v/v/v | HPLC | MS/MS | (Steiner, Sulyok, et al., 2020) |
| mycotoxins, bacterial metabolites, plant toxins (>500) | wheat, maize, figs, dried grapes, walnuts, pistachios, almonds | Dilute & shoot acetonitrile/water/acetic acid 79:20:1, v/v/v | HPLC | MS/MS | (Sulyok et al., 2020) |
| pesticides, mycotoxins, plant toxins (389) | leek, wheat, tea | QuEChERS | HPLC | HRMS/MS | (Dzuman et al., 2015) |
| veterinary drugs, mycotoxins, pesticides, hormones (226) | bovine & porcine muscle | SLE acetonitrile/ethanol 5:1, v/v, purification by low temperature and d-SPE | UHPLC | MS/MS | (Zhan et al., 2013) |
| pesticides, veterinary drugs (>350) | honey | SLE acetonitrile/1% formic acid | UHPLC | Exactive-Orbitrap | (Gómez-Pérez et al., 2012) |
| pesticides, plant alkaloids, veterinary drugs (118) | corn silage, muscle & liver tissue, whole milk | QuEChERS | UHPLC | Exactive-Orbitrap | (Filigenzi et al., 2011) |
| pesticides, mycotoxins, plant toxins, veterinary drugs (258) | compound feed, honey | Dilute & shoot water/acetonitrile/1% formic acid 5:15, v/v | UHPLC | MS/MS | (Mol et al., 2008) |

SPE = Solid-phase extraction, SLE = Solid-liquid extraction, (U)HPLC = (ultra) high performance liquid chromatography, MS/MS = tandem mass spectrometry, HRMS = high resolution mass spectrometry

**Table 8: Examples of multi-residue, multi-contaminant analytical methods in food analysis**

| Compounds | Matrix | Extraction protocol | Separation | Detection | Reference |
|---|---|---|---|---|---|
| | | *Multi-target approaches* | | | |
| veterinary drugs (105) | milk, meat, fish, egg, fat | QuEChERS | UHPLC | MS/MS | (Desmarchelier et al., 2018) |
| mycotoxins (295) | apple puree for infants, hazelnuts, maize, green pepper | Dilute & shoot<br>acetonitrile/water/acetic acid 79:20:1, v/v/v | HPLC | MS/MS | (Malachová et al., 2014) |
| pesticides (300) | cucumber, lemon, wheat flour, rocket salad, black tea | SLE<br>acetonitrile, water + acetonitrile for wheat flour and black tea | 2D-LC | MS/MS | (Kittlaus et al., 2013) |
| mycotoxins (191) | hazelnuts, almonds, pistachios, peanuts | SLE<br>acetonitrile/water/acetic acid 79:20:1, v/v/v | UHPLC | MS/MS | (Varga et al., 2013) |
| pesticides (166) | fruits & vegetables | QuEChERS | UHPLC | Q-Orbitrap | (Wang et al., 2012) |
| pesticides (212) | food plants | QuEChERS | UHPLC | TOF-MS | (Lacina et al., 2010) |
| veterinary drugs (>30) | animal tissue (chicken muscle) | QuEChERS | HPLC | MS/MS | (Stubbings & Bigwood, 2009) |
| veterinary drugs (>100) | meat | LLSE<br>acetonitrile | UHPLC | TOF-MS | (Kaufmann et al., 2008) |
| mycotoxins (33) | peanuts, pistachios, wheat, maize, cornflakes, raisins, figs | Dilute & shoot<br>acetonitrile/water 80:20, v/v | HPLC | MS/MS | (Spanjer et al., 2008) |

SLE = Solid-liquid extraction, LLSE = Liquid-liquid-solid extraction, (U)HPLC = (ultra) high performance liquid chromatography, 2D-LC = Two-dimensional chromatography, MS/MS = tandem mass spectrometry, Q = Quadrupole, TOF-MS = Time of flight mass spectrometry

## 3.2 Limitations of Multiclass Methods

Despite the improvement of detection and separation techniques, a major cornerstone within the analytical process is the sample preparation procedure, which ensures reliable results and maintains instrumental performance. In the last decade, sample extraction protocols mainly focused on a single class of analytes and typically followed solid-liquid extraction (SLE), solid-phase extraction (SPE), pressurised liquid extraction (PLE) or solid-phase microextraction (SPME) protocols and were usually combined with a selective clean-up step (Frenich et al., 2014; Ridgway et al., 2007). With an increasing number of analytes covered by one method, the trend in sample preparation in the past few years moved towards generic extraction procedures, applicable for a broad range of compounds from different substance classes (Frenich et al., 2014). These sample preparation techniques are characterised by small sample amounts and a reduction of organic solvents leading to a high sample throughput. One of the most frequently applied generic extraction technique is QuEChERS (quick, easy, cheap, effective, rugged and safe) which was developed in 2003 for the analysis of organic compounds such as pesticides in food (Anastassiades et al., 2003). An even further simplification of the sample treatment is given with the "dilute and shoot" approach which is suitable for multi-residue and multiclass analysis including pesticides, veterinary drugs and natural toxins. In 2008, Mol et al. proved the feasibility of a multiclass method based on such dilute and shoot procedure and found an acidified acetonitrile/water mixture to be the best compromise for solvent extract. This straightforward approach was revealed as the most suitable compared to several tested sample preparation protocols including QuEChERS (Mol et al., 2008). Although these generic extraction techniques are suitable for a wide range of applications, sample preparation remains the major bottleneck in the analytical procedure of multiclass method development which requires a willingness to compromise (Stubbings & Bigwood, 2009). With multiclass methods covering hundreds or thousands of compounds, individually low extraction yields must be expected. Additionally, within a generic extraction procedure, inherent matrix components can be co-extracted leading to an interference during the ionisation process (Frenich et al., 2014). Several strategies to mitigate these effects exist, but each approach has its own merits and shortcomings. A very common approach is the use of a matrix-matched calibration, but due to high sample heterogeneity, large variances between different lots of the same sample type can occur (Stadler et al., 2018; Varga et al., 2013). This is especially true for complex sample materials such as compound feed, which consists of multiple feed components and feed additives (Steiner, Krska, et al., 2020). The application of a standard addition is widely

applied in routine laboratories but is also associated with high costs for standards and an elevated turnaround time due to further individual sample treatment (O'Mahony et al., 2013). Also very expensive is the use of isotopically-labelled internal standards which are additionally limited in their commercial availability (Beltrán et al., 2013). Combining the extraction protocol with a subsequent sample clean-up step (e.g. d-SPE, PSA or C18) could minimise unwanted matrix effects but should be avoided since selective clean-ups are not compatible to all compounds, and analytes can be lost during this step. Diluting the extract with a complementary dilution solvent seems to be the most straightforward solution since matrix components are reduced significantly, which in turn is accompanied by a loss of sensitivity. However, by using the latest generation tandem mass spectrometric instruments, these losses can be tolerated, as the sensitivity of these instrument enables meeting the legislative limits despite large dilution factors (Frenich et al., 2014). Furthermore, targeted data acquisition within MS/MS detection expected to become a limiting factor due to the finite number of compounds which can be measured within one analytical run. A possible solution might be to switch to high resolution instruments in exact mass scan mode such as TOF-MS and developing screening methods based on full scan mass spectrometric detection techniques (Mol et al., 2008). Theoretically, these instruments are able to scan an unlimited number of analytes using an automated screening method. Additionally, data can be investigated retrospectively for substances which were excluded in the original list of target analytes. However, the application of such qualitative and semi-quantitative methods requires the development of a comprehensive database including accurate masses and information about characteristic insource fragments as well as retention time data. A major disadvantage regarding TOF-MS instruments is their low sensitivity compared to MS/MS devices (Fernández-Alba & García-Reyes, 2008). Comparable sensitivity to MS/MS configurations is given with current Orbitrap instruments. Based on a higher resolution in combination with mass accuracies which are independent of encountered ion abundance, Orbitraps ensure higher selectivity and consequently lower limits of detection and numbers of false positive findings. However, a method-transfer from TOF or MS/MS to Orbitrap seems to be critical since the analysis of crude and protein rich extracts is limited due to strong influence of interference compounds. A further clean-up step or re-development of the extraction procedure is therefore required which clearly limits the broad applicability of these devices. A higher matrix tolerance is given for matrices with high water content such as fruits and vegetables, indicating that HRMS based devices will dominate the field of pesticide analysis in the near future. Nevertheless, software improvements will be necessary in order to increase the process speed and ensure flexible report generation (Kaufmann et al., 2011).

## 3.3 Challenges in Multiclass Method Development and the Author's Contributions

The routine application of a multiclass method covering several hundred compounds require reliable and precise standard management. In order to keep the total effort for the preparation of single standard and mixed working solutions low, the investigation of suitable solvents is necessary. The main goal in standard preparation is to find a final uniform solvent solution, applicable for all analytes with consideration of possible solvent-mediated degradation or epimerisation. However, due to different compound related physico-chemical properties such as polarity, pH optimum or the number of functional groups, a variety of solvent solutions are necessary to prepare individual standard stock solutions. The most common solvents for this work were acetonitrile (primarily), acetonitrile/water 1:1 (v,v), methanol, methanol/water 1:1 (v,v) and water. In order to maintain appropriate standard stabilities, intermediate mix-solutions containing e.g. 10 substances should be stored at high concentration levels (≥ 10 ppm) at their specific temperature optimum, which requires an investigation of their short- and long-term stability. Final multi-compound work solution should be prepared by mixing the individual intermediate solutions together. In case of a multi-component standard exhibiting analyte co-elution all over the chromatogram, a steady decline in the ionisation yield can be observed for compounds with increasing concentrations. This can be explained with the increasing number of competing analytes which influences the partition equilibrium between charged bulk and surface solution species. To avoid significant signal suppressions among the analytes, the working range of the calibration standards should be kept as low as possible, since the competition of co-eluting target compounds for charged surface sites decreases with lower concentration levels (Mol et al., 2008; Sojo et al., 2003). Beside proper standard management, a major bottleneck exists in the evaluation of the raw MS data. For the preparation of a positive peak list, peak detection has to be manually confirmed from the original data set especially at low levels near the limit of quantification (LOQ). With increasing number of target analytes, the preparation of positive peak lists and the final quantitative data evaluation are becoming more and more time-consuming procedures. Additionally, compounds present in low concentration levels close to the LOQ may perish within the electronical and chemical background noise. Software-related improvements in automated peak detection could lead to significant improvements of the overall turnaround time. However, to ensure an accurate and reliable quantification of low concentration levels, the system background noise should be reduced to a minimum by primarily optimising the acquisition parameters.

### 3.3.1 Acquisition Parameters

In LC-MS/MS, quantitative target analysis typically follows a conventional multiple reaction monitoring (MRM) algorithm. The acquisition rate within MRM is determined by the scan or Dwell time ($t_{Dwell}$) which represents the time needed for collecting one data point for a specific MS/MS transition, and the acquisition or cycle time ($t_{Cycle}$) which is the time needed to complete one cycle of all transitions. Hence, $t_{Dwell}$ represents a compromise between the number of data points that can be acquired and the signal-to-noise (S/N) ratio across a chromatographic peak (Hermes et al., 2018; O'Mahony et al., 2013). However, MS/MS based MRM data acquisition in multiclass method development is a challenging factor since the number of compounds which can be determined in one analytical run is limited (Mol et al., 2008).

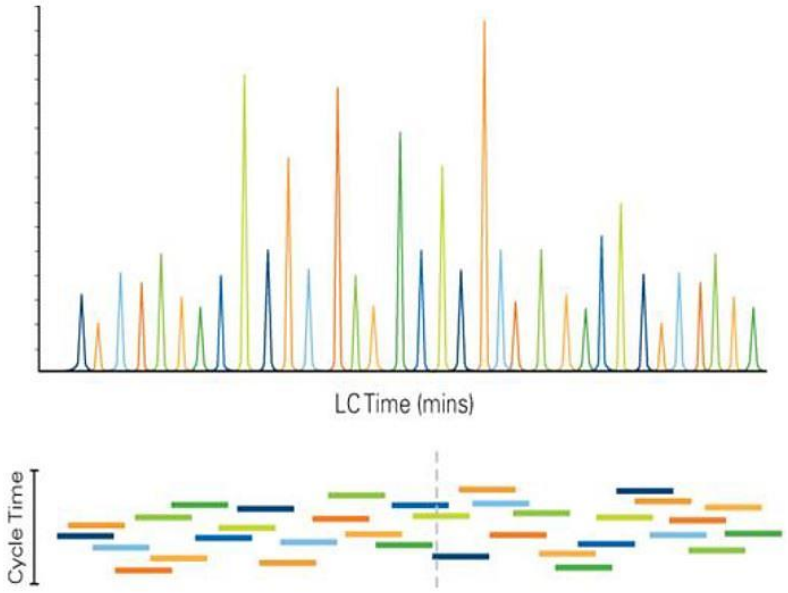



*Figure 12: Scheduled multiple reaction monitoring mode (AB Sciex, 2018)*

In order to significantly increase the detectable number of analytes (> 500), the acquisition algorithm can be changed to scheduled multiple reaction monitoring (sMRM) mode. (Figure 12) Within sMRM each compound is measured only within a defined time window ($t_{Window}$) covering the expected retention time, with $t_{Dwell}$ resulting from the cycle time divided by the number of concurrent MS/MS transitions at a particular retention time. To increase the method's sensitivity, $t_{Dwell}$ should be as high as possible since this decreases the background noise. One way to improve $t_{Dwell}$ is to increase $t_{Cycle}$, but this also reduces the number of data points per peak. Therefore, in multiclass method development it is necessary to find an optimal setting for $t_{Cycle}$ and $t_{Window}$ in order to maximise $t_{Dwell}$ and ensure appropriate amounts of data points per peak (Hermes et al., 2018). Typically, $t_{Dwell}$ may be ~25 ms for methods covering ~50 compounds, resulting in 10-15 data points per peak with a chromatographic peak width of 15 seconds. Significant losses in reproducibility are evident with $t_{Dwell}$ < 10 ms since the scan time is roughly proportional to the number of ions in the

quadrupole. As a result lowering the scan times reduces the number of detectable ions, leading to higher signal variations (Lu et al., 2008).

**Contribution of the author**

The final LC-MS/MS multiclass method covered >1,400 analytes and was split into two chromatographic runs (ESI pos/neg), about 1,000 (~2,000 MRMs) substances were measured in positive and the remaining 400 (~800 MRMs) in negative mode. Adjustments to the acquisition setup included changes to $t_{Cycle}$ and $t_{Window}$ parameters. The impact on repeatability was measured by repeated measurements of a multi-analyte standard near the instrumental LOQ and the results are shown in Figure 13.

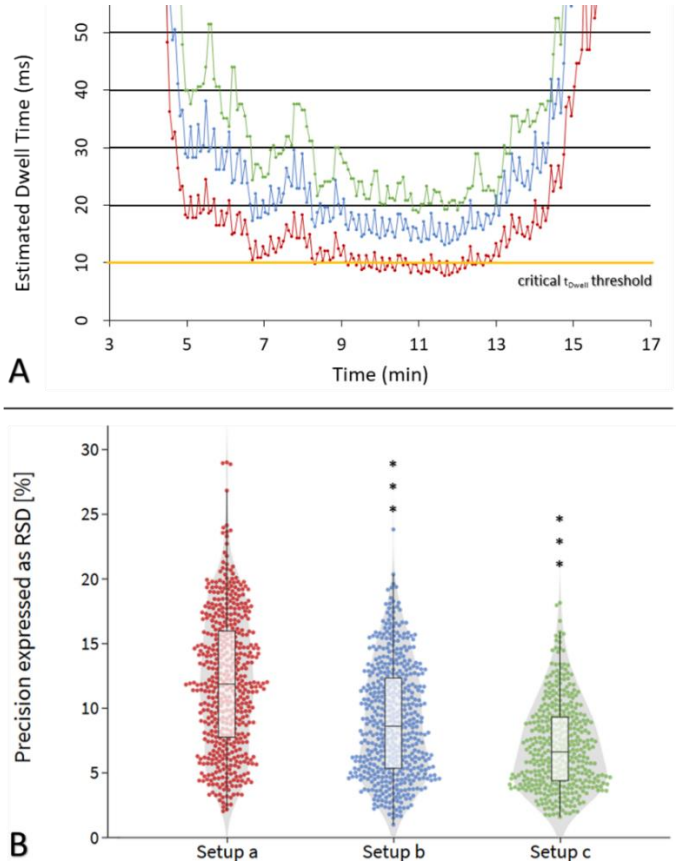



*Figure 13: Comparison of different acquisition setups. A represents a computational estimation of $t_{Dwell}$. B represents the repeatability (n=5) for a multi-analyte standard (instrumental LOQ). Tested setup configurations consist of $t_{Window}$ of 40, 40 and 30 sec, as well as $t_{Cycle}$ of 1.0, 1.5, 1.5 for setup a (red), b (blue) and c (green).*

A combination of 1.5 sec $t_{Cycle}$ with a $t_{Window}$ of 30 sec was revealed as the most suitable. Under these conditions, $t_{Dwell}$ was increased by a factor of 2 for substances in the most critical chromatographic time window while maintaining at least 10 data points per peak for a peak width of 15 seconds. The enhancement in $t_{Dwell}$ also resulted in higher S/N ratios which can be explained by a background noise reduction at the peak. Detailed experimental description and results are given in Publication #1.

### 3.3.2 Matrix Effects

Beside a significant deterioration in precision caused by overly minimised $t_{Dwell}$ times, matrix effects represent the major limiting factor in LC-ESI-MS/MS method development (Sulyok et al., 2020). These effects occur at the electrospray interface when matrix-inherent molecules co-elute with the compounds of interest and trigger an alteration in ionisation efficiency leading to a suppression or (more rarely) to an enhancement of the primary signal response of the target compound (Taylor, 2005).

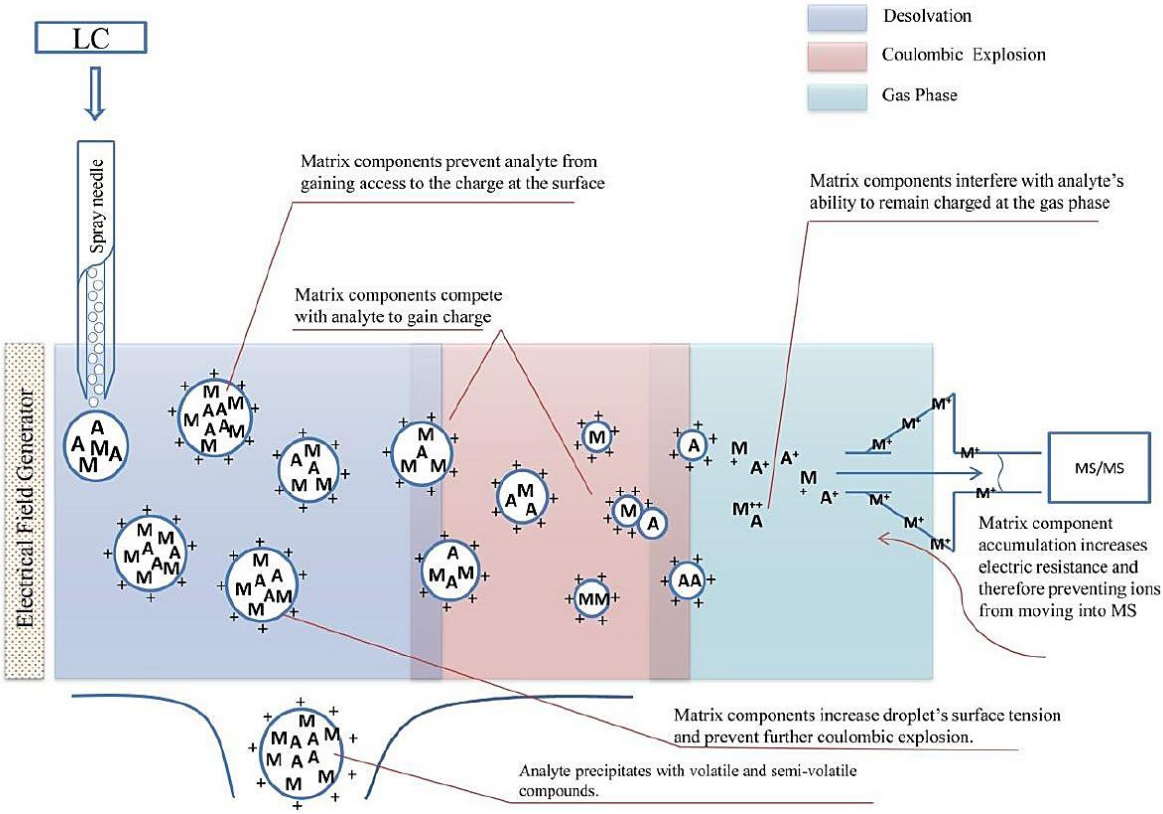



*Figure 14: The matrix effects mechanism in electrospray ionisation (Panuwet et al., 2016)*

The mechanism of matrix effects is depicted in Figure 14 which is in principle based on a competition between analyte ions and undetected non-volatile matrix components for accessing the droplet surface in order to enter the gas phase (King et al., 2000; Tang & Kebarle, 1993). This competition can lead to a significant decrease (ion suppression) or increase (ion enhancement) in ionisation efficiency of the target analyte and depends on the environment in which the evaporation process and ionisation take place. Consequently, large variations in matrix effects are possible, strongly depending on the type of matrix entering the ESI source, and on the type of ESI source (Taylor, 2005). Additionally, the degree of matrix effects is largely influenced by the chemical nature of the target compound. For example, the extent of ion suppression is elevated with increasing polarity of the target analyte. These findings of the

complex nature of matrix effects have important consequences in particular for optimising the sample preparation procedure and select a suitable clean-up technique (Bonfiglio et al., 1999). Hence, for LC-ESI-MS/MS based method development it is essential to understand and investigate these effects, since reliability in terms of accuracy and precision may be hampered by matrix effects leading to lower sensitivity and thus higher LOQs (Matuszewski et al., 1998).

**Contribution of the author**

As discussed in chapter 3.2, the applicability of conventional matrix effect reduction or compensation strategies such as matrix-matched calibration, the use of isotopically labelled internal standards, the combination of the sample extraction protocol with a selective clean-up procedure, or the conduction of a standard addition is rather limited. In order to minimise these unwanted adverse effects, optimisation of HPLC/UHPLC conditions including flow rate, injection volume and chromatographic column, as well as a comparison of unspecific clean-up procedures were conducted in this work. Experimental description and detailed results are provided in Publication #1. Briefly, a combination of a high flow rate and low injection volume under HPLC conditions was revealed as the most suitable configuration for the multiclass approach. Further, a comprehensive comparison between different QuEChERS approaches with several dilution steps was performed, results are illustrated in Figure 15.

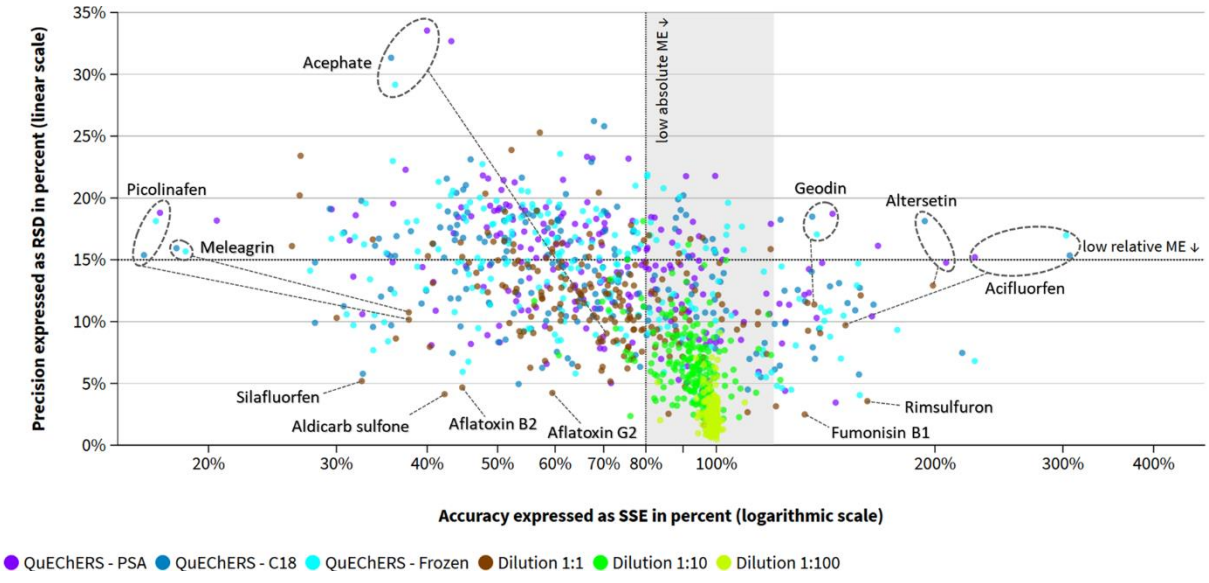



*Figure 15: Comparison of unspecific clean-up procedures including dilutions of 1:1, 1:10 and 1:100 as well as QuEChERS in combination with PSA (primary secondary amine), C18 and deep freeze treatment. (ME = matrix effect)*

The results indicated a straightforward 1:1 dilution as the best compromise in terms of matrix effect reduction while maintaining an appropriate sensitivity.

### 3.3.3 Sample Heterogeneity

Considering matrix effects, the main limitation is probably not related to the absolute effect of signal suppression or enhancement (SSE), but more related to relative matrix effects, as the latter cannot be compensated by a matrix-matched calibration (Sulyok et al., 2020). These effects are significant differences in SSE in different lots of the same matrix type (Matuszewski et al., 1998). Neglecting the matrix/sample heterogeneity by using technical replicates of a single or pooled sample within the method validation procedure (as suggested by most guidelines) can lead to a significant underestimation of the measurement uncertainty (Stadler et al., 2018). This is especially true for complex animal feed material where exact specifications of feed rations are often missing (Steiner, Krska, et al., 2020). The European Commission regulation 767/2009 divides animal feed into compound feed and feed materials. The latter are products of animal or vegetable origin which should meet the nutritional needs of food producing animals (European Commission, 2002c). Feed materials are used for the production of compound feed or are directly fed to animals with or without additional processing (European Commission, 2013).

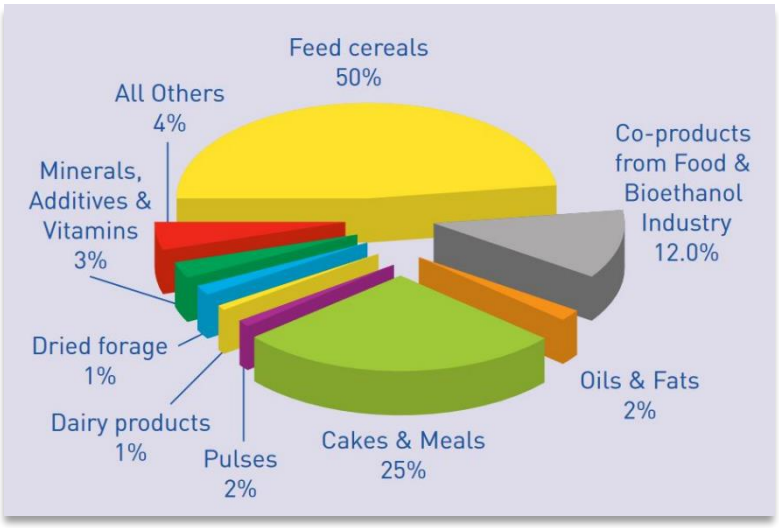



*Figure 16: Consumption of feed material by the compound feed industry in the EU-28 in 2018 (FEFAC, 2019)*

Based on the annual European Feed Manufacturers' Federation (FEFAC) report of 2018, the most highly consumed feed material of the compound feed industry in the EU-28 were cereals (e.g. barley, maize, wheat, triticale), accounting for 50%, followed by cakes and meals (e.g. sunflower cake, soybean meal) with 25% and co-products from the bioethanol industry (e.g. dried distillers' grains with solubles (DDGS)) with 12% (Figure 16). At least two single feed ingredients are combined for the preparation of compound feed, as well as possibly feed additives. Depending on its composition, compound feed is administered as complementary or complete feed. These feed rations are prepared in relation to animal specific physiological requirements (including growth status and species-specific properties), consequently high compositional differences exist between the individual feed formulas (FEFAC, 2018). As a standardisation in global feed production is not feasible, the sample

heterogeneity in terms of intra-matrix variations must be addressed within the protocol of validation. Additionally, future performance and validation guidelines should take these effects into account and consider an expansion of their scope with compound feed matrices as they are currently completely neglected (Steiner, Krska, et al., 2020).

**Contribution of the author**

Within a comprehensive pre-validation study, we evaluated analytical performance data including matrix effects, extraction efficiencies and apparent recoveries for a pilot set of representative analytes with different physico-chemical properties and an equal distribution over the entire chromatogram in 3 different compound feed matrices (cattle, chicken, pig) and their individual feed ingredients. The 12 single feed materials (alfalfa, barley, broad beans, DDGS, maize, rapeseed, soy, sunflower cake, triticale, wheat and wheat bran) complied with the regulated animal feed groups for validation purposes of multi-methods from the German national accreditation body (DAkkS) (Deutsche Akkreditierungsstelle, 2017). For validation purposes, artificial complex model matrices, composed of blank individual components were prepared in-house and the related numerical values for signal suppression/enhancement were modelled based on the data derived from the individual single feed ingredients. Comparability between model and real samples was statistically tested by comparing 7 different replicates for each matrix type. High matrix effects (both absolute and relative) were revealed as main negative contributor to the overall uncertainty. However, model matrices were less prone to influences of sample inhomogeneity due to the reduced natural background contamination. Additionally, compositional uncertainties of individual feed rations were eliminated by following the feed modelling approach. Therefore, we suggested a fit-for-purpose validation proposal (Figure 17) for LC-MS/MS multiclass methods in complex compound feed material based on 5 different replicates or lots of artificially prepared in-house samples. Study design and results are described in detail in Publication #2.

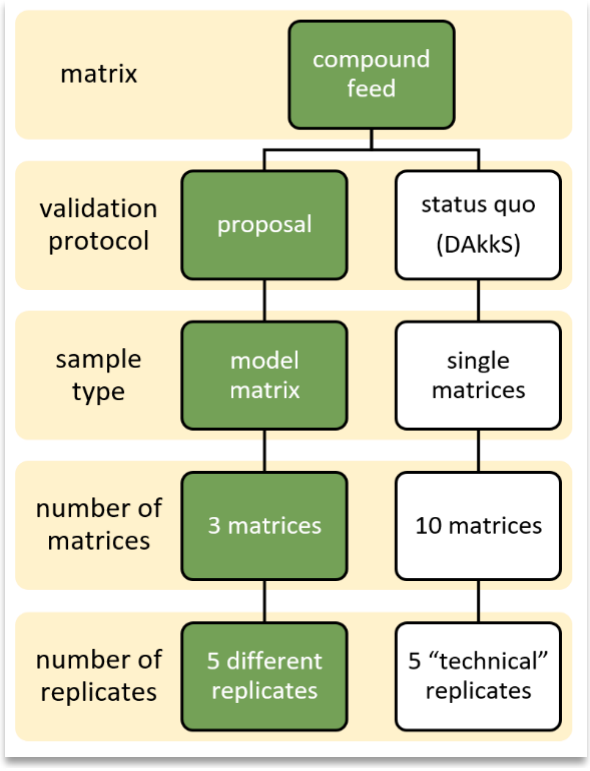



*Figure 17: Compound feed validation proposal*

### 3.3.4 Regulatory Limits

As described in chapter 1.3, maximum residue limits (MRLs) for different residue classes and maximum limits (MLs) for mycotoxins were set by the European Union. These legal limits define the maximum concentration of contaminants and residues permitted in feed and food to ensure the lowest possible consumer exposure (EFSA, 2014; Handford et al., 2015). Consequently, they represent indicators for quantification and detection limits of confirmatory methods of accredited national reference or official control laboratories. In other words, these values constitute minimum requirements in terms of method performance for different analyte matrix combinations. Partially, extremely low maximum values, like for aflatoxin $M_1$ in infant formulae (0.025 µg/kg), represent a major challenge with respect to the applicability of multiclass methods for the largest possible number of matrices. In addition, high variances in current regulatory limits, illustrated in Figure 18, pose a challenge with regard to method validation and application.

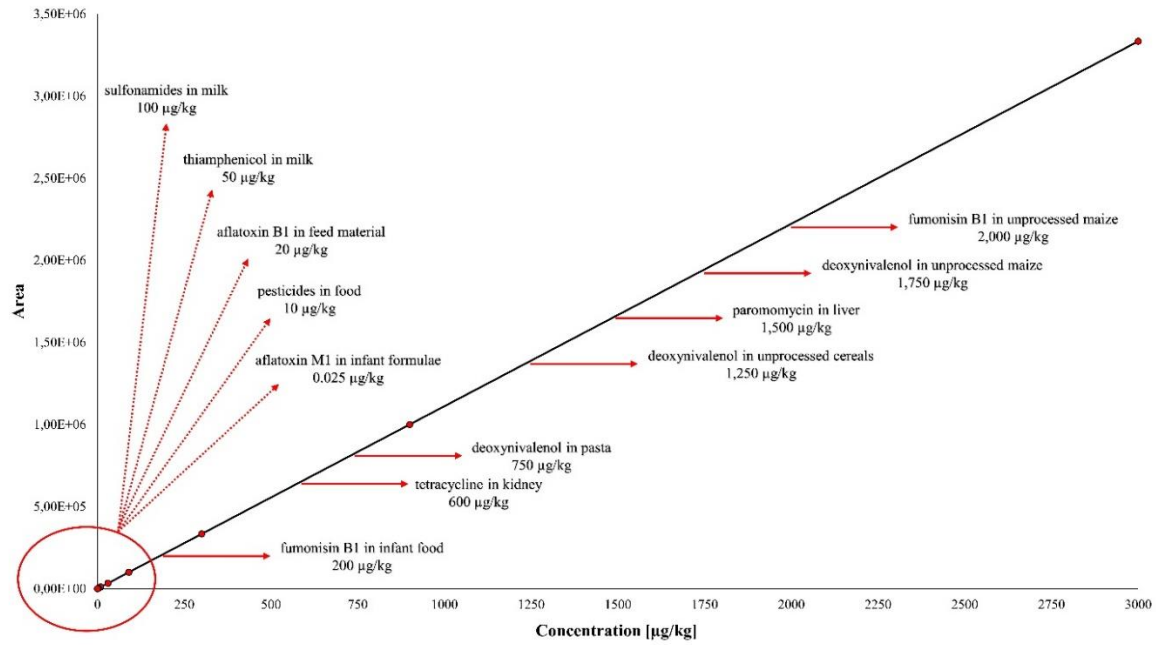



*Figure 18: Working/linear range related to existing regulatory limits for selected pesticides, mycotoxins and veterinary drugs. The y-axis represents the peak area, the x-axis the concentration in µg/kg.*

Thus, the wide application of these approaches faces not only the challenge in terms of limited sensitivity, but also very broad working ranges. Factors as high as 800-fold differences have to be considered within the method development process. It is obvious that multiclass approaches based on current LC-MS/MS technology are not able to comply with regulatory limits for all analyte/matrix combinations without additional adjustments to sample preparation, or chromatographic separation. Therefore, it is essential to define the scope of the multiclass approach prior validation and take existing regulatory limits into account.

## 3.4 Validation of Multiclass Approaches

As concerns the validation of multiclass approaches, there is a lack of guidance in the definition of performance criteria for the determination of multiple substance classes. Existing validation guidelines or documents refer primarily to a single substance class like the Commission Regulation (EC) No 401/2006 for mycotoxins. Additionally, the definition of relevant terms such as matrix effects or recovery are insufficient or imprecise, as in Commission Decision 2002/657/EC (European Commission, 2002a, 2006b). There is especially room for interpretation regarding the term "recovery" which refers to the acceptance criteria of 70-120% (Sulyok et al., 2020). IUPAC differentiates two different terms of recovery for distinct situations. First, the term "recovery" which is defined as "the yield of a preconcentration or extraction stage of an analytical process", and second the term "apparent recovery" which is "the quantity observed value/reference value, obtained using an analytical procedure that involves a calibration graph" (Burns et al., 2003). Based on these definitions, the term "recovery" is solely related to the extraction process, whereby the "apparent recovery" represents a combined measure between matrix effects and extraction losses. However, the lack of harmonisation in current validation and performance guidelines produces some uncertainties with respect to the proper definition of these terms. Therefore, the validation of the multiclass approach presented in this work took both definitions into account by investigating all relevant parameters such as matrix effects (SSE), extraction efficiencies (RE) and apparent recoveries (RA).

Evaluation of apparent recovery was carried out by comparing peak areas derived from spiked samples before extraction with peak areas of neat solvent standards.

$$\text{RA [\%]} = \frac{\text{area [spiked sample]}}{\text{area [standard]}} \text{ x } 100$$

Calculation of signal suppression or enhancement was performed by comparing peak areas from spiked sample extracts with peak areas of neat solvent standards.

$$\text{SSE [\%]} = \frac{\text{area [spiked extract]}}{\text{area [standard]}} \text{ x } 100$$

Extraction efficiency or "recovery" was calculated from the area of spiked samples before and after extraction.

$$\text{RE [\%]} = \frac{\text{area [spiked sample]}}{\text{area [spiked extract]}} \text{ x } 100$$

Since there is no validation guideline existing for animal feed in particular, the validation was carried out in accordance with SANTE/12682/2019 (European Commission, 2020). This guidance document specifies performance criteria for analytical quality control and multi-method validation procedures for pesticides and is applicable to food and feed. The assessment of the methods performance is based on the mean recovery (a measure for trueness and bias) and precision (repeatability), which are graphically demonstrated in Figure 19.

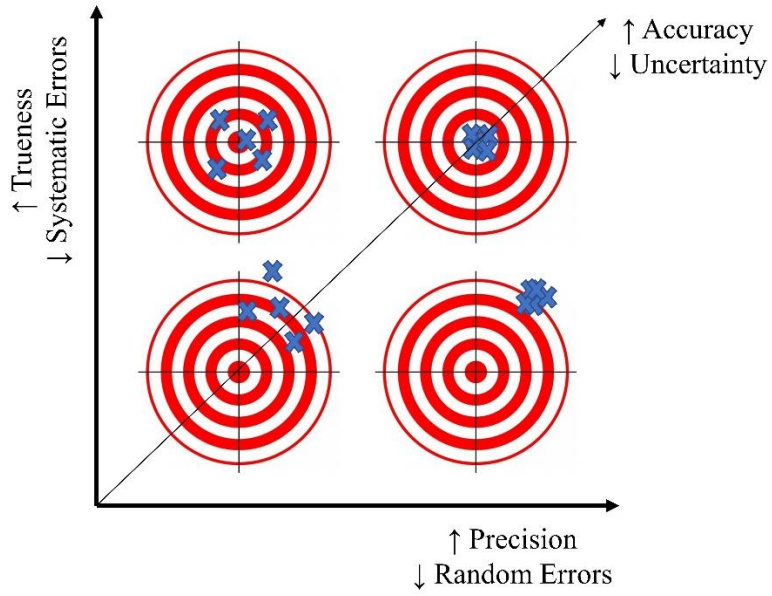



*Figure 19: Graphical illustration of the term's accuracy, precision and trueness*

The term accuracy describes the "closeness of agreement between an analytical result and the true, or accepted reference value". Applied to a set of results, it represents a combination of a systematic error (trueness or bias) and a random error (precision). The method's precision, on the other hand, is defined as "the closeness of agreement between independent analytical results obtained by applying the experimental procedure under stipulated conditions". Precision can be improved by lowering experimental errors. Acceptance criteria for individual recoveries are 70-120%. However, as noted in SANTE/12682/2019, a practical default range of 60-140% is accepted for individual recoveries in routine analysis. If the recovery rate is outside this range, it must be shown that the variance of the method allows a reliable quantitative statement. Acceptance criteria for repeatability, based on 5 sub-samples from 1 homogenised sample, is expressed as relative standard deviation (RSD) and should be ≤ 20% for each spike level tested ($RSD_r$), and for the within-laboratory reproducibility ($RSD_{WLR}$) (European Commission, 2020). However, as already discussed in chapter 3.3.3, the relative effects derived from intra-matrix variations are not yet considered by current validation guidelines. Recommendations for repeatability are based on identical test items/replicates or multiple (n = 18) aliquots of a blank

material (CEN/TR 16059, 2010; European Commission, 2002a, 2020). The investigation of relative matrix effects, which are the most pronounced in complex matrices such as compound feed, is fully neglected by this. Future guidance recommendations should therefore consider lots obtained from at least 5 individual samples per matrix type.

