THE CHALLENGE OF LC/MS/MS MULTI-MYCOTOXIN ANALYSIS –HERACLES BATTLING THE HYDRA?

Meri Kokkonen

Academic Dissertation

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Custos: Professor Vieno Piironen
Department of Food and Environmental Sciences
University of Helsinki
Helsinki, Finland

Supervisors: Professor Kimmo Peltonen
Chemistry and Toxicology Research Unit
Finnish Food Safety Authority
Helsinki, Finland

Doctor Marika Jestoi
Chemistry and Toxicology Research Unit
Finnish Food Safety Authority
Helsinki, Finland

Reviewers: Docent Tuulia Hyötyläinen
VTT Technical Research Centre of Finland
Espoo, Finland

Associate Professor Kristian Fog Nielsen
Department of Systems Biology
Technical University of Denmark
Lyngby, Denmark

Opponent: Associate Professor Alberto Ritieni
Department of Food Science
University of Naples Federico II
Portici, Italy

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ABSTRACT

Mycotoxins are secondary metabolites of filamentous fungi. They pose a health risk to humans and animals due to their harmful biological properties and common occurrence in food and feed. Liquid chromatography/mass spectrometry (LC/MS) has gained popularity as a feasible analytical technique in the analysis of food contaminants including mycotoxins. In this study, the applicability of the technique was evaluated in multi-residue methods aiming at simultaneous determination of several chemically different mycotoxins produced by fungal genera of *Aspergillus*, *Fusarium*, *Penicillium* and *Claviceps*.

Multi-residue methods were developed for determining mycotoxins from cheese, cereal based agar matrices and grains. A method for selective determination of ergot alkaloids from grains was also devised. The analytical procedures were simple allowing rapid analysis of the mycotoxins of interest. Analytes were extracted from the sample matrices with organic solvents. Minimal sample clean-up was carried out before the determination of the mycotoxins with reversed phase LC coupled to tandem MS utilizing electrospray ionization (ESI-MS/MS). The methods were in-house validated and applied for investigating mycotoxin levels in cheese and ergot alkaloid occurrence in grains. Additionally, the mycotoxin production of *F. sporotrichioides* and *F. langsethiae* isolated from grains was studied.

Nine mycotoxins could be determined from cheese matrix with the method developed. The limits of quantification (LOQ) allowed the quantification at concentrations varying from 0.6 to 5.0 μg/kg. The recoveries ranged between 96 and 143 %, and the within-day repeatability (as relative standard deviation, RSD_r) between 2.3 and 12.1 %. Roquefortine C and mycophenolic acid could be detected at levels of 300 up to 12000 μg/kg in the mould cheese samples analysed.

A total of 29 or 31 mycotoxins were included in the method developed for cereal based agar matrices and grains, respectively. The LOQs from agars ranged from 0.1 to 86 μg/kg while being from 1.0 to 1250 μg/kg from grains. The recoveries from both types of matrices ranged generally between 44 and 139 %, and the RSD_r between 2.0 and 38 %. The values of certain problematic compounds, such as fumonisins, were outside these ranges. Type-A trichothecenes and beauvericin were determined from the agar and grain cultures of *F. sporotrichioides* and *F. langsethiae*. T-2 toxin was the main metabolite of both species, with the average levels reaching 22000 μg/kg measured from the grain cultures after an incubation period of 28 days.

The method developed for the determination of ten ergot alkaloids from grains allowed their quantification at concentrations varying from 0.01 to 10 μg/kg. The recoveries ranged from 51 to 139 %, and the RSD_r from 0.6 to 13.9 %. Ergot alkaloids were measured in rye and barley samples at average levels of 720 and 59 μg/kg,

respectively. All ten ergot alkaloids could be detected, with the two most prevalent being ergocornine and ergocristine.

This study contributes to the area of mycotoxin analysis by presenting novel multi-residue methods. The methods developed employ LC-MS/MS techniques for the determination of chemically diverse mycotoxins from complex food and agar matrices. Rapid and simple analytical procedures with reduced sample pre-treatment were achieved. Generally, the performance of the methods was good, allowing reliable, repeatable and selective analysis of the mycotoxins of interest with sufficiently low quantification limits. With the aid of these methods, new information was obtained about the occurrence of mycotoxins in mould cheeses and ergot alkaloids in Finnish grains. In addition, the study revealed the high mycotoxin-producing potential of two common fungi in Finnish crops, *F. sporotrichioides* and *F. langsethiae*.

PREFACE

This study was carried out at Chemistry and Toxicology Research Unit at Finnish Food Safety Authority, Evira. The work was funded by Evira, Ministry of Agriculture and Forestry (Dnro 4759/501/2004 and 4775/501/2005) and the Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment (ABS). Additional funding was obtained from The Raisio plc Research Foundation, Walter Ehrström Foundation and Finnish Concordia Fund. The financial support of all these bodies is gratefully acknowledged.

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My deepest thanks are due to my family who has been supporting me in life in so many ways. I also thank my dear friends Helena, Minna-Maria and Sanna from the bottom of my heart for sharing the ups and downs in this process!

Greek mythology tells a story of Heracles' battle with the multi-headed Lernean hydra. The multi-mycotoxin analysis with LC-MS/MS often resembled that struggle; once you got one analytical problem solved (at least) two new emerged! And like it was with Heracles, sometimes a different strategy and help from friends had to be sought to accomplish the task. Finally however, both stories ended well.

Helsinki, September 2011

LIST OF ORIGINAL PUBLICATIONS

I Kokkonen, M., Jestoi, M. and Rizzo, A. 2005. Determination of selected mycotoxins in mould cheeses with liquid chromatography coupled to tandem with mass spectrometry. *Food Additives and Contaminants, 22 (5): 449-456.*

II Kokkonen, M. and Jestoi, M. 2009. A multi-compound LC-MS/MS method for the screening of mycotoxins in grains. *Food Analytical Methods, 2 (2):128-140.*

III Kokkonen, M., Ojala, L., Parikka, P. and Jestoi, M. 2010. Mycotoxin production of selected *Fusarium* species at different culture conditions. *International Journal of Food Microbiology, 143:17-25.*

IV Kokkonen, M., Laitila, A. and Jestoi, M. 2011. Mycotoxin production of *Fusarium sporotrichioides* and *Fusarium langsethiae* on cereal-based substrates. *Mycotoxin Research. Accepted manuscript.*

V Kokkonen, M. and Jestoi, M. 2010. Determination of ergot alkaloids from grains with UPLC-MS/MS. *Journal of Separation Science, 33(15):2322-2327.*

CONTRIBUTION OF THE AUTHOR IN PAPERS I-V

I Meri Kokkonen planned the study and prepared the manuscript together with the other authors. She carried out the experimental work and had the main responsibility for interpreting the results and hence she was the main author of the manuscript.

II Meri Kokkonen planned the study together with Dr. Marika Jestoi. Meri Kokkonen was responsible for the experimental part as well as for interpretation of the results. Thus, she was the main author of the manuscript.

III Meri Kokkonen, M. Sc. Päivi Parikka and Dr. Marika Jestoi planned the study together. M. Sc. Laura Ojala carried out most of the experimental work. The interpretation of the result data was the responsibility of Meri Kokkonen and she prepared the first draft of the manuscript and was therefore the main author of the manuscript.

IV Meri Kokkonen planned the study together with Dr. Arja Laitila and Dr. Marika Jestoi. The experimental work was carried out by Meri Kokkonen and M. Sc. student Sini Elonen. Meri Kokkonen had the main responsibility for interpreting the results and she prepared the first draft of the manuscript, and hence she was the main author of the manuscript.

V Meri Kokkonen planned the study and prepared the first draft of the paper together with Dr. Marika Jestoi. Meri Kokkonen was responsible for the experimental work as well as for the interpretation of the results. Thus, she was the main author of the manuscript.

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LIST OF ABBREVIATIONS

AcDON	acetyl deoxynivalenol
ACN	acetonitrile
ANT Y	antibiotic Y
AF	aflatoxin
API	atmospheric pressure ionization
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photo ionization
ASE	accelerated solvent extraction
a_w	water activity
BEA	beauvericin
CID	collision induced dissociation
CIT	citrinin
CPA	cyclopiazonic acid
CRM	certified reference material
d-SPE	dispersive solid phase extraction
DAS	diacetoxyscirpenol
DART	direct injection in real time
DCM	dichloromethane
DON	deoxynivalenol
EAMINE	ergotamine
EAMININE	ergotaminine
ECORN	ergocornine
ECORN-INE	ergocorninine
ECRIS	ergocristine
ECRIS-INE	ergocristinine
ECRYPT	ergocryptine
ECRYPT-INE	ergocryptinine
EMETR	ergometrine
ESINE	ergosine
ENN	enniatin
ERG	ergot alkaloid
EU	European Union
ESI	electrospray ionization
EtOAc	ethyl acetate
FB	fumonisin
F-X	fusarenon X
GLIO	gliotoxin

HPLC	high performance liquid chromatography
HT-2	HT-2 toxin
IA	immunoaffinity
i.d	internal diameter
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
MON	moniliformin
MPA	mycophenolic acid
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NEO	neosolaniol
NIV	nivalenol
OT	ochratoxin
PA	penicillic acid
PLE	pressurized liquid extraction
PSA	primary secondary amine
QuEChERS	quick, easy, cheap, effective, rugged and safe
QqQ	triple quadrupole
RM	reference material
ROQ C	roquefortine C
RP	reversed phase
RSD	relative standard deviation
SD	standard deviation
SPE	solid phase extraction
SSE	signal suppression/enhancement
T-2	T-2 toxin
TOF	time of flight
TR	trichothecene
UHPLC	ultra high performance liquid chromatography
ZEN	zearalenone

CONTENTS

1 INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, most commonly of the genera *Aspergillus*, *Fusarium* and *Penicillium*, other important producers being *Claviceps* and *Alternaria* (Bhat et al. 2010). Currently, several hundreds of mycotoxins have been characterized. They are low molecular weight compounds with great structural variation resulting in diverse physicochemical properties (Köppen et al. 2010). Mycotoxins pose a hazard to human and animal health due to the harmful biological effects they possess. These effects vary from general symptoms, like immunosuppression, weight loss and nausea, to more severe such as oestrogenic, teratogenic, mutagenic and carcinogenic effects (Prelusky et al. 1993; D'Mello et al. 1999).

Mycotoxins occur mostly in agricultural products but may be carried over also to animal products due to consumption of contaminated feed. Small-grain cereals and maize are susceptible to fungal and mycotoxin contamination, and especially aflatoxins, ochratoxin A, fumonisins, zearalenone and trichothecenes are frequently encountered (Bhat et al. 2010). Different types of mycotoxins produced by a single or several fungal species may be present simultaneously in a commodity, which is of importance since exposure to mixtures of mycotoxins may have unexpected synergistic or additive toxic effects differing from those of an individual compound (Speijers and Speijers 2004). To protect consumers and to control the mycotoxin occurrence in products intended for human or animal consumption, regulatory authorities have set limits for maximum residue levels of several toxins (van Egmond et al. 2007). This work is on-going, for example new limits are under consideration in the European Union.

The effective monitoring and research into mycotoxins requires relevant and reliable analytical methods. The determination of mycotoxins from food and feed is challenging as the compounds are typically present at low concentrations in complex sample matrices (Köppen et al. 2010). A wide range of analytical methods have been developed for the analysis of mycotoxins over the years but until recently, the methods have been limited to single compounds or at best to groups of structurally related compounds (Zöllner and Mayer-Helm 2006; Köppen et al. 2010). However, with the development of analytical instruments, multi-residue methods with the aim of simultaneous detection of a large number of co-occurring, chemically diverse compounds have gained popularity in food safety research, and this extends to mycotoxins (Malik et al. 2010). With these types of methods, comprehensive information about the quality of a sample can be obtained with a single method.

Additionally, the objectives of the methods are the high throughput of samples and the savings in costs and time when compound or group specific methods can be replaced with a multi-residue method (Mol et al. 2008).

High performance liquid chromatography (HPLC), or the recently introduced ultra high performance LC (UHPLC), coupled to mass spectrometry (MS) has become the method of choice in multi-residue analysis due to the high selectivity and sensitivity of the technique (Malik et al. 2010). Liquid chromatography is a suitable method for separating complex mixtures of compounds with a wide range of chemical polarities and molecular masses, and mass spectrometry enables the detection with high sensitivity and selectivity (Boyd et al. 2008). The applicability of the LC/MS based techniques is recognized, for example in separating and detecting up to 106 mycotoxins from various commodities, most typically from small-grain cereals, maize and related products (e.g. Sulyok et al. 2006;2010; Spanjer et al. 2008). Versatile analytical approaches have been described in the multi-mycotoxin methods in the literature. Sample treatment typically involves liquid extraction of analytes with the aid of polar solvents and clean-up of the extract with techniques based on solid phase extraction (e.g. Lattanzio et al. 2007; Monbaliu et al. 2009; Zachriasova et al. 2010). One novel trend is to attempt to reduce sample pre-treatment, and to inject food and feed extracts with minimum or no clean-up (Sulyok et al. 2006). Commonly, LC coupled to tandem MS (MS/MS) utilizing an electrospray ionization interface and quadrupole based mass analysers has been found to be reliable for the quantitative, targeted determination of the mycotoxins of interest at sufficiently low concentration levels (e.g. Sulyok et al. 2006; Spanjer et al. 2008; Martos et al. 2010). Methods based on time of flight and Orbitrap detectors have also been introduced, and these high mass resolution-high mass accuracy instruments are likely to become more common in the future (Tanaka et al. 2006; Malik et al. 2010; Zachariasova et al. 2010).

This literature review provides an overview of the chemistry, biological effects and occurrence of mycotoxins as well as considers the analytical challenges encountered in the multi-residue determination of mycotoxins. Approaches to sample preparation, and separation and detection of mycotoxins with multi-residue methods based on the LC/MS technique will be discussed. The methods used in mycotoxin research need to be improved constantly utilizing the modern techniques in order to improve the method performance and the usability in routine analysis. This research contributes to the area by presenting novel analytical methods capable of providing reliably and rapidly information on a number of toxins simultaneously. In the experimental part, the development of LC-MS/MS based multi-mycotoxin methods for the determination of toxins of *Aspergillus*, *Fusarium*, *Penicillium* and *Claviceps* is described and the suitability of these methods for quantitative analysis is evaluated. The methods were applied in food safety applications where co-

occurrence of several mycotoxins in samples was expected, i.e. to examine mycotoxin occurrence in commodities and to study toxin production by *Fusarium* fungi on agar substrates and grains.