For multi-methods (>300 compounds), a minimum of 75% of all active substances must comply with the corresponding performance criteria in order to meet current accreditation standards, otherwise restrictions apply to the method (Deutsche Akkreditierungsstelle, 2017). Method performance in terms of sensitivity is expressed as limit of quantification and limit of detection (LOD). Based on the calculation scheme recommended by EURACHEM (Figure 20), the LOQ corresponds to a relative standard deviation of 10%.

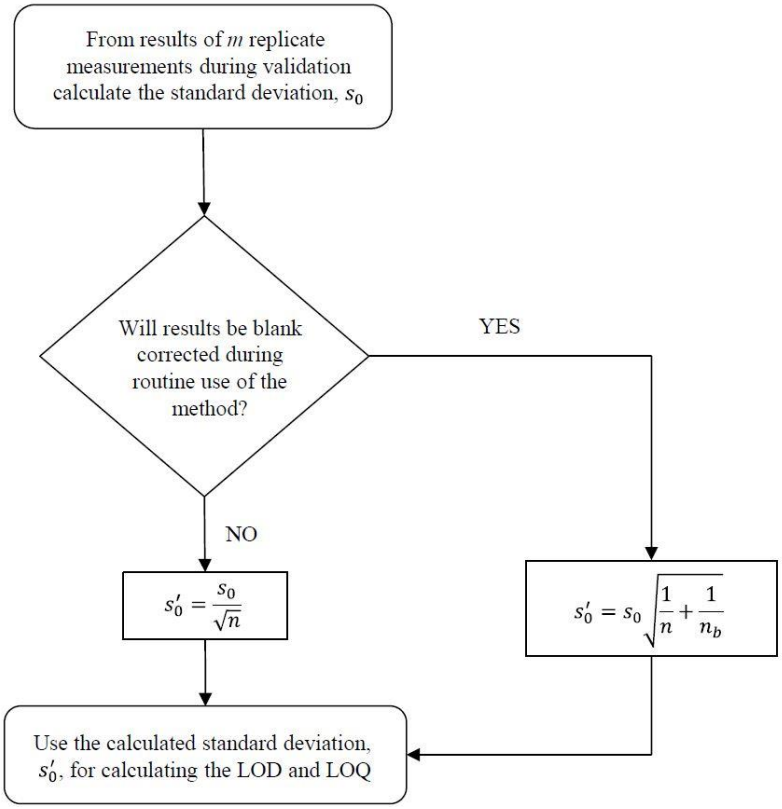
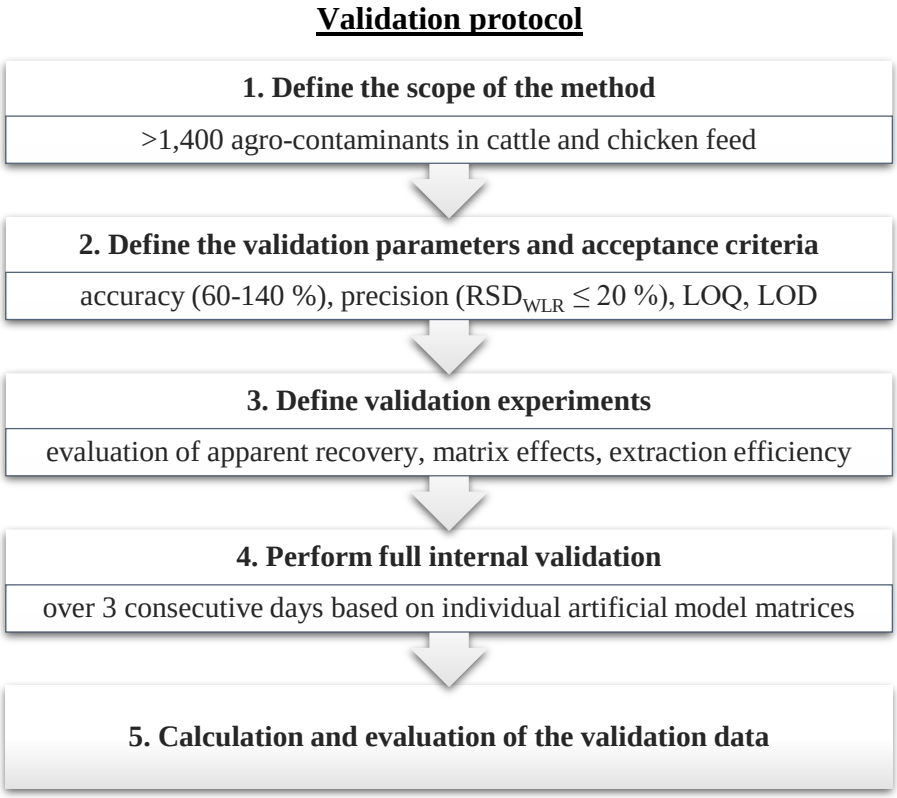



*Figure 20: Calculation of the standard deviation s'$_0$ for the determination of LOQ and LOD. ($s_0$ = estimated standard deviation of m single result at or near zero concentration, $s'_0$ = standard deviation for LOQ, LOD calculation, n = number of replicate observations averaged when reporting results where each replicate is obtained following the entire measurement procedure, $n_b$ = number of blank observations averaged when calculating the blank correction according to the measurement procedure)*

The obtained standard deviation ($s_0$) near zero concentration is multiplied by a factor of 3 for LOD and 10 for LOQ. With this approach, the conventional evaluation of signal/noise ratio was replaced (Wenzl et al., 2016). Following the regulations from SANTE and EURACHEM, the validation protocol was structured as follows:




## Contribution of the author

A concise overview of the multiclass validation data is provided in Publication #1. Briefly, apparent recoveries for 60% in cattle and 79% in chicken feed complied with the acceptance criteria of 60-140%. Extraction efficiencies ranged from 60 to 140% for 98-99% of all investigated compounds in cattle and chicken feed, revealing matrix effects as the main reason for the deviation from the target range. A fraction of 99% of analytes in chicken and 96% of analytes in cattle feed met the target criteria of $RSD_{WLR} \leq 20\%$ set for intermediate precision, which reflects the precision obtained within a single laboratory over an extended period of time. Limits of quantification between 1-10 µg/kg were achieved for the vast majority of compounds and complied with existing regulatory limits.

## 3.5 Application of the Multiclass Method

Since an employment of internal standards or a matrix matched calibration was omitted due to the wide range of physico-chemical properties and intra-matrix variations, the developed approach cannot be considered as official reference method and is not intended for measurements of residues and contaminants on the highest metrological level. However, external quality assurance based on >300 proficiency test (PT) results (Figure 21) for mycotoxins in compound and single feed ingredients (including bran, maize, triticale, wheat etc.), obtained between 2010-2019, with an overall success rate of >94%, indicates the competitiveness of this approach with other methods of the PT-participants (including accredited methods based on stable isotope dilution assays) (Sulyok et al., 2020).

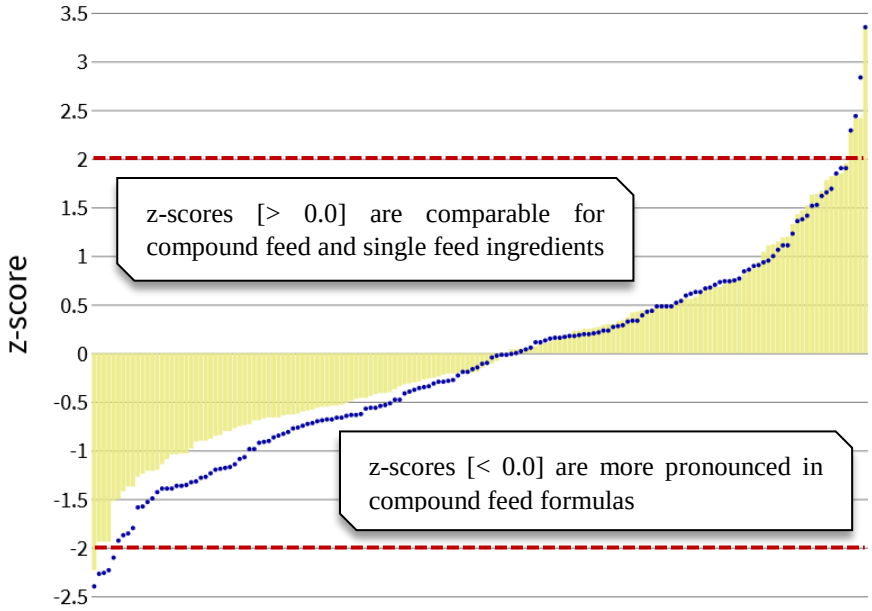



*Figure 21: Compilation of z-scores for mycotoxins in compound feed (blue dots, n = 160) and single feed ingredients (yellow bars, n = 160), 2010 to 2019*

The applicability of the multiclass method was tested within 2 studies. The first study was focusing on the investigation on the presence of regulated (Publication #3) and non-regulated (Publication #4) mycotoxins in maize harvested in the Republic of Serbia in the period of 2012-2015. Within the study number 2 (Publication #1) the focus was set on generating first insights into co-exposure scenarios of agro-contaminants in chicken and cattle feed.

### 3.5.1 Study 1 – Mycotoxins in Maize

The objective of this study was an investigation of the influence of changing weather conditions, including extreme drought (2012), hot and dry (2013, 2015) conditions and extreme

precipitation (2014), to the occurrence of mycotoxins in maize harvested in the Republic of Serbia. In total 204 maize samples were collected in the above-mentioned time period from the Autonomous Province of Vojvodina (Northern Serbia).

As described in detail in Publication #3, weather conditions in the four-year period of investigation had a significant influence on the presence and absence of 20 mycotoxins. A high aflatoxin prevalence was observed under conditions of extreme hot (deviation of +3°C of average temperature between June and July) and prolonged drought in the year 2012. High precipitation in 2014 (average of 10 days with precipitation between June and July), on the other hand led to an increase of deoxynivalenol and zearalenone. Only fumonisins were detected with high prevalence (76-100%) in all samples of the investigated time period. In addition, a significant number of maize samples contained mycotoxins at levels exceeding their regulatory limits. These results indicate that in particular an increase in the occurrence of aflatoxins and fumonisins in maize could pose a serious food safety problem in this region in the future. Moreover, the co-occurrence with other *Fusarium* toxins excluded from the regulatory framework might increase the potential risk of consumers in this part of Europe. For the first time, the natural occurrence of non-regulated fungal metabolites in this region was investigated using the same sample set. The results are highlighted in Publication #4. In total we have detected 109 different non-regulated fungal metabolites with 13-55 contaminants in every single maize sample. As already observed with the regulated mycotoxins, the non-regulated fungal metabolite profile of maize samples was strongly depending on weather conditions. Very wet and rainy conditions in 2014 were most favorable for the production of *Fusarium* metabolites, whereby *Aspergillus* metabolites were rarely detected in this year. Higher prevalence of *Aspergillus* metabolites was observed under hot and dry conditions especially in the year 2012. In addition, a very high frequency of *Penicillium* metabolites was detected in samples from the entire time period. Based on the results obtained in this study and taking climate change predictions (discussed in chapter 2.3) into account, significant improvements in maize production in Serbia will be necessary, including a multi-disciplinary integration of all participants in the feed and food chain, as well as management practices and in particular control strategies, in order to mitigate mycotoxin contamination (exceeding the regulatory limit) to ensure food safety in this region and for regions where Serbian maize is exported.

### 3.5.2 Study 2 – Multiclass Contaminants in Compound Feed

Study number 2 was focusing on the co-occurrence of all agro-contaminants covered by the multiclass approach in compound feed matrices. Detailed results are provided in Publication #1. In total 132 samples were investigated including 64 cattle and 68 chicken compound feed samples. In order to ensure high intra-matrix-variations, the sample material was collected from 15 different countries (Austria, Brazil, Colombia, Germany, Hungary, Italy, Kenya, Mozambique, Russia, South Africa, Switzerland, Tunisia, Turkey and Ukraine) between 2019 and 2020. All samples were tested positive for secondary fungal metabolites with a co-contamination (≥LOQ) of 12-87 compounds. In addition, we found representatives from the following substance classes: phytoestrogens, bacterial metabolites, veterinary drugs and plant toxins with an average co-occurrence (including secondary fungal metabolites) of 56 compounds in chicken and 45 compounds in cattle feed samples.

High co-contamination of estrogenic mycotoxins such as zearalenone and alternariol with phytoestrogens like genistein and daidzein were observed in 91% of chicken and 58% of cattle feed samples. Phytoestrogens are particularly prevalent in soy and alfalfa which explains their occurrence in chicken (includes fractions of soy) and cattle feed (includes fractions of alfalfa) (Hwang et al., 2001). Since these compounds are functionally and/or structurally similar to placental and ovarian estrogens this combination is of particular interest (Liu et al., 2010). Although the presence of phytoestrogens is mainly positive connotated within the majority of scientific publications, Stopper et al. described possible adverse genotoxic effects of phytoestrogens on humans (Stopper et al., 2005). These effects might be enhanced by the co-presence of mycoestrogens such as zearalenone as combinatory effects between these substances has already been described by Vejdovszky et al. 2017 (Vejdovszky et al., 2017).

These results indicate that a sole focus on regulated compounds, as usually practised within the analytical service of routine laboratories, relevant co-contaminations might be neglected and overlooked. More holistic approaches, as presented in this thesis, are necessary in order to simultaneously monitor the vast majority of chemical pollutants and derive relevant information of co-contamination patterns to better understand synergistic, additive and antagonistic effects.

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# Original Works

## List of Selected Publications

**Method development and validation**

Publication #1: **David Steiner**, Michael Sulyok, Alexandra Malachová, Anneliese Müller, Rudolf Krska (2020): Realizing the simultaneous liquid chromatography-tandem mass spectrometry based quantification of >1,200 biotoxins, pesticides and veterinary drugs in complex feed.
**Journal of Chromatography A** 1629: 461502

Publication #2: **David Steiner**, Rudolf Krska, Alexandra Malachová, Ines Taschl, Michael Sulyok (2020): Evaluation of Matrix Effects and Extraction Efficiencies of LC-MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed.
**Journal of Agricultural and Food Chemistry** 68 (12): 3868-3880

**Application of the method with respect to climate change**

Publication #3: Jovana Kos, Elizabet Janić Hajnal, Alexandra Malachová, **David Steiner**, Milena Stranska, Rudolf Krska, Birgit Poschmaier, Michael Sulyok (2020): Mycotoxins in maize harvested in Republic of Serbia in the period 2012-2015. Part 1: Regulated mycotoxins and its derivatives.
**Food Chemistry** 312: 126034.

Publication #4: Elizabet Janić Hajnal, Jovana Kos, Alexandra Malachová, **David Steiner**, Milena Stranska, Rudolf Krska, Michael Sulyok (2020): Mycotoxins in maize harvested in Serbia in the period 2012-2015. Part 2: Non-regulated mycotoxins and other fungal metabolites.
**Food Chemistry** 317: 126409.

Publication #1

# Realizing the simultaneous liquid chromatography-tandem mass spectrometry based quantification of >1,200 biotoxins, pesticides and veterinary drugs in complex feed.

**David Steiner**, Michael Sulyok, Alexandra Malachová, Anneliese Mueller, Rudolf Krska

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# Realizing the simultaneous liquid chromatography-tandem mass spectrometry based quantification of >1200 biotoxins, pesticides and veterinary drugs in complex feed

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ABSTRACT

The first quantitative multiclass approach enabling the accurate quantification of >1200 biotoxins, pesticides and veterinary drugs in complex feed using liquid chromatography tandem mass spectrometry (LC–MS/MS) has been developed. Optimization of HPLC/UHPLC (chromatographic column, flow rate and injection volume) and MS/MS conditions (dwell time and cycle time) were carried out in order to allow the combination of five major substance classes and the high number of target analytes with different physico-chemical properties. Cycle times and retention windows were carefully optimized and ensured appropriate dwell times reducing the overall measurement error. Validation was carried out in two compound feed matrices according to the EU SANTE validation guideline. Apparent recoveries matching the acceptable range of 60-140% accounted 60% and 79% for all analytes in cattle and chicken feed, respectively. High extraction efficiencies were obtained for all analyte/matrix combinations and revealed matrix effects as the main source for deviation of the targeted performance criteria. Concerning the methods repeatability 99% of all analytes in chicken and 96% in cattle feed complied with the acceptable RSD ≤ 20% criterion. Limits of quantification were between 1-10 µg/kg for the vast majority of compounds. Finally, the methods applicability was tested in >130 real compound feed samples and provides first insights into co-exposure of agro-contaminants in animal feed.



## 1. Introduction

Multiple factors, such as global trade, technological and socio-economic development, agricultural land use, and in particular climate change will affect food and feed safety in the coming century [1]. Due to climate change scenarios, crop growth and its interaction with pathogenic and beneficiary microorganisms vary from year to year, revealing the agricultural sector as the most vulnerable field [2]. Consequently, agricultural adaptions will be necessary, including changes in the geographical range of crop production. This may result in new interactions between plants and fungi, and a change in mycotoxin patterns [1]. Additionally, adverse conditions to the plant (via drought, pest attack, poor nutrition etc.) triggered by increasing temperatures may lead to increased mycotoxin production by fungi compared to favorable conditions [1]. Since the prevalence of plant pests and related diseases will increase, the use of pesticides and pesticidal activity will change considerably. Due to the limited activity of many pesticides under dry conditions, more frequent applications and/or higher dosage will be necessary to protect crops [3]. Beside agricultural crop production, the quality of food of animal origin is rising concern to public health organizations. In order to meet the challenges of providing adequate amounts of animal based foodstuff

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for the growing world population, veterinary drugs have played a key role in agro-industry and animal husbandry [4]. Hence, the worldwide application of veterinary drugs in animal production will inevitably increase in the next decades, leading to antimicrobial resistance of animal pathogens and subsequently impacts on the human resistome [5]. With the rising number of different agricultural contaminants, the potential of combinatory effects within [6], and in particular between [7] the respective substance classes may be enhanced. In order to assess these effects, an extensive data collection of various physical and chemical external exposures is mandatory. In recent years, the development of highly sensitive and selective, tandem mass spectrometric (MS/MS) and high-resolution mass spectrometric (HRMS) approaches, combined with advanced chromatographic technologies, enabled the development of such multi-methods. However, chromatography based quantitative multiclass approaches which enable the determination of more than two classes of contaminants and residues are still comparatively scarce [8]. Only a very limited number of real multiclass approaches, covering around 300 compounds, were developed so far [9–13]. Existing methods revealed targeted data acquisition within MS/MS detection as a limiting factor for the quantification of the rising number of analytes that can be determined in one analytical run [13].

This work presents the development and validation for a comprehensive quantitative LC–MS/MS based approach, covering a variety of the most important agro-contaminants from several substance classes in animal feed matrices. The applicability of this fully in-house validated MS/MS based approach covering a number of analytes which by far exceeds previous methods was demonstrated during the analysis of >130 real compound feed samples. Consequently, this method enables the construction of a prevalence data base for the investigation of combinatory effects from co-occurring compounds. We further highlight limitations of the current generation of the LC–MS/MS instruments with respect to the high number of target compounds measured within one chromatographic run.

## 2. Material and methods

### 2.1. Chemicals and reagents

In this work, 1467 analytes including 739 secondary fungal metabolites, 504 pesticides, 162 veterinary drugs, 47 plant toxins and 15 bacterial metabolites, were included. According to the availability of the analytical standards, the final validation was carried out for 1347 analytes. A list of all compounds including the LC–MS/MS acquisition parameters is covered in the supplemental material in Table S1. The majority of the reference standards were obtained commercially. In some cases, the standards were synthesized in-house or obtained as gifts from various research groups.

### 2.2. Preparation of stock and working solutions

LC gradient-grade acetonitrile and methanol as well as MS-grade glacial acetic acid (p.a.) and ammonium acetate were purchased from Sigma-Aldrich (Vienna, Austria). For further purification of reverse osmosis water, a Purelab Ultra system (ELGA Lab Water, Celle, Germany) was used. Reference standards were purchased from Romer Labs Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland), NEOCHEMA GmbH (Bodenheim, Germany), Restek GmbH (Bad Homburg, Germany), BioAustralis (Smithfield, Australia), AnalytiCon Discovery (Potsdam, Germany), Adipogen AG (Liestal, Switzerland), and LGC Promochem GmbH (Wesel, Germany). For each analyte, stock solutions were prepared by dissolving the solid standards in acetonitrile (primarily), acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v), or water. In total, 74 combined working solutions were prepared for biotoxins including fungal- and bacterial metabolites as well as plant toxins, 9 working solutions for pesticides, and 8 for pharmaceutical active agents. The combined working solutions were stored at −20°C.

### 2.3. Spiking protocol

For spiking purposes, a liquid multi-analyte standard was freshly prepared by combining the intermediate working mixtures. The final spike solution contained a concentration of 0.2 mg/l for pesticides and the majority of veterinary drugs and between 0.003 – 22.2 mg/l for biotoxins. An overview about the exact spike concentrations is provided in the supporting information in Table S2. Validation was performed at two different concentration levels with a factor of 5 difference, taking the high (ranged between level 2 and 3 of the calibration curve) as well as low (matched level 4) part of the linear range into account. To 0.25 g of homogenized samples, 50 µl and 10 µl of the multi-analyte spike solution were added for the high and low concentration level, respectively. The miniaturization of the spiking procedure was carried out for the economical use of standards. In order to avoid an analyte degradation and to ensure solvent evaporation, the spiked samples were stored in darkness and at room temperature overnight. For post extraction spiking experiments, 5 g of each sample material was extracted with 20 ml extraction solvent and the extracts were fortified with an appropriate amount of spiking solution, and dilution solvents. A detailed description of the post spiking procedure is described in the supplemental material in Table S3.

### 2.4. Data evaluation and quantitation

For the preparation of six external neat solvent calibration standards, a serial dilution of 1:3, 1:10, 1:30, 1:100, 1:300, and 1:1000 in acetonitrile/water/formic acid (49.5/49.5/1, v/v/v) was performed with a multi-analyte standard working solution. For pesticides and veterinary drugs, the calibration curve ranged between 0.1 – 31 µg/l, while for biotoxins no default calibration range could be applied. A detailed overview is provided in the supporting information in Table S2. Linear calibration curves for the neat solvent standards were prepared by using 1/x weighing. Peak integration and the construction of calibration curves was performed by using MultiQuant 3.0.3 (SCIEX, Foster City, CA, USA). The final data evaluation and calculations were carried out in Microsoft Excel 2013. Preparation of graphical content was performed by using the open access visualization software Flourish (Kiln Enterprises Ltd, London, UK).

### 2.5. Samples

Cattle and chicken compound feed matrices were used in this work. In order to maximize the challenge of repeatability of matrix effects and the extraction protocol, five different compound feed formulas were prepared in-house for each matrix type. The advantages of in-house matrix modelling for compound feed were described by us in [14]. For the preparation of the individual lots, single feed material including alfalfa, barley, corn, horse bean, rapeseed, soybean, sunflower cake, triticale, wheat, and wheat bran were used. The set of individual raw samples was provided by the companies Garant-Tiernahrung GmbH (Pöchlarn, Austria), BIOMIN GmbH (Getzersdorf, Austria), LVA GmbH (Klosterneuburg, Austria), and Bipea (Paris, France). Real compound feed samples were provided by BIOMIN GmbH (Getzersdorf, Austria). Pre-validation and optimization experiments were carried out with lots from the

same compound feed samples. Detailed information regarding the composition of the compound feed material and description of real samples is covered in the supplemental material in Table S4-5.

### 2.6. Sample preparation strategies

The initial evaluation of the sample preparation protocol included a comparison of different unspecific clean-ups, in order to determine a suitable procedure to reduce matrix effects. In all cases the samples were homogenized using an Osterizer blender. Five grams of each feed sample were extracted with 20 ml of extraction solvent (acetonitrile/water/formic acid 79:20:1, v/v/v) and shaken for 90 min under horizontal conditions by using a rotary shaker. The final sample extracts were either diluted or treated by an additional QuEChERS step and the subsamples were spiked with an appropriate amount of a multi-analyte standard.

#### 2.6.1. Dilute and shoot approach

Dilutions of 1:1, and 1:10, and 1:100 of the final extracts were prepared by mixing appropriate amounts of spiking solutions, raw extracts and dilution solvents. A mixture of acetonitrile/water/formic acid 20:79:1 (v/v/v) was used as dilution solvent for the 1:1 dilution, and acetonitrile/water/formic acid 49.5:49.5:1 (v/v/v) for the 1:10 and 1:100 dilution steps, respectively.

#### 2.6.2. QuEChERS approach

Modified QuEChERS procedures were performed based on the original protocol described in [15]. To 5 ml sample extract, 2 g of anhydrous $MgSO_4$, and 0.5 g of sodium chloride were added and shaken vigorously for 1 min. The mixture was centrifuged (5 min, 2400× *g*) and separated into 3 aliquots of 1 ml each. One set of aliquots were frozen overnight at -20°C in order to ensure a precipitation of lipid components from the feed matrix. To the remaining aliquots either 25 mg of PSA, or $C_{18}$ as cleanup sorbent were added, shaken for 1 min and centrifuged (5 min, 2400× *g*). Finally, supernatants were transferred into autosampler vials.

### 2.7. Liquid chromatography tandem mass spectrometry (LC−MS/MS) analysis

Initial LC–MS/MS optimization steps included column, injection volume, flow rate, dwell and cycle time investigations. The performance of the LC system under UHPLC and HPLC conditions was compared by evaluating the extent of matrix effects in spiked cattle feed extracts using a Kinetex UHPLC C18-column (1.7 µm 2.1 × 100 mm), and a Gemini HPLC C18-column (5 µm 150 × 4.6 mm) both from Phenomenex. Flow rate investigations were conducted between 0.5 to 1 ml/min and injection volume trials between 1 and 20 µl. Dwell and cycle time optimization steps were performed with a neat solvent multi-analyte mix standard solution and included a cycle time range between 1.0 to 1.5 s and retention windows from 30 to 40 s.

#### 2.7.1. HPLC instrumental conditions

The sSRM detection window of each analyte in the final method was set to the respective retention time ± 30 s. The target scan time was set to 1.5 s. The settings of the ESI source were as follows: source temperature 550°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage −4500 V and +5500 V, respectively, collision gas (nitrogen) medium. Column temperature was set at 25°C.

#### 2.7.2. UHPLC instrumental conditions

Under UHPLC conditions, the sSRM detection window of each analyte was set to the respective retention time ± 15 s. The target scan time was set to 0.8 s. The settings of the ESI source were as follows: source temperature 500°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 60 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 60 psi (551.6 kPa of nitrogen), ion-spray voltage −4500 V and +5500 V, respectively, collision gas (nitrogen) medium. Injection volume was set to 1 µl combined with a flow rate of 0.3 ml/min. Column temperature was set at 25°C.

#### 2.7.3. Final LC–MS/MS instrumental method

Detection and quantification of the final LC–MS/MS method was performed with a QTrap 5500 MS/MS system (SCIEX, Foster City, CA, USA) equipped with a TurboV source and an electrospray ionization (ESI) probe coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was performed on the previously mentioned Gemini C18-column at 25°C, equipped with a C18 security guard cartridge (4 × 3 mm i.d.) from Phenomenex. An injection volume of 5 µl was chosen for the autosampler program combined with a flow rate of 1 ml/min. Elution was carried out in a binary gradient mode consisting of methanol/water/acetic acid 10:89:1 (v/v/v) representing mobile phase A, and methanol/water/acetic acid 97:2:1 (v/v/v) representing mobile phase B, both contained 5 mM ammonium acetate buffer. The starting gradient conditions were set at 100% A after an initial time of 2 min and the proportion of B was increased linearly to 50% after 3 min. Mobile phase B was increased to 100% within 9 min followed by a hold time of 4 and 3.5-min column re-equilibration at 100% A. Two successive chromatographic runs in positive and negative ionization mode were carried out for the analytical measurement using a scheduled multiple reaction monitoring (sMRM) algorithm with a total run time of 21 min each. For increased confidence in compound identification, two sMRM transitions per analyte (with the exception of 3-nitropropionic acid, moniliformin, 4-chlorophenoxyacetic acid, bromoxynil, diclofop, ethoprophos, flumetralin, fluotrimazole, haloxyfop, isoxaflutol, MCPA, mecoprop-P, phorat, diclazuril-methyl, and levamisole which each exhibit only one fragment ion) were acquired.

### 2.8. Validation protocol

Method validation was performed according to SANTE/12682/2019 validation guideline criteria [16]. For two compound feed matrices, subsamples of 0.25 g were fortified with a multi-compound spiking solution covering all target analytes. This was carried out using 5 individual samples per matrix at two concentration levels (factor 5 difference). Lower concentration ranges of samples were adjusted to cover the respective limits of detection of each compound, and legislation limits of regulated mycotoxins following Directive 2002/32/EC [17]. For pesticides and veterinary drugs the low concentration levels were < 0.01 mg/kg. The fortified samples were extracted by following the protocol mentioned above, using 1 ml of extraction solvent and combined with a 1:1 dilution step. Within the LC–MS/MS sequence, the five sample extracts of each matrix were bracketed by the external neat solvent calibration standards and a control solvent standard at the same concentration. This control standard was analyzed for verification of linearity against response. Determination of the intermediate precision was carried out on three different days. Investigation of matrix effects, expressed as signal suppression/enhancement (SSE) and extraction efficiencies were conducted by spiking the diluted blank extracts of each model matrix at the concentration range matching the external standards

of the high concentration level. Determination of the limit of quantification (LOQ) and limit of detection (LOD) was performed according to EURACHEM guide [18]. Based on EURACHEM, the LOQ represents the lowest level at which the performance is acceptable for a typical application. The LOQ evaluation involved replicate measurements (n = 5) of individual samples spiked with a low concentration of analytes to determine the standard deviation $s_o$ expressed as concentration units. The LOQ and LOD were obtained after multiplication of $s_o$ with a factor of 10 and 3, respectively. Criteria for identification evidence were set in accordance to SANTE/12682/2019 and included an ion ratio deviation of 30 % and a retention time tolerance of 0.03 min.

## 3. Results and discussion

To the best of our knowledge, this work represents the first quantitative LC–MS/MS based method covering such a vast amount of natural and anthropogenic agro-contaminants and consequently enables the construction of a prevalence data base for the investigation of a "cocktail" of co-occurring compounds from different contaminant classes. As matrix effects and acquisition parameters (dwell time and cycle time) are considered to be the main limitation of such a method, several experiments were conducted in order to optimize the methodological procedure with respect to the mentioned limitations.

### 3.1. LC–MS/MS optimization

The original LC–MS/MS setup was designed for the determination of mycotoxins in cereal based material [19], and was optimized during the different development stages of this novel multiclass approach.

#### 3.1.1. Adjustment of acquisition parameters

Within every MRM scan each substance is monitored intermittently and requires a specific amount of dwell time ($t_{Dwell}$) which usually accounts ~25 ms for the simultaneous measurement of ~50 compounds, in order to ensure a sufficient number (10-15) of data points per peak with a chromatographic peak width ($t_{Window}$) of ≥15 s [20]. Within a scheduled MRM mode, $t_{Dwell}$ is automatically adjusted to the number of concurrent MRM transitions within the related cycle. Consequently, the reliability of peak quantitation decreases due to the rising number of contemporary transitions, since these determines the time needed to complete all transitions ($t_{Cycle}$) and data points per peak [21]. We further assume, that falling below a critical $t_{Dwell}$ threshold of 10 ms [22], causes a comparable deterioration in precision and leads to an increase of the measurement error. Therefore, we have compared different acquisition settings with varying $t_{Cycle}$ and $t_{Window}$ in order to obtain sufficient $t_{Dwell}$ and data points per peak. As shown in Fig. 1, an increase of $t_{Cycle}$ and a reduction of $t_{Window}$ led to a considerable improvement of $t_{Dwell}$. Critical $t_{Dwell}$ values (< 10 ms) were increased by a factor of ~2 in the critical chromatographic time window (8-13 min), covering the highest amount of concurrent MRM transitions. The average number of data points per peak was reduced by a third from 15 to 10 data points per 15 s peak width. However, sacrificing some data points in order to increase $t_{Dwell}$ had no negative impact on the methods precision measured by repeated injections (n = 5) of a multi-analyte standard close to the expected instrumental LOQ. On the contrary, the increased $t_{Dwell}$ budget led to a significant ($\alpha$ = 0.05) improvement in repeatability. This can be explained by a noise reduction on the baseline and the peak [21], and was confirmed by an enhancement of the signal-to-noise (S/N) ratio. Average S/N values (obtained by manual investigation) for 40 compounds amounted 12 (a), 22 (b), and 27 (c). However, this acquisition setup requires very stable retention times in order to prevent peaks shifting out of the target retention window. For routine purposes, a frequent change of methods and eluents in the LC–MS/MS system should therefore be avoided. Data recorded for the adjustment of the acquisition parameters are provided in the supplemental material in Table S6-8, and Fig. S1-2.

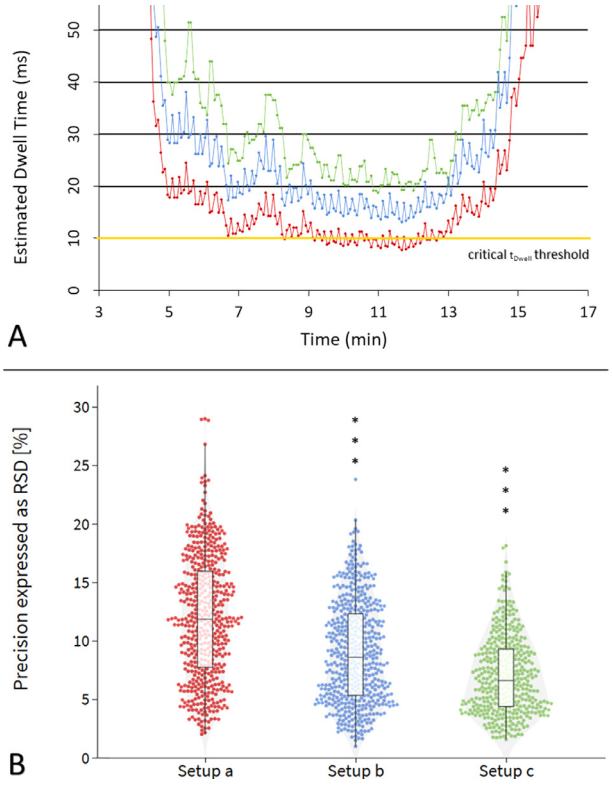


**Fig. 1.** Acquisition setup configurations consist of $t_{Cycle}$ 1.0, 1.5, and 1.5 s as well as $t_{Window}$ of 40, 40, and 30 s for setup a (red), b (blue) and c (green). A represents a computational estimation of $t_{Dwell}$ in positive ionization mode (y-axis). The x-axis shows the duration of the chromatographic run in minutes. B represents the repeatability ($n$ = 5) expressed as relative standard deviation in percent for a multi-analyte standard (instrumental LOQ). The outlier-corrected box plot includes an interquartile range of 1.5. Statistical significance was tested based on F-test statistics. Data evaluation was carried out for 400 target compounds with a concentration range of 0.008 µg/l (ergometrinine) and 33 µg/l (culmorin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

#### 3.1.2. HPLC versus UHPLC

In routine analysis, an increased throughput, speed, efficiency, and reduced analysis costs are essential features. Ultra-high performance liquid chromatography (UHPLC) is characterized by an ultra-high-pressure system which enables the use of columns with small diameter and particle size in order to reduce analysis time and improve efficiency, expressed as height equivalent of theoretical plates (HETP) [23]. Since the resolution is proportional to the square root of the column efficiency [24], UHPLC columns with small particle size should provide a benefit with respect to matrix effects, through an improved separation and lowering the potential of target analytes overlapping with co-eluting matrix components [25]. Therefore, we have evaluated matrix effects of five fortified cattle feed extracts for 200 compounds, once tested under HPLC conditions with a chromatographic runtime of 21 min and once under UHPLC conditions with a run time of 10.5 min. A detailed data overview on the column comparison experiments is given in Table S9-10 and Fig. S3 of the supplemental material. As assumed, peak resolution and peak shape was improved considerably on UHPLC. The average peak width at 50% was reduced by a

factor of ~2 from 0.21 min (HPLC) to 0.11 min (UHPLC). However, as considers matrix effects no significant ($\alpha = 0.05$) differences were observed neither for relative ($P_{(F<=f)} = 0.42$), nor for absolute matrix effects ($P_{(T<=t)} = 0.22$). These results indicate that the benefits of an UHPLC system with respect to matrix effects may be lost, as the increased peak resolution does not prevent co-elution between some of the hundreds of target compounds (being distributed over the whole chromatogram) and matrix components. Although UHPLC provides a better resolution and narrower peaks, we have decided to validate the method under HPLC conditions for several reasons. Narrowing the peak shape within UHPLC reduces the cycle time and evokes the problem of achieving appropriate dwell times and number of data points per peak [26,27]. Since the compatibility of UHPLC columns to turbid samples is limited compared to HPLC [27], the use of microfilters is necessary in order to prolong the life time of the UHPLC column. This additional step during sample preparation can be avoided by using HPLC, leading to an economization of time and resources. Consequently, as UHPLC did not reveal an advantage compared to HPLC, we abandoned this approach due to practical reasons.