2. LITERATURE REVIEW

2.1 MYCOTOXINS

Mycotoxins are natural substances that are produced by filamentous fungi and that can evoke a toxic response in higher vertebrates and other animals when consumed even at low concentrations (D'Mello et al. 1999; Bhat et al. 2010). These compounds are usually not required for the growth of fungi and are therefore considered as secondary metabolites (Bhat et al. 2010). A number of fungal genera are able to synthesize mycotoxins. The three genera of *Aspergillus*, *Penicillium* and *Fusarium* comprise the largest number of mycotoxin producing species. Other toxigenic genera include *Claviceps* and *Alternaria* (Bhat et al. 2010). The exact function of mycotoxins for fungi is not clearly understood, but their production is associated with the ecology and survival of the organism under different conditions (Magan and Aldred 2007). The growth and toxin production of fungi may take place at different stages of crop production; either in field before harvest or during storage and transportation if the conditions are favourable (Bhat et al. 2010). The secondary metabolism of the fungi is regulated by genetic mechanisms which respond to stimuli from the environment (Shwab and Keller 2008), temperature with water activity being the main environmental factor affecting the production of mycotoxins (Sanchis and Magan 2004).

2.1.1 CHEMISTRY AND BIOLOGICAL EFFECTS

Several hundreds of mycotoxins have been characterized so far, and they show significant diversity in their molecular structures (Figure 1) and chemical properties (Bhat et al. 2010). A broad range of secondary metabolites is derived from common biosynthetic origins. Most of the known mycotoxins originate from the three main pathways (or their combinations), which are 1) the polyketide pathway, 2) the isoprenoid pathway and 3) amino acid metabolism (ApSimon 1994; Huffman et al. 2010). Even though certain mycotoxins may share a common pathway, they can be structurally very different. For example, zearalenone (ZEN), fumonisins (FBs) and moniliformin (MON) produced by *Fusarium*, and aflatoxins (AFs) and ochratoxins (OTs) of *Aspergillus* and *Penicillium* genera all originate from the polyketide pathway (ApSimon 1994; Huffman et al. 2010). These mycotoxins are derived

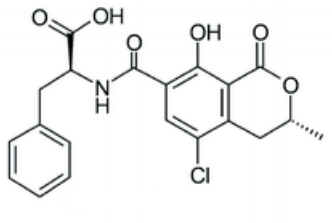
by repetitive condensation of acetate units or other short carboxylic acids, which subsequently undergo reductive and cyclization processes catalysed by polyketide synthase enzymes (Huffman et al. 2010). Fumonisins are polar, relatively large mycotoxins (molecular weight > 700 Da) consisting of C-20 diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane with a primary amino group (Figure 1). Moniliformin is also a highly polar compound, but it has a molecular weight of only 98 Da and a structure of a 3-hydroxycyclobut-3-ene-1,2,-dione (Figure 1). MON occurs usually as a potassium or sodium salt. Further, zearalenones are defined as macrocyclic lactones, whereas aflatoxins are difuranocoumarin derivatives and ochratoxins are isocoumarins linked to L-β-phenylalanine (Figure 1). These compounds have moderate polarity and molecular weights of around 300 to 400 Da.

Trichothecenes (TRs), which are produced mainly by *Fusarium* species, originate from the isoprenoid biosynthetic pathway (ApSimon 1994). They are formed from cyclisation of farnesyl pyrophosphate to trichodiene followed by a number of oxygenations, isomerisations, cyclisations and esterifications to form eventually tricyclic sesquiterpenes that possess a 12,13-epoxy-trichothec-9-ene ring (Tamm and Breitenstein 1980; Kimura et al. 2007). Trichothecenes are a large group of compounds, of which the type-A and B classes are the most commonly occurring in nature, generally with molecular weights between 300 and 500 Da. The difference in their structure is the carbonyl group in the position C-8 of the type-B structure that is missing in the type-A TRs (Figure 1).

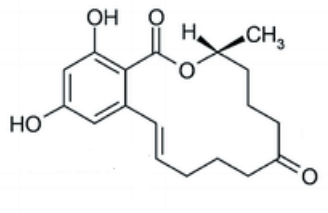
The amino acid derived mycotoxins are exemplified by the *Fusarium* metabolites enniatins (ENNs) and the closely related beauvericin (BEA) (ApSimon 1994) as well as ergot alkaloids (ERGs) produced by *Claviceps* (Floss and Anderson 1980). ENNs and BEA are cyclic hexadepsipeptides consisting of alternating D-α–hydroxy-isovaleryl and amino acid units (L-phenylalanine in BEA and valine or isoleucine in ENNs) (Figure 1). They are apolar compounds with molecular weights of > 640 Da. Ergot alkaloids are derivatives of the tetracyclic ergoline (Floss and Anderson 1980). The most pharmacologically active forms are amides of D-lysergic acid, which can be either 1) non-peptide amides of lysergic acid (e.g. ergometrine, syn. ergonovine, molecular weight 325 Da) or 2) peptide alkaloids (e.g. ergosine, ergocristine, molecular weight ≥ 547 Da) which have a peptide moiety linked to the tetracyclic ergoline (Figure 1). The lysergic acid/ergoline ring structure is formed from tryptophan and a five-carbon isoprene unit derived from mevalonate and the N-methyl group supplied by methionine, whereas the peptide moiety consists of a proline unit and other amino acids incorporated into the amino acid and hydroxyamino acid positions (Floss and Anderson 1980). Ergot alkaloids have a chiral carbon atom in the C-8 position leading to natural occurrence of two epimers, i.e. (*R*)- and (*S*)-diastereomers of which the former are the biologically more active forms (Berde and Stürmer 1978).

Mycotoxins are known to exert a broad range of biological effects in humans and animals (summarized in Table 1), and this subject has been reviewed comprehensively by several authors (e.g. D'Mello et al. 1999; Hussein and Brasel 2001; Pfohl-Leszkowicz and Manderville 2007; Jestoi 2008). Mycotoxins can be toxic either acutely or on chronic exposure. The magnitude of their toxicity depends on several factors e.g. the level, duration and route of exposure as well as mechanism of action and metabolism in the body, and sensitivity of the target animal (Hussein and Brasel 2001). Several mycotoxins can impair the immune system of humans and animals, which may consequently lead to exposure to other diseases and mask underlying toxicoses (Prelusky et al. 1993). Mycotoxicoses are generally difficult to diagnose since the biological effects of mycotoxins are diverse and often unspecific, causing symptoms such as nausea, loss of appetite, reduced feed intake and reduced bodyweight (Prelusky et al. 1993).

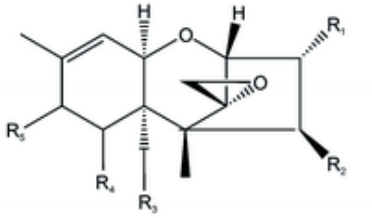
The toxicological effects of several mycotoxins, such as aflatoxins, fumonisins, deoxynivalenol (DON) and ochratoxin A (OTA) are well-known (Hussein and Brasel 2001), whereas the toxic mechanisms have not been elucidated thoroughly for the fungal metabolites characterized more recently, including MON, BEA and enniatins (Jestoi 2008). With respect to MON, symptoms of acute toxicity have been demonstrated, including muscular weakness, respiratory stress and degeneration and histopathological changes of cardiac muscle and other organs. The toxicity of the less widely investigated BEA and ENNs has been considered principally due to their antimicrobial and insecticidal properties and the effects demonstrated mostly only at cell level (reviewed in Jestoi 2008; Peltonen et al. 2010). On the other hand, AFs, FBs and OTA are regarded as the most toxic of the known mycotoxins. They are mutagenic and teratogenic, i.e. they may cause alterations in genetic material and impair the development of embryo or foetus (EFSA 2004b;2005; Pfohl-Leszkowicz and Manderville 2007). AFs have been demonstrated to cause liver cancer and are classified as human carcinogens belonging to Group 1 according to International Agency for Research on Cancer (IARC 1993;2002). Fumonisins and OTA are probable human carcinogens of group 2B (IARC 1993;2002). FBs have been linked to liver cancer in rats and epidemics of human oesophageal cancer, whereas OTA may cause tumours in kidneys and urinary tract (Pfohl-Leszkowicz and Manderville 2007; van der Westhuizen et al. 2010).



a) Ochratoxin A

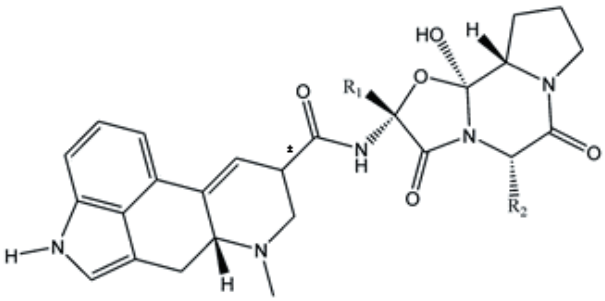


b) Zearalenone

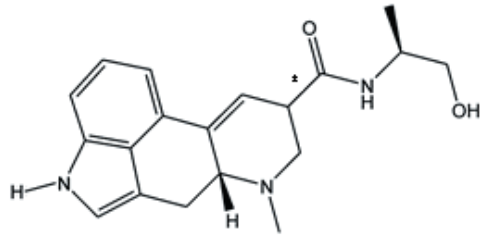


c) Trichothecenes

Trichothecene		R1	R2	R3	R4	R5
Type A	T-2 toxin	OH	OAc	OAc	H	OCOi-Bu
	HT-2 toxin	OH	OH	OAc	H	OCOi-Bu
	Diacetoxyscirpenol	OH	OAc	OAc	H	H
	Neosolaniol	OH	OAc	OAc	H	OH
Type B	Deoxynivalenol (DON)	OH	H	OH	OH	=O
	Nivalenol	OH	OH	OH	OH	=O
	3-Acetyl-DON	OAc	H	OH	OH	=O
	15-Acetyl-DON	OH	H	OAc	OH	=O
	Fusarenon-X	OH	OAc	OH	OH	=O

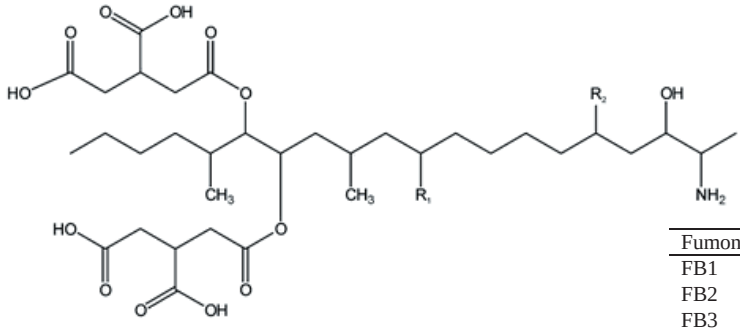


d) Ergot alkaloids (*stereo bond)



e) Ergometrine (*stereo bond)

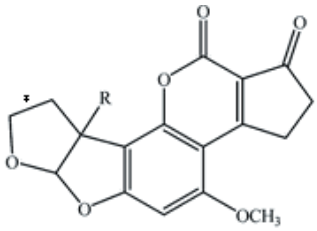
Ergot alkaloid	R1	R2
Ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergoristine	$CH(CH_3)_2$	$CH_2C_6H_5$
Ercocryptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergosine	CH_3	$CH_2CH(CH_3)_2$
Ergotamine	CH_3	$CH_2C_6H_5$



Fumonisin (FB)	R1	R2
FB1	OH	OH
FB2	H	OH
FB3	OH	H

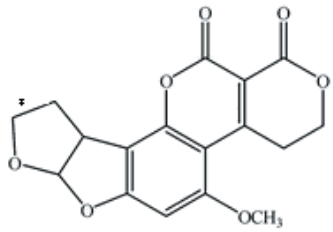
f) Fumonisins

Figure 1. Molecular structures of some mycotoxins.



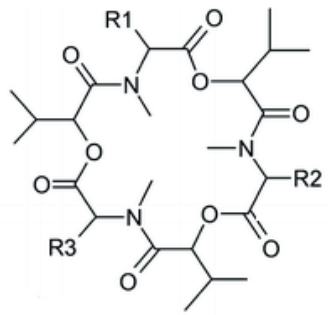
g) Aflatoxins B and M

Aflatoxin (AF)	R	Bond type*
AF B1	H	double
AF B2	H	single
AF M1	OH	double
AF M2	OH	single



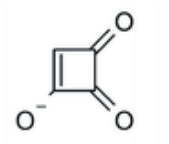
h) Aflatoxins G

Aflatoxin (AF)	Bond type*
AF G1	double
AF G2	single



Enniatin (ENN) / Beauvericin (BEA)	R1	R2	R3
ENN A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
ENN A1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
ENN B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
ENN B1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$	$CH(CH_3)_2$
BEA	$CH_2C_6H_5$	$CH_2C_6H_5$	$CH_2C_6H_5$

i) Enniatins and beauvericin



j) Moniliformin

Figure 1. (continued)

It has been demonstrated that mixtures of mycotoxins can exert combined toxicological effects which cannot be necessarily predicted on the basis of the known toxicity of the single substances (D'Mello et al. 1999; Speijers and Speijers 2004). Until recently, toxicity, exposure and risk assessments have focused on one chemical and one exposure route at a time. However, it is increasingly recognized that every day we are exposed to numerous chemicals by ingestion, inhalation and dermal absorption (Borchers et al. 2010). Mixture toxicology, that investigates these complex interactions of chemicals, is emerging in the field of mycotoxicology although there are still few well designed, systematic studies to assess the combined toxic effects (Tammer et al. 2007). However, the findings indicate that the effects of mycotoxins can be additive to each other or they can interact in synergistic (potentiating) or antagonistic way (Speijers and Speijers 2004).

The studies of combined toxic effects have been performed using animal models (Smith et al. 1997; Theumer et al. 2003; Wangikar et al. 2004; Grimes et al. 2010; Tan et al. 2011). However, these studies often lack the explicit theory about what is an additive or synergistic effect. It has been demonstrated that the combined impact of mycotoxins on histopathological changes, feed intake and weight gain can be more severe than that of individual toxins (Smith et al. 1997; Grimes et al. 2010). OTA and citrinin (CIT) have been suggested acting synergistically or additively on renal carcinogenicity in mice and toxicity on chicken embryos (Vesela et al. 1983; Kanisawa 1984), whereas an antagonistic effect has been reported for AF B1 and OTA on teratogenicity in rabbits (Wangikar et al. 2004). Furthermore, both ZEN and T-2 toxin (T-2) have been associated with reproductive problems and thus have been suggested to act synergistically on the fertility of the animals (D'Mello et al. 1999).

In vitro tests have also been used for investigating mycotoxin mixture toxicity (Thuvander et al. 1999; Tajima et al. 2002; Heussner et al. 2006; Tammer et al. 2007; Mwanza et al. 2009; Sugiyama et al. 2011). For example, cytotoxicity has been demonstrated as being additive for OTA, OTB, CIT and patulin (PAT) in a porcine renal cell line. The synergistic effect of CIT and OTA was also clear (Heussner et al. 2006). A synergistic effect of OTA and FB1 on the decrease of viability of human and pig lymphocytes has been demonstrated (Mwanza et al. 2009). Furthermore, an additive immunotoxic effect of DON and nivalenol (NIV) was reported in mouse macrophages (Sugiyama et al. 2011). The toxicity of fungal extracts and cereal sample extracts with a known content of mycotoxins has been examined in cell assays (Morrison et al. 2002; Jestoi et al. 2004b; Uhlig et al. 2006). These types of studies provide an estimate of the total toxicity of the extract but it may be difficult to demonstrate the correlation between the toxicity and the mycotoxin concentrations.

Controversy of which fungal metabolites should be called as mycotoxins exists due to lack of evidence of the stability, bioavailability and toxicity of these compounds *in vivo*, i.e. via natural route of exposure. For example, the term mycotoxin when

referring to enniatins or *Alternaria* toxins could be questioned in this respect. However in this thesis, the term mycotoxin is used in a broad sense to encompass various fungal metabolites due to simplicity and the fact that the main perspective is on the analytical aspects of these compounds.

2.1.2 OCCURRENCE IN COMMODITIES AND LEGISLATION

Mycotoxins occur in a wide range of commodities globally, with the highest prevalence being encountered in agricultural products. Small-grain cereals and maize, and products produced from these materials, are frequently contaminated, most commonly with aflatoxins, ochratoxin A, fumonisins, zearalenone and trichothecenes (Table 1). The prevalence and contamination levels of mycotoxins vary greatly according to several factors e.g. geographic location, commodity and harvest year (Gareis et al. 2003; Binder et al. 2007; Scudamore and Patel 2009). In Europe, small-grain cereals often contain trichothecenes and ZEN (Placinta et al. 1999; Eskola et al. 2001; Schollenberger et al. 2006). In particular, T-2 and HT-2 toxins are becoming prevalent in the temperate regions of Europe, and especially oats can contain high levels (Edwards 2009; Edwards et al. 2009). In addition, OTA can be detected in European crops, although the levels are relatively low (Miraglia et al. 2002). Other mycotoxins prevalent especially in Northern European climate include BEA, ENNs and MON (Uhlig et al. 2007). In Norwegian and Finnish small grain cereals, median levels of <3 to 240 μg/kg of MON and BEA have been reported, and concentrations of individual ENNs up to 18300 μg/kg have been found (Uhlig et al. 2007). The recently collected data demonstrate that also ERGs can be encountered in grains, the highest levels in Europe reaching mg/kg level, although the mean levels have been lower generally (Battilani et al. 2009). In European produce, AFs and FBs are detected principally in maize and maize products. The levels reported for AFs are moderate but FBs have been determined at high concentration levels, up to 10200 μg/kg (Gareis et al. 2003; Binder et al. 2007).

Table 1. Some common mycotoxins and their producing organisms and occurrence in Europe (modified from Köppen et al. 2010)

Mycotoxins	Examples of producing organisms	Toxic properties	Examples of commodities where encountered	Occurrence in cereals (including maize) in Europe (μg/kg)	References for occurrence data
Aflatoxins: AF B1, AF B2, AF G1, AF G2	*Aspergillus flavus, A. parasiticus, A. nomius*	Carcinogenic, teratogenic, hepatotoxic, nephrotoxic, mutagenic, immunosuppressive, haemorrhage of intestinal tract and kidneys, liver disease	Maize, wheat, rice, spices, sorghum, ground nuts, tree nuts, almonds, oilseeds, dried fruits, spices, eggs, meat	656 (max) 12 to 19 (median) (AF B1 in maize and feed)	Binder (2007)
Aflatoxins: AF M1, AF M2			Milk and milk products	mostly < 0.05 (AF M1 in milk)	EFSA (2004)
Fumonisins: FB1, FB2, FB3	*Fusarium proliferatum, F. verticillioides*	Carcinogenic (liver tumours), hepatotoxic, cerebral oedema, cause necrosis, immunotoxic	Maize, maize based products, sorghum, asparagus, rice, milk	5 to 10200 (min/max) < 0.3 to 4815 (median)	Gareis et al. (2003)
Type-A trichothecenes: T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol	*F. sporotrichioides, F. poae, F. langsethiae*	Immunotoxic, haematotoxic, gastrointestinal haemorrhaging, neurotoxic, inhibition of protein synthesis[a]	Cereals, cereal based products	4 to 7584 (min/max) 10 to 151 (median) (T-2 or HT-2 toxin)	van der Fels-Klerx (2010)
Type-B trichothecenes: nivalenol, deoxynivalenol (DON), 3-Acetyl-DON, 15-Acetyl-DON, fusarenon X	*F. graminearum, F. culmorum, F. sporotrichioides, F. cerealis*	Immuno-depressants, neurotoxic, gastrointestinal haemorrhaging, vomiting, feed refusal, reduced weight gain, inhibition of protein synthesis[b]	Cereals, cereal based products	1.7 to 50000 (min/max) 1 to 2000 (median) (DON)	Gareis et al. (2003)
Zearalenone	*F. graminearum, F. culmorum, F. equiseti, F. crookwellense*	Estrogenic activity (infertility, vulval oedema, vaginal prolapse, mammary hypertrophy in females, feminisation of males)	Barley, oats, wheat rice, sorghum, sesame, soy beans, cereal based products	1 to 6492 (min/max) 0.08 to 689 (median)	Gareis et al. (2003)
Ochratoxins: OTA, OTB	*A. niger, A. ochraceus, Penicillium verrucosum, P. viridicatum*	Carcinogenic (urinary tract tumours), mutagenic, nephrotoxic, hepatotoxic, teratogenic, immuno-depressant, inhibition of protein synthesis	Cereals, dried vine fruit, wine, coffee, oats, spices, rye, raisins, grape juice	0.005 to 33.3 (min/max) 0.01-2.0 (median) (OTA)	Miraglia et al. (2002)
Ergot alkaloids: ergocornine, ergocristine ergocryptine, ergosine, ergotamine	*Claviceps purpurea, Claviceps paspali, Claviceps africana*	Gangrenous form: vasoconstrictive activity (oedema of legs, paraesthesia, gangrene at tendons) Convulsive form: gastrointestinal symptoms (nausea, vomiting), effects on the central nervous system (drowsiness, ataxia, convulsions, blindness, paralysis)	Wheat, rye, barley, millet, oats, sorghum, triticale, cereal based products	2 to 3280 (min/max) 1 to 850 (median) (total ergot alkaloids)	Battilani et al. (2009)

a=references: Köppen et al (2010), EFSA (2001), b=references: Köppen et al (2010), EFSA (2004a)

Other plant products like pulses, nuts and oilseeds may contain various types of mycotoxins, including AFs, ZEN and *Alternaria* toxins (Schollenberger et al. 2005;2007; Logrieco et al. 2009; Njobeh et al. 2010). Additionally, spices and dried fruits are frequently contaminated with AFs and OTA (Miraglia et al. 2002; Bircan 2009). Furthermore, OTA is often found in coffee, tea and cocoa (Miraglia et al. 2002). Mycotoxins can be detected also from fresh vegetables and products made from these foodstuffs. For example, PAT is a common contaminant in apples and juices and OTA may be detected in grapes and wines (Blesa et al. 2006; Valero et al. 2008;). In addition, *Alternaria* toxins can be present in various vegetables and fruits (Logrieco et al. 2009; Asam et al. 2010).

Mycotoxins may occur in food and feed also as their metabolites or conjugated forms called as masked or bound mycotoxins (Berthiller et al. 2009). These compounds can be either soluble or associated with macromolecules, and they may emerge through the metabolism of fungi, other micro-organisms, plants and mammals or appear during food processing (Berthiller et al. 2009). The area of this type of altered mycotoxins has not been investigated extensively so far, but it is believed that they contribute significantly to the total prevalence of mycotoxins and thereby to food safety. Some known mycotoxin conjugates are the plant-derived glucosides of ZEN and DON. For example, zearalenone and zearalenol 4-glucosides and DON 3-glucoside have been detected occurring naturally in maize and small-grain cereals (Schneweis et al. 2002; Berthiller et al. 2005;2009).

Certain mycotoxins may be carried over to animal products from contaminated feed. Milk may contain AF M1, a hydroxylated form of AF B1, which is excreted in milk of lactating animals that have consumed contaminated feed (van Egmond 1989). In addition, ZEN, OTA and FBs can be transmitted into milk but generally, the levels found have been low (EFSA 2004b; Boudra et al. 2007; Gazzotti et al. 2009). Eggs have been reported as containing μg/kg or lower levels of AFs, DON and its metabolite de-epoxy-DON, ZEN and the metabolites α-zearalenol (ZOL) and β-ZOL (Sypecka et al. 2004; Valenta and Dänicke 2005; Garrido Frenich et al. 2011). Additionally, the frequent occurrence of BEA, ENN B and ENN B1 has been demonstrated in Finnish eggs at trace levels (Jestoi et al. 2009). OTA may be transferred to internal organs of animals, and low levels have been detected in pig kidneys and liver (Milicevic et al. 2009).

A number of different mycotoxins may be found in the same product since a single species of fungi can produce several toxic metabolites, or several species that produce different toxins can be present simultaneously. Although the samples may contain only relatively low levels of individual mycotoxins, co-occurrence is of concern due to possible interactive toxic effects of the mixtures of toxins. Mycotoxin co-occurrence has been reported frequently in cereals. Trichothecenes and ZEN are commonly simultaneously present in small-grain cereals (Placinta et al. 1999;

Eskola et al. 2001). In addition to these, BEA, ENNs and MON may occur in the same samples (Jestoi et al. 2004a). Maize is typically contaminated with both AFs and FBs, and additionally DON, NIV and ZEN may be present (Ali et al. 1998, Sangare-Tigori et al. 2006; Njobeh et al. 2010). BEA, MON, and fusaproliferin have also been found to be co-occurring in maize (Logrieco et al. 1993; Munkvold et al. 1998). Similarly, rice, nuts, dried fruits and spices may be contaminated with various combinations of mycotoxins (Sangare-Tigori et al. 2006; Ghali et al. 2008; Bircan 2009; Njobeh et al. 2010). Feeds, that can be mixtures of different raw materials, frequently contain various types of mycotoxins (Binder et al. 2007; Richard et al. 2007; Driehuis et al. 2008). For example, fermented silage may contain toxins of both pre- and post-harvest fungal species. CIT, DON and gliotoxin (GLIO) have been detected in maize silage and roquefortine C (ROQ C) and mycophenolic acid (MPA) were determined from a silage made of maize and grass (Richard et al. 2007; Driehuis et al. 2008).

The driving forces for mycotoxin research have been food safety concerns due to the toxicity of these secondary metabolites, but there is also an economic aspect due to losses of harvest (Bhat et al. 2010). In attempts to control the mycotoxin occurrence and protect consumers, regulatory authorities in several countries have set limits for maximum residue levels (MRL) for several mycotoxins (van Egmond et al. 2007). In the European Union (EU), maximum limits have been established for aflatoxins, OTA, fumonisins, DON and PAT in various food commodities, and for AF B1 in feed and feed raw materials (European Union 2002b;2006c;2007) as summarized in Table 2. Additionally, recommendations for the maximum allowable levels have been provided for certain mycotoxins in feed (Table 2) (European Union 2006a). MRLs for ergot sclerotia have been set for EU intervention grain and feeds (European Union 2002b;2006b), but there is no guidance for ergot alkaloid concentration levels. These, as well as regulatory limits for T-2 and HT-2 toxins are currently under discussion in the EU.

Table 2. Maximum allowable or recommended residue levels of mycotoxins in foodstuffs and animal feed in the EU.

Mycotoxin	Commodity	Maximum allowable residue levels in food (μg/kg)	Maximum allowable or recommended* residue levels in feed (μg/kg)
Sum of aflatoxins	Dried fruit, nuts, groundnuts, cereals, maize, cereal products	4 - 15	
Aflatoxin B1	Cereal based infant and dietary foods, groundnuts, nuts, spices, cereals, maize, cereal products	0.1 - 8.0	
Aflatoxin B1	Feed and related raw materials		5 - 50
Aflatoxin M1	Milk based infant and dietary food, milk	0.025 - 0.05	
Ochratoxin A	Cereal based infant and dietary foods, dried fruit, cereals, cereal products, coffee, wine, grape juice	0.5 - 10	
Ochratoxin A	Feed materials and feeding stuffs		50 - 250*
Patulin	Infant food, apple and fruit juices, various other apple drinks and products	10 - 50	
Deoxynivalenol	Cereal based infant foods, various cereal products, cereals, unprocessed cereals and maize	200 - 1750	
Deoxynivalenol	Feed materials and feeding stuffs		900 - 12000*
Zearalenone	Cereal and maize based infant foods, cereal and maize products, unprocessed cereals and maize	20 - 400	
Zearalenone	Feed materials and feeding stuffs		100 - 3000*
Sum of fumonisin B1 and B2	Maize based infant foods, maize products, unprocessed maize	200 - 4000	
Sum of fumonisin B1 and B2	Feed materials and feeding stuffs		5000 - 60000*
Ergot sclerotia	Intervention grain	0.05 % w/w (=500 mg/kg)	
Rye ergot	Feeding stuffs		1000 mg/kg

2.2 LC/MS IN FOOD SAFETY AND MYCOTOXIN RESEARCH

Reliable determination of chemical residues and contaminants from food and feed requires high performance methods as the compounds are typically present in complex sample matrices at low concentrations, i.e. at the µg/kg level (Malik et al. 2010). Additionally, national and international legislative framework places requirements on the performance of the analytical methods used for monitoring of various undesirable compounds. For example in the EU, the MRLs set for various foreign substances and the minimum required performance limits (MRPL) established for methods to be used in the control of residues in animal products require a certain level of method performance with regard to the detection capability, recovery and precision (European Union 2002a). In the Decision 2002/657/EC, techniques based on MS have been stated as being appropriate to reach the confidence required (as so called identification points) from the confirmatory methods (European Union 2002a). Due to the high selectivity, sensitivity and level of confidence of identification, MS techniques have gained great popularity not only in residue control of animal products, but also throughout the whole area of food safety research (Malik et al. 2010).