#### 3.1.3. Injection volume and flow rate

Matrix effects (ME) of five fortified extracts of cattle and chicken feed samples were evaluated for 50 selected compounds and detailed results of injection volume and flow rate investigations are provided in the supplemental material in Table S11-13 and Fig. S4-6. Based on the assumption that under lower flow, smaller ESI-droplets can be formed and the competition between analyte and matrix components at the droplet surface is reduced, decreased flow rates should have a beneficial effect on matrix effects [28]. Contrary to this assumption, an increase of the flow rate by a factor of 2 (from 0.5 to 1 ml/min) led to a reduction of matrix effects by 14% in cattle and 13% in chicken feed extracts. Since the size of the spray droplet released from the Taylor Cone not only depends on the flow rate but also on the capillary diameter, obviously the design of the ionization source is also influencing the magnitude of matrix effects [29,30]. Sensitivity was measured by the peak height and were accompanied by a constant decline of ~3.5% per 0.1 ml/min flow increase. The comparison of injection volumes was carried out with 1, 5, 10, and 20 µl and were compared to manual dilution series including dilution factors of 2, 5, 10, 20, and 100. Matrix effects in the range of 30-40% were reduced considerably (ME $\leq$ 20%) by applying a dilution factor of 10, while matrix effects >40% tend to require a further increase of dilution in order to comply with the ±20% criterion for ME [16]. In addition, a decrease of the injection volume by a factor of 5 reduces ME by ~20%. However, a general dilution factor cannot be derived for several reasons: depending on the analyte/matrix combination, the magnitude of matrix effects varies very strongly and requires individual dilutions. Additionally, it seems not appropriate to define a general dilution factor if matrix effects up to 20% are accepted [30]. Based on these results, an injection volume of 5 µl combined with a flow rate of 1 ml/min pointed out as the most suitable combination in order to ensure an appropriate instrumental dilution factor, and to achieve a satisfying sensitivity.

### 3.2. Sample preparation for multiclass analysis

In recent years, sample preparation procedures for multicompound determination reported by literature were primary dedicated to pesticide analysis in vegetables, fruits or cereals. The most frequently used protocols were based on a QuEChERS approach following a partitioning step with acetonitrile, which was developed for the reduction of the solvent volume in order to improve laboratory efficiency [31,32]. Similar approaches exist in the field of veterinary drug analysis mainly described for animal tissues [8], or animal based products such as meat, and milk [33,34]. In the area of mycotoxin analysis, extraction procedures consist of mixtures of acetonitrile, water or methanol, with and without acidification [26,35]. Multiclass approaches covering several hundred compounds from different substance classes follow a more generic sample preparation protocol. High extraction yields for a variety of mycotoxins, pesticides, plant toxins and veterinary drugs were obtained with acidified extraction solvents while avoiding phase separation [13]. On the basis of the literature, a solid liquid sample preparation protocol (see chapter 2.6) was used for extraction. Since relative matrix effects represent the major limitation of multi-analyte approaches [26], the extraction protocol was combined with further dilution steps as well as modified QuEChERS protocols in order to reduce these undesired effects. The initial comparison of all sample preparation experiments was conducted for 100 fungal metabolites with a concentration range of 0.27 – 571 µg/l and for 100 pesticides at 10 µg/l in cattle feed extracts. Detailed data description is covered in the supplemental material in Table S14 and Fig. S7. As highlighted in Fig. 2, the modified QuEChERS based approaches showed no considerable advantages with respect to absolute and relative matrix effects compared to dilute and shoot. Low matrix effects (ME <20%) were obtained only for 28.5%, 20%, 22%, and 21.5% of analytes (including e.g. 2,4-DB, calphostin, fellutanine A, fipronil sulfide, haloxyfop, metaflumizone, novaluron, oligomycin B, and usnic acid) following the QuEChERS combinations with PSA, $C_{18}$, deep freezing, and the 1:1 dilution, respectively. However, high matrix effects (ME >40%) were observed for acephate, acifluorfen, altersetin, geodin, meleagrin, and picolinafen in at least two QuEChERS combinations. Additionally, fumonisins were lost during the PSA purification step due to the acidic properties of these compounds which results in an irreverible binding to the PSA sorbent [36]. Based on the 1:1 dilution, high absolute matrix effects were observed for aflatoxin $B_2$, aflatoxin $G_2$, aldicarb sulfone, fumonisin $B_1$, rimsulfuron, and silafluorfen but with an evident consistency (RSD <5%). In general, the QuEChERS approaches showed a higher susceptibility to relative matrix effects (RSD >15%) [37]. Furthermore, the results showed that an increase of the dilution factor led to a significant reduction in both, absolute and relative matrix effects, but this is inevitably accompanied by a loss of sensitivity. As all of the investigated modified QuEChERS approaches showed limited improvement in terms of matrix effects, the final decision was made to use a straightforward 1:1 dilution approach, which represents the best compromise in terms of sensitivity and matrix effect reduction. However, for the screening of substances occurring at high concentrations, a further dilution would be the straightforward solution.

### 3.3. Method validation of complex compound feed

Currently, there is no particular guidance or directive existing for the validation of analytical methods with regard to the determination of multiple substance classes. Although some guidance documents are providing requirements and performance parameters for analytical method development, these are either only referring to a certain substance class such as the Commission Regulation (EC) No 401/2006 [38] for mycotoxins, or are insufficient in terms of the definition of matrix effects and recovery such as the Commission Decision 2002/657/EC [39]. Therefore, the validation of the given multiclass method was carried out according to SANTE/12682/2019 [16], since it is applicable for feed matrices and it takes real-life conditions of routine orientated laboratories into account. Low concentration levels were adjusted to existing regulatory limits for pesticides [40], mycotoxins [17], and veterinary drugs [41]. An overview of the validation performance including apparent recoveries ($R_A$), signal suppressions and enhancements

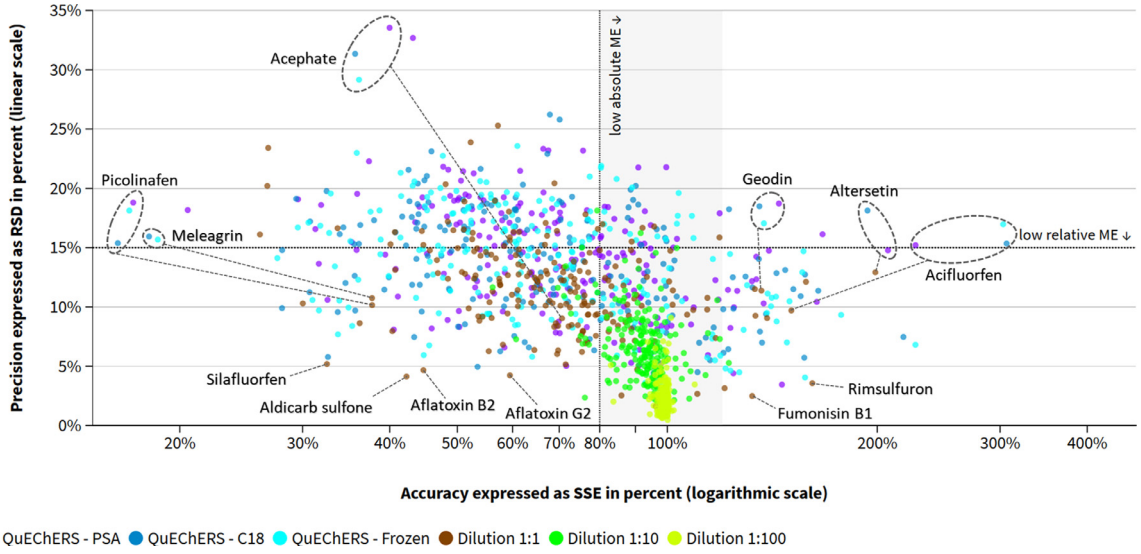


**Fig. 2.** Quadrant chart illustrating the accuracy expressed as signal suppression enhancement in percent in logarithmic scale (*x*-axis) and precision expressed as relative standard deviation (derived from 5 individual cattle feed lots) in percent in linear scale (*y*-axis). Each target analyte is depicted by a colored dot. Different colors represent the tested sample preparation protocols. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(SSE), and extraction efficiencies ($R_E$) is depicted in Fig. 3. A comprehensive validation data description is additionally provided in the supplemental material in Table S15 and Fig. S8-12.

### 3.3.1. Method accuracy

As the applicability of a matrix matched calibration is not feasible for a couple of reasons (it is almost impossible to find a compound feed sample material which is entirely blank for this high amount of substances, and the high sample complexity in terms of varying feed rations cannot be covered by a single sample representative), validation was performed based on a neat solvent calibration. A range for the criteria "recovery" is set for 70-120% [16], but there is still a discrepancy with respect to the definition of this term [26]. Therefore, we have evaluated the methods accuracy based on the apparent recovery ($R_A$), representing a combined measure of matrix effects and losses during extraction, and the recovery from the extraction ($R_E$). According to this criterion, $R_A$ values at the high concentration level complied for 38.9% of analytes in cattle and for 62% of the analytes in chicken feed. However, in routine analysis a practical default range of 60-140% [16] for multi-compound determination can be applied, leading to 60.5% and 79.3% of analytes at high level and 60.6% and 78% of analytes at low level which were successfully validated in cattle and chicken feed, respectively. As highlighted in Fig. 3, the main cause triggering a deviation from the target recovery range are matrix effects. Strong signal suppressions were especially pronounced in cattle feed, which is mainly caused by green fodder components (alfalfa) in the compound feed rations [14]. SSE values <60% were accounting for 32.3% of analytes in cattle and 13.6% in chicken feed. In contrast, extraction efficiencies were very consistent in both feed types. In cattle, 97.9% and 99.3% of analytes in chicken, were in the range of 60-140%.

### 3.3.2. Method precision

Both the precision of the method as well as the within laboratory reproducibility ($RSD_{WLR}$) was proven by spiking a set of five different lots at high concentration level per matrix (in contrast to "identical test items" which are used in most published methods) on three different days, resulting in 15 total repetitions for $R_A$. Repeatability results of the extraction protocol ($RSD_{RE}$) and matrix effects ($RSD_{SSE}$) are based on five individual lots per matrix, spiked on one day. With 98.8% and 95.9% of analytes in chicken and cattle feed, most of the compounds complied with the $RSD_{WLR}$ criterion of RSD ≤20% [16]. As shown in Fig. 3, the methods precision in chicken feed was equally influenced by relative matrix effects with a median $RSD_{SSE}$ of 6.7%, and the variability of the extraction with 6.8% median $RSD_{RE}$. On the contrary, cattle feed showed a higher susceptibility to relative matrix effects with 11.3% median $RSD_{SSE}$ compared to 8.2% $RSD_{RE}$. High relative matrix effects are obviously a result of increased sample complexity in terms of composition, including the number and amounts of raw feed material used for the preparation of the compound feed formulas (see supporting information Table S4). As the results in the preliminary experiments have shown, a 1:10 dilution would reduce the relative matrix effects considerably. However, a compliance with the current limits of quantification, especially for pesticides and veterinary drugs could not be guaranteed due to an associated sensitivity loss.

### 3.3.3. Performance characteristics and applicability

The limits of quantification and limits of detection for all analytes were calculated according to the EURACHEM guideline [18]. As described in Section 2.8, the obtained standard deviation ($s_o$) at low concentration level is multiplied by a factor of 10 for LOQ and 3 for LOD. Consequently, this multiplier corresponds to a relative standard deviation of 10% for the LOQ. The numerical values for LOQs for all analytes in chicken and cattle feed are listed in Table 1.

No huge differences were observed comparing LOQs and LODs between cattle and chicken feed. The majority of compounds are in the LOQ range between 1-10 µg/kg, accounting for almost all pesticides and veterinary drugs. Lowest LOQs (<1 µg/kg) were in both matrices obtained for ergot alkaloids (e.g. dihydroergosine, ergocryptine, ergocornine, ergotamine, and ergine), some cyclic depsipeptides produced by *Fusarium* fungi (enniatin A, enniatin B2, enniatin B3), the bacterial metabolites nonactin and monactin and the aflatoxin $B_1$ precursor averufanin.

With respect to the compound identification, the analytes complied with a relative ion ratio deviation of 30 % based on the average ion ratio of all standards measured within one sequence. As considers the retention time tolerance, the compounds met the criteria of 0.03 min, which represents a stricter criterion compared to the legislative tolerance of 0.1 min [16].

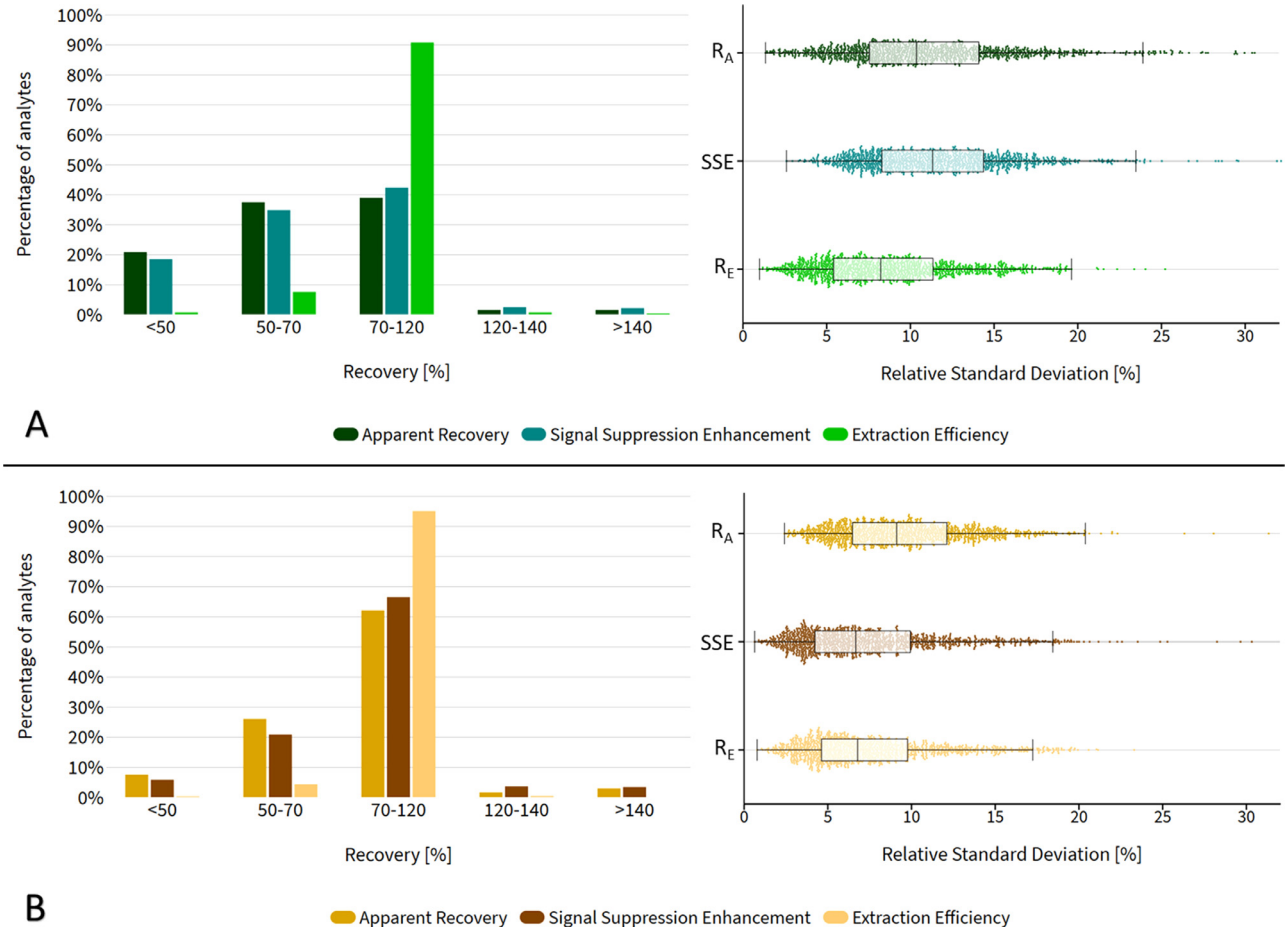


**Fig. 3.** Distribution of apparent recoveries (RA), signal suppressions and enhancements (SSE), and extraction efficiencies (RE) as well as associated relative standard deviations of all analytes in cattle (A) and chicken feed (B).

**Table 1**
Limits of quantification for all tested analytes in cattle and chicken feed.

| number of contaminants and residues LOQ in µg/kg ($n$ = 5) | | | | | | |
|---|---|---|---|---|---|---|
| matrix | class | < 1 | 1-10 | 10-50 | 50-100 | > 100 |
| chicken | FM | 26 | **402** | 115 | 17 | 10 |
| | P | 1 | **488** | 5 | 2 | 1 |
| | PT | 0 | **9** | 22 | 3 | 3 |
| | VD | 0 | **92** | 12 | 1 | 0 |
| | BM | 2 | **6** | 2 | 0 | 0 |
| cattle | FM | 23 | **387** | 123 | 17 | 12 |
| | P | 1 | **481** | 11 | 3 | 0 |
| | PT | 0 | **12** | 14 | 6 | 7 |
| | VD | 0 | **90** | 15 | 0 | 0 |
| | BM | 2 | **4** | 4 | 0 | 0 |

**FM** = fungal metabolite, **P** = pesticide, **PT** = plant toxin, **VD** = veterinary drug, **BM** = bacterial metabolite

*3.3.4. Application to real compound feed samples*

To prove the methods applicability in real compound feed material, chicken ($n$=68) and cattle feed ($n$=64) samples from 15 different countries were tested. An average co-contamination (≥ LOQ) of 45 compounds in cattle and 56 in chicken feed was observed, including representatives from almost all substance classes. In detail, we observed a high co-contamination of phyto- (e.g daidzein, genistein) and mycoestrogens (zearalenone, alternariol) in 91% of chicken, and 58% of cattle feed samples, which can be explained by the soy and alfalfa proportion in the respective feed formulas [42]. This combination is of particular relevance, since a mixture of phyto- and mycoestrogens may cause combinatory effects and could thus negatively impact on animal health [7].

## 4. Conclusion

For the first time the feasibility of the simultaneous quantitative determination of >1200 biotoxins, pesticides and veterinary drugs has been demonstrated for two different compound feed matrices. It has been shown that potential advantages of UHPLC with respect to matrix effects are diminished with increasing number of target analytes. A combination of a high flow rate with a low injection volume under HPLC conditions revealed as the most suitable combination in order to achieve a yet unknown ideal compromise between sensitivity and matrix effects. Adjustments including cycle time and retention window width are necessary to ensure appropriate dwell times in order to reduce the overall measurement error. Limits of quantification were < 10 µg/kg for the vast majority of analyte matrix combinations and complied with existing regulations for mycotoxins, pesticides, and veterinary drugs. Therefore, this fully in-house validated multiclass method enables the construction of a prevalence data base of co-occurring compounds from different contaminant classes on a quantitative basis, and reveals insights into metabolite profile changes due to climate change. Further possible applications include the improved risk assessment of co-occuring substances, such as phyto- and mycoestrogens which might act in a synergistic, additive, or antagonistic way. Additionally, the method can be transferred and applied to other

commodities e.g. from the food chain, which may provide relevant exposure data as part for the assessment of the dietary-exposome.

## Declaration of Competing Interest

The authors declare no competing financial interest.

## CRediT authorship contribution statement

**David Steiner:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Michael Sulyok:** Conceptualization, Methodology, Writing - review & editing. **Alexandra Malachová:** Conceptualization, Methodology, Writing - review & editing. **Anneliese Mueller:** Funding acquisition, Resources, Writing - review & editing. **Rudolf Krska:** Conceptualization, Methodology, Project administration, Supervision, Writing - review & editing.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461502.

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Publication #2

# Evaluation of Matrix Effects and Extraction Efficiencies of LC-MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed

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Article

# Evaluation of Matrix Effects and Extraction Efficiencies of LC−MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed

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**ABSTRACT:** This work provides a proposal for proper determination of matrix effects and extraction efficiencies as an integral part of full validation of liquid chromatography coupled to tandem mass spectrometry-based multiclass methods for complex feedstuff. Analytical performance data have been determined for 100 selected analytes in three compound feed matrices and twelve single feed ingredients using seven individual samples per matrix type. Apparent recoveries ranged from 60−140% for 52−89% of all compounds in single feed materials and 51−72% in complex compound feed. Regarding extraction efficiencies, 84−97% of all analytes ranged within 70−120% in all tested feed materials, implying that signal suppression due to matrix effects is the main source for the deviation from 100% of the expected target deriving from external calibration. However, the comparison between compound feed and single feed materials shows great variances regarding the apparent recoveries and matrix effects. Therefore, model compound feed formulas for cattle, pig, and chicken were prepared in-house in order to circumvent the issue of the lack of a true blank sample material and to simulate compositional uncertainties. The results of this work highlight that compound feed modeling enables a more realistic estimation of the method performance and therefore should be implemented in future validation guidelines.

**KEYWORDS:** *residues, mycotoxins, veterinary drugs, pesticides, compound feed, recovery, model matrices*



## 1. INTRODUCTION

A number of influencing factors such as storage and climate conditions, cultivation practices, and processing contribute to the presence of a large variety of undesired substances in the food and feed chain. Besides anthropogenic inputs, by purpose-related use such as pesticides and veterinary drugs, food safety is additionally challenged by the occurrence of natural contaminants such as secondary fungal metabolites or plant toxins.[1] Liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) gained more and more attention within the last decades and has become the instrumental technique of choice for a precise and reliable determination of trace compounds in complex food and feed material.[2] However, the high sample complexity and substance-related physicochemical diversity hamper quantitative extraction of target molecules from the sample material. Although in routine pesticide analysis modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction procedures are most commonly applied, recent sample preparation protocols in multiclass analysis follow an even more straightforward and economic extraction approach which is applicable for multiple analytes from various substance classes.[3,4] These generic extraction protocols are based on a simple dilution of the sample extract after a fast solid−liquid extraction, which represents an optimal compromise in terms of work and resource consumption, extraction efficiency, and analytical quality. To ensure advanced laboratory quality assurance measures in a routine-orientated environment, a precise characterization of analytical performance parameters in target matrices is inevitable. However, the maintenance of such extended quality assurance is significantly hampered by increasing sample heterogeneity. Particularly, in the field of animal feed analysis, the sample matrix is often characterized by a highly complex nature and exact specifications of feed rations are therefore not given. Based on the European Commission regulation 767/2009, animal feed is differentiated as feed materials and compound feed. Feed materials are defined as products of vegetable or animal origin, whose principal purpose is to meet animals' nutritional needs, in their natural state, fresh or preserved, and products derived from industrial processing.[5] These products are intended for use in oral animal feeding either directly as such, or after processing, or in preparation of compound feed. This category includes cereal grains (e.g., barley, maize, triticale, and wheat), oil seeds and oil fruits (rape seed, soy, sesame, and sunflower), legume seeds (horse beans, lentils, peas, and vetches), tubers and roots (sugar beet, beet pulp, carrots, and potato), other seeds and fruits (acorn, buckwheat, red clover, and fruit pulp), forages and roughage (beet leaves, alfalfa, silages, and straw), other plants (algae, barks, leaves, and mint), milk products (butter, casein, milk fat, and whey), as well as land animal products, fish products, minerals, and products obtained by fermentation

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Table 1. Overview of the Investigated Analytes, Categorized by the Substance Class, Including Spiking Concentrations in μg/kg[a]

| analyte | substance class | polarity | concentration [μg/kg] |
|---|---|---|---|
| lotaustralin | FM | neg | 604 |
| altertoxin-I | FM | neg | 388 |
| agistatin E | FM | neg | 287 |
| gibberellic acid | FM | neg | 259 |
| 3-nitropropionsäure | FM | neg | 223 |
| pseurotin A | FM | neg | 165 |
| alpha-zearalenol | FM | neg | 95 |
| macrosporin | FM | neg | 87 |
| cladosporin | FM | neg | 79 |
| moniliformin | FM | neg | 78 |
| alternariolmethylether | FM | neg | 53 |
| fusarenon-X | FM | neg | 51 |
| 3-acetyldeoxynivalenol | FM | neg | 51 |
| deoxynivalenol | FM | neg | 51 |
| nivalenol | FM | neg | 51 |
| zearalenone | FM | neg | 51 |
| averantin | FM | neg | 49 |
| norsolorinic acid | FM | neg | 48 |
| malformin C | FM | neg | 43 |
| curvularin | FM | neg | 34 |
| ternatin | FM | neg | 34 |
| altersetin | FM | neg | 30 |
| amidepsin B | FM | neg | 30 |
| andrastin A | FM | neg | 30 |
| averufin | FM | neg | 30 |
| dihydrocitrinone | FM | neg | 30 |
| meleagrin | FM | neg | 30 |
| phomalone | FM | neg | 30 |
| thielavin B | FM | neg | 30 |
| equisetin | FM | neg | 28 |
| fumiquinazolin A | FM | neg | 27 |
| ilicicolin A | FM | neg | 27 |
| cercosporamide | FM | neg | 27 |
| alternariol | FM | neg | 27 |
| emodin | FM | neg | 27 |
| pinselin | FM | neg | 24 |
| versicolorin A | FM | neg | 24 |
| cylindrocarpon A4 | FM | neg | 12 |
| atpenin | FM | neg | 10 |
| asperphenamate | FM | neg | 3 |
| bentazon | P | neg | 50 |
| dinoseb | P | neg | 50 |
| fluazinam | P | neg | 50 |
| novaluron | P | neg | 50 |
| sulfoxaflor | P | neg | 50 |
| carprofen | VD | neg | 50 |
| florfenicol | VD | neg | 50 |
| flumethasone | VD | neg | 50 |
| mefenamic acid | VD | neg | 50 |
| chloramphenicol | VD | neg | 34 |
| fumonisin B1 | FM | pos | 404 |
| fumonisin B2 | FM | pos | 400 |
| 15-acetyldeoxynivalenol | FM | pos | 286 |
| chetomin | FM | pos | 286 |
| neosolaniol | FM | pos | 191 |
| secalonic acid D | FM | pos | 145 |
| gliotoxin | FM | pos | 129 |
| fumigaclavine C | FM | pos | 121 |
| mycophenolic acid | FM | pos | 75 |
| 15-hydroxyculmorin | FM | pos | 73 |
| cytochalasin B | FM | pos | 72 |
| cytochalasin J | FM | pos | 72 |
| roquefortine C | FM | pos | 72 |
| griseofulvin | FM | pos | 65 |
| sulochrine | FM | pos | 65 |
| aflatoxin M1 | FM | pos | 52 |
| diacetoxyscirpenol | FM | pos | 51 |
| HT-2 toxin | FM | pos | 51 |
| T-2 toxin | FM | pos | 51 |
| monoacetoxyscirpenol | FM | pos | 42 |
| penitrem A | FM | pos | 40 |
| 3-methylsterigmatocystin | FM | pos | 39 |
| cyclopenin | FM | pos | 39 |
| ochratoxin A | FM | pos | 38 |
| brevianamid F | FM | pos | 36 |
| questiomycin A | FM | pos | 32 |
| sterigmatocystin | FM | pos | 27 |
| destruxin A | FM | pos | 24 |
| ochratoxin B | FM | pos | 20 |
| anisomycin | FM | pos | 18 |
| aflatoxin B1 | FM | pos | 17 |
| aflatoxin B2 | FM | pos | 17 |
| aflatoxin G1 | FM | pos | 17 |
| aflatoxin G2 | FM | pos | 17 |
| fungerin | FM | pos | 12 |
| quinolactacin A | FM | pos | 12 |
| herquline A | FM | pos | 8 |
| ergine | FM | pos | 3 |
| ergocristine | FM | pos | 3 |
| enniatin A1 | FM | pos | 0.55 |
| aspon | P | pos | 50 |
| cyromazine | P | pos | 50 |
| dithiopyr | P | pos | 50 |
| ethirimol | P | pos | 50 |
| permethrin | P | pos | 50 |
| prometon | P | pos | 50 |
| rofecoxib | VD | pos | 50 |
| sulfamethoxazole | VD | pos | 50 |
| tiamulin | VD | pos | 50 |
| tilmicosin | VD | pos | 50 |

[a]Fungal metabolite (FM), pesticide (P), and veterinary drug (VD).

using microorganisms.[6] In contrast, compound feed is defined as a mixture of at least two feed materials whether or not containing feed additives, for oral animal feeding in the form of complete or complementary feed. By reason of its composition, complete feed on the one hand is sufficient for a daily ration, whereby complementary feed on the other hand is only sufficient if used in combination with other feed sources.[5] Considering animal species-specific properties and growth status, the individual feed rations are prepared in order to meet the animal-related physiological requirements, leading to high compositional differences in feed formulas.[7] Because standardization of the global feed production is not feasible and the


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compound feed market is growing steadily (+58% compound feed production in EU28 between 1989 and 2018), extensive validation processes are necessary in order to meet the high demands on feed and food safety. So far, trace analysis in compound feed has been rather neglected, but the growing production figures show that there is a rising need for action in the field of method validation and guideline regulations.

Current validation guidelines of the German accreditation body (DAkkS) are exclusively focusing on the validation of single feed material, leading to a potential discrepancy between analytical performance measures derived from validation data and data based on real world samples.[8]

In this work, method performance data for 80 fungal metabolites, 11 pesticides, and 9 pharmaceutical active agents in three different compound feed and twelve different single feed matrices were determined. Based on these data, the applicability of the current animal feed validation guidelines to multiclass methods is discussed. This study presents the first comprehensive overview and comparison of analytical performance data in complex compound feed and its single feed ingredients and differs significantly from studies which exclusively evaluated data on individual feed components.

## 2. MATERIALS AND METHODS

**2.1. Chemicals and Reagents.** LC gradient-grade methanol and acetonitrile and MS-grade ammonium acetate and glacial acetic acid (p.a.) were purchased from Sigma-Aldrich (Vienna, Austria). A Purelab Ultra system (ELGA Lab Water, Celle, Germany) was used for further purification of reverse osmosis water.

Standards of fungal and bacterial metabolites, pesticides, and pharmaceuticals were either purchased from Romer Labs Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland), NEOCHEMA GmbH (Bodenheim, Germany), Restek GmbH (Bad Homburg, Germany), BioAustralis (Smithfield, Australia), AnalytiCon Discovery (Potsdam, Germany), Adipogen AG (Liestal, Switzerland), and LGC Promochem GmbH (Wesel, Germany) or were obtained as gifts from various research groups. Each analyte was dissolved in acetonitrile (primarily), acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1 (v/v), or water.

By mixing the stock solutions of the corresponding analyte, 74 combined working solutions were prepared for fungal toxins, 9 working solutions for pesticides, and 8 for pharmaceutical active agents and were stored at −20 °C. For spiking purposes, a liquid multi-analyte standard was freshly prepared by combining the intermediate working mixtures deriving from liquid stock solutions.

**2.2. Instrumental Conditions.** A detailed description of the analytical procedure for this study was published elsewhere.[9] Briefly, the detection was carried out with a QTrap 5500 MS/MS system (SCIEX, Foster City, CA, USA) equipped with a TurboV source and an electrospray ionization (ESI) probe coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini C18-column, 150 × 4.6 mm i.d. and a particle size of 5 μm (Phenomenex, Torrance, CA, US). The column was equipped with a C18 security guard cartridge, 4 × 3 mm i.d. (Phenomenex, Torrance, CA, US).

The autosampler program included an injection volume of 5 μL, and elution was carried out in the binary gradient mode following a flow rate of 1 mL/min. Mobile phase A was composed of methanol/water/acetic acid 10:89:1 (v/v/v) and mobile phase B was composed of methanol/water/acetic acid 97:2:1 (v/v/v). Both mobile phases contained 5 mM ammonium acetate. Gradient conditions started with 100% A after an initial time of 2 min. After 3 min, the proportion of B was increased linearly to 50%. Within 9 min, mobile phase B was increased to 100% followed by a hold time of 4 and 3.5 min column re-equilibration at 100% A.

The analytical measurement was carried out in two successive chromatographic runs in the positive and negative polarity mode following a scheduled multiple reaction monitoring (sMRM) algorithm with a run time of 21 min each. For increased confidence in compound identification, two sMRM transitions per analyte were acquired according to the SANTE/11813/2017 validation guideline.[10]

**2.3. Data Evaluation.** *2.3.1. Calibration and Quantitation.* External neat solvent calibration was performed by diluting suitable volumes of multi-analyte standard working solutions. The final calibrant solution contained 300 μL of multitoxin working solution, 120 μL of pesticide solution, 120 μL of veterinary drug solution, 20 μL of a certified liquid standard of fumonisin $B_1$ and $B_2$, and 20 μL of a certified liquid standard of fumonisin $B_3$. Because the concentration of fumonisins does not remain stable in the almost pure acetonitrile multi-analyte solution, they were added at this late stage.

Serial dilution was performed with acetonitrile/water/formic acid (49.5/49.5/1, v/v/v) to obtain calibration levels of 1:3, 1:10, 1:30, 1:100, 1:300, and 1:1000. Linear $1/x$ weighted calibration curves were obtained for the solvent standards in order to check the linearity of the response. MultiQuant 3.0.3 (SCIEX, Foster City, CA, USA) software was used to construct the calibration curve and perform peak integration. Final data evaluation was performed in Microsoft Excel 2013. Graphical content was prepared using the open access visualization tool Flourish (Kiln Enterprises Ltd, London, UK).

*2.3.2. Performance Parameters.* Performance characteristics of the method were evaluated by the apparent recovery ($R_A$), the matrix effects, expressed by signal suppression/enhancement (SSE), and the recovery of the extraction step ($R_E$). The parameters were calculated from the peak areas of the samples spiked before and after the extraction and the neat solvent standards.

$$R_A\ (\%) = \frac{\text{area(spiked sample)}}{\text{area(standard)}} \times 100$$

$$\text{SSE}\ (\%) = \frac{\text{area(spiked extract)}}{\text{area(standard)}} \times 100$$

$$R_E\ (\%) = \frac{\text{area(spiked sample)}}{\text{area(spiked extract)}} \times 100$$

**2.4. Set of Analytes.** The described analytical approach was originally designed for the determination of 39 mycotoxins in cereals in the year 2006.[11] Since then, the method has been extended and improved continuously to a wide range of additional secondary metabolites of fungi and bacteria, plant toxins, pesticides, and veterinary drugs.[9,12,13] In order to ensure an adequate workload and time management, a set of 100 analytes, including 80 secondary fungal metabolites (including all regulated mycotoxins), 11 pesticides, and 9 pharmaceutical active agents, was chosen. To guarantee a high representativeness, the selected analytes were evenly distributed over the whole chromatogram covering differences in physicochemical characterization such as acidity, hydrophobicity, functional groups, and ESI polarity. An overview of the selected set of representative analytes is depicted in Table 1.

**2.5. Spiking and Extraction Procedure.** The extraction procedure is used for the routine analysis of contaminated food and feedstuff, basically the animal feed material. Therefore, 5 g of the sample is extracted with 20 mL of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) and shaken using a rotary shaker (GFL 3017, Burgwedel, Germany) for 90 min under horizontal conditions. To improve the extraction for fumonisins, the pH value of the extraction solvent was lowered to pH 4 using formic acid instead of acetic acid, following the original dilution ratio. The improved extraction under strong acidic conditions is apparently structure-related because fumonisins contain several carboxyl groups.[14]

For spiking purposes, an appropriate amount of multi-analyte working solutions (50 μL of multi-toxin solution, 25 μL of pesticide solution, 25 μL of veterinary drug solution, and 20 μL of fumonisin solution) was added to 0.25 g of homogenized samples. The


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miniaturization of the spiking protocol was performed for the economical use of standards. In order to isolate matrix effects, the obtained spike concentrations were matched to calibrant standard dilution levels of the higher working range, such as 1:10 and 1:30. For mycotoxins addressed by regulatory limits, the spiking concentrations were far below the guidance values and in the range of the regulatory limit for aflatoxins in feed.[15,16] The difference between the lowest and highest concentration levels (0.55 μg/kg for enniatin A1 and 604 μg/kg for lotaustralin) investigated in this study amounted to a factor of 100.

To avoid analyte degradation and to ensure solvent evaporation, the spiked samples were stored in darkness and at room temperature overnight. This step ensures proper equilibration between matrix and analytes. On the next day, the samples were extracted using 1 mL of the extraction solvent and were shaken for 90 min using a rotary shaker. Finally, the samples were centrifuged at 3500 rpm for 5 min. After transferring the supernatant (300 μL) into high-performance liquid chromatography (HPLC) vials, the same volume of a complementary dilution solvent (acetonitrile/water/formic acid 20:79:1, v/v/v) was added and mixed properly. Finally, 5 μL of the diluted raw extract was injected into the LC−MS/MS system without further cleanup.

For post extraction spiking, 5 g of the sample was extracted with 20 mL of the extraction solvent. The supernatant (400 μL) was fortified with an appropriate amount of spiking solution (20 μL of multi-toxin solution, 10 μL of pesticide solution, 10 μL of veterinary drug solution, and 8 μL of fumonisin solution), diluted with 352 μL of the dilution solvent and injected as described above.

**2.6. Samples.** Three matrices of real and model compound feed (with distinct differences in their composition) and twelve matrices of single feed material including alfalfa, barley, maize, horse beans (broad beans), distiller's dried grains with solubles (DDGS), rapeseed, silage, soy, sunflower cake, triticale, wheat, and wheat bran were chosen for this study. Cattle feed was taken as a matrix with high amounts of forage crops. Matrices with high grain content were represented by pig and chicken feed. Between four and seven different lots of each matrix type were collected, in order to maximize the intrasubject variation and challenge the reproducibility of the extraction protocol. The heterogeneous set of individual raw samples was provided by the companies BIOMIN GmbH (Getzersdorf, Austria), LVA GmbH (Klosterneuburg, Austria), Garant-Tiernahrung GmbH (Pöchlarn, Austria), Romer Labs Diagnostic GmbH (Tulln, Austria), and Bipea (Paris, France). The model compound feed formulas were prepared following the information provided by our company partners BIOMIN GmbH and Garant-Tiernahrung GmbH and are illustrated in Table 2 (compositional information might vary from country to country and has to be collected by national feed producers in order to apply this approach in other laboratories). In total, 42 compound feed samples (21 real and 21 model) and 73 single feed matrix replicates were evaluated. The detailed model matrix composition is illustrated in the work sheet "samples" in the Supporting Information (Table S1). Homogenization of the samples was carried out using an Osterizer blender (Sunbeam Oster Household Products, Fort Lauderdale, FL, USA).

## 3. RESULTS AND DISCUSSION

**3.1. Validation of Multiresidue Methods in Feed.** Multimethods covering dozens or even hundreds of analytes are characterized using a high number of compounds, which differ in polarity, structural formulas, and physicochemical properties. With single-residue methods, compounds may be extracted almost quantitatively, and optionally, after clean up determined with the help of one and/or several specific detectors. In contrast, a high level of compromise is needed for the development of multiple methods, especially accounting for complex sample materials, in which the applicability of analyte-specific extraction and purification steps is extremely limited. Because of its compositional variability, feed represents one of the most complex sample materials and therefore requires powerful and reliable analytics. In routine laboratories, multiple methods are frequently covering more than 300 individual compounds which are subject to matrix validation procedures. Based on the validation guide from the German accreditation body for multiresidue methods in feed, the matrix validation can be conducted in groups for the specific feed type (Table 3). To obtain accreditation for feed matrices in general, the analysis of the active substances in each group must be validated by selecting at least one matrix from the corresponding feed group.[8] In order to include a multimethod in the scope of accreditation, the laboratory must be able to determine at least 75% of the target analytes with a satisfactory performance per group, following SANTE criteria for pesticide analysis in terms of reproducibility and repeatability.[8,17]

Related to the method performance, significant variations may occur because of the high number of analyte/matrix combinations.[9] Variations within the analytical performance data have to be collected in the validation process and, if necessary, reduced by adequate adaptations of the extraction step and/or chromatographic conditions.