Methods based on liquid chromatography coupled to different types of MS detectors (LC/MS) have been utilized widely for residues of pesticides, veterinary drugs and growth promoters, environmental contaminants, toxic compounds originating from food processing and packaging materials, as well as natural toxins such as marine biotoxins and mycotoxins (Malik et al. 2010). LC/MS methods have been devised for single analytes and for groups of chemically related compounds such as veterinary drug residues of selected classes (Bogialli and Di Corcia 2009; Malik et al. 2010). However, the fact that various contaminants may co-occur and that the same samples are often analysed multiple times for different purposes has encouraged investigators to develop multi-residue methods capable of detecting a larger number of chemically diverse compounds simultaneously, thereby achieving high throughput of samples as well as savings in costs and time (Mol et al. 2008). Examples of this type of LC/MS based multi-residue methods are the recently published methods for milk, eggs, fish and meat which can detect up to 150 different veterinary drugs or growth promoters (Ortelli et al. 2009; Peters et al. 2009) and the pesticide residue methods, the most extensive of which enable simultaneous determination of 160 or 297 chemicals from vegetables (Kmellár et al. 2008; Mezcua et al. 2009). The scope of the method can be further widened by inclusion of contaminants of diverse origins. These types of generic LC/MS methods have been developed and validated for a total of 93 and 258 analytes containing pesticides, veterinary drug residues, mycotoxins and plant toxins analysed from food and feed materials (Mol et al. 2008; Romero-González et al. 2011).

LC/MS based techniques have become common in mycotoxin research in the field of food and feed safety during the last two decades. Due to the large variety of these toxins and the different sample matrices in which they may be present, a range of methods exists (Zöllner and Mayer-Helm 2006; Malik et al. 2010). Applications have been presented for the analysis of single compounds, like MON, OTA or PAT (Jestoi et al. 2003; Blesa et al. 2004; Ito et al. 2004) and groups of chemically related mycotoxins, typical example being the determination of type-A and B trichothecenes from grains (Razzazi-Fazeli et al. 2002; Berthiller et al. 2005; Klötzel et al. 2005;2006). Other group-specific LC/MS analysis are those developed for aflatoxins, fumonisins, enniatins (including BEA) or ergot alkaloids (Vahl and Jørgensen 1998; Plattner 1999; Takino et al. 2004; Jestoi et al. 2005; Sewram et al. 2005; Krska et al. 2008b). Similarly to other areas of residue analysis, the multi-analyte approach is gaining popularity in mycotoxin research, and methods intended to analyse a wide range of toxins of different classes have been published. In this literature review, multi-mycotoxin methods for food and feed matrices comprising of 8 to 106 chemically diverse compounds (usually originating of several fungal genera) will be discussed (Table 3).

LC/MS multi-residue methods have been developed mostly for targeted analysis, i.e. for detecting and quantitating the selected mycotoxins of interest. The majority of the methods are intended for determining mycotoxins in the agricultural products where these compounds frequently occur, such as small-grain cereals, maize, feed, rice, soya, nuts, spices, dried fruit as well as fresh vegetables (Sulyok et al. 2006; Boonzaaijer et al. 2008; Spanjer et al. 2008; Garrido Frenich et al. 2009; Monbaliu et al. 2009; Desmarchelier et al. 2010). Typically, the methods include those toxins for which regulatory limits have been established or are planned in the EU. These methods comprise a total of 9 to 12 analytes including AFs, FBs, OTA, ZEN, DON, HT-2 and T-2 with the sample matrices being cereals, maize and feed (Lattanzio et al. 2007; Beltrán et al. 2009; Garrido Frenich et al. 2009; Capriotti et al. 2010; Desmarchelier et al. 2010). The other methods developed for various agricultural commodities comprise from 10 to 39 mycotoxins considered relevant. The analytes have typically included all or some of the "regulated" mycotoxins and additionally other *Fusarium* toxins (e.g. other TRs, MON, ENNs, BEA and derivatives of ZEN), *Alternaria* toxins (such as altenuene and alternariol), *Penicillium* toxins (e.g. sterigmatocysteine, ROQ C, cyclopiazonic acid, penicillic acid, MPA and PAT), and ergot alkaloids (Sulyok et al. 2006;2007b; Spanjer et al. 2008; Herebian et al. 2009; Martos et al. 2010; Monbaliu et al. 2009;2010a). Currently, conjugated and metabolised forms of mycotoxins are not widely included in the multi-mycotoxin methods, which is probably due to analytical challenges, such as lack of commercially available standards and difficulty in bringing the bound toxins into soluble forms (Berthiller et al. 2009).

Table 3. Multi-mycotoxin methods based on liquid chromatography-mass spectrometry in food and feed matrices comprising 8 to 106 mycotoxins or fungal metabolites.

Number of analytes	Type of analytes	Sample matrices	Sample clean-up	LC-MS instrumentation	Reference
13	*Penicillium* toxins (validation for ROQ C, griseofulvin, chaetoglobosin B, penitrem A, MPA, verruculogen)	Food mixture	Hexane defatting	HPLC-APCI-IT-MS, HPLC-APCI-IT-MS/MS	Rundberget and Wilkins (2002)
8	AF B1, OTA, OTα, ZEN, α-ZOL, α-ZOL, NIV, DON	Pig kidney	SPE (Oasis HLB)	HPLC-ESI-QqQ-MS/MS	Driffield et al. (2003)
18	TRs, ZEN, ZAN, (OTA and AFs for confirmation purpose)	Cereal based samples	SPE (Mycosep push-through)	HPLC-ESI-QqQ-MS/MS	Biselli et al. (2004)
18	OTA, ZEN, α-ZOL, α-ZOL, α-ZAL, α-ZAL, FBs, TRs, AF M1	Milk	SPE (Oasis HLB)	HPLC-ESI-QqQ-MS/MS	Sørensen end Elbæk (2005)
13	TRs, FBs, ZEN, α-ZOL	Corn meal	SPE (C18, Carbograph-4)	HPLC-ESI-QqQ-MS/MS	Cavaliere et al. (2005)
39	TRs, ZEN+derivatives, FBs, ENNs, ERGs, OTs, AFs, MON	Wheat, maize, rice, spelt, barley	Direct injection	HPLC-ESI-QIT-MS/MS	Sulyok et al. (2006); Sulyok et al. (2007a)
13	TRs, ZEN, AFs	Corn, wheat, cornflakes, biscuits	SPE (Multisep 226)	HPLC-APCI-TOF-MS	Tanaka et al. (2006)
87	Metabolites of diverse fungal genera	Mouldy food	Direct injection	HPLC-ESI-QqQ-MS/MS	Sulyok et al. (2007b)
17	TRs, ZEN, α-ZOL, α-ZOL, α-ZAL, α-ZAL, FBs	Maize	SPE (Carbograph-4)	HPLC-ESI-QqQ-MS/MS	Cavaliere et al. (2007)
11	AFs, OTA, FBs, TRs	Maize	SPE (IA, Myco-6-in-1)	HPLC-ESI-QIT-MS/MS	Lattanzio et al. (2007)
17	AFs, OTA, FBs, TRs, CIT, STE	Peanut butter, corn feed	SPE (Mycosep 226)	UHPLC-ESI-QqQ-MS/MS	Ren et al. (2007)
33	AFs, OTA, TRs, ZEN, α-ZOL, α-ZOL, α-ZAL, STE, CPA, PA, FBs, MPA, ERGs, ALT, AME	Peanut, pistachio, wheat, maize, cornflakes, raisin, figs	Direct injection	HPLC-ESI-QqQ-MS/MS	Spanjer et al. (2008)
258	Residues including 36 mycotoxins (AFs, ERGs, FBs, TRs, ZEN, α-ZOL, α-ZOL, OTA, STE)	Feed, honey	QuEChERS	HPLC-ESI-QqQ-MS/MS, UHPLC-ESI-QqQ-MS/MS, UHPLC-ESI-QTOF	Mol et al. (2008)
11	AFs, OTA, DON, NIV, T2, HT2, ZEN, FB1	Flavour ingredients, spices	Direct injection	HPLC-ESI-QqQ-MS/MS	Bonzaaijer et al. (2008)
12	AFs, OTA, DON, HT-2, T-2, FBs, ZEN	Maize, walnuts, biscuits, breakfast cereals	Direct injection	UHPLC-ESI-tQ-MS/MS	Garrido Frenich et al. (2009)
23	AFs, OTA, TRs, ZEN, FBs, ALT, AOH, AME, BEA, STE	Sweet pepper	SPE (NH_2, C18, SAX)	UHPLC-ESI-QqQ-MS/MS	Monbaliu et al. (2009)
11	AFs, OTA, DON, HT-2, T-2, FBs, ZEN	Maize, wheat pasta, cereal based babyfood	Direct injection	UHPLC-ESI-QqQ-MS/MS	Beltran et al. (2009)

Table 3. continued.

32	TRs, ZEN, α-ZOL, FBs, AFs, ERGs, PAT, CIT, OTs, MON, ALT, AOH, AME, ENN B, BEA, gibberellic acid	Wheat, maize	Direct injection	HPLC-ESI-QqQ-MS/MS, MicroLC-ESI-Orbitrap-MS	Herebian et al. (2009)
13	*F. avenaceum* metabolites (e.g. MON, ANT Y, ENNs, aurofusarin)	Apple	Direct injection	HPLC-ESI-QqQ-MS/MS	Sørensen et al. (2009)
12	AFs, OTA, DON, HT-2, T-2, FBs, ZEN	Beer	SPE (C18)	UHPLC-ESI-QqQ-MS/MS	Romero-Gonzalez et al. (2009)
23	TRs, AFs, FBs, OTA, ZEN, BEA, STE, ALT, AOH, AME	Food supplements	Hexane defatting, SPE (Oasis HLB)	UHPLC-ESI-QqQ-MS/MS	Di Mavungu et al. (2010)
23	AFs, OTA, TRs, ZEN, FBs, ALT, AOH, AME, ROQ C, STE	Feed	SPE (C18, Multisep 226), hexane defatting	UHPLC-ESI-QqQ-MS/MS	Monbaliu et al. (2010a)
26	AFs, TRs, FBs, ZEN, OTA, ALT, AME, STE, CIT, MPA, fumigaclavine, paxilline	Tea, herbal infusions	SPE (NH_2, C18)	UHPLC-ESI-QqQ-MS/MS	Monbaliu et al. (2010b)
106	Metabolites of diverse fungal genera	Food infected by moulds	Direct injection	HPLC-ESI-QqQ-MS/MS	Sulyok et al. (2010)
17	AFs, ZEN, FBs, TRs, OTA	Cereals, rice, maize, soya, infant food	QuEChERS, hexane defatting	HPLC-ESI-QqQ-MS/MS	Desmarchelier et al. (2010)
27	TRs, FBs, ENN B, GLIO, OTA, MPA, ROQ A and C, other *Penicillium* and *Alternaria* toxins	Maize silage	QuEChERS	HPLC-ESI-QqQ-MS/MS	Rasmussen et al. (2010)
11	TRs, ZEN, FBs	Cereal, cereal based products	QuEChERS or direct injection	UHPLC-ESI-TOF-MS, UHPLC-APCI-Orbitrap-MS	Zachariasova et al. (2010)
9	OTA, AFs, ZEN, DON, T-2, HT-2	Cereals	Direct injection	HPLC-APPI-QIT-MS/MS	Capriotti et al. (2010)
22	AFs, STE, CPA, TRs, OTA, FBs, ZEN, ERGs	Wheat, barley, oats, rye, maize	Direct injection	HPLC-ESI-QqQ-MS/MS	Martos et al. (2010)
11	DON, 3-AcDON, NIV, DON, F-X, DAS, ZEN, STE, ALT, AOH, AME	Wheat, maize, millet	Direct ionization from cereal extract	HPLC-DART-Orbitrap	Vaclavik et al. (2010)
10	TRs, MON, ZEN, ZAN, OTA, OTB	Rice, maize, millet, beans	SPE (mixed mode)	UHPLC-ESI-QqQ-MS/MS	Jin et al. (2010)
93	Residues including 7 mycotoxins (AFs, OTA, HT-2, T-2)	Wheat, cucumber, wine	QuEChERS	UHPLC-ESI-QqQ-MS/MS	Romero-González et al. (2011)
10	AFs, ENNs, CIT, OTA	Eggs	QuEChERS	UHPLC-ESI-tQ-MS/MS	Garrido Frenich et al. (2011)

AcDON=Acetyl deoxynivalenol, AF=aflatoxin, ALT=alternariol, ANT Y=antibiotic Y, AME=alternariol methyl ether, AOH=alternuene, APCI=atmospheric pressure chemical ionization, APPI=atmospheric pressure photoionization, BEA=beauvericin, CIT=citrinin CPA=cyclopiazonic acid, DAS=diacetoxyscirpenol, DART=direct injection in real time, DON=deoxynivalenol, FB=fumonisin, F-X=fusarenon X, ENN=enniatin, ERG=ergot alkaloid, ESI=electrospray ionization, HPLC=high performance liquid chromatography, HT-2=HT-2 toxin, IT=ion trap, LC=liquid chromatography, MON=moniliformin, MPA=mycophenolic acid, MS=mass spectrometry, NIV=nivalenol, OT=ochratoxin, PA=penicillic acid, PAT=patulin, QIT=quadrupole ion trap, QuEChERS=quick, easy, cheap, effective, rugged and safe, QqQ=triple quadupole, ROQ C=roquefortine C, SAX=strong anion exchange, SPE=solid phase extraction, STE=sterigmatocysteine, T-2=T-2 toxin, TOF=time of flight, tQ=tandem quadrupole, TR=trichothecene, UHPLC=ultra performance liquid chromatography, ZAN=zearalanone, ZAL=zearalanol, ZEN=zearalenone, ZOL=zearalenol

Sometimes the expected mycotoxin content requires specific analyte targeting. For example, a total of 27 metabolites of both pre- and post-harvest fungi of genera *Penicillium, Alternaria, Aspergillus* and *Monascus* were analysed from fermented maize silage (Rasmussen et al. 2010). Further, Sørensen et al. (2009) developed a method specifically for 13 diverse *F. avenaceum* metabolites that could occur in apples suffering from wet apple core rot. Quantitative methods have been presented also for the simultaneous analysis of several mycotoxins from honey and products of animal origin, such as meat, kidney, eggs, milk and cheese comprising from eight to 18 toxins and their metabolites that could carry over from feed, or originate from other contamination sources (Sørensen and Elbæk 2005; Mol et al. 2008; Garrido Frenich et al. 2011). The most comprehensive multi-mycotoxin method presented for food and feed matrices comprises a total of 106 analytes consisting of mycotoxins produced mainly by *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*, and some of their metabolised and conjugated forms (Sulyok et al. 2010). Although some analytes could be determined only qualitatively, the method is a practical tool for applications where a comprehensive idea of the quality of a product is needed rapidly, such as when identifying the agent causing food or feed contamination, or examining the toxin production capacity of fungal species. The applicability of the method was demonstrated in the screening of mycotoxins from a range of spontaneously molded foodstuffs, including bread, cheese, nuts, jam and wine (Sulyok et al. 2010).