**Table 2. Compositional Information of the Real-World Samples and in-House-Prepared Prehomogenized Model Matrices, Expressed as the Maximum Share in Percent[a]**

| | maximum share (%) | | | | | |
|---|---|---|---|---|---|---|
| | cattle | | pig | | chicken | |
| | real | model | real | model | real | model |
| additives | | | 7 (4) | | | |
| barley | 18 (6) | 24 (7) | 29 (4) | 30 (5) | | |
| broad beans | 22 (5) | 22 (7) | | | | |
| corn meal | 4 (1) | | | | | |
| DDGS | | | 35 (3) | 10 (4) | 10 (5) | 10 (5) |
| maize | 20 (3) | 20 (7) | 44 (4) | 44 (7) | 62 (7) | 74 (7) |
| peas | 7 (3) | | | | | |
| rapeseed | | | 0.8 (1) | | 3.5 (2) | 5 (7) |
| rice bran | | | | | 15 (1) | |
| rye | 25 (4) | | | | | |
| silage | 26 (7) | 26 (7) | | | | |
| soy | | | 27 (4) | 35 (7) | 30 (7) | 30 (7) |
| sunflower cake | 10 (5) | 10 (6) | | | | |
| triticale | 18 (4) | 21 (6) | 15 (1) | 10 (3) | | |
| unknown | 37 (6) | | 100 (4) | | 17 (6) | |
| wheat | | | 29.7 (4) | 30 (5) | 20 (1) | |
| wheat bran | 18 (6) | 20 (7) | | | | |

[a]Numbers in brackets represent the absolute prevalence of compound feed samples containing the respective individual single feed ingredient.

**3.2. Influencing Criteria on the Method Performance.** *3.2.1. Valid Analytical Methods.* For routine laboratories working in the food and feed sector, the use of confirmatory methods which comply with the requirements of international standardizing organizations, such as Codex Alimentarius Commission, the European Committee for Standardization (CEN), AOAC International (AOACI), or the European Union, is essential. Therefore, valid analytical methods require the determination of accuracy, covering trueness, and precision.[18] The accuracy is defined as the closeness of the measurement result to the true or accepted reference value and thus combines both, precision and trueness.[10] In this study, a comprehensive spike-and-recovery experiment was carried out in order to assess the accuracy of the method.


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**Table 3. Overview of Animal Feed Groups for Validation Purposes of Multimethods[8]**

| no. | feed group | characteristics | matrix example |
|---|---|---|---|
| F1 | cabbage vegetable | high water content | kale |
| | forage plant | | weeds, alfalfa, clover, rape |
| | leaves from root and tuber vegetables | | sugar beet leaves |
| | silage | | maize, clover, weeds |
| F2 | fruit pulp | high acidic and high water content | citrus fruits |
| F3 | extraction cake | high sugar and low water content | rape extraction cake |
| F4a | oils and oilseeds | high fat and very low water content | sunflower, rapeseeds |
| F4b | oil fruits | high fat and moderate water content | soybeans, olives |
| F5 | cereals | low water and low fat content, high starch, and/or protein content | wheat, rye, barley, maize, rice, oat grains |
| | hay | | weeds |
| | legumes | | horse bean, lenses |
| | straw | | wheat, rye, barley, oat |
| F6 | special matrices | | |
| F7 | meat, fish and shellfishes | animal-based compound feed | feed from fish farms |
| F8 | milk and milk products | | |
| F9 | eggs | | |
| F10 | fat from animal-based compound feed | fat-based compound feed | |

*3.2.1.1. Apparent Recovery.* The apparent recovery is a parameter combining the recovery of the analyte from the matrix by the sample extraction procedure and matrix effects and has also been termed as "process efficiency".[19] Generally, the apparent "recovery" should be in the range of 70−120%.[10,20] In routine analysis, recovery rates between 60 and 140% are still acceptable.[8] If the recovery rate is outside this range, it must be shown that the method variability allows a reliable quantitative statement.[8,9] In particular, low apparent recoveries show adverse effects on the accuracy, especially affecting the limit of quantification.[21]

The distribution of apparent recoveries for 100 analytes in 6 grain-based feed matrices (A) and 6 matrices including legumes, oilseeds, and forage crops (B) is displayed in Figure 1. Absolute apparent recoveries for each matrix commodity are expressed as average values of the individual lots measured under repeatability conditions. The variety of matrices allows a comprehensive collection of different matrix characteristics such as low water and low fat content, represented by group A commodities such as wheat, barley, maize, or triticale. In contrast, group B is characterized by matrices with high water content such as alfalfa and silages, high fat and very low water content such as sunflower cake and rapeseeds, high fat and moderate water content such as soybeans, and high starch and/or protein content such as horse beans. The spike concentration corresponds to a 1:10 and 1:30 dilution range of the final working solution of the analytical reference standards. $R_A$ values are expressed as the mean apparent recovery derived from 4 to 7 different lots of each feed type and were calculated according to equation $R_A$ described in 2.3.2. Regarding the $R_A$ results, 72% of analytes in maize, 89% in barley, 82% in wheat bran, 52% in DDGS, 88% in triticale, 84% in wheat, 66% in rapeseed, 52% in alfalfa, 52% in silage, 61% in sunflower, 56% in soy, and 84% in horse beans were in the range of 60−140% as described by DAkkS.[8] For the analytes outside the acceptance criteria, a combination of low extraction efficiency and high signal suppressions or enhancements was observed.

*3.2.1.2. Extraction Efficiency.* Currently, there is no official guidance document available which is focusing on the validation of analytical methods for the determination of multiple analytes in compound feed in general.[9] This nonavailability opens some gaps in the interpretation of results, which counts, in particular, for the definition of the term recovery. An exact definition is missing and therefore two possible interpretations exist. First, the previously described apparent recovery and the recovery of the analyte from the matrix using the sample extraction procedure.[19] Based on the DAkkS guideline, the recovery has to be determined using a single or multi-analyte standard prepared in the respective matrix, which implies the second definition mentioned above.[8]

The distribution of extraction efficiencies (according to equation $R_E$ described in 2.3.2) for 100 analytes in 12 tested feed materials is depicted in Figure 2. Absolute extraction recoveries for each matrix commodity are expressed as average values of the individual lots measured under repeatability conditions. Regarding the $R_E$ results, 94% of analytes in maize, 91% in barley, 89% in wheat bran, 90% in DDGS, 94% in triticale, 96% in wheat, 86% in rapeseed, 83% in alfalfa, 91% in silage, 90%, in sunflower, 83% in soy, and 89% in horse beans were in the range of 60−100%. Only 2−4% of analytes in group A (grains and byproducts) and 3−12% of analytes in group B (legumes, oilseeds, and forage crops) show lower extraction recovery than 60%. Low extraction efficiencies were especially observed for altersetin, andrastin A, chetomin, and

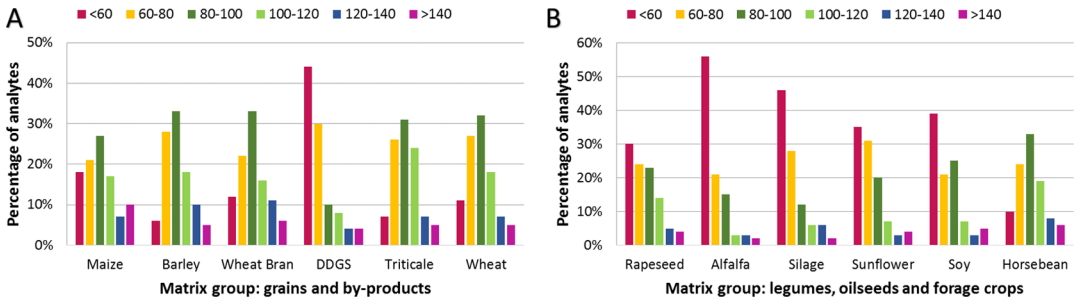


**Figure 1.** Distribution of apparent recoveries through the set of 100 analytes in grains and byproducts (A) and legumes, oilseeds, and forage crops (B).



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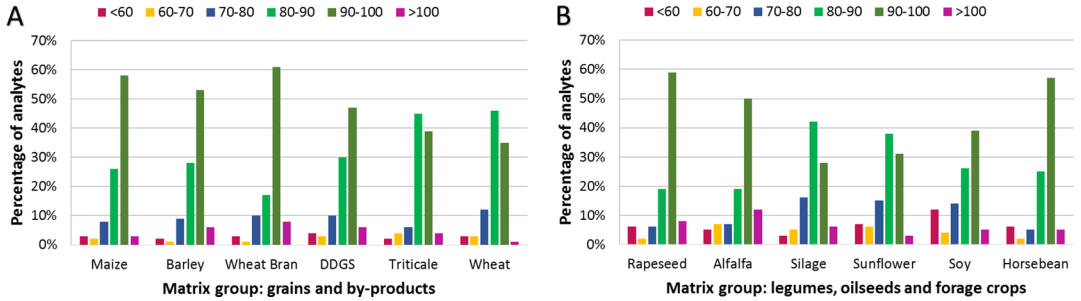


**Figure 2.** Distribution of extraction efficiencies through the set of 100 analytes in grains and byproducts (A) and legumes, oilseeds, and forage crops (B).

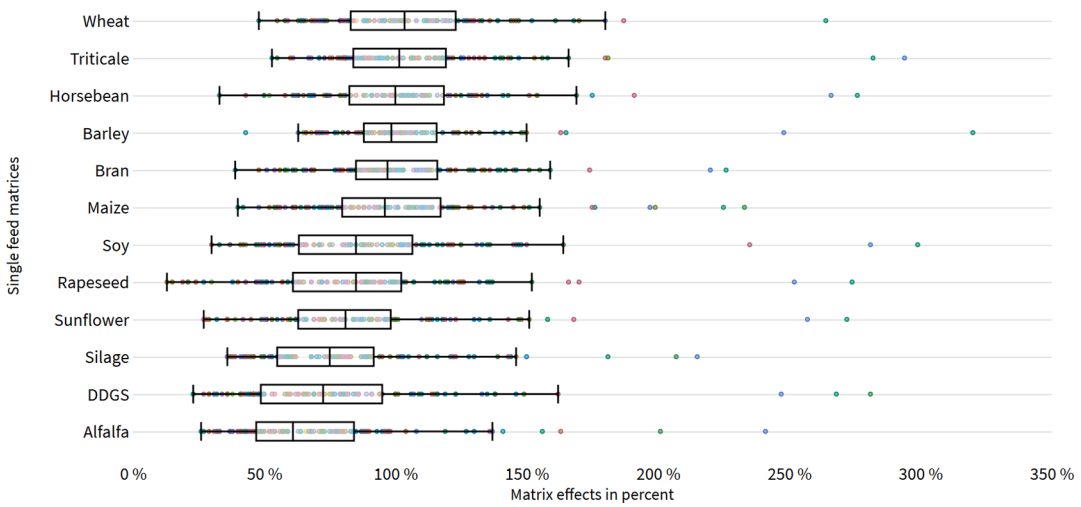


**Figure 3.** Scatter plot illustrating matrix effects in percent (*x*-axis) expressed as SSE for 12 single feed matrices (*y*-axis). Each target analyte is depicted by a colored dot. The outlier-corrected box plot includes an interquartile range of 1.5, representing John Tukey's standard value.[25]

cyromazine. These compounds share a number of specific alkaline functional groups which might decrease the solubility in the acidified apolar extraction mixture. Performing the extraction process at low pH is necessary for the majority of secondary fungal metabolites as approximately 40% of them contain an acidic moiety.[22] Nevertheless, excellent extraction recoveries were observed for the majority of compounds, leading to the conclusion that matrix effects are the main causes for not achieving the required method performance criteria of isolated analytes.

*3.2.1.3. Matrix Effects.* In HPLC−ESI−MS/MS, matrix effects are combined consequences between the influence of the matrix entering the electrospray ion source and the chemical nature of the target compound.[23,24] The heterogenous environment of feed matrices results in a competition between analyte ions and nonvolatile matrix components. This competition leads to an effective decrease (ion suppression) or increase (enhancement) in the ionization process, expressed as the absolute matrix effect and shows high analyte/matrix-dependent differences.[19]

An overview of absolute matrix effects in 12 single feed matrices is depicted in Figure 3.[25] Moderate absolute matrix effects were particularly observed in grain-based feed materials with median values of 104, 102, 99, 97, and 96% in wheat, triticale, barley, bran, and maize, respectively. In contrast, higher signal suppressions were observed in crops and oilseeds. With 85, 85, 81, 75, and 61% in soy, rapeseed, sunflower, silage, and alfalfa, respectively, matrix effects were considerably more-pronounced in this category. Contrasting effects within their specific feed group were observed for DDGS and horse bean with median values of 72.5 and 100%, respectively. Although the majority of compounds were primarily affected by signal suppressions, some compounds were influenced by an enhancement of the signal (>20%) in almost all matrices. In general, the ion enhancement can be caused by matrix components which act as a dopant, increasing the ionization efficiency of analytes with high ionization energy.[26] Furthermore, especially polar analytes in the positive ionization mode are more susceptible to undergo ion suppression.[27] The observed signal enhancements in this experiment were evident for rather apolar analytes in the negative ionization mode such as dihydrocitrinone ($R_t$: 10.0 min), amidepsin B ($R_t$: 11.1 min), cercosporamide ($R_t$: 11.5 min), carprofen ($R_t$: 12.3 min), dinoseb ($R_t$: 12.6 min), ternatin ($R_t$: 12.7 min), atpenin ($R_t$: 13.1 min), novaluron ($R_t$: 13.2 min), mefenamic acid ($R_t$: 13.4 min), fluazinam ($R_t$: 13.7 min), equisetin ($R_t$: 14.7 min), altersetin ($R_t$: 15.1 min), and norsolorinic acid ($R_t$: 16.6 min). Additionally, with moniliformin ($R_t$: 3.3 min) and gibberellic acid ($R_t$: 7.1 min), two polar representatives in the negative ionization mode showed similar signal enhancement patterns, which could be caused either by concomitant matrix components or target analytes in the same ion mode.[28] The work sheet "single feed material" in the Supporting Information (Table S1) gives a detailed overview about matrix effects, extraction recoveries, and apparent recoveries of the individual single feed matrices.

The obtained results for $R_A$ and SSE reflect the high variation in the exact composition of different lots/brands of


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animal feed which counts for both, single feed material and consequently also for complex feed. Because there is no uniform recipe in the production of complex compound feed, validation protocols of routine-based confirmation methods and scientific focus is mainly set on single feed matrices, for example, grains or silages, as described in several studies.[17,29−31] However, because of its variability in composition, complex feedstuff should also be considered in validation approaches for this matrix type. As the exemplary comparison of pseurotin A between real complex cattle feed samples and their main single ingredients in Figure 4 shows, great variances

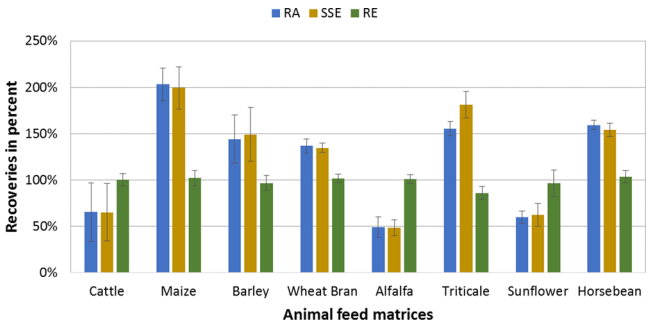


**Figure 4.** Comparison between real cattle feed and its main individual ingredients for pseurotin A. Apparent recoveries ($R_A$, blue bar), matrix effects (SSE, yellow bar), and extraction efficiencies ($R_E$, green bar) in percent include the error indicator expressed as the relative standard deviation.

in $R_A$ and SSE can be observed. The relative standard deviation derived from 7 different cattle feed lots either for $R_A$ (RSD: 32%) or SSE (RSD: 31%) indicates that validation data obtained from individual feed material cannot guarantee a correct and reliable estimation of complex animal feedstuff.

This is aggravated by the fact that a comprehensive validation of an analytical approach for animal feed is associated with a very high workload. A complete validation of an average multimethod in each of the listed feed groups in Table 3 would lead to an evaluation of about 60,000 signals (300 compounds × 200 chromatograms, deriving from 10 matrices × 5 lots × 2 concentration levels × 2 ($R_A$, SSE)), blank and calibration data excluded.

Therefore, reconsideration of the current analytical approach must be taken into account, including the economization of resources (standards, measurement time, workload, etc.) and the complexity of compound feed material.

**3.3. Preparation of Model Matrices.** In order to account for information gaps about the composition of complex feed, model matrices were prepared in-house for three different compound feed types (cattle, chicken, and pig) with seven different lots each. Information regarding the compositional nature of real compound feedstuff was provided by the companies listed in 2.5. In order to minimize the workload and because of the nonavailability of specific feed ingredients, only the main compound feed elements were used for modeling purposes. Furthermore, the proportions of unknown feed ingredients were complemented by increasing the share of the selected known elements.

Beside knowledge of the exact compositional formula, in-house matrix modeling has the advantage to use blank single feed material for the preparation of the individual lots because it is almost impossible to obtain complex feedstuff that is entirely free from charge of natural contaminants.

With seven individual ingredients, cattle feed was the most heterogeneous matrix representative. In contrast, chicken feed mainly consists of maize and soy, leading to the hypothesis that cattle feed is more prone to intrasubject variations than chicken, or pig feed, whose main components are maize, soy, and wheat. In general, no differences were expected between real and model samples in terms of $R_A$, SSE, and $R_E$. Furthermore, accurate intrasubject variations can be simulated by preparing nonidentical individual lots, which better reflect the real conditions in a routine-orientated laboratory, instead of using a single replicate prepared multiple times.

*3.3.1. Intrasubject Variation.* Multimethod validation procedures are commonly performed based on a single lot of a matrix because there are no particular regulations existing for this matter. However, not considering the intrasubject variation could lead to an additional component of uncertainty during the method validation process. Neglecting the intrasubject variation leads to an underestimation of the measurement uncertainty,[32] especially relevant for complex matrices such as compound feed, because of their heterogeneous composition. In official guidance documents, a statement of

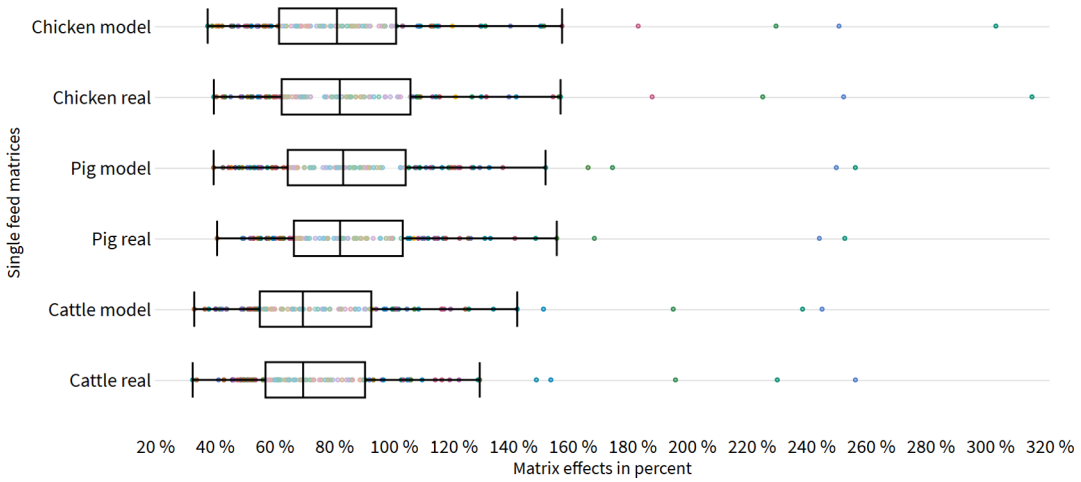


**Figure 5.** Box-plot comparison of matrix effects in complex compound feed. The *x*-axis represents the matrix effects expressed as SSE in percent, and the y-axis shows the different sets of real and model compound feed samples.



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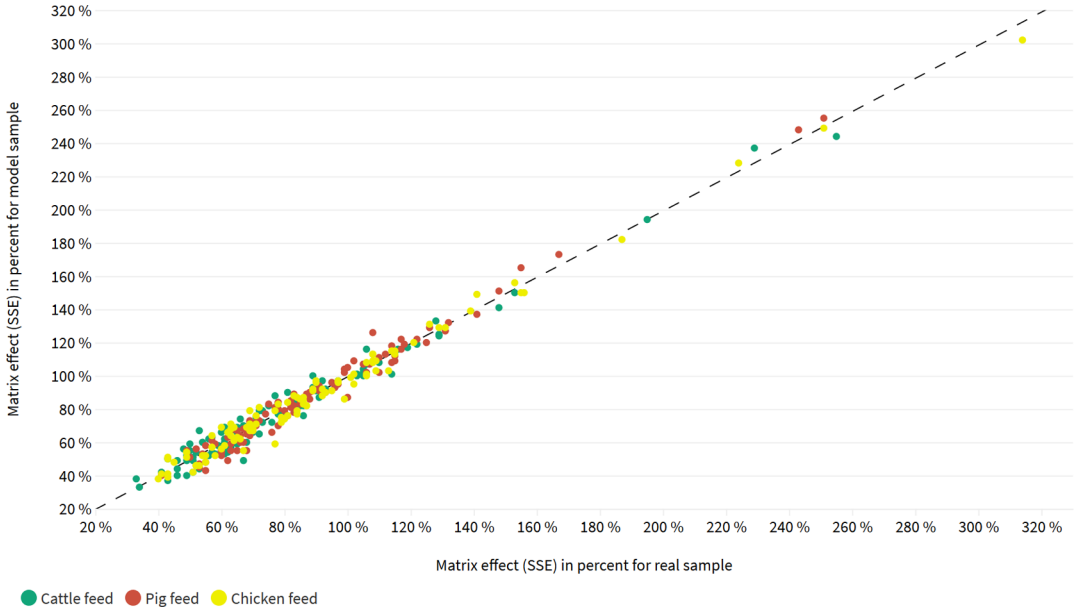


**Figure 6.** Basic scatter plot for correlation analysis between absolute matrix effects from real compound feed samples (*x*-axis) and model compound feed samples (*y*-axis). Analytes are represented by a colored dot. Cattle feed is pictured by green, pig feed is pictured by red, and chicken feed is pictured by yellow dots.

intrasubject variation or specific performance criteria for this parameter is either limited or completely missing. Only the validation guide of the US Food and Drug Administration for chemical methods requires a minimum number of three different sources per matrix type for the analysis of contaminants.[33] In the official validation guidelines of the European Union (EU), the phenomenon of a matrix mismatch is mentioned as a potential source of uncertainty; specific requirements, however, are not formulated.[34,35] To avoid an underestimation of the measurement uncertainty and to obtain an accurate estimation of the method performance, the aspect of intramatrix variations was implemented in this study by replicate analysis of seven different matrix lots.

*3.3.1.1. Absolute Matrix Effects.* Strong matrix effects (>20% SSE) were observed for all complex feed matrices. The distribution of SSE in real and model feed samples is visualized in Figure 5. A detailed overview of the numerical SSE values for real and model matrices is displayed in the work sheets "real compound feed" and "model compound feed" within Table S1. Smaller matrix effects were observed in pig and chicken feed. Concerning pig feed, 42% of analytes in real samples and 43% of analytes in model samples were suppressed/enhanced by <20%. In chicken feed, for both types of samples, 39% of analytes for model and real samples were in the SSE range between 80 and 120% and therefore not affected by matrix effects according to SANTE/11813/2017.[10] In contrast, higher matrix effects were observed in cattle feed. In this matrix, only 28% of analytes in real samples and 31% of analytes in model samples were not affected by SSE, indicating that the analysis of cattle feed suffers the most from matrix effects. In general, matrix-related signal suppression was observed more frequently than signal enhancement. A higher number of analytes were suppressed in pig (47% real and 44% model) and in chicken feed (48% real and 49% model) than enhanced in pig (11% real and 13% model) and chicken feed (13% real and 12% model). Furthermore, even more analytes were suppressed in cattle feed 63% (real) and 61% (model), compared to 9% (real) and 8% (model) of analytes showing an enhancement of the signal in this matrix. As already observed within the matrix categories of single feed material, signal enhancement is strongly correlated with compounds analyzed in the negative mode such as altersetin, equisetin, dihydrocitrinone, and fluazinam in all compound feed formulas. All average values for SSE, $R_A$, and $R_E$ for the positive and negative mode, respectively, are shown in the Supporting Information (Table S1).

However, model and real sample materials are well-comparable in terms of absolute matrix effects. Median values for SSE in chicken feed are at 82% in real samples and 81% in model samples. In pig feed, 82 and 83% median values were observed for real and model matrices, respectively, and 70% in each case for cattle feed. Furthermore, *T*-test statistics (Table S1, work sheet *t*-test and *F*-test statistics) revealed no significant difference between model and real samples for all species. Null hypothesis is not rejected because *t*-stats for cattle feed (0.616), pig feed (0.898), and chicken feed (1.611) are lower than the critical value 1.66. Additionally, $P_{T\leq t}$ values for cattle feed (0.270), pig feed (0.186), and chicken feed (0.055) are not falling below $\alpha$ (0.05).

A visualized correlation analysis between matrix effects derived from the sample sets of real and model matrices is displayed in Figure 6. With a Pearson correlation coefficient of 0.987 in cattle, 0.990 in pig, and 0.992 in chicken feed, all categories showed a high positive correlation, which indicates a strong connection between modeled and real matrices.

*3.3.1.2. Relative Matrix Effects.* A matrix mismatch is typically the result of the heterogeneous nature of the tested sample material. Analyte-specific variabilities in SSE in samples from different sources, but from the same type, can be considered as a measure of relative matrix effects.[36,37] In general, an acceptable deviation from a nominal value expressed as a percentage ($RSD_{SSE}$) should be ≤15% to be considered as not affected by intramatrix variations.[38] We observed the highest relative matrix effects in cattle feed, followed by pig and chicken feed. Concerning real samples, in cattle feed, 50% of analytes were affected by high intramatrix


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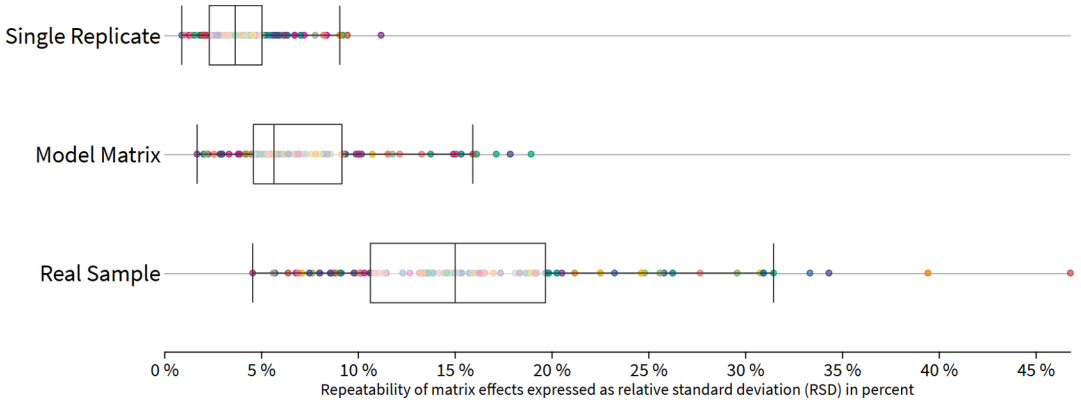


**Figure 7.** Scatter plot comparison of matrix effects (*x*-axis) for extracts from a single cattle feed replicate, as well as from a different model, and real cattle feed samples (*y*-axis) under repeatability conditions.

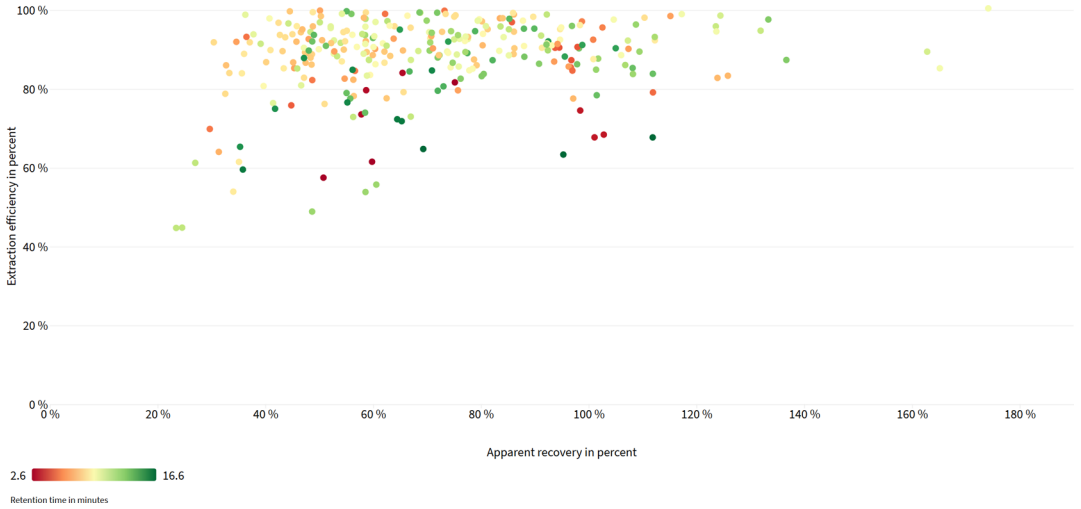


**Figure 8.** Basic scatter plot visualizing apparent recoveries (*x*-axis) and extraction efficiencies (*y*-axis) for selected analytes in complex model matrices. Each target analyte is represented by a colored dot. Retention times are reflected by different colors from purple (polar compounds) to green (apolar compounds).

variations, compared to 34 and 15% in pig and chicken feed. In contrast, model feed matrices were less prone to relative matrix effects. Only 7% of analytes in cattle and in each case, 4% of analytes in pig and chicken feed did not comply with the $RSD_{SSE}$ criterion of ≤15%. A summary of relative matrix effects for compound and single feed matrices is shown in the respective work sheet of Table S1.

The high intramatrix variability of the SSE in real samples, particularly in cattle feed, can be a result of the nature of the samples or by the feed ration, which may pose an interference. Because the model feed matrices were basically prepared using blank single feed ingredients, the relative matrix effects were significantly reduced. F-test statistics (Table S1, work sheet *t*-test and *F*-test statistics) gives a detailed explanation of the statistical characteristics for relative matrix effects in model and real compound feed samples. *F* values are higher for cattle feed (4.120), pig feed (2.428), and chicken feed (1.532), compared to the critical *F* value 1.394. Additionally, all $P_{F\leq f}$ values are lower for cattle feed ($6.78 \times 10^{-12}$), pig feed ($7.37 \times 10^{-6}$), and chicken feed ($1.74 \times 10^{-2}$), compared to $\alpha$ (0.05), indicating that the null hypothesis is rejected.

Thus, shown by the statistical *T*-test (Table S1/work sheet *t*-test and *F*-test statistics), modeling different feed lots reveals a suitable technique to obtain an accurate estimation of the method performance and ensure high compliance with validation acceptance criteria. In contrast, as shown by the statistical *F*-test (Table S1/work sheet *t*-test and *F*-test statistics), results obtained under repeatability conditions ($n = 7$) from one identical replicate indicate an overestimation of the method performance, graphically exemplified for cattle feed in Figure 7.

Consistently, strong relative matrix effects in real and model feed samples were observed for alternariol (20% real and 16% model), alternariolmonomethylether (16% real and 15% model), brevianamid F (19% real and 17% model), cytochalasin J (15% real and 18% model), ergine (26% real and 15% model), fumigaclavine C (17% real and 16% model), and ilicicolin A (20% real and 19% model), while these compounds were much less-affected under repeatability conditions based on an identical matrix replicate. In general, concerning $RSD_{SSE}$, we observed high differences between the different cattle feed sample sets. Median $RSD_{SSE}$ values of 3.7, 5.7, and 15% for a single sample replicate, model samples, and real samples, respectively, imply an increasing overestimation of the method performance through the application of replicates derived from a single sample material.

*3.3.1.3. Compatibility of the Extraction Protocol.* Apparent recoveries and extraction efficiencies for all three modeled


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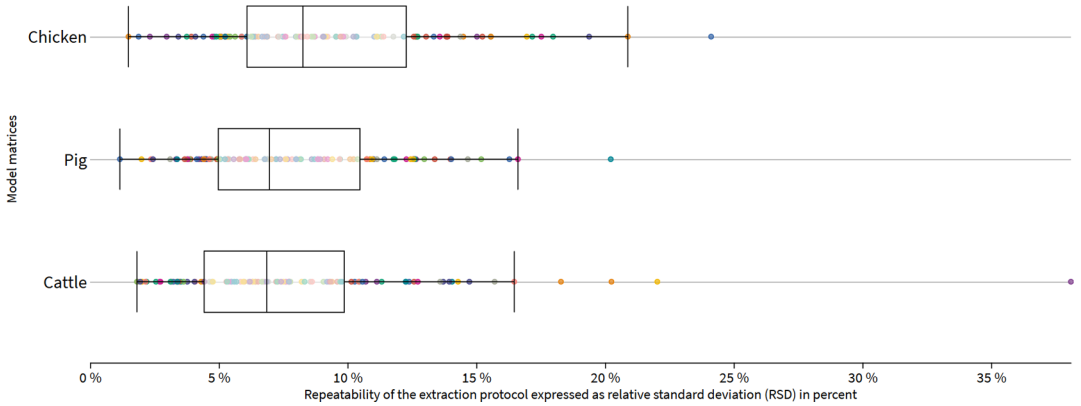


**Figure 9.** Scatter plot comparison of repeatability conditions of the extraction protocol (*x*-axis) for model compound feed matrices (*y*-axis).

compound feed formulas are depicted in Figure 8. Predominant extraction efficiencies between 60 and 100% prove the applicability of the extraction protocol with complex feed material, while, in particular, signal suppression leads to low numerical values of apparent recoveries for some analyte/matrix combinations. Lower extraction efficiencies (≤60%) were observed for cyromazin (57%), andrastin A (49%), and ilicicolin A (60%) in cattle feed. In pig feed, gliotoxin (54%), chetomin (45%), and andrastin A (54%) and in chicken feed, only chetomin (45%) and andrastin A (56%) showed $R_E$ values lower than 60%. Low extraction efficiencies for andrastin A, chetomin, and cyromazin were also observed in the single feed materials, while low extraction yield for ilicicolin A in cattle feed and for gliotoxin in pig feed is associated with a poor extraction efficiency of ilicicolin A in sunflower and gliotoxin in soy, as components of the respective compound feed formula.

Concerning $R_A$-values, 47% of analytes in cattle feed and 66 and 59% in pig and chicken feed were in the $R_E$ criteria range of 60−140%. This implies that deviations from 100% of the external calibration are, in particular, a result of adverse matrix contributions. For a significant reduction of these effects, validation guidelines recommend a preparation of calibration standards with the corresponding matrix extract. However, because of the high sample complexity, a correction between different matrix lots is not applicable, graphically illustrated in Figure 7. In addition, the natural sample background contamination complicates the applicability of this approach.[8]

The extraction variability under repeatability conditions for the model compound feeds is shown in Figure 9. With regard to the acceptance criteria of RSD ≤15%, extraction efficiency complies similarly to relative matrix effects. The fraction of analytes not complying to this criterion was 6, 4, and 10% for cattle, pig, and chicken feed, respectively. However, the majority of analytes show excellent extraction behavior under repeatability conditions, indicating the high efficacy of the extraction protocol for complex feed material.

### 3.4. Validation Proposal for Complex Feed Material.

Based on considerable analyte/matrix-dependent differences between performance criteria for compound feed formulas and their single feed ingredients, the requirements of future validation guidelines for feed should be extended.

Validation guidelines such as the DAkkS document (71SD4012) are exclusively focusing on the validation of single feed ingredients or are completely neglecting these matrices.[8] Therefore, we propose an extension of validation guidelines with the most important compound feed formulas, depicted in Figure 10. Based on the European animal feed production data provided by FEFAC, more than 90% of the

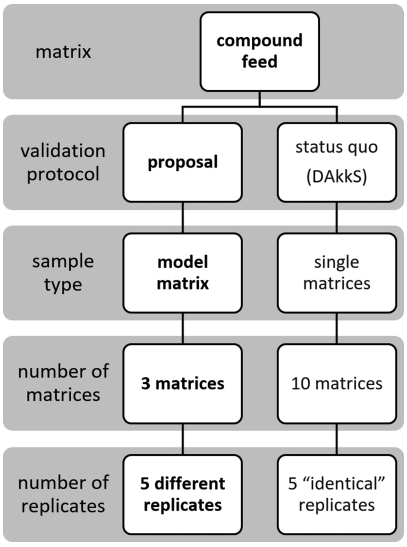


**Figure 10.** Validation proposal scheme for complex feed material.

total compound feed production (253.6 million tons in 2018) is accounting for chicken, pig, and cattle.[39] Taking the market share as a reference, these three compound feed types should be included within the validation scope of laboratories conducting routine analysis for animal feed material. Because the natural background contamination of compound feed possesses a particular problem in order to validate these matrices, we further propose to perform validation processes using in-house model matrices, based on true blank single feed ingredients. We have shown that there is no significant difference between real and model matrices with respect to absolute effects such as extraction efficiency and matrix effects.

In order to simulate the heterogenic nature of compound feed, we suggest preparing at least 5 lots with different compositional patterns. Feed formula variations for animals at different growth stages should be taken into account. As elaborated in chapter 3.3.1.2, simulating the intramatrix variation leads to a more realistic estimation of the method precision.

To conclude, this work presents the first comprehensive evaluation of analytical parameters for complex compound feed based on in-house-prepared model matrices in LC−MS/MS


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analysis. We have shown that substantial differences between $R_A$, SSE, and $R_E$ values occur, when comparing single feed material with complex compound feed formulas. A straightforward and economical procedure for the validation of compound feed was applied which ensures an accurate estimation of real-life conditions in routine-based laboratories. The method performance was estimated based on spiking experiments for a representative set of analytes in seven different lots (compound feed) of each matrix type. Performance criteria in current animal feed validation guidelines exclusively focus on single feed material without consideration of intramatrix variation, which facilitates the compliance of the corresponding criteria regarding trueness and precision. Discrepancies in $RSD_{R_A}$ and $RSD_{SSE}$ for compound feed and its single feed ingredients indicate a noncompliance of validation data based on individual feed material with complex feedstuff. However, recoveries outside the range of 70−120% can be accepted if they are consistent (RSD ≤20%) and a recovery correction is applied.[10] Model matrices for three different animal species (cattle, pig, and chicken) were prepared in-house based on the compositional information provided by animal feed producers. Analytical parameters for extraction efficiency, matrix effects, and apparent recovery were compared between modeled feed material and equivalent real samples. High absolute and relative matrix effects were the major negative contributor to the overall analytical outcome. Excellent comparability for absolute matrix effects between model and real samples was observed, while model matrices were less-prone to influences of sample inhomogeneity. It was further demonstrated that neglecting the intrasubject variation by following a validation protocol based on one single matrix replicate leads to an overestimation of the method performance and subsequently underestimates the measurement uncertainty. The major outcomes are summarized as follows

- in-house model matrices allow a high comparability of real-life conditions,
- background information about the individual ratios of ingredients in different lots of compound feed is required in order to prepare the model matrix for validation (may differ from country to country),
- ensure an accurate but not overestimated method performance,
- simulate intrasubject variations,
- economize workload and resources, and
- retain no uncertainties regarding the composition of the complex material.

In summary, the work describes a fit-for-purpose validation proposal for LC−MS/MS multiclass methods in complex feed matrices.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b07706.