2.3 ANALYTICAL ASPECTS OF LC/MS MULTI-MYCOTOXIN DETERMINATION

The LC/MS analysis of mycotoxins from food and feed matrices typically consists of multiple steps. These include sampling, homogenisation of the sample, extraction of the analytes, sample purification procedures and finally, the separation with liquid chromatography and the detection and quantification with a mass spectrometer (Köppen et al. 2010). Finally, the suitability of the method for the intended analytical purpose should be evaluated with properly selected quality assurance procedures (Thompson et al. 2002). In the following, some aspects of the various steps in multi-mycotoxin analysis are discussed. The main emphasis will be on the so called targeted methods intended primarily for routine analysis. At present, most of the multi-mycotoxin LC/MS methods regarding food and feed safety are of this type rather than screening methods aimed at gathering information on a wider range of both targeted and non-targeted compounds. The challenges of quantitative multi-mycotoxin determination arise from adjusting the conditions at each of the analytical steps for all the compounds of interest that can exhibit very different chemical

properties. Typically, optimal conditions cannot be found for all the analytes and often some of them need to be omitted due to e.g. insufficient extraction, poor chromatography or improper ionization in MS (Rundberget and Wilkins 2002; Sulyok et al. 2006). In any case, the final multi-mycotoxin method is likely to be a compromise of achievable method performance (Sulyok et al. 2006).

2.3.1. SAMPLING AND EXTRACTION

The distribution of mycotoxins in commodities is often heterogeneous and therefore, sampling is a critical part of their chemical determination (Whitaker 2006). With properly performed sampling, a representative sample can be obtained for the laboratory analysis. By increasing the number or size of the laboratory sample or by improving comminution of the sample, the total error related to the analysis can be further reduced (Whitaker 2006). The degree of comminution (or homogenization) should be paid attention to as it affects also the extraction efficiency of the analytes (Boyd et al. 2008). Criteria regarding sampling and performance of analytical methods for official control of certain mycotoxins have been established in the EC regulation EC/401/2006 (European Union 2006d). The regulation does not set requirements for the preparation or the size of a laboratory sample. However, the official or standardized analytical methods for mycotoxins from various commodities like grains, feed and milk powder recommend sample sizes of 10 to 50 g (AOAC International 2006; International Organization for Standardization. 2007; The European Committee for Standardization 2009a;2009b). In the published multi-mycotoxin studies, sample sizes from 10 to 25 g have been taken from cereals, nuts, dried fruit and feed (Tanaka et al. 2006; Lattanzio et al. 2007; Ren et al. 2007; Spanjer et al. 2008). However in several methods, the sample size has been reduced down to between 2 and 5 g, with the smallest sample sizes reported being 0.25 and 0.5 g of cereals (Sulyok et al. 2006; Herebian et al. 2009; Sørensen et al. 2009; Monbaliu et al. 2010a).

Extraction has been stated as the most challenging step in a multi-mycotoxin analysis as chemically different compounds need to be isolated (Lattanzio et al. 2007), and typically, the resulting extraction method is a compromise of achievable recoveries (Sulyok et al. 2006). Generally, the extraction of mycotoxins from diverse biological matrices has been achieved with liquid extraction using organic solvents and their mixtures (Zöllner and Mayer-Helm 2006). In multi-mycotoxin applications, acetonitrile (ACN) and methanol (MeOH) have been commonly chosen (Lattanzio et al. 2007; Ren et al. 2007; Spanjer et al. 2008; Martos et al. 2010). The majority of mycotoxins are soluble in these solvents, and additionally both are volatile and thus compatible with LC/MS analysis (Boyd et al. 2008), which is of

importance when sample extract is injected directly to the analytical instrument.

The improved efficiency of ACN based solvents in comparison to MeOH has been demonstrated in multi-mycotoxin extractions from cereals and feed (Sulyok et al. 2006; Beltrán et al. 2009; Garrido Frenich et al. 2009). For example, the best results for 34 chemically diverse mycotoxins extracted from spiked wheat were obtained with a high percentage of ACN (Sulyok et al. 2006). Recoveries were mostly between 87 and 111 % with ACN:water (75:25) with the exception of patulin and FBs (recoveries were 17-35 %), whereas the equivalent MeOH:water mixture yielded more variable recoveries from 27 to 111 % (with the values for PAT and ZEN-4-glucoside being outside this range). MeOH and ACN have rather equal polarity (Snyder et al. 1997). However, a plausible reason for the improved extraction efficiency of ACN could be its more suitable selectivity towards the non-ionic mycotoxins, as the selectivity of ACN is based more on dipole interactions rather than acid or basic functionalities (which are stronger for MeOH) (Snyder et al. 1997). On the other hand, Mol et al. (2008) showed that adequate recoveries could be achieved with acidified ACN, MeOH or acetone for 174 diverse residues from feed. In further extraction experiments, acetone ensured the best recoveries for the selected 34 target analytes from six different food and feed matrices. However, the use of ACN resulted in the best overall compromise due to the reduced extraction of matrix components and subsequently less severe signal suppression in MS in comparison to using MeOH and acetone.

The extraction of multiple mycotoxins has been performed mostly in a single step for the sake of simplicity within the analytical procedure (Spanjer et al. 2008) although extraction in several steps can be also a feasible option. Recoveries of ≥79 % were obtained for 11 mycotoxins from maize with a double extraction procedure using first phosphate buffered saline (PBS) followed by MeOH:water (70:20) and combining these extracts for the subsequent clean-up procedure and LC/MS analysis (Lattanzio et al. 2007). Sometimes it may not be possible to extract all the desired compounds sufficiently. For example, none of the solvent compositions tested resulted in satisfactory extraction of PAT from wheat and therefore, the compound was excluded from the method (Sulyok et al. 2006). Several studies have also highlighted the challenges of extracting the acidic fumonisins. It has been demonstrated that these compounds can be extracted only in a high water content or at a low pH (e.g. Sulyok et al. 2006; Beltrán et al. 2009; Desmarchelier et al. 2010; Monbaliu et al. 2010a). However, the increase of the proportion of water may result in reduced recoveries of other mycotoxins of interest (Sulyok et al. 2006; Beltrán et al. 2009; Garrido Frenich et al. 2009). Therefore, the adjustment of pH has been proved as a more feasible solution. At a low pH, the four carboxylic groups of FBs are protonated (i.e. neutral) leading to less interaction and binding to the sample matrix (Mol et al. 2008). Recoveries of over 43 % have been obtained for FBs from milk and cereal based matrices when the pH of ACN or MeOH solvent has

been adjusted with sulphuric acid or an organic acid, such as formic or acetic acid (Sørensen and Elbæk 2005; Sulyok et al. 2006; Beltrán et al. 2009; Herebian et al. 2009; Desmarchelier et al. 2010; Monbaliu et al. 2010a; Zachariasova et al. 2010).

Liquid extraction in multi-mycotoxin methods has been performed generally by conventional methods like shaking e.g. with a horizontal shaker (e.g. Sulyok et al. 2006; Herebian et al. 2009) or simultaneously with Ultra Turrax homogenization (Martos et al. 2010). In some applications, extraction has been enhanced with ultrasonic agitation (Sørensen et al. 2009; Capriotti et al. 2010). Probably due to difficulties e.g. in optimization and in routine use, as well as the need to invest in special equipment, other enhanced liquid extraction methods, such as pressurized liquid extraction (PLE), microwave extraction or supercritical fluid extraction have not gained popularity in multi-mycotoxin methods. Only Desmarchelier et al. (2010) have described a method utilizing PLE by employing the automated ASE® instrument (Accelerated Solvent Extraction, Dionex, Sunnyvale, CA, United States) for extracting several mycotoxins from wheat. In PLE, organic solvents are used at elevated temperatures above their atmospheric boiling points, which is achieved by raising the pressure in a closed flow-through system. The enhanced extraction is based largely on the increase in temperature which results in lower surface tension and viscosity of the solvent allowing it to wet and penetrate the sample matrix more readily (Carabias-Martínez et al. 2005; Boyd et al. 2008). A drawback of PLE can be excessive co-extraction of matrix components and subsequent interfering signals in LC/MS, as was reported when applying temperatures above 60 °C by Desmarchelier et al. (2010). Therefore, the ASE instrument was operated at room temperature, still obtaining adequate recoveries (from 49 to 88 %) for the 17 analytes of interest (Desmarchelier et al. 2010). The overall method performance was comparable with a procedure employing QuEChERS (acronym for the words quick, easy, cheap, effective, rugged and safe) extraction. Despite the fitness-for-purpose, and the possibility of automatization of the ASE extraction, the method was considered tedious for routine work due to filling and washing of the extraction cartridges.

2.3.2 SAMPLE CLEAN-UP

Liquid extraction of chemical residues from food and feed samples cannot be adjusted so that only the desired analytes will be isolated selectively and thus, the extracts usually contain many matrix components such as lipids, proteins, carbohydrates and pigments that may interfere with the analysis (Anastassiades et al. 2003; di Mavungu et al. 2009; Monbaliu et al. 2009). In order to purify the sample extract from the remaining interferences, clean-up procedures of different types may need to be adopted. Enrichment of the analytes is also typically performed

at this step (Snyder et al. 1997). Sample preparation has been considered as an essential part of an analytical procedure, as it will affect the quality of the final results and is also advantageous for the maintenance of the analytical instrument (Boyd et al. 2008). In MS analysis, matrix components present in the final sample frequently cause suppression or enhancement of analyte signal (discussed more in-depth in 2.3.4.3). However, with sample clean-up these undesired matrix effects can be reduced (Boyd et al. 2008).

The role of sample purification has changed with the developments in LC/MS technology, and extensive clean-up is not always necessary due to the high selectivity and sensitivity of modern analytical instruments (Malik et al. 2010). This has made possible the development of methods with reduced sample clean-up and injection of unpurified sample extracts. With this approach, straightforward, rapid methods have been developed also in the area of mycotoxin analysis (Sulyok et al. 2006; Herebian et al. 2009; Martos et al. 2010). On the other hand, optimal method performance may not be achieved and for this reason, several investigators have regarded sample clean-up as being essential in multi-mycotoxin analysis (Monbaliu et al. 2009; Desmarchelier et al. 2010). The challenges of utilizing clean-up arise from optimizing the procedure for chemically diverse analytes without losing any of them and still achieving a feasible method for routine use (Monbaliu et al. 2009). The sample clean-up procedures used in the published methods include different forms of solid phase extraction (SPE) and the so called QuEChERS extraction.

2.3.2.1 Solid phase extraction

Solid phase extraction (SPE) is an established sample purification method that relies on the same chromatographic principles as HPLC, i.e. partition of solutes between a liquid and a solid phase (Snyder et al. 1997). The SPE sorbent phases exploit the same functionalities that are used in HPLC, although the separation efficiency is usually lower (Boyd et al. 2008). Some common SPE sorbents include reversed and normal phase, ion exchange and (immuno)affinity sorbents as well as so called mixed mode columns combining e.g. reversed phase (RP) and ion exchange functionalities (Snyder et al. 1997; Boyd et al. 2008). With the correct choice of the SPE stationary phase, the selectivity of the extraction can be adjusted leading to retention of either matrix interferences or the analytes thus enabling the separation of the two (Boyd et al. 2008). Purification can be improved by using multiple columns with different retention properties (Boyd et al. 2008). In multi-mycotoxin applications, RP columns have been commonly used, either alone or in conjunction with mixed-mode and ion exchange columns (Cavaliere et al. 2005; Monbaliu et al. 2009; Romero-González et al. 2009). Additionally, Multisep® and

Mycosep® cartridges (Römer Labs, Tulln, Austria) intended for the analysis of selected groups of mycotoxins, as well as specific immunoaffinity (IA) columns have been demonstrated as being applicable (Tanaka et al. 2006; Lattanzio et al. 2007; Ren et al. 2007).

The applicability of reversed phase SPE sorbents, such as a polymer based Oasis® HLB (Waters, Manchester, UK) and silica based octadecyl silane (C18) were demonstrated for adsorbing eight to 23 chemically diverse mycotoxins from food extracts and beer (Driffield et al. 2003; Sørensen and Elbæk 2005; di Mavungu et al. 2009; Romero-González et al. 2009). In these methods, the retention of the analytes to the column was achieved when loading of the sample was performed in high water content. The use of 100 % of organic polar solvent, i.e. MeOH or its mixtures with dichloromethane (DCM) or ACN, ensured the elution of the analytes of interest.

C18 columns have been combined in tandem with other types of sorbents to improve the selectivity of the SPE in multi-mycotoxin determinations from corn and feed (Cavaliere et al. 2005;2007; Monbaliu et al. 2010a). In the first step, the nonpolar functionality of C18 sorbent can be utilized to retain lipid components of sample matrices. This has been achieved by passing the sample extract through the column in a high percentage of ACN (75 % or 79 % with acetic acid). In the method of Cavaliere et al. (2005), further clean-up of the eluate diluted to high water content was performed in graphitized carbon black. This can be considered a mixed-mode SPE material as it is based mainly on RP retention but has also polar interaction and ion exchange properties (Andreolini et al. 1987; Altenbach and Giger 1995). The selected 13 *Fusarium* toxins could be retained, and the elution from the column was performed with a solvent of high elution strength (DCM:MeOH) that was acidified to ensure the release of the ionic FBs. In the application of Monbaliu et al. (2010a), the purification of the eluate obtained from the C18 column was continued with hexane defatting and subsequently passing it through a Mycosep® 226 column (Römer Labs, Tulln, Austria). All the 23 mycotoxins of interest could be recovered except for FBs. To include these compounds in the analysis, a portion of the defatted eluate was filtered through a glass filter, and the two portions were combined for LC/MS determination.

Clean-up procedures taking advantage of the different selectivities of C18 reversed phase and amino (NH2) normal phase sorbents for retaining sample matrix components have been described in the methods for detecting 23 and 26 mycotoxins from sweet pepper or tea (the dry product) (Monbaliu et al. 2009;2010b). First, the acidified ethyl acetate extract was allowed to let flow through NH2 column retaining polar matrix components, like pigments (Monbaliu et al. 2009;2010b). After evaporation and reconstitution of the sample in ACN:water (84:16, v:v), the eluate was cleaned in a C18 column with the aim of adsorbing more interferences. Since fumonisins were retained in the NH2 column, they were either analysed from

an unpurified extract (Monbaliu et al. 2010b), or isolated separately utilizing strong anion exchange (SAX) cartridges (Monbaliu et al. 2009). The SAX sorbent could retain the FBs in their anionic form. Consequently, acidified MeOH was used as an eluent to release these compounds from the column (Monbaliu et al. 2009). Apart from this application, the ion exchange mechanism has not been utilized widely in multi-mycotoxin analysis although the methods often comprise ionic analytes. The potential of using the orthogonal SPE, i.e. taking advantage of the different selectivity of anion and cation exchange and RP mixed mode columns, has been shown in the cleaning-up of microbial extracts for the screening and characterizing of metabolites (Månsson et al. 2010). This type of systematic approach might hold potential also in targeted multi-mycotoxin analysis although it would require careful preliminary optimization and the use of several columns to extract groups of analytes according to their polarity.