Title page; full list of investigated analytes including following information: quantifier/qualifier, substance class, polarity, retention, and acquisition parameter (q1, q3, DP, CE, and CXP); compositional information about tested real and model compound feed samples, graphical illustration of model feed samples, and list of investigated single feed material with origin information; overview on data of $R_A$, SSE, and $R_E$ and the respective RSD values for tested real compound feed samples; overview on data of $R_A$, SSE, and $R_E$ and the respective RSD values for tested model compound feed samples; overview on data of $R_A$, SSE, and $R_E$ and the respective RSD values for tested single feed material; *T*-test and *F*-test statistics for the comparison of model and real compound feed samples; relative standard deviations for the extraction protocol for single feed material and model compound feed; and overview on data of $R_A$, SSE, and $R_E$ and the respective RSD values for the tested single technical replicate of real cattle feed (PDF)

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### Notes

The authors declare no competing financial interest.

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Publication #3

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# Mycotoxins in maize harvested in Republic of Serbia in the period 2012–2015. Part 1: Regulated mycotoxins and its derivatives

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ABSTRACT

The main objective of this study was to apply a liquid chromatography–tandem mass spectrometric method to investigate the presence of 20 mycotoxins in 204 maize samples harvested in Northern Serbia in the period 2012–2015, including seasons with extreme drought (2012), hot and dry conditions (2013 and 2015) and extreme precipitation (2014). Between 2 and 20 mycotoxins contaminated examined samples. In samples collected from each year, all of six examined fumonisins were detected with very high prevalence (from 76% to 100%). Aflatoxin $B_1$ was detected in 94% and 90% maize samples from 2012 and 2015, respectively. In samples from year 2014, deoxynivalenol, zearalenone and its derivatives were detected in 100% of samples. Furthermore, ochratoxin A (25%) was the most predominant in samples from 2012. The obtained results indicate that changes in weather conditions, recorded in the period of four years, had significant influence on the occurrence of examined mycotoxins in maize.

## 1. Introduction

Maize represents one of the most widely used staple food and feed ingredients in the world. It is well known that consumption of maize provides significant amounts of nutrients, vitamins and minerals, and also frequent consumption of maize may have several health benefits on human and animal organism. In Republic of Serbia, maize is mostly used for animal feeding (80%), while the remaining amount is mainly intended for human consumption and food industry. About 40% of total planted area of field crops in Serbia is covered with maize; while around 70% of the total maize production in Serbia is located Northern Serbia. With the approximate amount of 6.5 million tons per year, Serbia is one of the largest maize producers in Europe. The overall amount of produced maize in Serbia is higher than domestic consumption; therefore a considerable fraction of produced maize is exported to European Union, neighboring as well as to Mediterranean countries, with a tendency to expand the market to China and Indonesia. An annual amount of 1.5 million tons of exported maize classified Serbia among leader countries for maize exports in Europe, as well as in the whole world (Maslac, 2018). Although, in the past decade, maize represents one of the most significant agricultural product and export items of Serbia, maize yield as well as maize quality and safety highly depend on weather conditions during the maize growing season (April–September). Maslac (2018) reported that only about 7–9% of arable land in Serbia is irrigated. This fact should be considered with a great attention due to the recent increases of air temperature by 1.4 °C as well as number of tropical days for 50% during maize growing seasons in Serbia (Matović, Gregorić, & Glamočlija, 2013).

Beside influence on maize yield, weather conditions during maize growing season represent factor with the very strong influence on the occurrence of mycotoxins in maize. Mycotoxins are secondary metabolites which are very often produced by toxigenic fungi in response to stress caused by environmental extremes (Medina, Rodríguez, & Magan, 2015). Furthermore, besides weather conditions, fungal infection and subsequent synthesis of mycotoxins also depend on agronomic factors (type of hybrid, soil, tillage, and previous crop), biological factors (susceptible crops), storage conditions (temperature, humidity, handling, presence of insects, rodents and birds), as well as storage time (Pepeljnjak & Šegvć, 2004). More than 400 mycotoxins are currently identified worldwide, but the most important groups of mycotoxins that are of major health concern for humans and animals are: aflatoxins

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(AFs), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUMs); due to their teratogenic, nephrotoxic, hepatotoxic, neurotoxic, mutagenic immunosuppressive characteristics, etc. (Creppy, 2002; Eriksen & Pettersson, 2004)

The International Agency for research on Cancer classified AFs ($AFB_1$, $AFG_1$, $AFB_2$, $AFG_2$, $AFM_1$) in the first group as human carcinogens, while fumonisin $B_1$ ($FB_1$) and OTA belong to the group 2B as possible carcinogenic compound to humans. Based on limited data and evidence in humans and animals, DON and ZEA are classified in group 3 (IARC, 2012).

AFs are a group of mycotoxins produced by *Aspergillus* (*A.*) species, particularly *A. flavus*, *A. parasiticus* and *A. nomius*. Among the approximately twenty AFs identified, five of them occur naturally and they are significant contaminants of a wide variety of food and feed: $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, and $AFM_1$ (Creppy, 2002). $AFB_1$ is highly toxic, classified as the most potent naturally occurring chemical liver carcinogen known (IARC, 2012).

Ochratoxins are a group of mycotoxins that includes at least nine different compounds. OTA is the most prevalent and toxic compound of the group. Ochratoxin B (OTB) is the non-chlorinated ester of OTA, which could be transformed to OTA even though at a low level. OTA and OTB often co-exist in food raw materials, easily transforming to each other under special environment conditions (El Khoury & Atoui, 2010).

DON belongs to the type B trichothecenes and their occurrence is primarily associated with *Fusarium* (*F.*) *graminearum* and *F. culmorum*. Occurrence of those fungus and DON in cereals is characteristic for regions with lower air temperature and higher amount of precipitation. Although DON is among the least toxic of the trichothecenes, it is the most frequently detected one throughout the world, and its occurrence is considered to be an indicator of the possible presence of other, more toxic trichothecenes (Eriksen & Pettersson, 2004). DON very often co-occurs in cereals with its acetylated derivatives 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON). In the recent years deoxynivalenol-3 glucoside (DON-3G), a so called masked form of DON is frequently detected in some cereals and cereal derived products (Maresca, 2013).

ZEA is produced by numerous *Fusarium* species, including *F. roseum*, *F. tricinctum*, *F. sporotrichioides*, *F. oxysporum* and *F. moniliforme*. In comparison to many other mycotoxins, ZEA has a lower acute toxicity. However, ZEA is a powerful estrogen, with hormonal activity exceeding that of most other naturally occurring non-steroidal oestrogens. Alpha zearalenol (α-ZOL) and beta zearalenol (β-ZOL) are reductive metabolites of ZEA, which are very often formed in plant and fungal metabolism. Whereas β-ZOL is less toxic than ZEA, α-ZOL possesses an about 10-fold higher estrogenicity than ZEA. Under microbial activity ZEA could be transformed to a zearalenone-sulfate (ZEA-S) (Zinedine, Soriano, Molto, & Manes, 2007).

So far, about sixteen different FUMs have been isolated and identified. The predominant mycotoxin is $FB_1$, produced by *F. verticillioides* and *F. proliferatum*, followed by $FB_2$, $FB_3$, $FB_4$, $FA_1$ and $FA_2$. $FB_1$ is considered to be the most toxic of these compounds (Butkeraitis et al., 2004). The endophytic nature of the fumonisin-producing maize pathogen *F. verticillioides*, resulted in natural occurrence of FUMs in maize and maize derived products worldwide. High prevalence of FUMs in maize have been noticed in many countries all over the world (Lino, Silva, Pena, & Silveira, 2006), and this is the reason why, from the past decades until today, there is constant tendency to minimize their occurrence in maize.

Previously published studies, related to the presence of mycotoxins in maize from Serbia, were mainly restricted to investigation of presence of one or a few mycotoxins. Previous studies reported high presence of AFs (Kos et al., 2018; Kos, Mastilović, Hajnal, & Šarić, 2013), and DON (Kos et al., 2017) in Serbian maize samples. Based on these reports it could be noticed that in the recent years human population as well as livestock in Serbia were exposed to high risk of certain mycotoxins. Furthermore, considering that in the recent years Serbia was faced with climate changes, and that maize and maize derived products are consumed almost on a daily basis, the primary aim of this work was to investigate the influence of weather conditions recorded in four different years (2012–2015) on the occurrence of the following 20 fungal metabolites: aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$), ochratoxins (OTA, OTB), fumonisins ($FB_1$, $FB_2$, $FB_3$, $FB_4$, $FA_1$, $FA_2$), ZEA, ZEA-S, α-ZOL, β-ZOL, DON, DON-3G, and 15-ADON. To the best of author's knowledge, this study represents the first report from Serbia, as well as from neighboring countries, related to the occurrence of all regulated mycotoxins and its derivatives in maize samples collected in four different years.

## 2. Materials and method

### 2.1. Samples

A total of two hundred and four (n = 204) maize samples were collected in the period of four years, 2012–2015. Every year after harvest, 51 maize samples were collected from the Northern Serbia (Autonomous Province of Vojvodina). Most commercial maize hybrids, currently grown in Northern Serbia, were included in this study. Maize samples were selected to be representative for every investigated year, which means that maize samples were systematically taken from the entire investigated area.

In order to overcome irregular mycotoxins distribution, sampling was performed by official controllers according to European Union (EU) requirements (European Commission, 2006a). Particular numbers of incremental samples were combined in order to obtain aggregate samples of approximately 10–15 kg. After collection, maize samples were transported to the laboratory of the Institute of Food Technology, University of Novi Sad. Total amount of aggregate samples was homogenized using a Nauta mixer (model 19387, Nauta patenten, Netherlands). After homogenization, each aggregate sample was quartered to get 500–1000 g of a representative laboratory sample. The representative maize samples were ground to a 1 mm particle size using laboratory mill (KnifetecTM 1095 mill, Foss, Hoganas, Sweden) and refrigerated at −20 °C. In September 2017, laboratory samples were taken from freezer, again homogenized (Nauta mixer, model 11102, Nauta patenten, Netherlands) and quartered to get sub-samples of 15 g. A total of 204 sub-samples were placed in marked zip lock bags and transported into a cooler in Austria (Department for Agrobiotechnology, IFA-Tulln, Austria). In IFA, maize samples were prepared and analyzed. In order to maintain the originality of the samples, each manipulation step (homogenization, grinding, quartering, packaging and preparation) was done by qualified and experienced laboratory technicians.

### 2.2. Sample preparation and LC–MS/MS analysis

Sample preparations as well as LC–MS/MS analysis of maize samples were conducted in accordance to method published by Malachová, Sulyok, Beltrán, Berthiller, and Krska (2014); without any modifications, as well as with the same equipment and chemicals.

Five grams of maize samples were extracted with 20 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v). The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently centrifuged for 2 min at 3000 rpm (radius 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). The extracts were transferred into glass vials using Pasteur pipettes, and 350 μL aliquots were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). After appropriate mixing, 5 μL of the diluted extract was injected into the LC–MS/MS system without further pre-treatment.

Detection and quantification was performed with a QTrap 5500 MS/MS system (Applied Biosystems, Foster City, CA), equipped with a

**Table 1**
Method performance characteristics for regulated mycotoxins and its derivatives in maize samples.

| Analyte | LOD (μg/kg) | Apparent Recovery ± RSD (%) |
|---|---|---|
| Aflatoxin $B_1$ | 0.25 | 58.2 ± 10.9 |
| Aflatoxin $B_2$ | 0.4 | 61.0 ± 14.8 |
| Aflatoxin $G_1$ | 0.3 | 84.7 ± 10.6 |
| Aflatoxin $G_2$ | 0.8 | 68.3 ± 14.6 |
| Aflatoxin $M_1$ | 0.4 | 84.7 ± 7.2 |
| Ochratoxin A | 0.4 | 87.8 ± 3.4 |
| Ochratoxin B | 1.6 | 86.5 ± 6.3 |
| Fumonisin $B_1$ | 3.2 | 54.6 ± 4.9 |
| Fumonisin $B_2$ | 2.4 | 62.0 ± 4.6 |
| Fumonisin $B_3$ | 2.4 | 64.1 ± 5.9 |
| Fumonisin $B_4$* | 2.4 | 100 |
| Fumonisin $A_1$ | 2 | 100 |
| Fumonisin $A_2$* | 1.5 | 100 |
| Zearalenone | 0.1 | 85.0 ± 8.8 |
| Alpha Zearalenol | 0.8 | 71.1 ± 13.4 |
| Beta Zearalenol | 0.8 | 129.6 ± 7.5 |
| Zearalenone-sulfate* | 92 | 1.60 ± 8.8 |
| Deoxynivalenol | 1.2 | 80.0 ± 12.0 |
| Deoxynivalenol-3-glucoside | 0.8 | 101.7 ± 18.0 |
| 15-acetyldeoxynivalenol | 12 | 89.2 ± 6.1 |

LOD: limit of detection (μg/kg).
RSD: relative standard deviation.
* Semi-quantified using response factor of a structurally related analyte.

TurboV electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® $C_{18}$-column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a $C_{18}$ security guard cartridge, 4 × 3 mm i.d. (all from Phenomenex, Torrance, CA, US). Elution was carried out in a binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively.

Quantification was based on linear, 1/x weighed calibration using serial dilutions of an external multicomponent stock solution. Determined concentrations of 20 fungal metabolites were corrected for apparent recovery (Table 1), while for the metabolites where standard were not available (ZEA-S, $FB_4$, and $FA_2$), semi-quantification was performed using the response factor of structurally related compound. The accuracy of the method is continuously verified by participation in inter-laboratory comparison studies including a regular scheme organized by BIPEA (Gennevilliers, France). Thus for maize, 160 of the 167 submitted results to BIPEA studies were in the satisfactory range (z-scores between −2 and 2). Limit of detection presented in Table 1 was determined following the EURACHEM guide (Magnusson & Örnemark, 2014).

### 2.3. Weather conditions analysis

Weather conditions parameters related to the monthly average air temperatures and sum of precipitation, palmer drought severity index (PDSI), number of days with precipitation, reserve of moisture and deviation of average air temperatures, were provided from the Republic Hydrometeorological Service of Serbia (2012–2015). The listed weather conditions data were collected for Northern Serbia for the entire period of maize growing season (from 1st April to 30th September) in four different years (2012–2015). Deviations of weather conditions parameters were determined in comparison to the data recorded in the long-term period (1981–2010).

### 2.4. Statistical analysis

Descriptive statistics for data related to the mycotoxins concentrations were expressed as the mean ± standard deviation (Std) using

**Table 2**
Occurrence of regulated mycotoxins and its derivatives in maize samples collected in Northern Serbia in the period 2012–2015.

| Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|
| Aflatoxin $B_1$ | 2012 | 48 (94) | 0.6–205 | $44^{b}$ ± 49 | 26 |
| | 2013 | 17 (33) | 0.5–48 | $8^{a}$ ± 11 | 5 |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 46 (90) | 0.4–41 | $8^{a}$ ± 9 | 4 |
| Aflatoxin $B_2$ | 2012 | 36 (71) | 0.7–22 | $5^{b}$ ± 5 | 3 |
| | 2013 | 5 (10) | 0.7–2 | $1^{a}$ ± 0.5 | 1 |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 8 (16) | 0.8–2 | $2^{a}$ ± 0.4 | 2 |
| Aflatoxin $G_1$ | 2012 | 23 (45) | 0.4–141 | $10^{b}$ ± 29 | 1 |
| | 2013 | 1 (2) | 3 | nd | nd |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 7 (14) | 0.3–1 | $0.8^{a}$ ± 0.3 | 0.9 |
| Aflatoxin $G_2$ | 2012 | 6 (12) | 2–73 | 16 ± 28 | 4 |
| | 2013 | nd | nd | nd | nd |
| | 2014 | nd | nd | nd | nd |
| | 2015 | nd | nd | nd | nd |
| Aflatoxin $M_1$ | 2012 | 29 (57) | 0.5–7 | $2^{a}$ ± 2 | 0.9 |
| | 2013 | 1 (2) | 0.6 | nd | nd |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 4 (8) | 0.5–0.7 | $0.6^{ab}$ ± 0.1 | 0.6 |
| Ochratoxin A | 2012 | 13 (25) | 2–318 | $53^{b}$ ± 108 | 6 |
| | 2013 | 1 (2) | 1 | nd | nd |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 9 (18) | 0.5–27 | $6^{a}$ ± 8 | 5 |
| Ochratoxin B | 2012 | 3 (6) | 3–8 | 5 ± 4 | 4 |
| | 2013 | nd | nd | nd | nd |
| | 2014 | nd | nd | nd | nd |
| | 2015 | 1 (2) | 2 | nd | nd |
| Fumonisin $B_1$ | 2012 | 51 (100) | 211–13396 | $4121^{b}$ ± 2172 | 3667 |
| | 2013 | 51 (100) | 88–16187 | $4690^{bc}$ ± 4280 | 3478 |
| | 2014 | 51 (100) | 193–27103 | $5846^{c}$ ± 5461 | 4053 |
| | 2015 | 51 (100) | 192–4253 | $1905^{a}$ ± 1058 | 1881 |
| Fumonisin $B_2$ | 2012 | 51 (100) | 72–3118 | $828^{ab}$ ± 525 | 706 |
| | 2013 | 50 (98) | 20–3811 | $967^{b}$ ± 926 | 586 |
| | 2014 | 50 (98) | 160–4651 | $1463^{c}$ ± 1296 | 1003 |
| | 2015 | 51 (100) | 46–1019 | $431^{a}$ ± 257 | 395 |
| Fumonisin $B_3$ | 2012 | 51 (100) | 29–1293 | $408^{b}$ ± 223 | 364 |
| | 2013 | 47 (92) | 30–1511 | $450^{b}$ ± 374 | 325 |
| | 2014 | 47 (92) | 38–2603 | $454^{b}$ ± 414 | 357 |
| | 2015 | 51 (100) | 38–2603 | $183^{a}$ ± 108 | 167 |
| Fumonisin $B_4$* | 2012 | 50 (98) | 89–1003 | $266^{ab}$ ± 187 | 222 |
| | 2013 | 50 (98) | 12–1156 | $339^{b}$ ± 318 | 266 |
| | 2014 | 51 (100) | 29–1682 | $498^{c}$ ± 423 | 360 |
| | 2015 | 49 (96) | 12–409 | $146^{a}$ ± 97 | 127 |
| Fumonisin $A_1$ | 2012 | 50 (98) | 2–70 | $12^{bc}$ ± 10 | 10 |
| | 2013 | 44 (82) | 2–24 | $9^{ab}$ ± 6 | 7 |
| | 2014 | 47 (92) | 2–40 | $14^{c}$ ± 10 | 12 |
| | 2015 | 44 (82) | 2–19 | $6^{a}$ ± 4 | 5 |
| Fumonisin $A_2$* | 2012 | 50 (98) | 4–157 | $22^{b}$ ± 22 | 19 |
| | 2013 | 44 (82) | 2–37 | $13^{a}$ ± 10 | 11 |
| | 2014 | 46 (90) | 2–58 | $23^{b}$ ± 16 | 19 |
| | 2015 | 39 (76) | 2–40 | $12^{a}$ ± 8 | 10 |
| Zearalenone | 2012 | 6 (12) | 5–67 | $26^{a}$ ± 25 | 18 |
| | 2013 | 19 (37) | 1–97 | $17^{a}$ ± 24 | 9 |
| | 2014 | 51 (100) | 15–2596 | $598^{b}$ ± 670 | 346 |
| | 2015 | 27 (53) | 1–58 | $14^{a}$ ± 16 | 6 |
| Zearalenone-sulfate* | 2012 | 7 (14) | 9–90 | $34^{a}$ ± 36 | 16 |
| | 2013 | 18 (35) | 3–128 | $32^{a}$ ± 38 | 10 |
| | 2014 | 51 (100) | 5–5650 | $906^{b}$ ± 1069 | 593 |
| | 2015 | 21 (41) | 8–72 | $27^{a}$ ± 17 | 22 |
| Alpha zearalenol | 2012 | 1 (2) | 15 | nd | nd |
| | 2013 | 1 (2) | 5 | nd | nd |
| | 2014 | 31 (61) | 2–27 | 7 ± 6 | 6 |
| | 2015 | nd | nd | nd | nd |

(continued on next page)

**Table 2** (*continued*)

| Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|
| Beta zearalenol | 2012 | 2 (4) | 3–12 | $7^{a}$ ± 6 | 7 |
| | 2013 | nd | nd | nd | nd |
| | 2014 | 49 (96) | 0.7–27 | $7^{a}$ ± 6 | 5 |
| | 2015 | nd | nd | nd | nd |
| Deoxynivalenol | 2012 | 32 (63) | 10–1855 | $128^{a}$ ± 325 | 31 |
| | 2013 | 18 (35) | 21–436 | $120^{a}$ ± 102 | 104 |
| | 2014 | 51 (100) | 428–16350 | $3522^{b}$ ± 2668 | 3331 |
| | 2015 | 32 (63) | 22–460 | $84^{a}$ ± 94 | 44 |
| Deoxynivalenol-3 glucoside | 2012 | 9 (18) | 3–162 | $41^{a}$ ± 56 | 8 |
| | 2013 | 6 (12) | 7–23 | $12^{a}$ ± 6 | 11 |
| | 2014 | 51 (100) | 48–946 | $244^{b}$ ± 172 | 218 |
| | 2015 | 8 (16) | 8–31 | $14^{a}$ ± 9 | 11 |
| 15-Acetyl deoxynivalenol | 2012 | 1 (2) | 51–308 | $188^{a}$ ± 133 | 196 |
| | 2013 | 1 (2) | 48 | nd | nd |
| | 2014 | 50 (98) | 80–1181 | $323^{a}$ ± 193 | 285 |
| | 2015 | 1 (2) | 40 | nd | nd |

N (%): number (percentage) of contaminated sample.
nd: not detected i.e., less than limit of detection (LOD).
Min-Max: minimum and maximum concentrations (μg/kg).
Mean ± Std: mean concentration (μg/kg) ± standard deviation (μg/kg).
Median: median concentration (μg/kg).
Different letters in the same column indicate significant differences ($P < 0.05$) between values according to the post-hoc Tukey's HSD test.
* Semi-quantified using response factor of a structurally related analyte.

STATISTICA software version 13.2 (StatSoft, 2016). The significant differences between samples were calculated according to post-hoc Tukey's HSD (honestly significant differences) test, for unequal sample sizes, at a $P < 0.05$ significance level, 95% confidence interval. Statistical analysis of variance for weather conditions data was carried out by Duncańs multiple comparison tests using the same STATISTICA (StatSoft, 2016) software, ($P < 0.05$ was regarded as significant).

## 3. Results and discussion

### 3.1. Occurrence of mycotoxins in maize samples

Results for the analysis of 20 fungal metabolites in 204 maize samples collected in the Northern Serbia in the period of four years (2012–2015) are shown in the Table 2. From the obtained results it could be noticed that significant differences exist in mycotoxins occurrence as well as in the range of determined concentrations in samples originated from different years. In the recent years, many authors indicated that weather conditions (especially air temperature and amount of precipitation) and climate changes represent factors with the strongest influence on the occurrence of mycotoxins in maize (Medina et al., 2015). Therefore, for the better interpretation of the obtained results in this study, related to the influence of changeable weather conditions on the occurrence of 20 different maize contaminants, detailed analysis of weather conditions parameters was conducted.

### 3.2. Weather conditions in Serbia, 2012–2015

Weather conditions parameters for the period of maize planting, growing and harvesting (April–September 2012–2015) in Northern Serbia are summarized and shown in Fig. 1. Average air temperatures (a) as well as sum of precipitation (b) are shown in the relation to average values of these parameters from the long-term period (1981–2010). Drought indicators were analyzed for the period of generative phase of maize from June to August (2012–2015), and shown in the Table 3.

In general, the climate of Northern Serbia is described as moderate-continental with more or less pronounced local characteristics. However, weather conditions (Fig. 1 and Table 3), indicate that

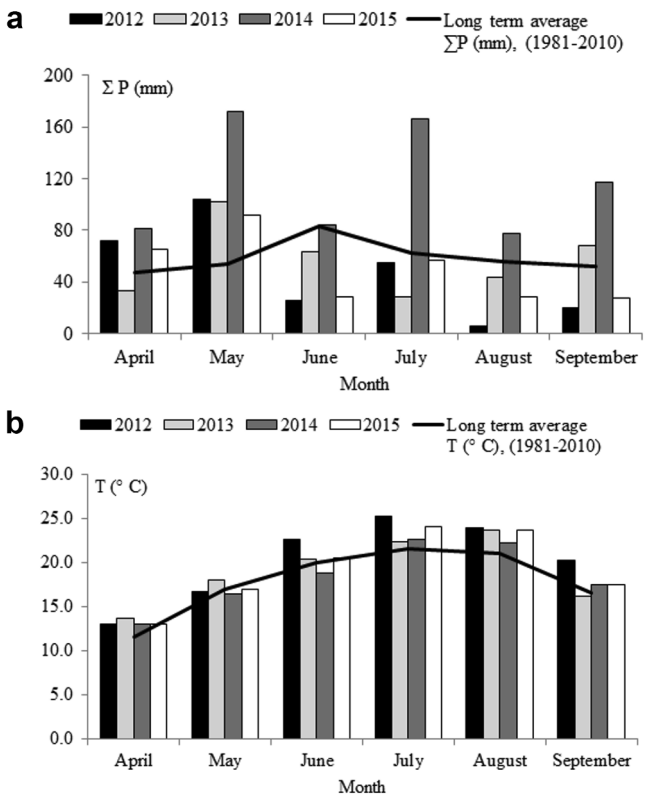


**Fig. 1.** Monthly average a) air temperatures and b) sum of precipitation in Northern Serbia in the period 2012–2015 (April–September) in comparison with the long term average values for the period 1981–2010.

**Table 3**
Drought indicators for the June, July and August in Northern Serbia in the period 2012–2015.

| Month | Year | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2012 | | 2013 | | 2014 | | 2015 | |
| | Palmer Drought Severity Index | | | | | | | |
| June | −4.2 | ED | 0.6 | N | −1.2 | N | −0.2 | N |
| July | −4.5 | ED | −1.1 | MID | 1.1 | N | −3.0 | SD |
| August | −4.5 | ED | −2.4 | MD | 1.7 | N | −2.6 | MD |
| | Number of days with precipitation | | | | | | | |
| June | $6^{a}$ | | $10^{b}$ | | $6^{a}$ | | $6^{a}$ | |
| July | $5^{a}$ | | $5^{a}$ | | $15^{b}$ | | $3^{a}$ | |
| August | $1^{a}$ | | $6^{b}$ | | $9^{c}$ | | $7^{b}$ | |
| | Reserve of moisture (mm) | | | | | | | |
| June | $1.4^{a}$ | | $20.1^{c}$ | | $20.9^{c}$ | | $10.4^{b}$ | |
| July | $6.7^{a}$ | | $5.0^{a}$ | | $71.1^{b}$ | | $3.0^{a}$ | |
| August | $1.7^{a}$ | | $30.2^{b}$ | | $41.6^{c}$ | | $27.8^{b}$ | |
| | Deviation of average temperature (°C) | | | | | | | |
| June | $2.6^{c}$ | | $0.4^{b}$ | | $-1.2^{a}$ | | $0.5^{b}$ | |
| July | $3.6^{c}$ | | $0.8^{a}$ | | $1.0^{a}$ | | $2.5^{b}$ | |
| August | $2.9^{b}$ | | $2.6^{b}$ | | $1.2^{a}$ | | $2.6^{b}$ | |

N: normal; MID: mild drought; MD: moderate drought; SD: severe drought; ED: extreme drought.
Different letters in the same row indicate significant differences ($P < 0.05$) between values in different years, according to the Duncan's multiple range test.

examined four years significantly deviated from long-term weather patterns.

In particular, the 2012 maize growing season was characterized by the highest air temperatures and the lowest amount of precipitation compared to the other years investigated, as well as in comparison to

average values from the long-term period (1981–2010). From June to August, minimum precipitation and very high daily temperatures (around 40 °C) indicate extreme drought conditions, as indicated by the PDSI. As a result, soil-water content and moisture reserve were at its lowest level throughout these investigated years.

Hot and dry weather conditions were also dominant during the majority of the maize growing season in 2013. As can be seen from the Fig. 1, air temperatures were around or above long-term average air temperatures in April, May, June, July, and August. Furthermore, precipitation was higher than average value only in May and September, while it was lower in April, June, July and August. The PDSI (Table 3) categorizes June 2013 as normal, while July and August are categorized as mild and moderate drought, respectively.

Contrary to the hot and dry weather conditions recorded in years 2012 and 2013, spring and summer in 2014 were characterized by changeable and particularly wet weather conditions. Maize growing season in 2014 was characterized by extreme high amount of precipitation. Especially high amount of precipitation was recorded in May and July. During the whole maize growing season in 2014 recorded sum of precipitation (780 mm) was significantly different in comparison to the other investigated years, and this value represents the maximum recorded value of precipitation since meteorological observations exist in Serbia. Such high amount of precipitation resulted in floods in many parts of Serbia which was associated with an increase in the amount of reserve of moisture in soil. Monthly average values of air temperatures in 2014 were around the average values for the same months in the long-term period (1981–2010).

After the rainy 2014 year, hot and dry weather conditions were again recorded in the maize growing season in 2015. According to the PDSI, June was characterized as normal, while conditions in July and August were characterized with severe and moderate drought conditions, respectively. Recorded higher air temperatures as well as smaller amount of precipitation during maize growing season in 2015 indicated that trend of climate changes followed by hot and dry conditions is continued.

### 3.3. Aflatoxins

Under agroecological conditions of Serbia around 30 different species of the genus *Aspergillus* have been identified. In the period 1967–2008 frequency of *A. flavus* in maize from Serbia varied from 3% to 16% (Lević et al., 2013). Contrary to this, the same group of authors reported that in year 2012 *A. flavus* was registered on maize with incidence of even 95% (Lević, Stanković, Krnjaja, Bočarov-Stančić, & Ivanović, 2012). Such high incidence of *A. flavus* could be explained by extremely stressful agrometeorological conditions recorded in 2012. It is well known that maize consumes great amounts of water due to its large vegetative mass, high yields and long growing season. Due to the described extreme drought conditions in maize growing season in 2012, maize was exposed to water stress which further resulted in appearance of various pests, especially European corn borer (ECB) (*Ostrinia Nubilalis*), rootworm (*Diabrotica Virgifera*), and corn damage (Lević et al., 2012). All listed factors influenced that maize became particularly prone to infection by *Aspergillus* species and aflatoxins synthesis (Medina et al., 2015).

Obtained results in this study confirmed that conditions of extreme drought in 2012 had a great influence on the presence of AFs, since $AFB_1$, $AFB_2$, $AFM_1$, $AFG_1$ and $AFG_2$ detected in even 94%, 71%, 57%, 45% and 12% of examined maize samples (Table 2), respectively. The highest determined concentration of the most toxic $AFB_1$ was 205 μg/kg, while the mean level of its determined concentrations was 44 ± 49 μg/kg.

According to the literature data $AFM_1$ is the 4-hydroxy derivative of $AFB_1$, which is formed in liver and excreted into the milk in the mammary glands of human and lactating animals that have been fed with $AFB_1$ contaminated diet (Fallah, Rahnama, Jafari, & Saei-Dehkordi, 2011). Hence, occurrence of $AFM_1$ is mainly related to milk and other products of animal origin. However, obtained results in this study confirmed previous findings that $AFM_1$, beside in animal products, could also be detected in plants with concentrations of a few percentage compared to that of AFB1 in the same sample. In maize samples from year 2012, $AFM_1$ was detected in more than half (57%) of the examined maize samples. The range and the mean value of the determined concentrations of $AFM_1$ were 0.5–7 μg/kg and 2 ± 2 μg/kg, respectively. High contamination frequency of AFs in maize samples from year 2012 had a great influence on the presence of AFs in the almost entire food and feed chain in Serbia, at the end of 2012 as well as in year 2013 (Kos, Lević, Đuragić, Kokić, & Miladinović, 2014). During that period, Serbia was faced with, aflatoxins crisis“ which was followed by protest of agricultural workers and farmers, replacement of Minister of Agriculture, great economical losses (about a hundred million dollars), several changes in Regulation related to the ML of $AFM_1$ in milk, confusion between consumers and decrease in purchase of milk and dairy products.

The absence of prolonged and extreme drought conditions during the maize growing season in year 2013, as well as recorded smaller deviation of air temperatures in June and July, and greater amount of reserve of moisture (especially in August) could have contributed to lower contamination frequency of AFs in maize samples from year 2013, in comparison to maize samples from 2012. $AFB_1$ and $AFB_2$ were detected in 33% and 10% of examined maize samples, respectively. Furthermore, $AFG_1$ and $AFM_1$ have occurred in only one sample (2%), while none of the analyzed maize samples from 2013 was contaminated with $AFG_2$. As can be seen from the Table 2, mean level of detected $AFB_1$ and $AFB_2$ were significantly lower in comparison to concentrations determined in maize samples from year 2012.

Contrary to the previous two years, none of the 51 maize samples from 2014 was contaminated with examined AFs. Obtained results could be explained by extreme rainy conditions recorded during the maize growing season in 2014 which were unfavorable for the growth of certain *Aspergillus species* and AFs synthesis.

After the absence of AFs in maize samples from year 2014, they were again detected in maize samples from 2015. Even though $AFB_1$ was detected with a high prevalence (90%), detected mean concentration of $AFB_1$ (8 ± 9 μg/kg) was significantly lower in comparison to mean concentration (44 ± 49 μg/kg) detected in maize samples from 2012. Furthermore, percentage of contaminated samples as well as determined concentrations of other investigated AFs were also lower in comparison to detected concentrations in samples from 2012. Differences in contamination level contribute to differences in weather conditions between years 2012 and 2015, especially in terms of deviations of air temperatures (in June and July), reserve of moisture (in June and August) and PDSI values.

Combination of different aflatoxins in maize samples collected from four different years was also examined in this study and shown in the Table 4. As can be seen, in maize samples from year 2012, every of investigated combinations of AFs were detected. The most frequently occurring AFs combination was $AFB_1$ + $AFB_2$ which were present in 71% of samples. Furthermore, occurrence of $AFB_1$ + $AFM_1$ and $AFB_1$ + $AFG_1$ was detected in 57% and 45% of maize samples, respectively. Prevalence of all four regulated aflatoxins was registered in 12% samples, while all five examined AFs were detected in 8% of analyzed maize samples from 2012. Contrary to the frequent occurrence of different AFs in maize samples from 2012, in maize samples from other investigated years, combination of different aflatoxins was less common. With occurrence of 10% and 16%, $AFB_1$ + $AFB_2$ was the most prevalent combination of AFs in maize samples from 2013 and 2015, respectively.

Previously, authors claimed that AFs rarely occurred in maize as well as in other agricultural products from Serbia (Kos et al., 2013). However, recent changes in weather conditions in Serbia influenced more frequent prevalence of AFs in maize. The findings obtained in this

**Table 4**
Combination of different mycotoxins and its derivatives in maize samples collected in Northern Serbia in the period 2012–2015.

| Mycotoxins | Year N (%) | | | |
|---|---|---|---|---|
| | 2012 | 2013 | 2014 | 2015 |
| $AFB_1$ + $AFB_2$ | 36 (71) | 5 (10) | nd | 8 (16) |
| $AFB_1$ + $AFG_1$ | 23 (45) | 1 (2) | nd | 7 (14) |
| $AFB_1$ + $AFG_2$ | 6 (12) | nd | nd | nd |
| $AFB_1$ + $AFM_1$ | 29 (57) | 1 (2) | nd | 1 (2) |
| $AFB_1$ + $AFB_2$ + $AFG_1$ | 19 (37) | nd | nd | 1 (2) |
| $AFB_1$ + $AFB_2$ + $AFG_1$ + $AFG_2$ | 6 (12) | nd | nd | nd |
| $AFB_1$ + $AFG_1$ + $AFB_2$ + $AFM_1$ | 12 (23) | nd | nd | 1 (2) |
| $AFB_1$ + $AFG_1$ + $AFG_2$ + $AFB_2$ + $AFM_1$ | 4 (8) | nd | nd | nd |
| OTA + OTB | 3 (6) | nd | nd | 1 (2) |
| DON + DON-3G | 9 (18) | 6 (12) | 51 (100) | 6 (12) |
| DON + 15-ADON | 4 (8) | 1 (2) | 50 (98) | 1 (2) |
| DON-3G + 15-ADON | 2 (4) | 1 (2) | 50 (98) | 1 (2) |
| DON + DON-3G + 15-ADON | 2 (4) | 1 (2) | 50 (98) | 10 (20) |
| ZEA + ZEA-S | nd | 15 (29) | 51 (100) | 20 (39) |
| ZEA + α-ZOL | 1 (2) | 1 (2) | 30 (59) | nd |
| ZEA + β-ZOL | 2 (4) | nd | 49 (96) | nd |
| ZEA + ZEA-S + α-ZOL | 1 (2) | 1 (2) | 30 (59) | nd |
| ZEA + ZEA-S + β-ZOL | 1 (2) | nd | 49 (96) | nd |
| ZEA + ZEA-S + α-ZOL + β-ZOL | 1 (2) | nd | 29 (57) | nd |
| $FB_1$ + $FB_2$ | 51 (100) | 50 (98) | 50 (98) | 51 (100) |
| $FB_1$ + $FB_2$ + $FB_3$ | 51 (100) | 47 (92) | 49 (96) | 51 (100) |
| $FB_1$ + $FB_2$ + $FB_3$ + $FB_4$ | 50 (98) | 46 (90) | 49 (96) | 49 (96) |
| $FB_1$ + $FB_2$ + $FB_3$ + $FB_4$ + $FA_1$ | 50 (98) | 42 (82) | 46 (90) | 41 (80) |
| $FB_1$ + $FB_2$ + $FB_3$ + $FB_4$ + $FA_1$ + $FA_2$ | 50 (98) | 40 (78) | 46 (90) | 36 (71) |
| AFs* + OTA + DON + ZEA + $FB_1$ + $FB_2$ | 4 (8) | 1 (2) | nd | nd |
| AFs* + DON + ZEA + $FB_1$ + $FB_2$ | 6 (12) | 8 (16) | nd | 30 (59) |
| AFs* + $FB_1$ + $FB_2$ | 48 (94) | 17 (33) | nd | 46 (90) |
| DON + ZEA + $FB_1$ + $FB_2$ | 5 (10) | 15 (29) | 50 (98) | 24 (47) |

N (%): number (percentage) of contaminated sample.
nd: not detected.
* At least one of the analyzed aflatoxins from B or G groups.

study indicate that occurrence of AFs in maize from Serbia is variable from year to year and highly dependent on the weather conditions.

### 3.4. Ochratoxins

Under hot and dry conditions, OTA is mainly produced by *Aspergillus* species, while in the regions characterized by a low-temperature climate OTA is very often produced by species belonging to the *Penicillium* genera (El Khoury & Atoui, 2010).

Among 51 examined maize samples, from each year in the period 2012–2015, OTA was detected in 13 (25%), 1 (2%) and 9 (18%) maize samples from years 2012, 2013 and 2015, respectively. The highest detected contamination frequency (25%) of OTA was recorded in maize samples from 2012, which means that prolonged drought was the most favorable conditions for the growth of certain *Aspergillus* species and synthesis of OTA. The range and the mean value of the determined concentrations of OTA in maize samples from 2012 were 2–318 and 53 ± 108 μg/kg, respectively. Non-chlorinated ester of OTA, OTB was detected in only 6% of maize samples from 2012 and in 2% of samples from 2015. Due to the fact that none of the analyzed maize samples from year 2014 was contaminated with OTA and OTB, it could be noticed that recorded rainy and wet conditions were not favorable for synthesis of ochratoxins. Furthermore, even though OTA is suspected to be the cause of the human fatal disease known as Balkan Endemic Nephropathy, an interstitial chronic disease affecting the south-eastern population of Europe, including Serbia (El Khoury & Atoui, 2010), obtained results in this study indicate that OTA is not very often detected in maize from Serbia.

### 3.5. Fumonisins

Results obtained in this study confirmed that among examined mycotoxins in this study, FUMs were the most prevalent mycotoxins group. Even though maize growing seasons in 2012, 2013 and 2015 were characterized by hot and dry conditions and maize growing season in 2014 was extremely wet, $FB_1$ was present in 100% of the samples from each year. As can be seen in Table 2, the highest level of mean $FB_1$ concentration of 5846 ± 5461 μg/kg was detected in maize samples from year 2014, followed by mean $FB_1$ concentrations of 4690 ± 4280 μg/kg, 4121 ± 2172 μg/kg and 1905 ± 1058 μg/kg in maize samples from 2013, 2012 and 2015 years, respectively. Based on the obtained results it could be noticed, that under wet conditions in year 2014, *F. verticillioides* produce the highest amount of $FB_1$. $FB_2$ was the second most common detected FUMs, with detected frequency of 100% in maize samples from years 2012 and 2015, and 98% in maize samples from years 2013 and 2014. The highest detected concentration of $FB_2$, as well as of $FB_1$, was detected in maize samples from year 2014. Prevalence of other examined FUMs was also very high in maize samples from each of the four investigated years. In the period of four years 2012–2015, prevalence of $FB_3$, $FB_4$, $FA_1$ and $FA_2$ was detected in the following range of percentage: 92–100%, 96–100%, 82–98% and 76–98%, respectively.

Combination of different investigated FUMs in maize samples from all four investigated years was also very frequent. Incidence of $FB_1$ and $FB_2$ was the most frequently found combination, and dependent on the year it was vary in between 98% and 100% of samples. As can be seen from the Table 4, combination of $FB_1$ + $FB_2$ + $FB_3$ was also very common (from 92% to 100%). Furthermore, combination of four, five and six different FUMs in maize samples from the period 2012–2015

were detected with the following prevalence 90–98%, 80–98% and 71–98%, respectively.

The findings obtained in this study indicate that high prevalence of FUMs were detected in maize samples from each of the four examined years regardless of different weather conditions recorded in examined period of four years. Regarding this issue it could be assumed that Serbia may become susceptible to problems concerning FUMs in maize. Moreover, some authors already reported that high prevalence of FUMs in maize, in the recent years resulted in high prevalence of FUMs in maize food products (Torović, 2018), and also had a great influence on toxicosis in horses from Serbia (Jovanović et al., 2015)

### 3.6. Deoxynivalenol and its derivatives

The obtained data (Table 2) indicate significant differences in the occurrence of DON and its derivatives in maize samples collected over the period of four years. In maize samples from maize growing season 2014, which was described as extreme rainy and wet, prevalence of DON and its derivatives was very high, since DON, DON-3G and 15-ADON were detected in even 100%, 100% and 98% of analyzed maize samples, respectively. Furthermore, determined concentrations of DON, DON-3G and 15-ADON in maize samples from 2014 were significantly different and higher in comparison to determined concentrations in maize samples from other investigated years. In maize samples from 2014, DON was detected in the concentration range from 428 to 16350 μg/kg, DON-3G from 48 to 946 μg/kg, and 15-ADON from 80 to 1181 μg/kg. Such high contamination frequency of DON, DON-3G and 15-ADON in maize samples from 2014 was associated with recorded high amount of precipitation. Extreme precipitation led to increase of moisture amount in the grain (above 20%) which resulted in favorable conditions for the growth of certain *Fusarium* species and synthesis of high concentrations of DON, DON-3G and 15-ADON.

Contrary to this, under the hot and dry conditions recorded in years 2012, 2013 and 2015, synthesis of DON, DON-3G and 15-ADON was less pronounced. Even though DON was detected in 63%, 35% and 63% of examined maize samples from years 2012, 2013 and 2015, it could be noticed that determined mean concentrations 128 ± 325, 120 ± 102 and 84 ± 94 μg/kg were significantly lower in comparison to detected concentration (3522 ± 2668 μg/kg) in year 2014, respectively.

As can be seen from the Table 4, when DON was detected with high contamination frequency their derivatives were also present with high prevalence and in high concentrations. DON and DON-3-G were detected in each sample (100%) from 2014 year, while other three combinations were detected in even 98% of samples. In maize samples from others investigated years, DON and DON-3-G was also the most frequently detected combination, while other examined combination were significantly less detected.

Based on the findings in this study, it can be noticed that DON is a frequent contaminant of maize from Serbia, but it should be emphasized that its concentration highly depends on the amount of precipitation during the maize growing season.

### 3.7. Zearalenone and its derivatives

According to previous studies, high concentrations of ZEA are very often found in maize grown under wet conditions. Hence, rainy conditions during maize growing season in year 2014, beside for DON and its derivatives were also favorable for synthesis of examined ZEA, α-ZOL, β-ZOL and ZEA-S. ZEA and ZEA-S were detected in 100% of examined maize samples from year 2014, while β-ZOL was detected in 96% and α-ZOL in 61% of samples. Furthermore, detected concentrations of ZEA (598 ± 670 μg/kg) and ZEA-S (906 ± 1069 μg/kg) in maize samples from year 2014 were higher as well as significantly different in comparison to detected concentrations of those toxins in maize samples from other investigated years. Based on the obtained results it could be noticed that high amount of precipitation recorded in maize growing season in 2014 had a great influence on synthesis of ZEA and its derivatives. On the other hand, high air temperatures as well as lack of precipitation recorded during maize growing seasons in 2012, 2013 and 2015 resulted in lower level of contamination. In years 2012, 2013 and 2015 ZEA was detected in 12%, 37% and 53% of examined maize samples with mean concentrations of 26 ± 25 μg/kg, 17 ± 24 μg/kg and 14 ± 16 μg/kg, respectively. α-ZOL was detected in only 1 (2%) sample from years 2012 and 2013, while β-ZOL was detected in 2 (4%) samples from 2012.

Similar to DON and its derivatives, extreme wet weather conditions recorded in maize growing season in 2014 were the most favorable conditions for synthesis of ZEA and its derivatives. It should be emphasized that in maize samples from 2014, combination of ZEA and ZEA-S (Table 4) were detected in each sample, while occurrence of ZEA + β-ZEA and ZEA + ZEA-S + β-ZEA was noticed in even 96% of samples. In more than half (59%) of the analyzed maize samples from 2014 occurrence of ZEA + α-ZEA and ZEA + ZEA-S + α-ZEA were detected, while combination of all four contaminants was detected in even 57% of samples. In 29% and 39% of analyzed maize samples from years 2013 and 2015, occurrence of ZEA and ZEA-S was detected. On the other hand, only one (2%) or two (4%) maize samples from years 2012, 2013 and 2015 were contaminated with ZEA and some of its derivatives.

### 3.8. Co-occurence of investigated mycotoxins

In the recent decade there is a great demand for investigation of co-occurrence of mycotoxins in different samples, since mixture of different mycotoxins may generate additive or synergistic effect in humans and animals (Njobeh et al., 2010). Hence, co-occurrence of investigated mycotoxins in maize samples collected from four different years was examined in this study and shown in the Table 4.

In terms of co-occurrence of regulated mycotoxins in maize samples, it could be noticed that maize samples from years 2012 (94%) and 2015 (90%), were mixture of AFs and FUMs, while the most frequently occurring combination in maize samples from 2014 was a combination of DON, ZEA and FUMs (98%). Due to the rare occurrence of OTA in examined maize samples, all regulated mycotoxins were detected in only 8% and 2% of maize samples from 2012 and 2013 years, respectively. Co-occurrence of all regulated mycotoxins, except OTA, occurred in 12%, 16% and 59% of maize samples from years 2012, 2013 and 2015, respectively. Furthermore, co-occurrence of regulated *Fusarium* mycotoxins (DON, ZEA, $FB_1$ and $FB_2$) were detected in 10%, 29%, 98% and 47% of samples from 2012, 2013, 2014 and 2015 years, respectively.

The obtained findings indicate that maize samples from each of the investigated year represent mixture of different mycotoxins. Determined high incidences of co-contamination in the same sample should be considered with a great attention, due to the possible strong synergistic or at least additive effects, which can be observed in simultaneous presence of several mycotoxins in one sample. Obtained results represent an important factor in upgrading of existing data related to the mycotoxins co-occurrence in maize and also could be useful as an important issue for risk assessment. Moreover, it should be emphasized that co-occurrence of mycotoxins in maize from Serbia has rarely been investigated, and therefore this study represents the first report related to the co-occurrence of regulated mycotoxins and its derivatives in maize samples from Serbia.

### 3.9. Occurrence of non-compliance levels of mycotoxins

The Regulation of Serbia (Serbian Regulation, 2011) on the control of mycotoxins in food was harmonized with the Regulation of European Union (European Commission, 2006c) and adopted in year 2011. Until then, maize intended for human consumption in Serbia had to be tested

**Table 5**
Frequency of the occurrence of non-compliance levels of mycotoxins in maize samples collected in Northern Serbia in the period 2012–2015 observed in accordance to Serbian and European Regulations.

| Mycotoxins | ML* | Maize intended for human consumption | | | | ML** EU/RS | Maize intended for animal consumption | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | N (%) Year | | | | | N (%) Year | | | |
| | | 2012 | 2013 | 2014 | 2015 | | 2012 | 2013 | 2014 | 2015 |
| Aflatoxin $B_1$ | 5 | 41 (80) | 7 (14) | nd | 21 (41) | 20/30 | 31 (61)/22 (43) | 1 (2)/1 (2) | nd/nd | 6 (12)/2 (4) |
| Aflatoxins | 10 | 36 (71) | 3 (6) | nd | 8 (16) | | | | | |
| Ochratoxin A | 5 | 10 (20) | nd | nd | 4 (8) | 250 | 2 (4) | nd | nd | nd |
| Zearalenone | 350 | nd | nd | 26 (51) | | 2000/4000 | nd/nd | nd/nd | 4 (8)/nd | nd/nd |
| Deoxynivalenol | 1750 | 1 (2) | nd | 43 (84) | nd | 8000 | nd | nd | 3 (6) | nd |
| Fumonisins | 4000 | 34 (67) | 31 (61) | 26 (51) | 6 (12) | 60,000 | nd | nd | nd | nd |

N (%): number (percentage) of contaminated sample with concentrations higher than maximum level.
ML: maximum level (μg/kg).
nd: not detected.
Aflatoxins: sum of $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$.
Fumonisins: sum of $FB_1$ and $FB_2$.
ML*: according to European (2006/1881/EC) and Serbian Regulation (35/2017).
ML**: according to European (2002/32/EC, 2006/576/EC) and Serbian Regulations (54/2017).

only for the presence of AFs and OTA. In terms of maize, which will be used in animal nutrition, there are still several differences between Serbian (Serbian Regulation, 2010) and European Regulations (European Commission, 2002, 2006b). Maximum levels (MLs) of all regulated mycotoxins in maize samples intended for human and animal consumption, as well as frequency of occurrence of non-compliance levels of investigated mycotoxins in maize samples are shown in the Table 5.

As can be seen from the Table 5, even 80% and 71% of maize samples from year 2012 were not suitable for human nutrition, since concentrations of $AFB_1$ and AFs were above MLs of 5 and 10 μg/kg, respectively. Considering that high concentrations of $AFB_1$ were detected, 61% (ML is 20 μg/kg) of samples could not be used for animal consumption according to European Regulation (European Commission, 2002), while 43% (ML is 30 μg/kg) had higher AFs concentrations than ML defined by Serbian Regulation (Serbian Regulation, 2010). OTA and FUMs concentrations exceeded ML in 20% and 67% of maize samples from 2012, making them unsuitable for human nutrition, respectively. In terms of OTA concentration, only two samples (4%) was unsuitable for animal consumption, while detected concentrations of DON, ZEA and FUMs in maize samples from 2012 were lower than MLs for maize intended for animal nutrition.

Among 51 examined maize samples from year 2013, 14% had $AFB_1$ and 6% had AFs concentrations greater than MLs, which is significantly lower in comparison to percentage of non-compliance samples from year 2012. Furthermore, exceeded FUMs concentrations were noted in even 61% of maize samples from 2013. Due to the high $AFB_1$ concentration, only one sample (2%) from 2013 was unsuitable for animal consumption according to the EU and Serbian regulations; while detected concentrations of other examined mycotoxins in maize samples from 2013, satisfied criteria for feeding animals.

High level of detected *Fusarium* toxins in maize samples from 2014 influenced that even 84%, 51% and 51% of samples contained DON, ZEA and FUMs in concentrations greater than MLs, respectively. In terms of maize intended for animal nutrition, 8% of samples had ZEA concentrations higher than ML according to the EU Regulation, while in 6% of samples concentrations of DON exceeded ML according to the both EU as well as Serbian Regulations.

Among 48 contaminated maize samples with $AFB_1$ from 2015, in 21 (41%) concentrations of $AFB_1$ were greater than 5 μg/kg. Furthermore, concentrations of AFs were higher than ML in 16% of maize samples from year 2015, while OTA concentrations exceeded ML in 8% of samples. In 6 and 2 maize samples from 2015, detected concentrations of $AFB_1$ were above ML for maize intended for animal consumption, according to the EU and Serbian Regulations, respectively. Furthermore, detected concentrations of other investigated mycotoxins, in maize from 2015, were lower than MLs for maize which will be used in animal diet.

### 3.10. Comparative study

The change in climate, followed by trend of warming around the globe, is a widely acknowledged fact as well as significant impact of climate changes on fungal growth, distribution, and mycotoxins synthesis. All of the above has the potential to increase the risks of mycotoxins occurrence and consequently to affect food and feed safety. Therefore, with the aim to introduce a comparative insight into the occurrence of regulated mycotoxins and its derivatives in maize from other leading maize producing countries from Europe, results from available studies are investigated. To the best of author's knowledge, there are no many published scientific data from Europe related to the occurrence of regulated mycotoxins and its derivatives in maize collected from different years.

In the review paper, about mycotoxins risks under a climate change scenario in Europe, Moretti, Pascale, and Logrieco (2019) reported that in the last 15 years several hot and dry seasons led to severe *A. flavus* infections of maize in several countries in Europe, including Italy, Romania, Serbia and Spain. In the respect to the predicted +2 °C climate change scenario Moretti et al. (2019) observed that there is a clear increase in aflatoxin risk in areas such as Central and Southern Spain, the south of Italy, Greece, Northern and South-eastern Portugal, Bulgaria, Albania, Cyprus, and European Turkey. Furthermore, low and medium aflatoxin risk was predicted for the four main maize producing countries in Europe (Romania, France, Hungary, and Northeast Italy). Furthermore, the authors concluded that further impact of climate change on infection of crops with *Aspergillus* and *Fusarium* species could result in a dramatic increase of mycotoxins, with the highest food safety risks for human and animals due to high levels of AFs and DON.

Annual reports published by Biomin company represent one of the most comprehensive available worldwide data related on the mycotoxins occurrence (www.biomin.net). Every year Biomin company conducts research related to the occurrence of mycotoxins in maize as well as in other cereals and feed materials. For the period of four years, 2012–2015, which was investigated in our study, Bimoni's reports show that DON and FUMs were the most prevalent mycotoxins in maize from Europe, followed by ZEA. As in our research, the highest incidence of AFs and DON was registered in European maize in 2012 and 2014 years, respectively. Furthermore, according to the EFSA report

published in year 2014, the incidence of mycotoxins in maize, particularly DON, ZEA and FUMs was increased from 1.3 to 6 times in comparison to mean levels from the previous assessments. This statement was based on the official monitoring programs conducted in more than 21 European countries (without Serbia), over a period of 5 to 10 years, between 2000 and 2012 year. The greatest number of data in EFSA report was coming from France, which is one of the largest maize producers in Europe. Beside France, Romania is also in the top three maize producers in Europe. There are two studies related to the presence of certain regulated mycotoxins in maize from Romania (Gagiu et al., 2018; Tabuc, Taranu, & Calin, 2011). The both studies applied Enzyme Linked Immunosorbent Assay (ELISA) method. Furthermore, in the both studies authors investigated presence of regulated mycotoxins in different cereal samples, without separate data processing for maize. Tabuc et al. (2011) reported that, among 21 examined maize samples in the period 2008–2010, the most frequent fungal contaminants of maize belonged to the *Aspergillus* and *Fusarium* genera, and that 38% of maize samples were contaminated with AFB1 and 57% with FUMs. In the same period (2012–2015), as in our study, Gagiu et al. (2018) examined presence of AFs, OTA, ZEA and DON in cereals samples collected in Romania. Authors reported that among the examined cereals, maize was the most contaminated materials with mycotoxins. As the main reason, for such high contamination of maize, the authors highlighted extreme weather conditions. Romania is neighboring country to Serbia, and that therefore weather conditions in the both countries were quite similar in the investigated period. The greatest AFs concentration (82.9 μg/kg) was detected in Romanian maize samples harvested in year 2012, which was characterized with high temperatures and drought conditions, as in the same period in Serbia. Furthermore, DON and OTA occurrence were significantly correlated with the cumulative precipitation in all investigated years. Determined ZEA concentrations were significantly lower than ML. It should be point out that this study included smaller number of maize samples in comparison to our study. Furthermore, results from Northern Italy conducted in the period 2009–2011 indicate that that meteorological conditions represents major factor with the greatest influence on the presence of AFB1 and fumonisins (FB1 and FB2) in maize (Camardo Leggieri, Bertuzzi, Pietri, & Battilani, 2015). Among 140 examined maize samples, FUMs were detected with very high prevalence (from 97.8% to 100% in different years), while occurrence of AFB1 varied between years, from 58.7% to 95.6%. DON was occurred in the percentage range from 8.7% to 32.6%, while ZEA contaminated 4 among 140 examined maize samples. Kovalsky et al. (2016) reported an extensive study which includes analysis of regulated, masked and emerging mycotoxins in 1926 samples from 52 countries. This study included 161 maize samples from 26 European countries, without Serbia. The authors confirmed our findings and reported that very high concentrations and prevalence of DON and ZEA were observed in maize samples from 2014. They also concluded that there is a constant need for new available emerging research that go hand-in-hand with increasing frequency of extreme weather events and a changing climate.

## 4. Conclusion

Considering the findings of the present study it could be noticed that among 20 examined fungal metabolites, 20, 17, 13, and 17 were detected in maize samples collected from 2012, 2013, 2014 and 2015 years, respectively. Furthermore, the obtained results indicate that recorded differences in weather conditions in investigated period of four years had a great influence on the presence or absence of certain mycotoxins in maize. On the one hand, conditions of extreme and prolonged drought in 2012 resulted in high prevalence of AFs, while on the other hand extreme precipitation in 2014 influenced high prevalence of DON and ZEA. Despite of recorded differences in weather conditions in the observed period of four years, FUMs were detected with high prevalence in maize samples from each of the investigated years. Therefore, apparent increase in the occurrence of AFs and FUMs in maize and their co-occurrence with others *Fusarium* toxins may become a food safety problem in this part of Europe in the near future. High prevalence of co-occurrence of detected metabolites was also detected in maize samples, which means that in the observed period of four years maize from Serbia was concentrated source of mycotoxins mixture. Furthermore, significant number of maize samples contained regulated mycotoxins in concentrations which exceeded MLs defined by different Regulations. Due to the fact that in Serbia maize and maize derived products are consumed almost on a daily basis it could be assumed that in the recent years human population as well as livestock were exposed to high levels of certain regulated mycotoxins and its derivatives. Contamination of maize with mycotoxins must be consider with a greater concern in Serbia, due to the fact that contaminated maize could be potential risk for further contamination of the almost whole food and feed chain.

Moreover, global climate change predictions indicate that increasing trend of air temperature will be continued in the next years, with a particularly pronounced influence on crop production in Northern Serbia (Olesen et al., 2011). Based on the results obtained in this study, maize production in Serbia requires significant improvement in terms of control strategy, management practices as well as multidisciplinary integration and education of all participants in the food and feed chain for improved resilience against climate change impacts. For example, prolonged drought in 2012 influenced that maize yield as well as the amount of maize intended for export were approximately 50% and 70% lower than average values of these parameters in the recent years, respectively. Contrary to this, high amount of precipitation during maize growing season in 2014 resulted in record amount of maize yield in some parts of Serbia, and in that year Serbia exported a record quantity of 3 million tons of maize.

Production of high quality maize may therefore become a major future challenge for maize producers in Serbia. Therefore, there is a need for continuous monitoring of the impact of weather variability and climate changes on maize yield, quality and safety. From an economic point of view, systematic and constant investments and improvement of maize production is very important, in order to avoid losses caused by mycotoxins presence in maize. On the other hand, tendency for permanent monitoring and control of different mycotoxins in maize in order to protect human and animal population against the unallowable risk of mycotoxins contamination is required. Based on everything stated above, it could be assumed that production of high quality maize may become a big future challenge for maize producers in Serbia. Furthermore, since mycotoxins represent one of the most toxic chemical contaminants of food, the authors consider that results from this study contributing to the increase of knowledge in the field of food security.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Publication #4

# Mycotoxins in maize harvested in Serbia in the period 2012-2015. Part 2: Non-regulated mycotoxins and other fungal metabolites.

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# Mycotoxins in maize harvested in Serbia in the period 2012–2015. Part 2: Non-regulated mycotoxins and other fungal metabolites

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ABSTRACT

The main objective of this study was to screen, for the first time, the natural occurrence of non-regulated fungal metabolites in 204 maize samples harvested in Serbia in maize growing seasons with extreme drought (2012), extreme precipitation and flood (2014) and moderate drought conditions (2013 and 2015). In total, 109 non-regulated fungal metabolites were detected in examined samples, whereby each sample was contaminated between 13 and 55 non-regulated fungal metabolites. Moniliformin and beauvericin occurred in all samples collected from each year. In samples from year 2012, oxaline, questiomycin A, cyclo (L-Pro-L-Val), cyclo (L-Pro-L-Tyr), bikaverin, kojic acid and 3-nitropropionic acid were the most predominant (98.0–100%). All samples from 2014 were contaminated with 7-hydroxypestalotin, 15-hydroxyculmorin, culmorin, butenolid and aurofusarin. Bikaverin and oxaline were quantified in 100% samples from 2013 and 2015, while 3-nitropropionic acid additionally occurred in 100% samples from 2015.

## 1. Introduction

Maize (*Zea mays*) is one of the most important agricultural crops in Serbia concerning quantities produced, and also represents the most important agricultural product intended for export, as was indicated in detail by Kos, Janić Hajnal et al. (2020). In addition to the quantity produced, the food safety aspects must be considered in order to protect human and animal health. During cultivation maize is exposed to numerous biotic and abiotic stress factors (Benbrook, 2005). As a result of stress factors, mainly weather conditions, maize can be contaminated with a large number of different fungal secondary metabolites (Medina, Rodríguez, & Magan, 2015; Van der Fels-Klerx, van Asselt, Madsen, & Olesen, 2013). Although Serbia is classified as moderate-continental with more or less pronounced local characteristics (Smailagić, Savovći, Marković, & Nešić, 2013), it faced extreme weather conditions throughout the last years; from extreme drought in 2012 maize growing season to excessive precipitation and flooding in 2014. Such variations in weather conditions during maize cultivation had significant influence on the mycotoxins' profile of the produced maize (Janić Hajnal et al., 2017; Kos et al., 2017). A multi-mycotoxin method based on high performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) is an effective tool for providing information about a wide range of non-regulated fungal metabolites (Streit et al., 2013; Abia et al., 2013; Shephard et al., 2013; Malachová, Sulyok, Beltrán, Berthiller, & Krska, 2014; Oliveira, Rocha, Sulyok, Krska, & Mallmann, 2017; Abdallah, Girgin, Baydar, Krska, & Sulyok, 2017; Getachew et al., 2018). Currently, knowledge about mycotoxin-profiles of maize from Serbia is limited just to a few regulated mycotoxins (Janić Hajnal et al., 2017; Kos et al., 2014, 2017; Kos, Mastilović, Hajnal, & Šarić, 2013; Torović, 2018a, 2018b), despite the development of methods for multi-mycotoxins determination that allows to detect the presence of non-regulated mycotoxins and other fungal metabolites. On the other hand, so far only limited information of some of the non-regulated fungal metabolites are available, especially on their toxic effects on human and animal health, which are summarized mainly in EFSA's scientific reports (EFSA (European Food Safety Authority), 2017; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2012a, 2014a, 2014b, 2018; EFSA Panel on contaminates in the Food Chain (CONTAM), 2011a; EFSA Panel on contaminants in the Food Chain (CONTAM), 2011b). Limited available information on toxicity and on toxicokinetics of moniliformin indicated haematotoxicity and cardiotoxicity as major adverse health effects in experimental and farm animals, while no

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relevant human epidemiological data on moniliformin were identified (EFSA, 2018). HT-2 and T-2 toxins have been assessed as immunotoxic, genotoxic and neurotoxic, occurring mainly in oats (EFSA Panel on contaminates in the Food Chain CONTAM, 2011a), while citrinin exhibits a nephrotoxic effect (EFSA Panel on Contaminants in the Food Chain CONTAM, 2012a). According EFSA scientific report (EFSA Panel on contaminats in the Food Chain CONTAM, 2011b) ergot alkaloids show vasoconstrictive effects. Further, the CONTAM Panel concluded that acute exposure to beauvericin and enniatins do not indicate concern for human health (EFSA Panel on Contaminants in the Food Chain CONTAM, 2014a), but, again, the available occurrence data were not adequate to carry out a dietary exposure assessment, neither for humans nor for animals. Regarding *Alternaria* toxins, some of them have been described to possess genotoxic and mutagenic properties; they show cytotoxic, fetotoxic and/or teratogenic activity; they are mutagenic, clastogenic and oestrogenic in microbial and mammalian cell systems and they inhibit the cell proliferation (EFSA Panel on contaminats in the Food Chain CONTAM, 2011b). As in the case of other groups of new non-traditional mycotoxins, relevant occurrence and exposure data, as well as, their negative impact on human and animal health still does not exist. Last but not least, attention of EFSA Panel on Contaminants in the Food Chain CONTAM (2014b) is paid also for mycotoxins conjugates, so called 'masked' mycotoxins, as metabolites of mycotoxins being produced through the defence mechanism of plants (glycosylated deoxynivalenol, zearalenone, T-2, or HT-2 toxins). They are of a great relevance because of their possible hydrolyses yielding the parent toxins during digestion (EFSA Panel on Contaminants in the Food Chain CONTAM, 2014b). Finally, co-occurred fungal metabolites can exhibit a synergistic and/or additive harmful effect on human and animal health.

Therefore, the present study aimed to screen for the first time, the presence and level of *Aspergillus, Fusarium, Penicillium, Alternaria* and other non-regulated fungal metabolites which existing in Serbian maize grains collected from four different maize growing seasons (2012–2015).

## 2. Materials and methods

### 2.1. Samples collection and weather conditions analysis

In the period of four years (2012–2015) a total of two hundred and four (n = 204) maize samples were collected. Every year after harvest 51 maize samples were collected from Northern Serbia (Autonomous Province of Vojvodina). The details related to the procedures of the collection of maize samples, preparation of representative laboratory samples, transport of the samples to the laboratory in Austria (Department for Agrobiotechnology, IFA-Tulln, Austria), as well as the weather conditions parameters for the four maize growing seasons (2012–2015) are described by Kos et al. (2020) (see Fig. 1 and Table 3 in the Part 1). In addition, for the better interpretation of the obtained results in this study, it is necessary to emphasize that maize growing season in year 2012 was characterized by extreme drought, 2014 by record amount of precipitation and flood, while hot and dry conditions were dominant during maize growing seasons in 2013 and 2015.

### 2.2. Sample preparation and LC-MS/MS analysis

Sample preparations, instrumental parameters and LC-MS/MS analysis of maize samples were conducted in accordance to method published by Malachová et al. (2014), without any modifications, as well as with the same equipment, chemicals and reagents. Furthermore, sample preparation as well as LC-MS/MS parameters of applied method are in detail described in PART 1 (Kos et al. 2020). Limits of detection (LOD) was determined following the EURACHEM guide (Magnusson and Örnemark, 2014). The method validation procedures and the method validation data for determination of non-regulated mycotoxins and

**Table 1**
Method performance characteristics for non-regulated mycotoxins and other fungal metabolites.

| Analyte | LOD (μg/kg) | Apparent Recovery ± RSD (%) |
|---|---|---|
| Nivalenol | 1.2 | 71.9 ± 4.7 |
| T-2 toxin | 0.8 | 96.8 ± 5.1 |
| HT-2 toxin | 3.2 | 81.8 ± 10.4 |
| HT-2-glucoside | 1.6 | 89.0 ± 10.5 |
| Monoacetoxyscirpenol | 1.6 | 83.3 ± 10.5 |
| Diacetoxyscirpenol | 0.4 | 76.0 ± 13.7 |
| Neosolaniol | 1.6 | 55.7 ± 17.1 |
| Culmorin | 1.6 | 102.1 ± 6.6 |
| 5-Hydroxyculmorin | 20 | n.d. |
| 15-Hydroxyculmorin | 4 | 100.1 ± 8.4 |
| Moniliformin | 1.6 | 81.0 ± 15.1 |
| Beauvericin | 0.008 | n.d. |
| Enniatin A1 | 0.03 | 91.3 ± 5.6 |
| Enniatin B | 0.024 | 91.2 ± 5.6 |
| Enniatin B1 | 0.04 | 79.8 ± 9.2 |
| Fusaproliferin | 40 | n.d. |
| Aurofusarin | 2.4 | 61.0 ± 12.1 |
| Bikaverin | 8 | 71.3 ± 12.3 |
| Butenolid | 5.6 | 79.9 ± 7.6 |
| Apicidin | 0.12 | 126.1 ± 8.3 |
| Chrysogin | 0.4 | 82.7 ± 4.0 |
| Fusapyron | 0.8 | 89.5 ± 4.5 |
| Fusarin C | 4.8 | 82.8 ± 19.3 |
| Aflatoxicol | 1 | 79.3 ± 9.4 |
| Sterigmatocystin | 0.08 | 104.1 ± 7.0 |
| seco-Sterigmatocystin | 0.24 | 168.8 ± 8.7 |
| O-Methylsterigmatocystin | 0.06 | 72.9 ± 19.6 |
| Methoxysterigmatocystin | 0.04 | 91.1 ± 5.1 |
| Versicolorin A | 0.04 | 84.4 ± 9.3 |
| Versicolorin C* | 0.04 | – |
| Nidurufin | 0.1 | 81.5 ± 9.0 |
| Averantin | 0.04 | 84.0 ± 5.3 |
| Averufin | 0.064 | 82.9 ± 8.3 |
| Norsolorinic acid | 0.8 | 63.8 ± 6.8 |
| Kojic acid | 16 | 91.3 ± 7.2 |
| 3-Nitropropionic acid | 0.8 | 75.5 ± 14.1 |
| Cyclopiazonic acid | 12 | 150 |
| Malformin A | 0.8 | 129.9 ± 7.5 |
| Malformin A2* | 0.8 | – |
| Malformin C | 0.16 | 108.3 ± 8.00 |
| Pseurotin A | 8 | 216.9 ± 96.8 |
| Citrinin | 0.16 | 27.7 ± 13.7 |
| Dihydrocitrinone | 2 | 147.3 ± 36.6 |
| Mycophenolic acid | 0.4 | 95.7 ± 6.5 |
| Mycophenolic acid IV | 0.8 | 79.7 ± 15.6 |
| Citreoviridin | 4 | 86.7 ± 8.9 |
| Roquefortine C | 2 | 63.7 ± 10.6 |
| Meleagrin | 6 | 154.9 ± 13.4 |
| Questiomycin A | 1.6 | 85.1 ± 10.0 |
| Oxaline | 0.08 | 91.4 ± 6.3 |
| Pestalotin | 0.4 | 88.1 ± 6.1 |
| 7-Hydroxypestalotin | 0.4 | 102.1 ± 7.8 |
| Secalonic acid D | 8 | 95.3 ± 11.0 |
| Flavoglaucin | 0.25 | 87.2 ± 58.8 |
| Quinolactacin A | 0.01 | 86.7 ± 6.1 |
| Aurantiamin A | 0.65 | 66.9 ± 3.9 |
| Aurantine | 0.3 | 77.9 ± 6.8 |
| Andrastin A | 0.16 | 48.7 ± 38.6 |
| Andrastin B | 0.4 | 72.5 ± 51.0 |
| Cycloaspeptide A | 0.1 | 93.9 ± 5.2 |
| Cyclopenin | 0.16 | 88.0 ± 5.3 |
| Cyclopenol | 1.6 | 92.6 ± 11.6 |
| Demethylsulochrin | 0.6 | 264.5 ± 42.3 |
| Desoxyverrucosidin* | 2 | – |
| Norverrucosidin | 1.5 | 118.3 ± 6.9 |
| O-Methylviridicatin | 0.16 | 90.6 ± 12.6 |
| Pinselin | 0.7 | 81.1 ± 8.8 |
| Purpuride | 0.24 | 83.7 ± 15.9 |
| Verrucofortine | 0.024 | 94.0 ± 5.1 |
| Viridicatol | 2.4 | 104.2 ± 5.2 |
| Griseofulvin | 1.2 | 80.9 ± 9.1 |
| Griseophenone B | 2 | 102.0 ± 18.2 |
| Griseophenone C | 0.24 | 104.4 ± 4.8 |

(*continued on next page*)

**Table 1** (*continued*)

| Analyte | LOD (μg/kg) | Apparent Recovery ± RSD (%) |
|---|---|---|
| Anacin | 6.4 | 98.3 ± 1.2 |
| Chanoclavin | 0.08 | 73.3 ± 22.6 |
| Citreohybridinol | 0.25 | 78.4 ± 10.4 |
| Tenuazonic acid | 8 | 82.7 ± 50.9 |
| Alternariol | 0.4 | 45.9 ± 20.6 |
| Alternariolmethylether | 0.032 | 106.7 ± 11.8 |
| Tentoxin | 0.08 | 120.9 ± 4.0 |
| Infectopyron | 8 | 82.0 ± 13.2 |
| Ascochlorin | 0.1 | 97.6 ± 7.0 |
| Monocerin | 0.4 | 92.0 ± 6.4 |
| Cladosporin | 1.6 | 79.4 ± 10.9 |
| Asperglaucide | 0.08 | 91.2 ± 5.7 |
| Asperphenamate | 0.04 | 131.8 ± 148 |
| Brevianamid F | 0.16 | 79.7 ± 9.0 |
| Citreorosein | 0.64 | 99.6 ± 10.5 |
| cyclo(L-Pro-L-Tyr) | 0.8 | 90.6 ± 5.6 |
| cyclo(L-Pro-L-Val) | 0.64 | 104.2 ± 10.9 |
| Emodin | 0.06 | 90.1 ± 5.3 |
| Endocrocin | 5 | 57.6 ± 5.7 |
| Fallacinol | 0.2 | 94.8 ± 14.5 |
| IsoRhodoptilometrin | 0.07 | 114.4 ± 8.1 |
| *N*-Benzoyl-Phenylalanine | 0.8 | 80.4 ± 7.8 |
| Neoechinulin A | 0.64 | 56.3 ± 15.1 |
| Rugulusovin | 0.24 | 79.3 ± 7.1 |
| Skyrin | 0.4 | 61.9 ± 6.6 |
| Tryptophol | 8 | 76.0 ± 9.8 |

LOD: limit of detection (μg/kg); RSD: relative standard deviation;
n.d.: not determined due to lack of blank samples/sufficiently large concentration in the spiking solution; an apparent recovery of 100% was assumed.
* semi-quantified using response factor of a structurally related analyte.

other fungal metabolites for the method used in this study are described in detail for maize as a matrix in previously published work by Malachová et al. (2014). Quantification was performed using an external calibration based on serial dilutions of a multi-analyte stock solution. As no quantitative standard was available for andrastin C, andrastin D, verrucosidin, siccanol, asparason A, nigragillin, aurasperon B, aurasperon C, aurasperon G and aspulvinone E, the presence of these fungal metabolites in the Section 3 were marked as detected and they are not shown in the Tables 1–4. Further, since standards were not available for versicolorin C, malformin A2 and desoxyverrucosidin, semi-quantification was performed using the response factor of structurally related compounds. The performance characteristics (LOD and Apparent Recovery) of the applied analytical method for non-regulated mycotoxins and other fungal metabolites are presented in Table 1. Quantified concentrations of fungal metabolites were corrected for the apparent recovery.

### 2.3. Statistical analysis

Descriptive statistics for data related to the mycotoxins concentrations were expressed as the mean ± standard deviation (Std) using STATISTICA software version 13.2 (StatSoft, Inc., 2016, USA). The significant differences between samples were calculated according to post-hoc Tukey's HSD (honestly significant differences) test, for unequal sample sizes, at a $P < 0.05$ significance level, 95% confidence interval.

## 3. Results and discussion

### 3.1. The overall average incidence of non-regulated fungal metabolites

A total of 129 fungal secondary metabolites (regulated mycotoxins and its derivatives, non-regulated mycotoxins and other fungal metabolites) were detected and/or quantified in 204 examined maize samples from Serbia collected in the investigated period 2012–2015. The occurrence of regulated mycotoxins and its derivatives in the same maize samples was discussed in detail by Kos et al. (2020).

Tables 2, 3 and 4 shows that the frequency and the level of each detected and/or quantified non-regulated mycotoxin varied by production years. In general, among of total of 109 detected and/or quantified non-regulated mycotoxins in the maize samples from investigated period 2012–2015, 38 belongs to *Penicillium* metabolites, 24 to *Fusarium* and 23 to *Aspergillus*, followed by 16 unspecific metabolites, 5 metabolites of *Alternaria* and 3 of the other fungal metabolites.

Among 204 maize samples the overall average contamination of detected and quantified *Penicillium* metabolites (Table 2) was as follows: questiomycin A (96%), oxaline (95%), 7-hydroxypestalotin (93%), pestalotin (85%), flavoglaucin (69%), quinolactacin A (53%), secalonic acid D (41%), meleagrin (38%), verrucosidin (33%), mycophenolic acid (32%), roquefortine C (28%), aurantine (22%) and citreohybridinol (17%).

The overall average incidence of *Fusarium* metabolites (Table 3) in the investigated four-year period was as follows: 100% of moniliformin and beauvericin (each), 99% of bikaverin and siccanol (each), 78% of aurofusarin, 77% of fusaproliferin, 68% of fusapyron, 60% of culmorin, 59% of 15-hydroxyculmorin, and 45% of butenolid. Furthermore, < 40% of the maize samples were contaminated with the following *Fusarium* metabolites: 5-hydroxyculmorin (31%), enniatin B (29%), T-2 toxin (24%), chrysogin (21%), enniatin B1 (12%) and nivalenol (12%).

Regarding *Aspergillus* metabolites (Table 3) the overall average incidence in tested maize samples was as follows: 75% of maize samples contained nigragillin, 73% 3-nitropropionic acid, 72% kojic acid, 55% averufin, 43% versicolorin C, 37% aurasperon C, 34% malformin A and aurasperon G (each), 30% versicolorin A, 23% malformin C, 22% norsoloric acid, O-methylsterigmatocystin and nidurufin (each), 18% averantin, 17% cyclopiazonic acid and aurasperon B (each), and 16% aspulvinone E.