Multisep® and Mycosep® (Römer Labs, Tulln, Austria) SPE cartridges intended for purification of food and feed extracts for the analysis of selected groups of mycotoxins have been demonstrated as being applicable in multi-mycotoxin analysis. Multisep 226® and Mycosep 226® Aflazon+ sorbents were applied in the determinations of 13 and 17 mycotoxins from cereal based products and peanut butter, respectively (Tanaka et al. 2006; Ren et al. 2007). According to the manufacturer, the cartridges consist of silica based sorbent having both polar and nonpolar functionalities able to remove matrix interferences while the selected analytes, i.e. AFs, ZEN, PAT and TRs, come through unretained. In the multi-mycotoxin applications, a portion of the sample extract in ≥ 85 % ACN was loaded to the cartridge achieving adsorption of interferences and direct elution of AFs, ZEN and TRs (Tanaka et al. 2006; Ren et al. 2007). Ren et al. (2007) showed that in addition, verrucarol, CIT and STE could be collected in the eluate. Both groups reported overall method recoveries of ≥70.6 % for the mycotoxins investigated (Tanaka et al. 2006; Ren et al. 2007).

SPE sorbents based on immunoaffinity (IA) chromatography have been used commonly in analyte and group specific mycotoxin extractions from various commodities (Zöllner and Mayer-Helm 2006; Senyuva and Gilbert 2010). The immunosorbents contain immobilized antibodies which specifically bind the analytes (i.e. antigens) thus providing a very selective method for isolating the compounds of interest from a sample extract (Senyuva and Gilbert 2010). However, commercial IA columns containing a mixture of antibodies designed for the determination of co-occurring but structurally different mycotoxins are not widely available. The IA column AOZ™ of Vicam (Waters corp., Manchester, UK) developed for extracting aflatoxins, OTA and ZEN has been employed in food analysis in conjunction with LC coupled to a fluorescence detector (Chan et al. 2004; Göbel and Lusky 2004; Ibañez-Vea et al. 2011). At present, the most comprehensive multi-mycotoxin application based on IA clean-up and LC/MS determination has been reported for 11 toxins which were isolated from a maize extract with a Myco6in1® column (Vicam/Waters

corp., Manchester, UK) containing antibodies of AFs, OTA, FBs, ZEN, DON, T-2 and HT-2 (Lattanzio et al. 2007). The analytes were loaded in a PBS:MeOH solution and released from the immunosorbent with pure MeOH. The loading volume and the percentage of MeOH were reported as being critical for any successful extraction of DON in particular. Antibodies in IA sorbents are generally known to be susceptible to organic solvents. Hence, it was hypothesized that the MeOH present at the loading step partially denaturated the DON antibodies, leading to unsatisfactory retention (Lattanzio et al. 2007). After optimization of the extraction and clean-up steps, total recoveries in a range of 79 to 104 % were obtained.

2.3.2.2 QuEChERS extraction

A recently introduced sample clean-up procedure called QuEChERS (quick, easy, cheap, effective, rugged and safe) initially developed for pesticide analysis from vegetables and fruit by Anastassiades et al. (2003) has been introduced also in multi-mycotoxin methods for cereal and feed samples (Mol et al. 2008; Desmarchelier et al. 2010; Rasmussen et al. 2010; Zachariasova et al. 2010). The feasibility of this procedure in simultaneous determination of contaminant residues of diverse classes has also been demonstrated (Mol et al. 2008; Romero-González et al. 2011). The QuEChERS approach is based on the extraction (usually with ACN) of analytes of different polarities and a simultaneous partitioning of the organic and water phases (Lehotay et al. 2010). The partitioning is performed with the aid of salts and buffering agents to induce the transfer of the analytes into the organic phase which is thereafter purified by dispersive solid phase extraction (d-SPE) using an SPE material of choice, such as primary secondary amine (PSA) (Lehotay et al. 2010). The separation of the two phases and the partitioning of the analytes between them (i.e. achievable recovery), are optimized with the type and amount of salts added (Anastassiades et al. 2003).

In the multi-residue methods for mycotoxins, a modified QuEChERS procedure has been utilized performing only the extraction/partitioning step but excluding the subsequent d-SPE clean-up. The mycotoxins were extracted from sample matrices with ACN which was acidified to ensure the extraction of FBs (Mol et al. 2008; Desmarchelier et al. 2010; Rasmussen et al. 2010; Zachariasova et al. 2010). ACN buffered with sodium acetate was advantageous for maize silage samples to compensate for the variation of pH (Rasmussen et al. 2010). The phase separation and transfer of the mycotoxins into the organic phase has been induced either with 4 g of magnesium sulphate ($MgSO_4$) alone or together with 1 g of sodium chloride (Desmarchelier et al. 2010; Vaclavik et al. 2010; Zachariasova et al. 2010), which is in accordance with the original QuEChERS procedure optimized for pesticides

(Anastassiades et al. 2003). $MgSO_4$ and the buffering of the solvent with sodium acetate has also been used (Mol et al. 2008; Rasmussen et al. 2010; Romero-González et al. 2011). The advantage of buffering was not discussed but a similar approach was introduced by Lehotay et al. (2005) to achieve acceptable recoveries for certain pH-dependent pesticides.

The d-SPE step has been omitted from most of the multi-mycotoxin methods because PSA effectively removes polar compounds thereby creating a risk of losing some of the analytes of interest (Anastassiades et al. 2003). In particular, fumonisins can bind to PSA, hindering their extraction (Desmarchelier et al. 2010; Zachariasova et al. 2010). PSA was not incorporated by Rasmussen et al. (2010) due to the risk of losing the acidic mycotoxins relevant in silage. After the QuEChERS extraction, the resulting ACN phase was directly subjected to the MS analysis (Mol et al. 2008; Desmarchelier et al. 2010; Rasmussen et al. 2010; Zachariasova et al. 2010; Romero-González et al. 2011). One exception was the method of Desmarchelier et al. (2010), who considered the cleaning effect of QuEChERS as being inadequate and added a defatting step with hexane to reduce matrix effects in MS. The d-SPE purification with PSA after the partitioning step has been employed in one application determining mycotoxins from cereals (Vaclavik et al. 2010). The effect of the QuEChERS procedure on method performance was not discussed as the challenges were associated more with the ionization of the analytes with the direct analysis in real time (DART)-MS. However, satisfactory recoveries (from 100 to 108 % when using internal calibration) were obtained for those 11 analytes that could be ionized and included in the method (Vaclavik et al. 2010).

2.3.2.3 Simplified sample-clean up

Injection of a crude or diluted sample extract without clean-up has been introduced in several multi-mycotoxin LC/MS analysis (e.g. Sulyok et al. 2006; Herebian et al. 2009; Martos et al. 2010). Most of the published applications are intended for cereals and feed but this approach has been utilized also for a food mixture (Rundberget and Wilkins 2002). In this case, hexane de-fatting was performed simultaneously with sample extraction to reduce the amount of lipids in the final sample. This was the only sample purification step, followed by concentration of the extract with evaporation, and reconstitution in a solvent compatible with the LC mobile phase used. Cereal and feed extracts have been injected to LC/MS as such or after concentration and reconstitution (Garrido Frenich et al. 2009; Herebian et al. 2009; Capriotti et al. 2010). The disadvantages of this practice are the extensive matrix effects and instrument contamination that may be encountered because of the high amount of matrix extracts present in the sample injected in relation to

solvent (Garrido Frenich et al. 2009).

The undesired matrix effects can be overcome by diluting the extract before injection. Sulyok et al (2006) demonstrated that matrix effects were not significant from diluted wheat and maize extracts for most of the 39 mycotoxins analysed. Furthermore, as the matrix effects were repeatable and could be compensated by using matrix-assisted standards, then the use of the dilute-and-shoot method was justified (Sulyok et al. 2006). A similar approach has been applied in several other studies investigating cereal, nut and dried fruit extracts (Spanjer et al. 2008; Beltrán et al. 2009; Herebian et al. 2009; Martos et al. 2010). However, in some cases, dilution of the extract led to the increase of limit of quantification (LOQ) of some of the analytes thus favouring the injection of crude extract (Garrido Frenich et al. 2009; Herebian et al. 2009).

Generally, the method performance, particularly the LOQs, have to be compromised when reducing sample purification (Sulyok et al. 2006). One drawback may also be the contamination of the LC/MS instrument. For example, a gradual increase in the MS response due to contamination has been reported when injecting repeatedly diluted cereal extracts (Sulyok et al. 2007b). In addition, contrary findings have been reported. Despite the visual contamination observed, the sensitivity of the instrument was not affected by over 200 injections of unpurified cereal extracts (Herebian et al. 2009). Some researchers have employed a washing run after each injection to avoid response variation caused by matrix related contamination (Rundberget and Wilkins 2002; Rasmussen et al. 2010).

The direct injection may be the only practical approach to include a large number of chemically diverse mycotoxins in a single analysis (Sulyok et al. 2006). Additionally, the approach enables an easy inclusion of new analytes and matrices, assuming that the previously optimized extraction and chromatographic conditions are applicable (Sulyok et al. 2007a;2007b). For example, Sulyok et al. (2006;2007a;2010) gradually expanded their method from 39 to 106 mycotoxins and from cereal matrices to a wide range of food matrices, such as honey, jam and wine. Furthermore, screening of unknown metabolites can be carried out only when injecting crude extract since any kind of sample preparation could result in the loss of interesting compounds (Herebian et al. 2009). Simplicity and high throughput are often listed as objectives of the multi-analyte methods, and these may be accomplished only with direct injection of a sample extract (Mol et al. 2008; Garrido Frenich et al. 2009; Martos et al. 2010).

2.3.3 LC AS A SEPARATION TECHNIQUE IN MULTI-MYCOTOXIN ANALYSIS

Trace analysis typically encompasses the use of some form of high performance separation technique after the preceding sample pre-treatment steps (Boyd et al. 2008). The separation of analytes in the multi-mycotoxin methods has been achieved with high performance liquid chromatography (HPLC) or the more recently emerged technique, ultra high performance liquid chromatography (UHPLC). In liquid chromatography, the separation is based on partitioning of the analytes between liquid mobile phase and solid stationary phase. The technique has wide applicability in the separations of compounds of diverse molecular weight and physico-chemical properties (Snyder et al. 1997), and has been proved as a feasible technique also in mycotoxin analysis (Zöllner and Mayer-Helm 2006).

The role of chromatography has changed with the emergence of highly selective, fast data acquisition MS instruments, and the need for complete separation of analytes is not a prerequisite anymore. Despite this, chromatography is generally utilized in trace analysis to separate the analytes from matrix components to reduce their negative impact on MS response (Boyd et al. 2008). In addition, chromatographic resolution of close eluting compounds is advantageous. First of all, the retention time is an important identification parameter of analytes (Snyder et al. 1997; European Union 2002a). Additionally, MS data acquisition may be enhanced since resolution of peaks helps acquiring enough data points across each peak and thereby achieving good sensitivity and peak appearance. Furthermore, baseline resolution is desirable to perform MS ionization polarity switching between two adjacent peaks (Lattanzio et al. 2007). The chromatographic performance can be affected by the choice of stationary and mobile phases (Snyder et al. 1997). In the multi-residue analysis, the challenges may be encountered when selecting and adjusting these in order to achieve adequate retention, required resolution and good peak appearance for analytes exhibiting diverse chemical properties.

2.3.3.1 Stationary phases

A range of stationary phases with different chemistries are available to carry out liquid chromatographic separation. Commonly used retention mechanisms include reversed phase, normal phase, ion exchange, ion pair, chiral and affinity chromatography (Snyder et al. 1997; Boyd et al. 2008). In this review, only RP chromatography is presented in more detail since it is used exclusively in multi-mycotoxin LC/MS determinations (e.g. Sulyok et al. 2006; Spanjer et al. 2008; Desmarchelier et al. 2010). RP is mainly based on hydrophobic interactions between the analyte and the stationary phase, ensuring retention for a wide range of chemical compounds (Snyder et al. 1997). Generally, RP is a suitable technique for separating

mixtures containing both neutral and ionic compounds of low molecular weight (< 2000 Da) that dissolve in water-organic solvents (Snyder et al. 1997). The most often used columns in multi-mycotoxin separations are those based on silica particles with octadecyl (C18) bonded phase that provide a rugged and highly retentive sorbent (Snyder et al. 1997). Additionally, C14 and C6 phenyl phases with somewhat different RP retention selectivity have been used (Sørensen et al. 2009; Desmarchelier et al. 2010; Rasmussen et al. 2010). The Zorbax Bonus-RP C14 column with embedded amide groups providing improved peak shape for basic compounds at neutral or low pH values (Agilent Technologies, Santa Clara, CA, United States) was used by Desmarchelier et al. (2010) but the advantages of the column choice were not discussed. The Gemini C6 phenyl column designed specifically for the separation of aromatic and polar compounds (Phenomenex, Torrance, CA, United States) was reported to achieve good retention for the aromatic fungal metabolite chrysogine as well as for the highly polar MON (Sørensen et al. 2009).

The dimensions of the columns used in multi-mycotoxin separations based on HPLC/MS have been mostly 100 or 150 mm of length and 2 or 2.1 mm of internal diameter (i.d), although larger diameter columns of 4.6 mm i.d with 150 mm length have also been employed (Rundberget and Wilkins 2002; Sørensen and Elbæk 2005; Sulyok et al. 2006). The typical particle size in the columns was 3 or 5 μm. The columns of these dimensions generally provide enough theoretical plates to ensure high separation efficiency (Snyder et al. 1997). For example, resolution of structurally close compounds, such as α- and β-isomers of zearalenol and zearalanol or the diastereomers of ergot alkaloids have been achieved in multi-mycotoxin separations performed in C18 columns (Sørensen and Elbæk 2005; Sulyok et al. 2006;2007a). The flow rates with the 2 mm i.d. columns have been from 0.2 or 0.3 ml/min, which are compatible with the MS instruments. However, with the larger columns, flow rates of 0.6 to 1.0 ml/min have been used, with the option being the splitting of the flow before the MS instrument (Rundberget and Wilkins 2002; Sørensen and Elbæk 2005; Sulyok et al. 2006).

UHPLC is being employed increasingly in food safety applications (Malik et al. 2010), and some methods have been published also for multi-mycotoxin analysis (Ren et al. 2007; Romero-González et al. 2009; Jin et al. 2010; Zachariasova et al. 2010). The UHPLC technique takes advantage of columns packed with small particles of sub-2 μm (Swartz 2005). In addition, higher flow rates compared to the conventional HPLC/MS are commonly used (Swartz 2005). The small particle size increases the column efficiency improving resolution and sensitivity as the chromatographic peaks are narrower. Since the column efficiency is not diminished when working with a high flow rate, the speed of analysis can be improved when the number of peaks resolved per unit time is increased (Swartz 2005). However, the reduction of particle size leads to increased back pressure in the UHPLC system requiring a special design of the instrumentation and the columns. Therefore, high

performance pumps that can deliver up to 15000 psi of pressure and columns with stationary phases able to withstand high mechanical forces are employed (Swartz 2005).

RP columns based on ethylene bridged-silicon hybrid particle (BEH) and high strength silica (HSS) sorbents introduced by Waters (Manchester, UK) have been used in multi-mycotoxin separations (Ren et al. 2007; Beltrán et al. 2009; Garrido Frenich et al. 2009; Jin et al. 2010; Monbaliu et al. 2010a; Zachariasova et al. 2010). Kinetex® columns (Phenomenex, Torrance, CA, USA) also utilize small particles (1.7 and 2.6 μm) which are made of non-porous silica core and a porous silica layer. These so called core shell particles have high mechanical strength, making the columns suitable for UHPLC applications. However, until today no reports exist about the use of these types of columns in mycotoxin analysis with UHPLC although an HPLC/MS based application employing a C18 Kinetex column (100 x 2.1 mm, 2.6 μm) using a flow rate of 0.2 ml/min has been described (Capriotti et al. 2010).

Studies carried out in the field of pesticides have demonstrated the benefits obtained from replacing an HPLC/MS multi-residue method with a UHPLC/MS method. For example, improved detection capability and reduced analysis time and solvent consumption were achieved in the analysis of 17 pesticides from apples (Kovalczuk et al. 2006). For mycotoxins, no such comparative studies have been published. However, the separation efficiency of the sub-2 μm particle columns was demonstrated in a multi-mycotoxin application (Ren et al. 2007). Improved resolution was achieved in a shorter run-time for the 19 mycotoxins of interest with Acquity C18 and C8 BEH columns (100 or 150 x 2.1 mm, 1.7 μm) in comparison to conventional C18 HPLC columns (150 x 2.1 mm, 5 μm particles) under equivalent conditions employing a UHPLC/MS system. The high separation efficiency of HSS T3 stationary phase was demonstrated by Jin et al. (2010). Improved resolution of the ten selected mycotoxins was achieved with this sorbent in comparison to BEH columns of equivalent dimensions (100 x 2.1 mm). In addition, the position isomers 3-Acetyl deoxynivalenol (3-AcDON) and 15-AcDON could be separated in the HSS T3 column although these peaks overlapped in the BEH columns. However, regarding the peak shapes and intensities, the two stationary phases ensured equal performance.

2.3.3.2 Mobile phases

The mobile phase used in a liquid chromatographic separation should be chosen on the basis of the analyte properties (e.g. solubility) as well as the desired retention and selectivity of the analytes (Snyder et al. 1997). In LC/MS applications, the eluent composition needs to be considered in conjunction with the MS analysis; one

prerequisite is that the reagents are volatile and thus compatible with the ionization (Boyd et al. 2008). Similarly to the extraction, mixtures of water and organic solvents, most commonly ACN and MeOH have been used in multi-mycotoxin LC separations. These two solvents are compatible with both RP chromatography and MS (Boyd et al. 2008). The physico-chemical properties of ACN and MeOH differ and therefore, the two provide somewhat differing retention and selectivity in chromatographic separation (Snyder et al. 1997). Although satisfactory chromatography can be achieved with either of these eluents or their mixture, MeOH has been chosen as the mobile phase in the majority of the multi-mycotoxin separations (e.g. Sulyok et al. 2006; Tanaka et al. 2006; Martos et al. 2010; Monbaliu et al. 2010a). One reason could be the weaker solvent strength of MeOH in C18 (Boyd et al. 2008). This is of importance especially at the beginning of gradient elution since increased retention of the highly polar mycotoxins can be achieved with MeOH in comparison to ACN when used at the same percentage. Thus, there is no need to increase the proportion of water, which would be disadvantageous for ionization process in MS (Boyd et al. 2008). In general, the effect of solvent on the MS response of the analytes has probably been a more decisive factor when choosing the eluent, as decreased ionization and sensitivity have been reported using ACN as compared to MeOH (Ren et al. 2007; Garrido Frenich et al. 2009).

Volatile mobile phase modifiers are used to improve both the chromatographic and mass spectrometric behaviour of analytes (Boyd et al. 2008). Organic acids (mostly acetic and formic acid) and their ammonium salts are commonly used modifiers in LC/MS, and have been frequently added to the eluents in multi-mycotoxin applications (e.g. Sulyok et al. 2006; Desmarchelier et al. 2010; Jin et al. 2010). With regard to the chromatography, the additives are utilized to adjust pH and thereby they can affect the retention of the analytes that have ionic groups (Snyder et al. 1997). In RP chromatography, improved retention is achieved when the compounds are in their neutral form, and pH should be adjusted taking into account the pKa values of the analytes (Snyder et al. 1997). Nonetheless, in multi-analyte separations, it may not be possible to adjust pH optimally for all the analytes. Acidic conditions have been reported as being advantageous in several multi-mycotoxin methods, a typical example being the fumonisins for which retention and acceptable peak shapes have been achieved only under acidic conditions when the four carboxylic groups are in the neutral form (Cavaliere et al. 2005; Sulyok et al. 2006; Lattanzio et al. 2007).

Despite using a low pH, broad or tailing peaks have been observed for some other acidic mycotoxins such as cyclopiazonic acid (CPA), CIT, fusaric acid and tenuazonic acid (Sulyok et al. 2007a; Spanjer et al. 2008; Beltrán et al. 2009; Sørensen et al. 2009). It is possible that the pH was not sufficiently low to ensure that the functional groups possessing a low pKa were in their neutral forms, causing improper or no retention. In addition, MON has a low pKa (1.7), but acceptable retention and peak

appearance have been achieved in C18 and C-6 phenyl phases (Sulyok et al. 2007a; Sørensen et al. 2009). However, Sulyok et al. (2007a) postulated that in contrast to the other acidic mycotoxins, ion-pairing and ionic interactions were involved instead of hydrophobic retention mechanism. On the other hand, Sørensen et al. (2009) suggested that the silica solid support of the column used in these studies (i.e. Gemini® particle manufactured by Phenomenex, Torrance, CA, USA), and not the bonded phase, was responsible for the retention.

The LC separation of chemically diverse mycotoxins has been accomplished by using a gradient elution with increasing the solvent strength, i.e. the proportion of the organic component in RP chromatography. With gradient, the peak shapes and elution of strongly retained, late eluting compounds can be promoted and reasonable run-times achieved (Snyder et al. 1997). In the most comprehensive multi-mycotoxin method, 85 analytes (of the total of 106) could be retained and separated in a single HPLC analysis using a flow rate of 1 ml/min and a C18 column (150 x 4.6 mm, 5 µm) with a gradient elution starting from MeOH:water:acetic acid (10:89:1+5 mM ammonium acetate). Some retention was obtained for the very polar analytes (e.g. MON, 3-nitropropionic acid and kojic acid), and the final compound (emodin) eluted at a retention time of 16.24 min (Sulyok et al. 2006;2007a;2010). In general, the HPLC-based separations of nine to 85 mycotoxins has been completed with run-times ranging from 21 to 44 min using flow rates of 0.2 to 1 ml/min (Sulyok et al. 2006;2010; Capriotti et al. 2010; Rasmussen et al. 2010). Shorter run-times (6.5 to 25 min) were needed for separating 10 to 26 mycotoxins with the UHPLC methods applying flow rates of 0.25 to 0.55 ml/min (Ren et al. 2007; Jin et al. 2010; Monbaliu et al. 2010b). A disadvantage of a gradient run can be the incompatibility of the initial mobile phase composition to that of the injected sample. Distortion or broadening of the peaks of early eluting mycotoxins has been reported when samples have been injected in a solvent with a higher organic content than that present at the initial gradient conditions (Sulyok et al. 2006; Ren et al. 2007; Zachariasova et al. 2010). The problem has been overcome by dilution of the sample with water or by reconstituting it into a solvent composition closer to the aqueous conditions at the beginning of the LC analysis (Sulyok et al. 2006; Ren et al. 2007; Zachariasova et al. 2010).

It may not be possible to analyse all the compounds of interest in a single analysis due to their differing requirements for LC/MS conditions. Sulyok et al. (2006) reported that 39 mycotoxins were too many to allow ionization polarity switching within a single run. In this case, it may be practical to divide the analytes on the basis of their ionization behaviour into two chromatographic runs which utilize either positive or negative ionization mode solely (Sulyok et al. 2006; Herebian et al. 2009; Rasmussen et al. 2010). This approach eliminates the problem arising from co-elution of two analytes with opposite MS ionization polarity needs. Generally, dividing the analytes into more than one analytical run offers more flexibility when

optimizing the LC/MS conditions for a greater number of analytes. For example, Rasmussen et al. (2010) used a mobile phase containing ammonia and formic acid for the pH dependent (i.e. ionic) compounds, improving their chromatography as well as the MS response of those analytes that formed ammonium adducts. However, some of the analytes suffered from signal suppression due to ammonium formate, and another LC analysis with formic acid as the only modifier was used.

2.3.4 MS detection in multi-mycotoxin analysis

Mass spectrometry (MS) has become a popular detection technique in trace analysis (Malik et al. 2010). The MS detectors are sensitive and capable of detecting compounds at low concentration levels (Boyd et al. 2008). They are also highly selective as the identification of analytes is based on molecular mass, and additional structure specific information can be obtained e.g. via specific fragmentation reactions (Boyd et al. 2008). The coupling of a chromatograph and an MS instrument is performed through an interface, the purpose of which in LC is to remove the excess of the mobile phase by evaporation and to ionize the analytes so that they can be transferred to the mass analyser section of the MS operated at vacuum (Boyd et al. 2008). In the mass analyser, the ions are separated according to their mass-to-charge ratio (m/z) (Boyd et al. 2008).

The atmospheric pressure ionization (API) techniques dominate modern on-line LC/MS equipment (Boyd et al. 2008), and these have been also used in the published multi-mycotoxin methods (e.g. Sulyok et al. 2006; Tanaka et al. 2006; Capriotti et al. 2010). The most common mass analysers have been the quadrupole based instruments like triple quadrupole (QqQ) and quadrupole ion trap (QIT) enabling tandem MS (MS/MS) (e.g. Sulyok et al. 2006; Martos et al. 2010; Monbaliu et al. 2010a; Rasmussen et al. 2010). Some applications utilizing single MS instruments have been also presented (Tanaka et al. 2006; Herebian et al. 2009; Zachariasova et al. 2010). The challenge of MS detection arises from finding suitable conditions to ionize the analytes of interest efficiently and to produce product ions of sufficient response for successful quantification. In addition, the negative effects of matrix interferences on analyte signal (i.e. matrix effects) need to be examined and compensated.

2.3.4.1 Ionization

The atmospheric pressure ionization (API) techniques dominate modern on-line LC/MS equipment (Boyd et al. 2008). The three commonly used API techniques,

electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) have all been applied in multi-residue applications of mycotoxins (e.g. Sulyok et al. 2006; Tanaka et al. 2006; Capriotti et al. 2010). The API interfaces have similar design features as they all accommodate liquid flow by making use of heating and/or nebulization aids often involving inert gases to aid desolvation. Additionally, they include optics for transferring ions from the atmospheric pressure ion source into the vacuum (Boyd et al. 2008). However, the physico-chemical processes that lead to ion formation are different between ESI, APCI and APPI.

In ESI, the ionization occurs in liquid phase, and an aerosol of droplets carrying a net electrical charge is formed by nebulizing the solution entering to the ion source through a capillary held at high voltage. The increase in electric field density of droplets leads to decrease in surface tension and in droplet size. This process is repeated until charged molecular ions have been produced in gas phase. Both heat and inert gas (usually nitrogen) are used to aid in desolvation of solvent (Boyd et al. 2008). APCI is a form of chemical ionization which is induced in a gas phase (i.e. in ambient air). APCI is used in combination with a heated pneumatic nebulizer which achieves nebulization of the liquid sample and evaporation of eluent droplets in a heated region before the ionization is achieved by subjecting the sample to a high potential applied through a so called corona discharge needle. Following the discharge, protonated (or deprotonated) molecules are formed from the eluent components. These subsequently ionize the analytes primarily through proton transfer reactions (Boyd et al. 2008). APPI is performed in a manner similar to APCI by replacing the corona discharge needle with a photoionization lamp. The ionization process in APPI is complex, involving the use of dopants such as acetone or toluene (Boyd et al. 2008). First, dopants are ionized by photons forming molecular ions. These react with eluent through proton transfer and subsequently, eluent can transfer protons to analytes. Dopant may also react directly with analytes through charge exchange mechanism (Boyd et al. 2008).

The ionization of a compound in an ion source is related to various factors: the operation parameters of the MS (e.g. ion source temperature, flow rate of the eluent and capillary voltage or corona discharge potential), the properties of the eluent (such as volatility, surface tension, proton affinity, conductivity, polarity and pH) as well as the nature of the analyte (e.g. pKa value, proton affinity, polarity and molecular mass) (Boyd et al. 2008). Even if these are known, the processes in the ion source may not be predictable and one will need to undertake experimental testing to determine the optimal conditions for the given analytes (Boyd et al. 2008). The studies have demonstrated that with optimization of the conditions and operating parameters, all API interfaces are suitable for multi-mycotoxin analysis (e.g. Sulyok et al. 2006; Tanaka et al. 2006; Capriotti et al. 2010). However, the majority of the multi-mycotoxin applications take advantage of ESI (e.g. Sulyok et al. 2006;2010;

Spanjer et al. 2008; Martos et al. 2010), which is probably due to applicability of ESI as it can ionize compounds with a wide range of chemical polarities and molecular masses. For example, the technique was suitable for ionizing the 106 different mycotoxins included in the method of Sulyok et al. (2010). Generally, ESI is considered a better choice for polar and ionic compounds in comparison to APCI or APPI (Boyd et al. 2008). Additionally, it can be used for thermally unstable compounds unlike APCI which requires some thermal stability (Boyd et al. 2008). In ESI, fragmentation of compounds does not usually take place during ionization process (Boyd et al. 2008). Furthermore, ESI can be performed over a wider range of flow rates than APCI. At the flow rates typically used in HPLC, ESI works as a concentration dependent instrument and a linear response is obtained. However, the dynamic range of ESI can be limited compared to APCI (Boyd et al. 2008).