In terms of contamination of maize samples with *Alternaria* toxins (Table 4), in investigated period of four years, it can be noted, that 36%, 12%, 40%, 5% and 2% of maize samples were contaminated with tenuazonic acid, alternariol, alternariol methylether, tentoxin and infectopyron, respectively. Cladosporin, ascochlorin, and monocerin (metabolites from other fungal genera) were quantified in 18%, 9%, and 2% of maize samples, respectively.

In the investigated period of four years (2012–2015) examined maize samples were also contaminated with the following unspecific metabolites: cyclo (L-Pro-L-Val) (89%), asperglaucide (78%), cyclo (L-Pro-L-Tyr) (76%), asperphenamate (71%), citreorosein (51%), neoechinulin A (51%), isorhodoptilometrin (49%), emodin (43%), *N*-benzoyl-Phenylalanine (39%), tryptophol (22%), fallacinol (17%) and brevianamid F (10%).

Generally, in maize samples from the 2012 maize growing season, occurrence of 24 *Penicillium* metabolites, 23 *Fusarium* metabolites, 20 *Aspergillus* metabolites, 15 unspecific metabolites, 5 *Alternaria* metabolites and 2 of other fungal metabolites was shown. Furthermore, in maize samples originated from 2013 maize growing season, 33 *Penicillium* metabolites, 19 *Fusarium* metabolites, 17 *Aspergillus* metabolites, 12 unspecific metabolites, 4 *Alternaria* metabolites and 2 of other fungal metabolites were detected. Regarding occurrence of quantified fungal metabolite in maize samples originated from 2014 maize growing season, prevalence of 23 *Fusarium* metabolites, 19 *Penicillium* metabolites, 12 unspecific metabolites, 6 *Aspergillus* metabolites, 4 *Alternaria* metabolites and 3 of other fungal metabolites was noted. In maize samples, from the last examined year (2015), occurred 30 *Penicillium* metabolites, 20 *Aspergillus* metabolites, 18 *Fusarium* metabolites, 15 unspecific metabolites, 4 *Alternaria* metabolites, and 3 of other fungal metabolites.

The predominate metabolites in maize samples from 2012 production year belonging to the genus *Penicillium, Fusarium*, and *Aspergillus*. As can be seen, under the conditions of extreme drought (2012) and moderate drought (2013 and 2015) recorded during maize cultivations,

**Table 2**
Occurrence of non-regulated *Penicillium* metabolites in maize samples collected in Republic of Serbia in the period 2012–2015.

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| *Penicillium* | Citrinin | 2012 | 4 | 10–48 | 11[a] ± 3 | 11 |
| | | 2013 | 8 | 5–547 | 175[a] ± 2 | 73 |
| | | 2014 | 4 | 2–6 | 4[a] ± 3 | 4 |
| | | 2015 | 23 | 7–10058 | 950[b] ± 2872 | 61 |
| | Dihydrocitrinone | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 8 | 2–68 | 18 ± 33 | 2 |
| | Mycophenolic acid | 2012 | 6 | 0.8–16 | 6[ab] ± 9 | 2 |
| | | 2013 | 43 | 0.5–368 | 54[b] ± 91 | 11 |
| | | 2014 | 23 | 0.7–92 | 27[ab] ± 30 | 16 |
| | | 2015 | 55 | 0.5–33 | 7[a] ± 8 | 4 |
| | Mycophenolic acid IV | 2012 | nd | nd | nd | nd |
| | | 2013 | 6 | 2–3 | 2 ± 0.6 | 2 |
| | | 2014 | 2 | 4 | | |
| | | 2015 | 8 | 3–12 | 7 ± 5 | 5 |
| | Citreoviridin | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 2 | 19 | | |
| | Roquefortine C | 2012 | 80 | 38–197 | 97[a] ± 47 | 82 |
| | | 2013 | 27 | 12–950 | 137[a] ± 251 | 48 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 4 | 29–46 | 38[a] ± 12 | 38 |
| | Meleagrin | 2012 | 96 | 7–41 | 19[a] ± 9 | 16 |
| | | 2013 | 39 | 4–151 | 22[a] ± 32 | 12 |
| | | 2014 | 4 | 5–7 | 6[a] ± 1 | 6 |
| | | 2015 | 10 | 4–17 | 9[a] ± 6 | 5 |
| | Questiomycin A | 2012 | 100 | 9–87 | 33[ab] ± 20 | 32 |
| | | 2013 | 94 | 3–119 | 18[a] ± 20 | 12 |
| | | 2014 | 96 | 3–526 | 42[b] ± 76 | 25 |
| | | 2015 | 94 | 9–69 | 33[ab] ± 15 | 28 |
| | Oxaline | 2012 | 100 | 2–239 | 93[b] ± 54 | 82 |
| | | 2013 | 100 | 0.6–88 | 12[a] ± 17 | 7 |
| | | 2014 | 84 | 0.1–5 | 0.7[a] ± 1 | 0.2 |
| | | 2015 | 100 | 0.1–42 | 6[a] ± 9 | 3 |
| | Pestalotin | 2012 | 80 | 0.5–21 | 4[ab] ± 4 | 3 |
| | | 2013 | 86 | 0.6–9 | 3[a] ± 2 | 2 |
| | | 2014 | 96 | 1–17 | 6[c] ± 3 | 5 |
| | | 2015 | 74 | 1–18 | 5[bc] ± 3 | 4 |
| | 7-Hydroxypestalotin | 2012 | 94 | 0.6–12 | 3[a] ± 3 | 3 |
| | | 2013 | 84 | 0.8–16 | 3[a] ± 3 | 2 |
| | | 2014 | 100 | 1–24 | 6[b] ± 5 | 5 |
| | | 2015 | 96 | 0.5–8 | 3[a] ± 2 | 3 |
| | Secalonic acid D | 2012 | 96 | 32–547 | 176[b] ± 121 | 126 |
| | | 2013 | 47 | 16–390 | 81[a] ± 90 | 44 |
| | | 2014 | 6 | 16–72 | 48[a] ± 29 | 57 |
| | | 2015 | 14 | 23–99 | 39[a] ± 33 | 27 |
| | Flavoglaucin | 2012 | 65 | 0.3–1559 | 63[ab] ± 271 | 6 |
| | | 2013 | 72 | 0.4–113 | 22.45[a] ± 27 | 8 |
| | | 2014 | 57 | 0.6–209 | 33[a] ± 52 | 17 |
| | | 2015 | 80 | 0.4–680 | 145[b] ± 135 | 87 |
| | Quinolactacin A | 2012 | 25 | 0.1–3 | 0.4[ab] ± 0.9 | 0.1 |
| | | 2013 | 86 | 0.02–1 | 0.1[a] ± 0.2 | 0.1 |
| | | 2014 | 8 | 0.02–0.05 | 0.03[ab] ± 0.01 | 0.1 |
| | | 2015 | 92 | 0.1–11 | 0.7[b] ± 2 | 0.2 |
| | Aurantiamin A | 2012 | nd | nd | nd | nd |
| | | 2013 | 14 | 2–61 | 27[a] ± 22 | 20 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 8 | 8–87 | 32[a] ± 37 | 16 |
| | Aurantine | 2012 | 6 | 2–4 | 3[a] ± 1 | 2 |
| | | 2013 | 45 | 0.7–39 | 7[a] ± 11 | 2 |
| | | 2014 | 6 | 3–7 | 5[a] ± 2 | 3 |
| | | 2015 | 29 | 0.4–25 | 5[a] ± 8 | 1 |
| | Andrastin A | 2012 | nd | nd | nd | nd |
| | | 2013 | 2 | 3 | | |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 18 | 2–294 | 49 ± 97 | 6 |
| | Andrastin B | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 8 | 2–28 | 11 ± 12 | 7 |
| | Cycloaspeptide A | 2012 | nd | nd | nd | nd |
| | | 2013 | 2 | 0.6 | | |
| | | 2014 | nd | nd | nd | nd |

(*continued on next page*)

**Table 2** (*continued*)

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| | | 2015 | nd | nd | nd | nd |
| | Cyclopenin | 2012 | 6 | 0.2–1 | 0.9[a] ± 0.6 | 1 |
| | | 2013 | 6 | 1.-4 | 2[a] ± 2 | 2 |
| | | 2014 | 2 | 0.4 | | |
| | | 2015 | 2 | 2 | | |
| | Cyclopenol | 2012 | 4 | 19–39 | 29[a] ± 14 | 29 |
| | | 2013 | 6 | 4–56 | 26[a] ± 27 | 18 |
| | | 2014 | 2 | 4 | | |
| | | 2015 | 6 | 4–15 | 9[a] ± 6 | 7 |
| | Demethylsulochrin | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 8 | 0.6–1 | 0.8 ± 0.2 | 0.8 |
| | Desoxyverrucosidin* | 2012 | 2 | 3 | | |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 2 | 2 | | |
| | | 2015 | nd | nd | nd | nd |
| | Norverrucosidin | 2012 | 6 | 3–2 | 5[a] ± 5 | 4 |
| | | 2013 | 10 | 3–7 | 5[a] ± 2 | 4 |
| | | 2014 | 2 | 1 | | |
| | | 2015 | 8 | 2–20 | 8[a] ± 8 | 4 |
| | O-Methylviridicatin | 2012 | 2 | 2 | | |
| | | 2013 | 6 | 0.7–4 | 2 ± 2 | 1 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 6 | 0.2–1 | 0.6 ± 0.7 | 0.2 |
| | Pinselin | 2012 | nd | nd | nd | nd |
| | | 2013 | 2 | 15 | | |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 2 | 7 | | |
| | Purpuride | 2012 | nd | nd | nd | nd |
| | | 2013 | 4 | 1–15 | 8 ± 10 | 8 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 7.8 | 0.4–1 | 0.9 ± 0.6 | 0.9 |
| | Verrucofortine | 2012 | nd | nd | nd | nd |
| | | 2013 | 16 | 0.03–0.5 | 0.1 ± 0.1 | 0.1 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Viridicatol | 2012 | 4 | 21–31 | 26 ± 7 | 26 |
| | | 2013 | 6 | 13–26 | 20 ± 6 | 21 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Griseofulvin | 2012 | 2 | 7 | | |
| | | 2013 | 4 | 18–40 | 29 ± 15 | 2 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Griseophenone B | 2012 | 2 | 13 | | |
| | | 2013 | 4 | 45–70 | 58 ± 18 | 58 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Griseophenone C | 2012 | 2 | 0.4 | 0.4 | |
| | | 2013 | 6 | 0.3–5 | 3 ± 2 | 3 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Anacin | 2012 | nd | nd | nd | nd |
| | | 2013 | 6 | 11–14 | 13 ± 2 | 14 |
| | | 2014 | 2 | 6 | | |
| | | 2015 | 2 | 11 | | |
| | Chanoclavin | 2012 | nd | nd | nd | nd |
| | | 2013 | 8 | 0.1–0.3 | 0.2 ± 0.1 | 0.2 |
| | | 2014 | 2 | 0.4 | | |
| | | 2015 | nd | nd | nd | nd |
| | Citreohybridinol | 2012 | 2 | 0.7 | | 0.7 |
| | | 2013 | 22 | 0.5–12.3 | 3[a] ± 4 | 0.9 |
| | | 2014 | ND | | | |
| | | 2015 | 43 | 0.6–38 | 8[a] ± 11 | 3 |

N (%): number (percentage) of contaminated sample.
nd: not detected i.e., less than limit of detection (LOD).
Min-Max: minimum and maximum concentrations (μg/kg).
Mean ± SD: mean concentration (μg/kg) ± standard deviation (μg/kg).
Median: median concentration (μg/kg).
Different letters in the same column indicate significant differences ($P < 0.05$) between values according to the post-hoc Tukey's HSD (honestly significant differences) test, for unequal sample sizes.
* Semi-quantified using response factor of a structurally related analyte.

**Table 3**
Occurrence of non-regulated *Fusarium* and *Aspergillus* metabolites in maize samples collected in Republic of Serbia in the period 2012–2015.

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| *Fusarium* | Nivalenol | 2012 | 6 | 19–41 | 27[a] ± 12 | 22 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 37 | 13–85 | 29[a] ± 20 | 21 |
| | | 2015 | 4 | 27–37 | 32[a] ± 7 | 32 |
| | T-2 toxin | 2012 | 18 | 1–99 | 14[a] ± 22 | 8 |
| | | 2013 | 37 | 1–32 | 8[a] ± 9 | 7 |
| | | 2014 | 35 | 1–59 | 10[a] ± 13 | 6 |
| | | 2015 | 6 | 1–2 | 2[a] ± 0.7 | 1 |
| | HT − 2 toxin | 2012 | 12 | 5–43 | 23[a] ± 16 | 24 |
| | | 2013 | 12 | 11–51 | 26[a] ± 17 | 17 |
| | | 2014 | 16 | 14–178 | 62[a] ± 59 | 36 |
| | | 2015 | nd | nd | nd | nd |
| | HT-2 glucoside | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 4 | 12–15 | 14 ± 2 | 14 |
| | | 2015 | nd | nd | nd | nd |
| | Monoacetoxyscirpenol | 2012 | 8 | 9–37 | 21[a] ± 13 | 19 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 14 | 5–17 | 10[a] ± 4 | 9 |
| | | 2015 | nd | nd | nd | nd |
| | Diacetoxyscirpenol | 2012 | 18 | 0.6–12 | 7[ab] ± 5 | 7 |
| | | 2013 | 4 | 1–2 | 2[a] ± 0.6 | 2 |
| | | 2014 | 10 | 7–20 | 12[b] ± 5 | 13 |
| | | 2015 | nd | nd | nd | nd |
| | Neosolaniol | 2012 | 2 | 5 | | |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Culmorin | 2012 | 45 | 8–160 | 55[a] ± 61 | 32 |
| | | 2013 | 27 | 15–138 | 69[a] ± 40 | 74 |
| | | 2014 | 100 | 144–3995 | 1287[b] ± 795 | 1109 |
| | | 2015 | 65 | 8–221 | 50[a] ± 46 | 43 |
| | 5-Hydroxyculmorin | 2012 | 14 | 34–155 | 118[a] ± 69 | 146 |
| | | 2013 | 4 | 125–253 | 189[ab] ± 90 | 189 |
| | | 2014 | 96 | 159–11040 | 1728[b] ± 1592 | 1664 |
| | | 2015 | 9 | 47–174 | 403[ab] ± 53 | 65 |
| | 15-Hydroxyculmorin | 2012 | 45 | 4–156 | 38[a] ± 41 | 18 |
| | | 2013 | 33 | 9–164 | 51[a] ± 44 | 38 |
| | | 2014 | 100 | 67–7974 | 1435[b] ± 1271 | 1156 |
| | | 2015 | 55 | 4–288 | 40[a] ± 55 | 24 |
| | Moniliformin | 2012 | 100 | 10–4113 | 1261[c] ± 707 | 1144 |
| | | 2013 | 100 | 22–8710 | 792[b] ± 1314 | 364 |
| | | 2014 | 100 | 8–2062 | 277[a] ± 337 | 144 |
| | | 2015 | 100 | 50–1624 | 380[a] ± 303 | 310 |
| | Beauvericin | 2012 | 100 | 0.3–89 | 21[a] ± 20 | 13 |
| | | 2013 | 100 | 0.4–571 | 40[b] ± 92 | 13 |
| | | 2014 | 100 | 0.3–94 | 11[a] ± 18 | 3 |
| | | 2015 | 100 | 0.3–94 | 7[a] ± 7 | 4 |
| | Enniatin A1 | 2012 | 2 | 0.2 | | |
| | | 2013 | 12 | 0.03–0.4 | 0.2[a] ± 0.1 | 0.1 |
| | | 2014 | 6 | 0.1–0.2 | 0.1[a] ± 0.03 | 0.1 |
| | | 2015 | 2 | 0.2 | | |
| | Enniatin B | 2012 | 51 | 0.03–0.4 | 0.1[a] ± 0.09 | 0.1 |
| | | 2013 | 37 | 0.03–4 | 0.4[a] ± 1 | 0.1 |
| | | 2014 | 18 | 0.03–0.5 | 0.1[a] ± 0.2 | 0.1 |
| | | 2015 | 10 | 0.06–0.5 | 0.2[a] ± 0.1 | 0.2 |
| | Enniatin B1 | 2012 | 20 | 0.3–1. | 0.4[a] ± 0.2 | 0.4 |
| | | 2013 | 16 | 0.2–1 | 0.5[a] ± 0.4 | 0.3 |
| | | 2014 | 8 | 0.2–0.5 | 0.4[a] ± 0.2 | 0.3 |
| | | 2015 | 6 | 0.3–0.6 | 0.5[a] ± 0.2 | 0.4 |
| | Fusaproliferin | 2012 | 94 | 99–16176 | 776[a] ± 2284 | 386 |
| | | 2013 | 63 | 64–2882 | 549[a] ± 659 | 330 |
| | | 2014 | 78 | 76–3416 | 791[a] ± 716 | 531 |
| | | 2015 | 71 | 42–956 | 373[a] ± 243 | 290 |
| | Aurofusarin | 2012 | 78 | 5–397 | 58[a] ± 77 | 28 |
| | | 2013 | 53 | 6–587 | 169[a] ± 165 | 121 |
| | | 2014 | 100 | 1645–59210 | 10671[b] ± 10350 | 8245 |
| | | 2015 | 80 | 4–462 | 106[a] ± 104 | 67 |
| | Bikaverin | 2012 | 98 | 49–325 | 158[b] ± 59 | 161 |
| | | 2013 | 100 | 17–700 | 131[b] ± 109 | 110 |
| | | 2014 | 98 | 53–364 | 155[b] ± 69 | 145 |
| | | 2015 | 100 | 23–160 | 80[a] ± 34 | 82 |
| | Butenolid | 2012 | 10 | 30–119 | 81[a] ± 35 | 82 |
| | | 2013 | 22 | 29–988 | 154[a] ± 279 | 59 |

(*continued on next page*)

**Table 3** (*continued*)

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| | | 2014 | 100 | 140–3259 | 1018[b] ± 615 | 953 |
| | | 2015 | 45 | 20–611 | 98[a] ± 121 | 68 |
| | Apicidin | 2012 | 4 | 1–1 | 1[a] ± 0.04 | 1 |
| | | 2013 | 4 | 1–1 | 1[a] ± 0.1 | 1 |
| | | 2014 | 8 | 1–7 | 4[a] ± 2 | 4 |
| | | 2015 | 4 | 1–2 | 2[a] ± 1 | 2 |
| | Chrysogin | 2012 | 10 | 4–15 | 7[a] ± 5 | 4 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 71 | 2–18 | 5[a] ± 3 | 5 |
| | | 2015 | 2 | 2 | | 2 |
| | Fusapyron | 2012 | 63 | 1–52 | 12[a] ± 13 | 8 |
| | | 2013 | 51 | 1–436 | 32[b] ± 86 | 9 |
| | | 2014 | 90 | 2–166 | 30[ab] ± 40 | 13 |
| | | 2015 | 67 | 1–55 | 9[a] ± 9 | 6 |
| | Fusarin C | 2012 | 18 | 70–201 | 128[a] ± 43 | 109 |
| | | 2013 | 2 | 454 | | 375 |
| | | 2014 | 10 | 239–1727 | 642[b] ± 630 | 337 |
| | | 2015 | nd | nd | nd | nd |
| *Aspergillus* | Aflatoxicol | 2012 | 18 | 1–5 | 2 ± 1 | 2 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Sterigmatocystin | 2012 | 12 | 0.2–0.3 | 0.2[a] ± 0.1 | 0.2 |
| | | 2013 | 6 | 0.1–31 | 12[a] ± 18 | 0.7 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 4 | 6–6 | 6[a] ± 0.1 | 6 |
| | seco-Sterigmatocystin | 2012 | nd | nd | nd | nd |
| | | 2013 | 4 | 0.4–2 | 1 ± 1 | 1 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 4 | 0.5–1 | 0.7 ± 0.3 | 0.7 |
| | O-Methylsterigmatocystin | 2012 | 61 | 0.3–2 | 0.8[a] ± 0.4 | 0.7 |
| | | 2013 | 14 | 0.3–16 | 3[a] ± 6 | 0.4 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 12 | 0.4–9 | 3[a] ± 4 | 1 |
| | Methoxysterigmatocystin | 2012 | nd | nd | nd | nd |
| | | 2013 | 8 | 0.5–22 | 6 ± 10 | 2 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 4 | 5–13 | 9 ± 5 | 9 |
| | Versicolorin A | 2012 | 84 | 0.2–3 | 1[a] ± 0.7 | 0.7 |
| | | 2013 | 14 | 0.2–3 | 1[a] ± 2 | 0.6 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 23 | 0.3–0.8 | 2[a] ± 0.2 | 0.6 |
| | Versicolorin C* | 2012 | 96 | 0.3–21 | 4[b] ± 4 | 3 |
| | | 2013 | 18 | 0.4–32 | 7[ab] ± 10 | 2 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 57 | 0.2–3 | 1[a] ± 0.9 | 0.8 |
| | Nidurufin | 2012 | 57 | 0.2–3 | 0.8[a] ± 0.6 | 0.5 |
| | | 2013 | 10 | 0.4–2 | 0.8[a] ± 0.7 | 0.5 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 20 | 0.2–3 | 0.9[a] ± 0.8 | 0.5 |
| | Averantin | 2012 | 55 | 0.2–2 | 0.6[a] ± 0.5 | 0.5 |
| | | 2013 | 4 | 0.2–14 | 7[b] ± 10 | 0.2 |
| | | 2014 | 2 | 0.2 | | |
| | | 2015 | 10 | 0.4–2 | 0.7[a] ± 0.7 | 0.4 |
| | Averufin | 2012 | 96 | 0.1–24 | 4[ab] ± 6 | 2 |
| | | 2013 | 31 | 0.1–42 | 5[b] ± 13 | 0.6 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 84 | 0.1–20 | 2[a] ± 4 | 0.3 |
| | Norsolorinic acid | 2012 | 74 | 2–18 | 4[a] ± 3 | 3 |
| | | 2013 | 6 | 2–10 | 5[a] ± 5 | 3 |
| | | 2014 | 2 | 2 | | |
| | | 2015 | 6 | 2–4 | 3[a] ± 1 | 3 |
| | Kojic acid | 2012 | 98 | 126–23091 | 9586[b] ± 5268 | 8303 |
| | | 2013 | 94 | 92–10000 | 1971[a] ± 2347 | 861 |
| | | 2014 | 6 | 44–236 | 108[a] ± 111 | 44 |
| | | 2015 | 90 | 103–11497 | 2664[a] ± 2459 | 1875 |
| | 3-Nitropropionic acid | 2012 | 98 | 9–879 | 303[b] ± 183 | 254 |
| | | 2013 | 86 | 2–345 | 13[a] ± 13 | 7 |
| | | 2014 | 10 | 2–35 | 3[a] ± 0.7 | 3 |
| | | 2015 | 100 | 5–453 | 78[a] ± 89 | 40 |
| | Cyclopiazonic acid | 2012 | 52 | 94–258 | 152[a] ± 43 | 149 |
| | | 2013 | 14 | 54–370 | 203[a] ± 110 | 180 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| *Aspergillus* | Malformin A | 2012 | 86 | 0.8–211 | 27[b] ± 50 | 5 |

(*continued on next page*)

**Table 3** (*continued*)

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| *niger* | | 2013 | 8 | 1–15 | $6^{ab}$ ± 7 | 4 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 41 | 0.8–21 | $4^{a}$ ± 3 | 3 |
| | Malformin A2* | 2012 | 12 | 0.8–3 | 2 ± 0.7 | 2 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| | Malformin C | 2012 | 67 | 0.5–40 | $7^{a}$ ± 11 | 0.7 |
| | | 2013 | 4 | 0.2–2 | $1^{a}$ ± 1 | 1 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 22 | 0.3–5 | $1^{a}$ ± 1 | 0.8 |
| | Pseurotin A | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 2 | 5 | | |

N (%): number (percentage) of contaminated sample.
nd: not detected i.e., less than limit of detection (LOD).
Min-Max: minimum and maximum concentrations (μg/kg).
Mean ± SD: mean concentration (μg/kg) ± standard deviation (μg/kg).
Median: median concentration (μg/kg).
Different letters in the same column indicate significant differences ($P < 0.05$) between values according to the post-hoc Tukey's HSD (honestly significant differences) test, for unequal sample sizes.
* Semi-quantified using response factor of a structurally related analyte.

20, 17 and 20 different *Aspergillus* metabolites occurred in investigated maize samples, respectively. Further, the most prevalent metabolites under moderate drought weather conditions in 2013 year, belonging to the genus *Penicillium*, followed by *Fusarium* and *Aspergillus*. On the other hand, high amount of precipitation and humid condition during maize growing season in 2014 resulted in maize samples contaminated a wide number of *Fusarium* and *Penicillium* metabolites, while metabolites of *Aspergillus* spp. rarely occurred. Finally, under moderate drought weather conditions in 2015, the most prevalent metabolites belong to the genus *Penicillium*, followed by *Aspergillus* and *Fusarium*. The overall findings show that high prevalence of non-regulated fungal metabolites were detected and/or quantified in maize samples, collected in Serbia in the period of four years (2012–2015).

### 3.2. Occurrence of Penicillium metabolites

Among of 38 detected and/or quantified *Penicillium* metabolites in maize samples from four-year period (2012–2015), 20 of them were quantified in maize samples collected from 2012 maize growing season. The most frequently present *Penicillium* metabolites, in maize samples from 2012, were questiomycin A and oxaline (100% each), meleagrin and secalonic acid D (96% each), followed by 7-hydroxypestalotin (94%), pestalotin and roquefortine C (80% each) and flavoglaucin (65%). It should be noted, that the mean concentration of secalonic acid D (176 ± 121 μg/kg) was the highest compared to the mean concentrations of above mention *Penicillium* metabolites, as well as significantly differed than its concentrations detected in maize samples from other maize growing seasons. As can be seen from the Table 2, the other *Penicillium* metabolites rarely were present in maize samples from 2012 maize growing season. A total of 29 *Penicillium* metabolites were present in maize samples which originated from the 2013 maize growing season. Since the weather conditions during 2013 maize growing season was characterised as a moderate drought, similar incidence of investigated *Penicillium* metabolites were observed, as in maize samples from extreme drought maize growing season (2012). Namely, oxaline was present in 100% of maize samples, while questiomycin A was quantified in 94% of samples. Furthermore, frequent contaminants of maize samples were pestalotin and quinolactacin A (86% each), 7-hydroxypestalotin (84%), flavoglaucin (72%), followed by secalonic acid D (47%), aurantine (45%) and mycophenolic acid (43%). The mean concentrations of above mentioned *Penicillium* metabolites were relatively low, with the exception for roquefortine C (137 ± 251 μg/kg), secalonic acid D (81 ± 90 μg/kg), mycophenolic acid (54 ± 91 μg/kg) and aurantine (7 ± 11 μg/kg). It should be highlighted, that obtained mean concentrations of roquefortine C, mycophenolic acid and aurantine were the highest compared to their mean concentrations in maize samples from other maize growing seasons. Therefore, only the mean concentration of mycophenolic acid significantly differed compared to its determined concentration from other production years. Further, the other *Penicillium* metabolites rarely were present in analysed maize samples from 2013 maize growing season. In maize samples which originated from 2014 maize growing season, characterized by record precipitation and flood, 17 different *Penicillium* metabolites were quantified (Table 2). The most of the quantified *Penicillium* metabolites occurred with low frequency. However, the following *Penicillium* metabolites occurred with high prevalence in maize samples from 2014 maize growing season: 7-hydroxypestalotin (100%), questiomycin A and pestalotin (96% each), oxaline (84%) and flavoglaucin (57%). The mean concentrations of above mentioned metabolites were the highest and significantly different compared to the other investigated years, with exception of flavoglaucin. In maize samples which originating from 2015 maize growing season, 27 *Penicillium* metabolites were quantified. The most frequently metabolites were oxaline (100%) 7-hydroxypestalotin (96%), questiomycin A (94%), quinolactacin A (92%), flavoglaucin (80%) and pestalotin (74%). It should be emphasized that the mean concentration of flavoglaucin was the highest (145 ± 135 μg/kg) and significantly different compared to other investigated maize growing seasons. In 2015 production year the highest frequency (55%) of mycophenolic acid was observed in comparison to frequency in other maize growing seasons. Similar situation was observed regarding occurrence of citreohybridinol. Citreohybridinol contaminated 43% of maize samples from 2015 production year and its obtained mean concentration was 8 ± 11 μg/kg. Additionally, it should be highlighted that the mean concentration of citrinin significantly differed and was the highest (950 ± 2872 μg/kg) in maize samples from 2015 maize growing season compared to other production years. This also applies to the frequency (23%) of its occurrence, as well as its quantified content (from 7 to 10058 μg/kg) in maize samples. Furthermore, it should be noted that the presented results are notably higher comparing to the data published by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2012a), EFSA Panel on contaminats in the Food Chain

**Table 4**
Occurrence of non-regulated *Alternaria* toxins and other fungal and unspecific metabolites in maize samples collected in Republic of Serbia in the period 2012–2015.

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| *Alternaria* | Tenuazonic acid | 2012 | 67 | 74–503 | $230^{b}$ ± 114 | 201 |
| | | 2013 | 18 | 37–18 | $87^{a}$ ± 48 | 75 |
| | | 2014 | 29 | 40–253 | $115^{a}$ ± 64 | 99 |
| | | 2015 | 27 | 20–218 | $92^{a}$ ± 55 | 71 |
| | Alternariol | 2012 | 6 | 4–8 | $5^{a}$ ± 2 | 5 |
| | | 2013 | 6 | 4–26 | $12^{a}$ ± 12 | 5 |
| | | 2014 | 23 | 2–134 | $22^{a}$ ± 36 | 11 |
| | | 2015 | 14 | 1–16 | $7^{a}$ ± 5 | 7 |
| | Alternariolmethylether | 2012 | 47 | 0.1–2 | $0.4^{a}$ ± 0.4 | 0.2 |
| | | 2013 | 18 | 0.2–2 | $0.6^{a}$ ± 0.7 | 0.3 |
| | | 2014 | 63 | 0.3–24 | $2^{a}$ ± 5 | 0.3 |
| | | 2015 | 33 | 0.1–12 | $2^{a}$ ± 3 | 0.3 |
| | Tentoxin | 2012 | 10 | 0.1–0.2 | $0.1^{a}$ ± 0.1 | 0.1 |
| | | 2013 | 6 | 0.1–0.5 | $0.3^{a}$ ± 0.2 | 0.5 |
| | | 2014 | 6 | 0.1–0.4 | $0.3^{a}$ ± 0.1 | 0.2 |
| | | 2015 | nd | nd | nd | nd |
| | Infectopyron | 2012 | 6 | 15–23 | 19 ± 4 | 18 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | nd | nd | nd | nd |
| Other fungal metabolites | Ascochlorin | 2012 | 6 | 0.1–0.002 | $0.1^{a}$ ± 0.03 | 0.1 |
| | | 2013 | 8 | 0.2–0.6 | $0.4^{a}$ ± 0.2 | 0.3 |
| | | 2014 | 2 | 0.2 | | |
| | | 2015 | 22 | 0.2–6 | $1^{a}$ ± 2 | 0.7 |
| | Monocerin | 2012 | nd | nd | nd | nd |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 8 | 0.7–3 | 2 ± 1 | 2 |
| | | 2015 | 2 | 0.5 | 0.5 | |
| | Cladosporin | 2012 | 10 | 4–26 | $11^{a}$ ± 10 | 6 |
| | | 2013 | 31 | 2–89 | $13^{a}$ ± 19 | 6 |
| | | 2014 | 4 | 9–29 | $19^{a}$ ± 14 | 19 |
| | | 2015 | 25 | 2–21 | $7^{a}$ ± 5 | 5 |
| Unspecific metabolites | Asperglaucide | 2012 | 82 | 0.1–82 | $8^{a}$ ± 19 | 3 |
| | | 2013 | 82 | 0.1–345 | $18^{a}$ ± 54 | 3 |
| | | 2014 | 65 | 0.2–104 | $9^{a}$ ± 19 | 2 |
| | | 2015 | 82 | 0.1–73 | $13^{a}$ ± 21 | 2 |
| | Asperphenamate | 2012 | 22 | 0.3–276 | $1.^{a}$ ± 0.8 | 1 |
| | | 2013 | 86 | 0.5–106 | $7^{ab}$ ± 18 | 2 |
| | | 2014 | 86 | 0.2–59 | $5^{ab}$ ± 10 | 2 |
| | | 2015 | 88 | 0.3–69 | $10^{b}$ ± 14 | 6 |
| | Brevianamid F | 2012 | 18 | 2–7 | $3^{ab}$ ± 2 | 3 |
| | | 2013 | 8 | 2–14 | $6^{b}$ ± 6 | 3 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 16 | 0.6–2 | $1^{a}$ ± 1 | 1 |
| | Citreorosein | 2012 | 92 | 0.7–7 | $2^{b}$ ± 1 | 2 |
| | | 2013 | 41 | 0.8–11 | $3^{b}$ ± 2 | 2 |
| | | 2014 | 12 | 0.7–3 | $2^{ab}$ ± 1 | 1 |
| | | 2015 | 59 | 0.7–4 | $1^{a}$ ± 0.8 | 1 |
| | cyclo(L-Pro-L-Tyr) | 2012 | 100 | 1–20 | $5^{b}$ ± 5 | 4 |
| | | 2013 | 88 | 1–12 | $4^{ab}$ ± 3 | 3 |
| | | 2014 | 35 | 1–17 | $4^{ab}$ ± 4 | 2 |
| | | 2015 | 76 | 0.9–7 | $2^{a}$ ± 2 | 2 |
| | cyclo(L-Pro-L-Val) | 2012 | 100 | 1–28 | $7^{bc}$ ± 6 | 4 |
| | | 2013 | 96 | 4–22 | $8^{c}$ ± 4 | 7 |
| | | 2014 | 82 | 0.7–13 | $3^{a}$ ± 3 | 2 |
| | | 2015 | 71 | 1–13 | $4^{ab}$ ± 3 | 3 |
| | Emodin | 2012 | 53 | 0.4–6 | $1^{a}$ ± 1 | 0.7 |
| | | 2013 | 96 | 0.3–2 | $1^{a}$ ± 0.4 | 0.8 |
| | | 2014 | 82 | 0.2–0.9 | $0.5^{a}$ ± 0.2 | 0.4 |
| | | 2015 | 71 | 0.3–1 | $0.5^{a}$ ± 0.2 | 0.5 |
| | Endocrocin | 2012 | 6 | 18–391 | 90 ± 156 | 59 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 16 | 19–45 | 32 ± 11 | 19 |
| | Fallacinol | 2012 | 4 | 1–10 | $6^{b}$ ± 6 | 6 |
| | | 2013 | 12 | 0.2–4 | $1^{a}$ ± 1 | 0.6 |
| | | 2014 | 8 | 0.3–2 | $1^{a}$ ± 0.6 | 1 |
| | | 2015 | 45 | 0.2–4 | $0.8^{a}$ ± 0.7 | 0.6 |
| | IsoRhodoptilometrin | 2012 | 92 | 0.1–0.7 | $0.3^{a}$ ± 0.1 | 0.2 |
| | | 2013 | 45 | 0.1–4 | $0.4^{a}$ ± 0.7 | 0.2 |
| | | 2014 | 12 | 0.1–1 | $0.5^{a}$ ± 0.5 | 0.2 |
| | | 2015 | 47 | 0.1–1 | $0.2^{a}$ ± 0.3 | 0.1 |
| | N-Benzoyl-Phenylalanine | 2012 | 10 | 1–6 | $3^{ab \pm 2}$ | 3 |
| | | 2013 | 72 | 1–29 | $7^{b}$ ± 6 | 4 |

(*continued on next page*)

**Table 4** (*continued*)

| Fungi | Mycotoxin | Year | N (%) | Min-Max | Mean ± Std | Median |
|---|---|---|---|---|---|---|
| | | 2014 | 23 | 1–7 | $3^{ab}$ ± 2 | 2 |
| | | 2015 | 49 | 1–11 | $3^{a}$ ± 3 | 3 |
| | Neoechinulin A | 2012 | 20 | 4–408 | $79^{a}$ ± 125 | 30 |
| | | 2013 | 71 | 6–1297 | $122^{a}$ ± 231 | 53 |
| | | 2014 | 49 | 6–490 | $92^{a}$ ± 118 | 57 |
| | | 2015 | 65 | 6–332 | $101^{a}$ ± 85 | 97 |
| | Rugulusovin | 2012 | 4 | 2–3 | 3 ± 1 | 3 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 8 | 1–2 | 1 ± 0.4 | 2 |
| | Skyrin | 2012 | 4 | 5–21 | 13 ± 11 | 3.18 |
| | | 2013 | nd | nd | nd | nd |
| | | 2014 | 4 | 3–27 | 16 ± 16 | 15.98 |
| | | 2015 | nd | nd | nd | nd |
| | Tryptophol | 2012 | 55 | 12–35 | $23^{a}$ ± 7 | 22 |
| | | 2013 | 4 | 17–53 | $35^{a}$ ± 25 | 35 |
| | | 2014 | nd | nd | nd | nd |
| | | 2015 | 27 | 11–91 | $34^{a}$ ± 30 | 18 |

N (%): number (percentage) of contaminated sample.
nd: not detected i.e., less than limit of detection (LOD).
Min-Max: minimum and maximum concentrations (μg/kg).
Mean ± SD: mean concentration (μg/kg) ± standard deviation (μg/kg).
Median: median concentration (μg/kg).
Different letters in the same column indicate significant differences ($P < 0.05$) between values according to the post-hoc Tukey's HSD (honestly significant differences) test, for unequal sample sizes.

(CONTAM) (2012b). According to the scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed the quantified content of citrinin in maize samples was ranged from 50 to 1500 μg/kg. As can be seen from the Table 2, the other quantified *Penicillium* metabolites were present with low frequency and with relatively low concentrations. Further, in examined maize samples the following *Penicillium* metabolites were also detected: andrastin C, andrastin D and verrucosidin. Among of the above *Penicillium* metabolites the most prevalent was verrucosidin with frequency of 6%, 12%, 2% and 12% in maize samples from 2012, 2013, 2014 and 2015 maize growing seasons, respectively. On the other hand, andrastin C and andrastin D were detected only in maize samples from 2015 maize growing season with the frequency of 6% and 4%, respectively.

### 3.3. Occurrence of Fusarium metabolites

Among of the total of 24 quantified *Fusarium* metabolites, moniliformin and beauvericin were present in 100% of maize samples collected from each of the four investigated maize growing seasons, while the highest concentrations of these metabolites were observed in year 2013 (Table 3). It should be noted, that the highest mean concentration of moniliformin (1267 ± 707 μg/kg) was observed in 2012, while beauvericin occurred with the highest mean content (40 ± 92 μg/kg) in maize from 2013 maize growing season. According to the scientific opinion on the risks to human and animal health related to the presence of moniliformin in food and feed published by EFSA (2018), in maize intendent for human consumption the maximum reported value was 2606 μg/kg, while the mean values ranged from 89 to 1127 μg/kg. In maize samples intended for animal feeding the maximum determined value was 3300 μg/kg. Compared to these data, it can be noted, that the maximum observed content of moniliformin in Serbian maize samples was much higher (8710 μg/kg) in 2013, while the maximum concentrations determined in other three years ranged from 1624 to 4113 μg/kg. Regarding beauvericin, the obtained concentrations in this study are relatively low in comparison to the highest mean (390 μg/kg) and maximum level (6402 μg/kg) reported by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2014a, 2014b). Additional, in 2013 and 2015 maize growing seasons, bikaverin was present in 100% of analysed maize samples. Also, in 2012 and 2014, its presence was recorded with very high frequency (98%). Further, siccanol was detected in every single samples originated from 2013, 2014 and 2015 maize growing seasons, while in maize samples from 2012 production year siccanol contaminated 96% of examined samples. In 2014 production year, which was characterized as year with record precipitation and flood, 100% of analysed maize samples were additionally contaminated with culmorin, 15-hydroxyculmorin, aurofusarin and butenolid. It should be noted, that at the same time the concentrations of the mentioned *Fusarium* metabolites in year 2014 were the highest and significantly differed from concentrations in other years, with mean concentration of 1287 ± 795 μg/kg, 1435 ± 1271 μg/kg, 10671 ± 10350 μg/kg and 1018 ± 615 μg/kg for culmorin, 15-hydroxyculmorin, aurofusarin and butenolid, respectively (Table 3). Furthermore, although the fact that moniliformin was quantified in 100% of maize samples from 2014, its mean concentration was the lowest and significantly differed from the determined levels of its content in maize samples from 2012 and 2013 years. Frequency of occurrence of other quantified *Fusarium* metabolites in maize samples differed by the production years, while statistically significant differences were not observed in their mean concentrations, with the exception for diacetoxyscirpenol and fusapyron (Table 3). Namely, diacetoxyscirpenol was present with the highest frequency in maize samples from 2012 maize growing season, while the highest mean concentration ($P < 0.05$) of diacetoxyscirpenol was observed in maize samples from 2014. With the highest frequency fusapyron occurred in maize samples originating from year 2014, while the significantly different mean content of this secondary metabolite was observed in maize samples from 2013. T-2 and HT-2 toxins occurred rarely and with relatively low concentration in maize samples during investigated period (Table 3). According to the scientific report published by EFSA (2017) within grains, the highest T-2 and HT-2 contamination frequency (78% and 80%, respectively) and the highest mean concentrations were measured in oats (124 μg/kg for T-2 and 307 μg/kg for HT-2), while barley, maize, rye, wheat and triticale were less contaminated (mean concentrations in the range of 0.07–6.40 μg/kg) with these fungal metabolites. However, the mean concentration of T-2 and HT-2 observed in this study (in the range of 2–14 μg/kg for T-2 and 23–62 μg/kg for HT-2), indicated that these fungal metabolites contaminated maize in Serbia with higher content in comparison to the published

data by EFSA (2017). Furthermore, HT-2 glucoside was present only in maize samples from 2014 maize growing season with low frequency (4%) and low mean content of 14 ± 2 μg/kg.

As can be seen from the Table 3 enniatins (A1, B and B1) were present whit low frequency as well as quantified amount in investigated maize samples comparing to the reported levels of these toxins by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2014a, 2014b). According to the scientific opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed by EFSA report (2014) the highest mean and maximum concentration of enniatin B were 577 μg/kg and 2960 μg/kg, and 89 μg/kg and 496 μg/kg for enniatin B1, respectively.

### 3.4. Occurrence of Aspergillus metabolites

Twenty different *Aspergillus* metabolites were detected and/or quantified in examined maize samples collected in 2012 production year. The most frequently present *Aspergillus* metabolites were kojic acid and 3-nitropropionic acid (98% each), followed by versicolorin C and averufin (96% each). Versicolorin A was frequently present (84%) in maize samples, but in very low concentrations. As can be seen from the Table 3, the quantified mean concentrations of kojic acid (9586 ± 5268 μg/kg) and 3-nitropropionic acid (303 ± 183 μg/kg) were the highest and significantly different in 2012 than their concentrations in maize samples from other production years. Although the fact that the highest incident of versicolorin C and averufin were observed in maize samples from 2013 maize growing season, these fungal metabolites occurred with relatively low levels in investigated maize samples from all maize growing seasons. From *Aspergillus niger* metabolites, malformin A (86%), and malformin C (67%) frequently contaminated maize samples in 2012 production year, with significantly higher mean concentrations in comparison with other production years. Maize samples from 2012 growing season were also frequently (61%) contaminated with O-methylsterigmatocystin but with relatively low concentration, while methylsterigmatocystin and seco-sterigmatocystin were not quantified in any of the analysed samples. Furthermore, only maize samples originated from the 2012 production year were contaminated with aflatoxicol. The frequency of occurrence of aflatoxicol was low (18%), as well as its mean concentration (2 ± 1 μg/kg). Further, sterigmatocystin was present also in maize samples which originated from extreme drought year (2012), as well as from moderate drought maize growing seasons (2013 and 2015). Additionally, maize samples originated from 2013 and 2015 were also highly contaminated with kojic acid (94% and 90%) and 3-nitropropionic acid (86% and 100%). Kojic acid was present with high content (1971 ± 2347 and 2664 ± 2459 μg/kg) in maize samples from both maize growing seasons (2013 and 2015), but its content did not differ significantly amongst 2013 and 2015 production years. Similar situation was observed concerning 3-nitropropionic acid, but its mean concentration in maize samples from years 2013 and 2015 was lower and significantly different compared to its content in maize samples from 2012 production year. Furthermore, it can be noticed, that the observed mean concentration of averufin was the highest in maize samples from 2013, and significantly different compared to its content in maize samples from other production years, although it was present with lower frequency (31%). However, as can be seen from the Table 3, several different *Aspergillus* metabolites contaminated maize samples from 2013 maize growing season, but their frequency of occurrence and their mean concentrations were very low.

Regarding contamination of maize samples which originated from 2014 maize growing season, characterized by record precipitation and flood, it should be highlighted that *Aspergillus* metabolites rarely occurred (< 10%). None of the *Aspergillus* metabolites, which are included in aflatoxin pathway, occurred in the examined maize samples from year 2014. Beside kojic acid (90%) and 3-nirtopropionic acid (100%), maize samples from 2015 maize growing season (moderate drought weather condition) were also frequently contaminated with averufin (84%) and versicolorin C (57%), but their mean concentrations were relatively low (2 ± 4 and 1 ± 0.7 μg/kg, respectively). Exclusively only one maize sample from 2015 production year was contaminated with pseurotin A (5 μg/kg). Additionally, the following *Aspergillus* metabolites were detected in maize samples analysed in this study: nigragillin, aurasperon B, aurasperon C, aurasperon G and aspulvinone E. Among the detected *Aspergillus* metabolites the most prevalent was nigragillin with frequency of 96%, 94%, 15% and 96% in maize samples from 2012, 2013, 2014 and 2015 maize growing seasons, respectively. Aspulvinone E was detected also in maize samples from each year, but with much less frequency: 2%, 29%, 10% and 27% in 2012, 2013, 2014, and 2015 years, respectively. This results indicated, that under the moderate drought conditions (2013 and 2015), aspulvinone E more frequently contaminated the maize crop. Aurasperon B, aurasperon C, and aurasperon G were not detected in maize samples from 2013 and 2014 maize growing seasons, while in extreme drought weather conditions during 2012, they were present in 39%, 81% and 76% of maize samples, respectively. Similar situation was recorded in 2015 maize growing season, since frequency of occurrence of aurasperon B, aurasperon C, and aurasperon G in maize samples were 27%, 67%, and 58%, respectively.

### 3.5. Occurrence of Alternaria toxins

The *Alternaria* metabolites alternariol, alternariol methylether, tenuazonic acid, tentoxin and infectopyron were quantified among 204 analysed maize samples. The frequency and the level of each metabolite varied by production years (Table 4), although a statistically significant differences in their content between the production years was not observed, with exception of tenuazonic acid and infectopyron. Namely, infectopyron was occurred in low frequency (6%) only in maize samples which originating from 2012 maize growing season with the mean concentration of 19 ± 4 μg/kg. On the other hand, the most prevalent *Alternaria* toxin was tenuazonic acid with the highest frequency (69%) and with the highest mean (230 ± 114 μg/kg) and maximum concentrations (503 μg/kg), in year 2012, which was characterized as extreme drought. The observed mean concentration of tenuazonic acid in maize samples from 2012 year statistically significantly differed from the mean concentration of this mycotoxin in maize samples from other production years. In maize samples which originating from 2012 maize growing season all five *Alternaria* toxins were quantified (Table 4). The highest frequency and mean concentrations of alternariol and alternariol methylether were recorded in maize samples from 2014 maize growing season (Table 4), which was characterized as extreme wet. In the investigated period, tentoxin was present in maize samples from each production year, with exception in maize samples from 2015. Although alternariol, alternariol methylether and tentoxin occurred in maize samples from each of the four examined maize growing seasons, they were detected in low concentrations. However, it can be noted that tenuazonic acid occurred only in maize samples from Serbia, in relation to the reported data of occurrence of four most common *Alternaria* toxins in maize samples (Abdallah et al., 2017; Getachew et al., 2018; Oliveira et al., 2017; Shephard et al., 2013), while presence of tentoxin (3% of analysed samples) was reported only in maize samples from Egypt (Abdallah et al., 2017). Alternariol and alternariol methylether more often and with higher concentrations contaminated maize samples in Serbia in comparison with so far published data (Abdallah et al., 2017; Getachew et al., 2018; Oliveira et al., 2017; Shephard et al., 2013). On the other hand, according to published data by Abdallah et al. (2017), Oliveira et al. (2017) and Shephard et al. (2013), macrosporin A was present with relatively low frequency in maize samples from Egypt, Brazil and South Africa, respectively, while this metabolite was not detected in Serbian maize samples.

### 3.6. Occurrence of other fungal metabolites

Concerning the other fungal metabolites, ascochlorin and cladosporin were quantified in maize samples from each production year. Monocerin was occurred in maize samples from 2014 and 2015, but with a low incidence and concentrations (Table 4). Cladosporin was quantified in maize samples from each year (from 4% to 31%), and there was no significant differences between determined concentrations. Quantified amount of ascochlorin was low in maize samples from every year. With the highest frequency of 22%, it was present in maize samples from 2015, while in other investigated years low incidence of ascochlorin was observed.

### 3.7. Occurrence of unspecific metabolites

Maize samples from each production year were contaminated with wide range of fifteen unspecific metabolites (Table 4). The most frequently (100%) occurred unspecific metabolites in maize samples from year 2012 were cyclo (L-Pro-L-Tyr) and cyclo (L-Pro-L-Val), followed by citreorosein (92%), isorhodoptilometrin (92%) and asperglaucide (82%). Under the weather conditions of extreme drought (2012) and hot and dry weather conditions (2013 and 2015), the following unspecific metabolites were quantified in maize samples: endocrocin (2012 and 2015), rugulusovin (2012 and 2015) and tryptophol (2012, 2013 and 2015). Regarding the maize samples originating from 2014 maize growing season, characterized by record precipitation and flood, the most common quantified unspecific metabolites were asperphenamate (86%), cyclo (L-Pro-L-Val) (82%) and emodin (82%). Additionally, in this year maize samples were also contaminated with the following unspecific metabolites: asperglaucide (65%), neoechinulin A (49%), cyclo (L-Pro-L-Tyr) (36%), *N*-benzoyl-bhenylalanine (23%), citreorosein (12%), isorhodoptilometrin (12%) fallacinol (8%) and skyrin (4%), while endocrocin, rugulusovin and tryptophol were not detected. It should be noted that, in maize samples from the whole investigated period 2012–2015, a mentioned metabolites were present in relative low concentrations with the exception of neoechinulin A, tryptophol and endocrocin. Furthermore, rugulusovin was present with low frequency and low concentration in maize samples from 2012 and 2015 maize growing seasons, while skyrin occurred with low frequency and low concentration in maize samples from 2012 (extreme drought) and 2014 (recorded high amount of precipitation) maize growing seasons. Fallacinol occurred with the lowest frequency in maize samples from 2012 production year, but the mean concentration of this metabolite was the highest in comparison to its concentration in maize samples from other production years. Additionally, asparason A was detected only in one maize sample from 2015 maize growing season.

### 3.8. Frequency of the most common fungal metabolites during the period 2012–2015

The results related to the differences in the frequency of the most common detected non-regulated mycotoxins, as well as other fungal metabolites per investigated year are summarized in Fig. 1.

It can be seen that the profile of the most common secondary fungal metabolites were different across the investigated period and highly dependent on weather conditions during maize growing seasons. Namely, aurofusarin, butenolid, culmorin and 15-hydroxyculmorin, were the most common detected non-regulated fungal metabolites in maize samples from 2014 production year (characterized by high amount of precipitation), while those metabolites were very rarely present in maize samples which originated from other maize growing sessions. Furthermore, their determined concentrations in 2014 were the highest and statistically different ($P < 0.05$) compared to their mean concentrations in maize samples from other investigated years (Table 3). On the other hand, from the Fig. 1 and Tables 2–4 it can be noted that the profile of the most common non-regulated fungal

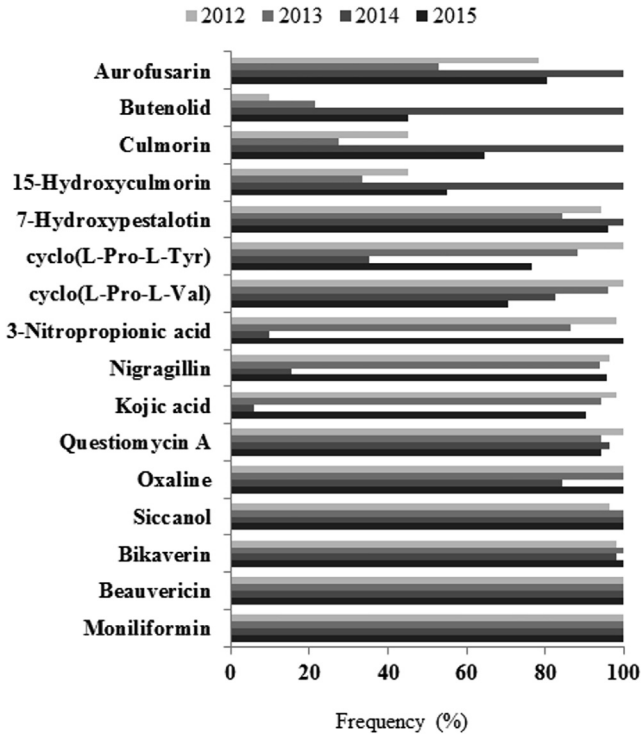


**Fig. 1.** Frequency (%) of the most prevalent non-regulated fungal metabolites in maize samples from Serbia in the period 2012–2015.

metabolites were very similar for maize samples which originated from 2012, 2013 and 2015 maize growing seasons (characterized by hot and dry weather conditions), but it should be highlighted that their mean concentrations in most cases significantly differed cross 2012, 2013 and 2015 maize growing seasons. The most dominant fungal metabolites in maize samples collected from these three years were kojic acid, 3-nitropropionic acid and nigragilin. However, even though weather conditions in the investigated period of four years were different, several following fungal metabolites were detected with very high prevalence in maize samples from each year: moniliformin, beauvericin, bikaverin, siccanol, oxaline, questiomzcin A and 7-hydroxypestalotin. The obtained results in this study could not be completely compared to the published data, since to the best of authors' knowledge there is no previously published data from Serbia, as well as from neighboring countries related to the occurrence of non-regulated mycotoxins and other fungal metabolites in maize. Furthermore, there is also a lack of data from Europe related to this topic. The greatest number of previously published data related to the non-regulated mycotoxins and other fungal metabolites in maize are from the countries which have significantly different conditions of maize cultivation, as well as climate in comparison to Serbia. As we already pointed out, regardless of different weather conditions, moniliformin and beauvericin were quantified in every maize sample from each of the four investigated years. Compared to the available published data it can be noted that 100% of maize samples from Cameroon (Abia et al., 2013), Ethiopia (Getachew et al., 2018), and South Africa (Shephard et al., 2013) were also contaminated with beauvericin, while 99% of maize samples from Brazil was contaminated with this fungal metabolite (Oliveira et al., 2017). On the other hand, moniliformin with high frequency (73%) occurred only in maize samples from Brazil (Oliveira et al., 2017), while rarely contaminated maize samples from Egypt (Abdallah et al., 2017) and South Africa (Shephard et al., 2013). Moreover, in investigated maize samples from Cameron (Abia et al., 2013) and Ethiopia (Getachew et al., 2018) moniliformin was not detected. The mean concentrations, as well as the maximum quantified amount (Table 3) of the both fungal metabolites in this study were higher in comparison to the published

**Table 5**
Average, minimum and maximum number of total fungal metabolites and non-regulated fungal metabolites which co-occurred in maize samples from Serbia in the period 2012–2015.

| Year | Number of total fungal metabolites | | | Number of non-regulated fungal metabolites | | |
|---|---|---|---|---|---|---|
| | $N_{ave}$ | $N_{min}$ | $N_{max}$ | $N_{ave}$ | $N_{min}$ | $N_{max}$ |
| 2012 | 46 | 23 | 66 | 37 | 20 | 55 |
| 2013 | 36 | 20 | 47 | 24 | 13 | 34 |
| 2014 | 36 | 18 | 57 | 29 | 15 | 48 |
| 2015 | 42 | 17 | 57 | 34 | 13 | 47 |

$N_{ave}$ – average number of total fungal metabolites represents the sum of average number of regulated mycotoxins and its derivates (Kos et al., 2020) and average number of non-regulated fungal metabolites; $N_{min}$-minimum number in sigle sample; $N_{max}$-maximum number in single sample.

data so far. Similar situation can be noted regarding contamination of maize samples with bikaverin, siccanol, oxaline, questiomycin A and cyclo (L-Pro-L-Tyr) compared to the other published data, since the above mentioned fungal metabolites rarely occurred in maize samples from other countries. However, bikaverin and cyclo (L-Pro-L-Tyr) highly contaminated maize samples from Brazil (93%) (Oliveira et al., 2017) and Egypt (100%) (Abdallah et al., 2017).

### 3.9. Co-occurrence of investigated fungal metabolites

The average number of total metabolites, as well as the average number of non-regulated fungal metabolites which co-occurred in maize samples collected in Serbia in the period 2012–2015 are shown in the Table 5.

As can be seen, in maize samples collected from maize growing season in 2012, the average (46), maximum (66) and minimum numbers (23) of detected metabolites, which co-occurred in single maize sample were the highest in comparison to the other years, as well as in comparison to the other published data. Namely, the maximum number of metabolites observed per sample from Egypt was 54 (Abdallah et al., 2017), while all of the examined samples were contaminated with at least 4 toxins. The largest number of fungal metabolites detected in one maize sample from Brazil being 51 (Oliveira et al., 2017), while all analysed maize samples were found to be contaminated by at least 10 different metabolites. Furthermore, as can be seen from the Table 5, average, minimum, as well as maximum numbers of detected total fungal metabolites were also very high in maize samples from 2013, 2014 and 2015 production years. Results related to the numbers of detected and/or quantified non-regulated fungal metabolites also indicated that average, maximum and minimum number of detected fungal metabolites per sample were very high. Among of 109 detected and/or quantified non-regulated fungal metabolites in maize samples from 2012, the average, maximum and minimum number of non-regulated fungal metabolites in the single sample were 37, 55 and 20, respectively. Similar situations was also observed in maize samples from the 2015 production year, since this maize growing season was also characterized as hot and dry. The great number of detected and/or quantified non-regulated fungal metabolites was also noticed in maize samples from 2013 and 2014 maize growing seasons. Generally, among all examined maize samples the minimum number of investigated non-regulated secondary fungal metabolites which were quantified in single maize samples from Serbia was 13 in 2013 and 2015, while the maximum number was 55 in 2012.

## 4. Conclusions

The present study represents the first report on the multi-occurrence of a board spectrum of mycotoxins, other fungal and unspecific metabolites in maize samples from Serbia (2012–2015), quantified by using a multi-mycotoxin method based on LC-MS/MS. The results obtained showed that regardless of the weather conditions during the production years, a board spectrum of mycotoxins, other fungal and unspecific metabolites co-occurred in the examined maize samples. None of the analysed maize samples was without any of the investigated fungal secondary metabolites. Among all examined maize samples the minimum number of investigated secondary fungal metabolites (regulated and non-regulated) which were quantified in single maize samples from Serbia was 17 in 2015, while the maximum number was 66 in 2012. Based on the findings obtained in this study, it could be noticed, that presence of certain fungal metabolites highly dependent on the weather conditions recorded during maize growing season; while on the other hand some fungal metabolites were detected with very high prevalence even though weather conditions in the examined period of four years were significantly different. For instance, rainy and wet conditions in year 2014 were the most favorable for certain *Fusarium* species and synthesis of *Fusarium* metabolites. Contrary to this, *Aspergillus* metabolites were very rarely detected in maize samples collected in year 2014. However, hot and dry conditions recorded in years 2012, 2013 and 2015 influenced higher prevalence of *Aspergillus* metabolites, especially in maize samples cultivated under extreme drought conditions in 2012. The most common *Penicillium* metabolites were detected with very high frequency in maize samples collected from each of the four years. Furthermore, taking into account the fact, that maize samples collected from four different years in Serbia, represent a mixture of different fungal metabolites, as well as the fact that co-occurred metabolites can exhibit a synergistic and/or additive effect; a great attention should be taken on non-regulated fungal metabolites, especially those, which frequently contaminated maize crop from Serbia. The obtained data of the occurrence, as well as relationships between various weather conditions (2012–2015) and levels of non-regulated mycotoxins and other fungal metabolites is a unique report about this topic from Serbia, as well as from the neighboring countries. Finally, it should be highlighted, that based on the climate change prediction and results obtained in this study, as well as, in the study by Kos et al. (2020), maize production in Serbia requires significant improvement in terms of control strategy, management practices, as well as, multi-disciplinary integration and education of all participants in the food and feed chain, as the most important mitigation measures of the contamination of maize by fungal metabolites.

## CRediT authorship contribution statement

**Elizabet Janić Hajnal:** Conceptualization, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Jovana Kos:** Conceptualization, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization, Project administration. **Alexandra Malachová:** Conceptualization, Methodology, Validation, Investigation, Writing - review & editing, Supervision, Project administration. **David Steiner:** Methodology, Validation, Writing - review & editing. **Milena Stranska:** Conceptualization, Investigation, Writing - review & editing, Project administration. **Rudolf Krska:** Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Michael Sulyok:** Methodology, Validation, Resources, Writing - review & editing, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Conclusion

Following the trend in multi-target analysis, we succeeded in developing and validating an LC-MS/MS based multiclass method for the quantitative determination of >1,400 agro-contaminants. To the best of our knowledge, this is the first comprehensive approach fully validated in two different feed matrices. Several impacts and applications are expected including 1) exact identification of exposures of agro-contaminants in different feed commodities 2) characterise the influence of climate change to future contamination patterns 3) preparation of a prevalence database for the investigation of potential synergistic, additive, or antagonistic effects 4) increase of consumer confidence in feed and subsequently also in food products.

This work was conducted within the Green Area of FFoQSI and consisted of two major objectives. The first aim was the development of a multiclass approach including a full in-house validation in complex feed (Publication #1). With this work, we have shown the possibility of developing modern LC-MS/MS based multi-analyte methods covering a vast majority of natural and anthropogenic contaminants, applicable even for challenging matrices. Previous multiclass methods were either severely limited in their number of analytes or in the number of substance classes included, and targeted data acquisition within MS/MS detection was highlighted as a limiting factor with the rising number of analytes that can be determined in one analytical run. Therefore, an emphasis has been put on the optimisation of HPLC/UHPLC including chromatographic column, flow rate and injection volume, as well as MS/MS conditions. Cycle times and retention windows were carefully optimised in an unprecedented way which ensured appropriate dwell times and reduced the overall measurement error. Pre-validation data revealed that benefits of an UHPLC system with respect to matrix effects may be lost with increasing number of analytes, as the increased peak resolution does not prevent target compounds from overlapping events with matrix components. Furthermore, the provision of sufficient dwell time compared to the increase in data points per peak is prioritised in order to reduce the overall method error. The validated approach was applied to a pilot set of real cattle and chicken feed samples and revealed first co-exposure data on agricultural contaminants.

The second aim was to develop a strategy for preventing compositional uncertainties within feed analysis. Existing recommendations for feed are exclusively based on single feed material

including 10 different feed groups. As a consequence, validation of feed matrices leads to an enormous effort and leaves uncertainties regarding the exact sample properties. A comprehensive feed study was conducted and revealed substantial differences between the analytical performance data when comparing complex compound feed with single feed ingredients. We have developed an economical and straightforward procedure based on the preparation of artificial model samples for the validation of complex feedstuff. This approach takes the heterogenic nature of compound feed into account and ensures a more realistic and accurate method performance. The comparability between model and real feed samples was statistically proven for chicken, pig and cattle feed. Therefore, we suggest a fit-for-purpose validation proposal for LC-MS/MS multiclass methods in complex feed (Publication #2).

The method's applicability was tested within two comprehensive occurrence studies. Focusing on the influence of climate change to the presence of mycotoxins, the first study was investigating the occurrence of regulated (Publication #3) and non-regulated (Publication #4) secondary fungal metabolites in maize samples collected in the Republic of Serbia between 2012 and 2015. Results revealed a significant influence on changing weather conditions to the presence of 20 mycotoxins. In addition, 109 non-regulated secondary fungal metabolites were detected in maize samples from this region for the first time. High contamination levels, exceeding the regulatory limit for aflatoxins and fumonisins in combination with co-occurrence of non-regulated metabolites might pose a potential threat for consumers in this region. The objective of the second occurrence study was to generate first insights into co-exposure of all implemented agro-contaminants in chicken and cattle feed. None of the samples were entirely free of contamination and contained compounds from 3 major substance classes. In particular, high co-occurrence of myco- and phytoestrogens was observed in 91% in chicken and 58% in cattle feed samples.

To conclude, within this doctoral thesis a worldwide unique multiclass method was developed and validated in two compound feed matrices. The validation was based on artificially prepared model samples in order to obtain a realistic picture of the method performance. Generated data from a pilot set of real feed samples revealed first insights into co-exposure patterns of different agro-contaminants. A method expansion and application to other food commodities could therefore serve as the base for assessment of the human dietary exposome.

# List of Scientific Contributions

## International Reviewed Publications

Publication #1

**David Steiner**, Michael Sulyok, Alexandra Malachová, Anneliese Müller, Rudolf Krska (2020) Realizing the simultaneous LC-MS/MS based quantification of >1,200 biotoxins, pesticides and veterinary drugs in complex feed. *Journal of Chromatography A* 1629: 461502

Publication #2

**David Steiner**, Rudolf Krska, Alexandra Malachová, Ines Taschl, Michael Sulyok (2020): Evaluation of Matrix Effects and Extraction Efficiencies of LC-MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed. *Journal of Agricultural and Food Chemistry* 68 (12): 3868-3880

Publication #3

Jovana Kos, Elizabet Janić Hajnal, Alexandra Malachová, **David Steiner**, Milena Stranska, Rudolf Krska, Birgit Poschmaier, Michael Sulyok (2020): Mycotoxins in maize harvested in Republic of Serbia in the period 2012-2015. Part 1: Regulated mycotoxins and its derivatives. *Food Chemistry* 312: 126034.

Publication #4

Elizabet Janić Hajnal, Jovana Kos, Alexandra Malachová, **David Steiner**, Milena Stranska, Rudolf Krska, Michael Sulyok (2020): Mycotoxins in maize harvested in Serbia in the period 2012-2015. Part 2: Non-regulated mycotoxins and other fungal metabolites. *Food Chemistry* 317: 126409.

Publication #5

Mari Eskola, Christopher T. Elliott, Jana Hajšlová, **David Steiner**, Rudolf Krska (2019): Towards a dietary-exposome assessment of chemicals in food: An update on the chronic health risks for the European consumer. *Critical Reviews in Food Science and Nutrition* 60 (11):1890-1911

Publication #6

Michael Sulyok, David Stadler, **David Steiner**, Rudolf Krska (2020): Validation of an LC-MS/MS-based dilute-and-shoot approach for the quantification of > 500 mycotoxins and other secondary metabolites in food crops: challenges and solutions. *Analytical and Bioanalytical Chemistry* 412: 2607–2620.

## Other Publications

Publication #7

**David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska (2019): LC-MS multi-class analytics goes > 1,000. *Chrom + Food Forum* 09: XXI-XXIII

Publication #8

**David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska (2019): LC-MS/MS-Multimethoden Analyse von Kontaminanten in der Lebens- und Futtermittelanalytik. *GIT Labor-Fachzeitschrift*: 9: 21-23; ISSN 0016-3538.

# Oral Presentations at Conferences and Seminars

1. **Seminar/IFA-Tulln**, Center for Analytical Chemistry, Tulln, AUSTRIA, May 18th 2017
   **David Steiner**, Andreas Gschaider, Sonja Sturmberger, Daniela Pantazi, Michael Gartner, Helmut Rost. Development and validation of routine-based confirmation methods in food analysis.

2. **The 6th International Scientific Meeting: Mycology, Mycotoxicology and Mycoses**, Novi Sad, SERBIA, September 27th 2017
   **David Steiner**, Michael Sulyok, Rudolf Krska. Application of LC-ESI-MS/MS based multi-class methods for complex feed matrices.

3. **5th Doc Day (PhD Conference)**, Tulln, AUSTRIA, October 17th 2017
   **David Steiner**, Michael Sulyok, Rudolf Krska. Development of a quantitative multi-class confirmation method based on LC-ESI-MS/MS for the determination of natural contaminants and anthropogenic residues in complex animal feed matrices.

4. **8th International Symposium on Recent Advances in Food Analysis (RAFA)**, Prague, CZECH REPUBLIC, November 9th 2017
   **David Steiner**, Michael Sulyok, David Stadler, Rudolf Krska. LC-ESI-MS/MS multi-class method for the analysis of complex animal feed: Evaluation and reduction of absolute and relative matrix effects.

5. **AGES Workshop nationaler Referenzlaboratorien für Mykotoxine und PAKs**, Linz, AUSTRIA, November 14th 2017
   **David Steiner**, David Stadler, Michael Sulyok. Zeitgemäß oder veraltet - Sind aktuelle Guidelines ausreichend für die Entwicklung von Multimethoden?

6. **3rd Linzer Kontaminantentagung**, Linz, AUSTRIA, December 5th 2017
   **David Steiner**, Michael Sulyok, Rudolf Krska. Simultane Bestimmung von natürlichen Kontaminanten und anthropogenen Rückständen in Tierfutter mittels LC-ESI-MS/MS.

7. **Seminar/IFA-Tulln**, Center for Analytical Chemistry, Tulln, AUSTRIA, January 17th 2018

   **David Steiner**, Uta Kachelmeier, Caroline Stadlmann, Wolfgang Kandler, Andrea Koutnik. Accreditation of the IFA-Test Systems PT-Scheme: Establishing a QM-System in accordance to ISO/IEC 17043.

8. **Food Integrity Postgraduate Workshop**, Belfast, NORTHERN IRELAND, February 13th 2018

   **David Steiner**, Michael Sulyok, Lidija Kenjerić, Rudolf Krska. Development and validation of a multi-class LC-ESI-MS/MS-based method (>1,400 analytes) in animal feed.

9. **Junganalytikerforum**, Vienna, AUSTRIA, May 4th 2018

   **David Steiner**, Michael Sulyok, Lidija Kenjerić, Rudolf Krska. Going beyond 1,000: pushing LC-MS/MS multi-targeted analysis to the next level.

10. **40th Mycotoxin Workshop**, Munich, GERMANY, June 12th 2018

    **David Steiner**, Michael Sulyok, Lidija Kenjerić, Rudolf Krska. Animal feed matrices: How to get control of a complex analytical problem in LC-MS/MS?

11. **Mycotoxin Summer Talks**, Tulln, AUSTRIA, July 6th 2018

    **David Steiner**, Michael Sulyok, Leonardo Mariño-Repizo, Lidija Kenjerić, Rudolf Krska. Challenges in development of multi-class methods for natural and anthropogenic contaminants in complex compound feed.

12. **4th International Congress on Food Technology, Quality and Safety**, Novi Sad, SERBIA, October 24th 2018

    **David Steiner**, Michael Sulyok, Leonardo Mariño-Repizo, Lidija Kenjerić, Rudolf Krska. The Matrix Effect Issue: Modern strategies for development of multi-targeted LC-MS/MS approaches.

13. **FFoQSI Annual Assembly**, Tulln, AUSTRIA, November 7th 2018

    **David Steiner**, Michael Sulyok, Rudolf Krska. Development, validation and application of advanced multi-toxin analytical methods with special focus on globalisation and climate change.

14. **2nd MultiCoop Training School on Challenges in Food and Feed Safety Research**, Prague, CZECH REPUBLIC, November 28th 2018
    **David Steiner**, Michael Sulyok, Leonardo Mariño-Repizo, Lidija Kenjerić, Rudolf Krska. Detection and elimination strategies of matrix effects in quantitative multi-target LC-ESI-MS/MS analysis.

15. **FFoQSI Annual Assembly**, Vienna, AUSTRIA, October 10th 2018
    **David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska. Advanced multi-toxin methods - development, validation and application, with special focus on globalisation and climate change.

16. **The World Mycotoxin Forum and the IUPAC International Symposium on Mycotoxins**, Belfast, NORTHERN IRELAND, October 16th 2019
    **David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska. LC-MS/MS based quantitative multi-target approach for food and feed: crossing the limit of 1000 metabolites.

17. **9th International Symposium on Recent Advances in Food Analysis (RAFA)**, Prague, CZECH REPUBLIC, November 6th 2019
    **David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska. Scratching on the edge: Development of a quantitative multi-target LC-MS/MS method for the determination of >1,400 pesticides, veterinary drugs, fungal metabolites and plant toxins in food and feed.

18. **4th Linzer Kontaminantentagung**, Linz, AUSTRIA, December 3rd 2019
    **David Steiner**, Michael Sulyok, Alexandra Malachová, Rudolf Krska. LC-MS/MS basierte analytische Lösungsansätze für die Validierung komplexer Futtermittel.

## Poster Presentations at Scientific Conferences

1. **10th World Mycotoxin Forum**, Amsterdam, NETHERLANDS, March 12th to 14th 2018
    **David Steiner**, Michael Sulyok, Lidija Kenjerić, David Stadler, Rudolf Krska. Quantitative analysis of natural contaminants and anthropogenic residues in animal feed by LC-ESI-MS/MS: Influence of absolute and relative matrix effects on the method performance.

# DAVID STEINER, M.SC.

## PERSONAL INFORMATION

DATE OF BIRTH:
September 26, 1986

PLACE OF BIRTH:
Wolfsberg, Carinthia

CITIZENSHIP:
Austria

## CONTACT

LINKEDIN:
https://www.linkedin.com/in/david-steiner-msc-1bb956142/

ORCID:
https://orcid.org/0000-0002-7941-2790

## DRIVING LICENCE

CLASS B

## MILITARY SERVICE

fulfilled (Oct. 2005 – Mar. 2006)

## EDUCATION

**PhD study of "Food Chemistry and Biotechnology" at University of Natural Resources and Life Sciences, Vienna**
since April 2017

Title of the thesis: "Development, validation and application of an advanced LC-MS/MS based multiclass method for the analysis of animal feed with a special focus on globalisation and climate change"

**Master study of "Nutritional Science" with special focus on molecular nutrition at University of Vienna**
January 2015 – January 2017

Title of the thesis: "Development and validation of an LC-MS/MS based analytical confirmation method for mycotoxins in plant-based foodstuff"

**Bachelor study of "Nutritional Science" at University of Vienna**
March 2007 – December 2014

Title of the thesis: "Toxicological characteristics of biogenic amines and their role in human nutrition"

**Business Academy, Wolfsberg**
September 2000 – June 2005

## WORK EXPERIENCE

**Romer Labs Diagnostic GmbH, Tulln Austria – Team Lead Mycotoxin Analytic**
since September 2020

- routine analysis of mycotoxins in feed and food by LC-MS/MS according to ISO 17025
- development of new and improvement of existing mycotoxin confirmatory methods
- customer communication and consultancy in selection of testing methods and interpretation of reports

**FFoQSI GmbH, Tulln Austria – Associate Research Scientist**
July 2017 – August 2020

- associate research scientist in work package: on-field/plant quality assurance – LC-MS/MS based enhanced toxin analysis
- leading researcher in multi-mycotoxin LC-MS/MS method development and transfer to the international pet food industry

## LANGUAGE SKILLS

GERMAN:

native language

ENGLISH:

business fluent

## TECHNICAL SKILLS

SOFTWARE SKILLS:

Agilent Mass Hunter
Sciex-OS, Multiquant, Analyst
MS Office 2013
Photoshop C6

INSTRUMENTAL SKILLS:

Agilent LC (1290 Infinity series)
Agilent TQMS (6470 & 6490)
SCIEX TQMS (QTrap 4000, 4500, 5500, 6500)
SCIEX QTOF (X500R)

## HOBBIES

TRAVELING:

especially city trips and "natural wonders" e.g. Aurora Borealis (Norway 2019), Iron Gate and Rajačke Pimnice (Serbia 2018), Krimmler Wasserfälle (Austria 2017), Koh Tao (Thailand 2013)

SPORTS:

Oct. 2010 – Oct. 2012
football youth coach at ASV 13, Vienna

Oct. 2003 – Jul. 2005
football player at SV Lavamünd, Carinthia

## HONORARY POSTS

since Dec. 2018
director of the national-wide theme initiative "environment and sustainability", Vienna

**University of Natural Resources and Life Sciences, Tulln Austria – Research Associate & Project coordinator**
April 2017 – September 2018

- global pattern assessment of fungal metabolites in a variety of plant-based matrices in cooperation with industrial partner Biomin GmbH
- coordination of the successful accreditation processes for the IFA-Test systems PT scheme according to ISO 17043

**LVA GmbH, Klosterneuburg Austria – Analytical Specialist**
October 2015 – March 2017

- mycotoxin and pesticide LC-MS/MS based method development and analysis following ISO 17025 within the R&D and residue analysis departments

## SECONDARY EMPLOYMENTS & INTERNSHIPS

**TBB Bauer & Bauer GmbH, Vienna Austria – Engineer for cleanroom technology**
December 2012 – January 2015 (*fulltime/intermittently*)

- qualification of RLT systems according to ÖNORM EN ISO 14644-1 and VDI 2083 at Octapharma GmbH, Vienna

**Uniport, Vienna Austria – Part-time worker**
August 2010 – November 2015

- team leader at career events
- coordination of on-campus promotional activities

**MONDI GmbH, Frantschach Austria**
July and August between 2009 – 2011

- project "PE-roadmap" within the R&D Innovation center

## TRAININGS AND SEMINARS

| | |
|---|---|
| June 2018 | Completion of the course "Environmental Politics" at the Vienna Education Academy (WBA) AUSTRIA |
| November 2018 | Completion of an Ion Mobility training course Prague CZECH REPUBLIC |
| March 2017 | Agilent Forum Analytik Vienna AUSTRIA |
| October 2016 | AGES workshop for national reference laboratories Linz AUSTRIA |
| April 2016 | Symposium on "Innovations in Food & Feed Safety Tulln AUSTRIA |
| November 2015 | AGES workshop for national reference laboratories Linz AUSTRIA |
| March 2015 | ANAKON 2015 Graz AUSTRIA |