APCI and APPI are best suited to medium or nonpolar compounds which may not be ionized efficiently with ESI (Boyd et al. 2008). The APCI interface has proved applicable in mycotoxin determinations of restricted group of analytes, such as trichothecenes (Razzazi-Fazeli et al. 2002; Berthiller et al. 2005). Its feasibility has been examined also in a few multi-mycotoxin analysis comprising of 13 to 18 chemically diverse analytes (Rundberget and Wilkins 2002; Biselli et al. 2004; Tanaka et al. 2006). However, better responses were obtained generally by employing ESI instead of APCI with MeOH as the mobile phase (Biselli et al. 2004). On the other hand, Tanaka et al. (2006) reported improved responses for trichothecenes and ZEN with APCI although ESI worked better for aflatoxins when MeOH with ammonium acetate was used as the mobile phase. APPI has been applied in the specific analysis of AFs and TRs (Takino et al. 2004; Tanaka et al. 2009) but until now, it has been a less widely used interface in multi-analyte methods. Capriotti et al. (2010) investigated the applicability of this technique in the analysis of nine mycotoxins from cereal matrices. It was demonstrated that improved responses for some of the analytes (e.g. AFs) could be achieved with APPI in comparison to the equivalent ESI method, thus demonstrating the potential of this ionization technique in multi-mycotoxin analysis. Normally, the matrix effects in APCI and APPI are less profound compared to ESI interface (Boyd et al. 2008). For example, it has been reported that matrix components did not affect the signal intensity of analytes determined from cereal extracts purified with Mycosep® 226 cartridge when using HPLC-APCI-MS (Tanaka et al. 2006). Similarly, Capriotti et al. (2010) reported that the matrix effects were not significant as determined with the APPI based method although unpurified cereal extracts were injected. In both studies, external calibration in standard solution could be used instead of matrix-assisted calibrants (Tanaka et al. 2006; Capriotti et al. 2010).

Generally, in the ionization processes of the API techniques, ionized intact analyte molecules of the form $[M+H]^+$ are produced. Additionally, various adduct ions e.g. with alkali metal ions (Na, K, Li), ammonia or chloride may be produced in the

positive ionization mode. In the negative mode, deprotonated molecular ions $[M-H]^-$ are formed, with formate and acetate adducts being also common (Boyd et al. 2008). The formation of the ions is application dependent due to various factors that affect the process and can be manipulated. Therefore, different ions have been produced and selected as precursor ions for mycotoxins in the published applications. Sulyok et al. (2006) stated that most of the 39 mycotoxins investigated produced reasonably high signal intensities in both ionization modes in the conditions applied. In this case, the polarity mode could be chosen on the basis of the best achievable response. Examples of compounds that can be ionized readily at both ESI polarities are OTA and ZEN which have been monitored as protonated or deprotonated molecular ions depending on the conditions (Lattanzio et al. 2007; Ren et al. 2007; Beltrán et al. 2009; Garrido Frenich et al. 2009). However, it seems that the majority of mycotoxins have a higher tendency to ionize in ESI+ and acquire a proton, thus forming protonated molecular ions (Sulyok et al. 2006;2007a;2010). Nonetheless, certain compounds ionize only in either of the ionization polarity modes. For example, MON can be ionized only in the negative mode as $[M-H]^-$ (Sulyok et al. 2006; Herebian et al. 2009), whereas ENNs and type-A TRs have been reported as ionizing only in the positive mode (Sulyok et al. 2006).

Although MS is considered a universal detector, sometimes it may not be possible to ionize certain analytes. For example, poor or no ionization has been reported for PAT and PA in ESI (Rundberget and Wilkins 2002; Beltrán et al. 2009; Herebian et al. 2009). Additionally, inadequate ionization of trichothecenes as protonated or deprotonated molecular ions with ESI is known. This problem has been overcome by monitoring adducts since the type-A TRs produce readily ammonium adducts in ESI+ and the type-B TRs form acetate and formate adducts in ESI- (Cavaliere et al. 2005; Sulyok et al. 2006; Garrido Frenich et al. 2009; Herebian et al. 2009). The formation of adducts in API can be enhanced by adding appropriate mobile phase modifiers (e.g. ammonia, organic acids and salts) if it is desirable to use the adducts as precursor ions (Boyd et al. 2008). It is important that only one form of the ion contributes significantly to the ion current derived from the analyte and that the concentration of the ion forming species is constant under the conditions being used (Boyd et al. 2008). This is also beneficial to avoid the formation of stable precursor ions such as sodium adducts which are commonly formed for the type-A and B TRs and AFs in ESI+ without yielding detectable product ions upon collision induced dissociation (CID) in the mass analysers (Sulyok et al. 2006; Beltrán et al. 2009; Garrido Frenich et al. 2009).

Both MeOH and ACN have suitable properties for API ionization (Boyd et al. 2008). However, MeOH has been reported as yielding better responses for mycotoxins as compared to ACN. This has been demonstrated for TRs, AFs, ZEN and OTA (Cavaliere et al. 2005; Ren et al. 2007; Garrido Frenich et al. 2009). One explanation could be that MeOH as a protic solvent is capable of donating a

proton and thus enhancing the response of $[M+H]^+$ ions in the positive mode of ESI. However, MeOH has also been proven as a more favourable solvent for the ionization of analytes ionized in ESI- (Cavaliere et al. 2005, Ren et al. 2007; Garrido Frenich et al. 2009). The composition of a mobile phase typically used in the RPLC is not necessarily optimal for ESI ionization. Ideally, chromatographic conditions could be chosen so as to create charged analytes, which would increase ion formation and therefore sensitivity (Boyd et al. 2008). However, improved retention in RP chromatography is achieved when analytes are in their neutral forms (Snyder et al. 1997). Furthermore, simultaneous separation of basic and acidic compounds does not enable adjusting pH to obtain charged species for both of these. In multi-mycotoxin methods, the conditions are usually adjusted to a low pH ensuring the retention of the acidic compounds and thus, their ideal ionization might be sacrificed. Additionally, the conditions at the beginning of a RP gradient elution do not favour ESI ionization, since a high concentration of water is present (Boyd et al. 2008). Usually the ionization is improved when a higher percentage of organic solvent is present, as the surface tension of the eluent is lower and the volatility higher (Boyd et al. 2008). For example, increased sensitivity of 12 mycotoxins was achieved in the higher contents of MeOH (from 5 to 40 % were tested) with a compromise of 25 % of MeOH ensuring both satisfactory MS responses and RP chromatography (Garrido Frenich et al. 2009).

Modifiers added to the mobile phase can affect negatively on the ionization of some analytes. If their concentration is too high, they can displace the analyte ions from the charged droplets formed in ESI leading to limited emission of the analytes to the gaseous phase thereby reducing their response (Boyd et al. 2008). Signal suppression was reported for early eluting compounds NIV, DON, PAT and GLIO if 0.4 mM ammonium formate was added (Rasmussen et al. 2010). Hence, these toxins were analysed without the buffer in a separate LC-MS/MS run with the negative mode of ESI. Ionization suppression with ammonium acetate concentrations higher than 0.5 mM was observed by Beltrán et al. (2009). In addition, formic acid has been reported to evoke signal suppression of ZEN and other *Fusarium* toxins. Addition of the acid at 0.1 % or 10 mM has been found to represent a suitable compromise (Cavaliere et al. 2005; Beltrán et al. 2009).

2.3.4.2 Mass analysers

In the mass analyser section of a mass spectrometer, the molecules are separated according to their mass to charge ratio (m/z). This can be performed either with the aid of magnetic or electronic field as in linear quadrupole or ion trap instruments, or by monitoring the flight of ions like in time of flight devices (Boyd et al. 2008). The

analysers differ in design and properties and hence, in their suitability for analytical purposes (Boyd et al. 2008). Several types of mass analysers have been employed in multi-mycotoxin applications, including triple quadrupole (QqQ), quadrupole ion trap (QIT), time of flight (TOF) and Orbitrap instruments (Sulyok et al. 2006; Tanaka et al. 2006; Herebian et al. 2009; Martos et al. 2010).

The most commonly used analysers in the multi-mycotoxin methods are the quadrupole based instruments, particularly the triple quadrupole (QqQ) analysers (e.g. Martos et al. 2010; Monbaliu et al. 2010a; Rasmussen et al. 2010). Some applications employing quadrupole ion trap (QIT) have also been presented (Sulyok et al. 2006; Lattanzio et al. 2007; Capriotti et al. 2010). The QqQ instruments enable tandem mass spectrometry, where the precursor ion selected in the first quadrupole is fragmented in a collision cell (i.e. the second quadrupole) by CID to analyte specific product ions which are selected in the third quadrupole and monitored with multiple reaction monitoring (MRM) data acquisition mode (Boyd et al. 2008). QIT are hybrid quadrupole/linear ion trap mass spectrometers and closely related to QqQ instruments in operating principles. In QIT, ions are trapped and stored within the trap where CID can be performed to produce MS/MS or multiple stage MS (MS^n) data (Boyd et al. 2008).

Triple quadrupole and quadrupole ion trap instruments are relatively low cost and easy to operate. The popularity of the MS/MS instruments is based on their applicability in quantitative analysis. QqQ analysers in particular have been proved capable of providing the selectivity and sensitivity required from the most rigorously validated methods (Boyd et al. 2008). Collecting MS/MS data enhances the selectivity of the analysis when structure specific product ions are monitored leading to higher confidence in the identification of the analytes (Boyd et al. 2008). Furthermore, the relative abundance ratios of the product ions can be used for additional confirmation (European Union 2002a). Chemical background is also reduced in MS/MS providing an improved signal-to-noise ratio (S/N) compared to single MS (Boyd et al. 2008). For the identification of the analytes, it is desirable to monitor structure specific ion transitions and avoid products resulting from water or adduct loss (Beltrán et al. 2009; Rasmussen et al. 2010). However, obtaining two specific ions may not be always achieved. In the case of the type-B TRs that ionize as acetate adducts $[M+CH_3COO]^-$, abundant deprotonated molecular ions and acetate ions are produced upon fragmentation (Sulyok et al. 2006). Despite being non-specific products, these have been used for quantification with a third fragmentation added for confirmation (Sulyok et al. 2006).

Generally, the QqQ instruments have adequately high data acquisition rates for quantitative multi-residue analysis. These kinds of applications frequently involve co-eluting compounds requiring fast switching between precursor or product ion pairs but still providing enough data points across the chromatographic peak (Boyd et al. 2008). For example, isotope labeled structural analogues used as internal

standards elute simultaneously with the target analyte but need to be distinguished (Boyd et al. 2008; Zachariasova et al. 2010). Additionally, isomers 3-AcDON and 15-AcDON and AF M1 and AF G1 produce the same precursor ion in ESI and have been reported frequently to co-elute (Cavaliere et al. 2005; Sørensen and Elbæk 2005; Garrido Frenich et al. 2009; Monbaliu et al. 2009). Fast data acquisition has enabled monitoring analyte-specific fragment ions allowing the reliable identification of these mycotoxin pairs. Nevertheless, older generation QqQ instruments may have limited applicability in large-scale multi-residue analysis due to moderate scan speed/dwell times, restricting the number of compounds that can be simultaneously determined (Boyd et al. 2008). Other disadvantages of QqQ and QIT analysers are their low mass resolving power and low mass accuracy. However generally, these are adequate to achieve the selectivity required from quantitative trace analysis (Boyd et al. 2008). The limitation of the targeted multi-mycotoxin LC-MS/MS methods performed with the aid of QqQ and QIT instruments is their inability to provide data on non-target compounds that may be present in samples. If this type of information is needed, it has to be gathered in separate full scan analyses (usually of low sensitivity). On the other hand, the unique scanning functions of QqQ (i.e. neutral loss and precursor scans) and the MS^n possibility of QIT can be used for gathering additional structural information of analytes or non-target compounds (Boyd et al. 2008).

Time of flight and Orbitrap analysers have been utilized in a few multi-mycotoxin applications (Tanaka et al. 2006; Mol et al. 2008; Herebian et al. 2009; Vaclavik et al. 2010; Zachariasova et al. 2010). These are intrinsically single MS instruments that allow the detection of ionized precursor molecules in the full scan mode or the selected ion monitoring (SIM) mode (Orbitrap analysers) with the option being performing in-source CID to produce MS^n data (Boyd et al. 2008). Both analyser types have features that provide increased detection selectivity and confirmation of analyte identity, i.e. medium or high mass resolving power ($\geq 10^4$ measured as full peak width at half maximum, FWHM) as well as high mass accuracy (approximately 2 to 5 ppm) and precision (Boyd et al. 2008; Malik et al. 2010). The primary use of the TOF instruments has been for accurate mass measurements and elemental composition analysis (Boyd et al. 2008). They have rapid spectral acquisition speed that enable the recording of virtually unlimited number of compounds and the coupling with fast chromatography like UHPLC (Boyd et al. 2008; Malik et al. 2010). These properties make TOF as a suitable tool for profiling type of work in particular, when identification and confirmation of both targeted and non-targeted compounds in samples is needed (Malik et al. 2010). Earlier the limitation for the use of these instruments was the narrow dynamic linear range, but with the improvements in instrumentation, TOF has proved feasible also for quantitative work (Tanaka et al. 2006; Boyd et al. 2008). Orbitrap was introduced relatively recently and therefore, all the aspects of its applicability have not been elucidated yet but reports on its use

in trace analysis of food contaminants are emerging (Malik et al. 2010; Vaclavik et al. 2010; Zachariasova et al. 2010).

A method based on LC-ESI-TOF-MS has been described for profiling fungal metabolites (Senyuva et al. 2008). The identification was performed on the basis of a database of 465 secondary metabolites, and the utility of the approach was demonstrated in detecting fungal metabolites from agar cultures and spiked figs (a total of 20 mycotoxins were added). This type of approach holds potential in qualitative monitoring of a wide range of target and non-target fungal contaminants in food products. The utility of TOF in conjunction with HPLC-APCI and UHPLC-ESI has been demonstrated for quantitative determination of 13 and 11 mycotoxins, respectively (Tanaka et al. 2006; Zachariasova et al. 2010). Reliable and selective identification of the analytes was achieved with both techniques. It was also reported that when HPLC-APCI-TOF-MS was used for determining mycotoxins from cereal extracts purified with SPE, the influence of matrix interferences on the signal intensity and the mass accuracy was insignificant, allowing the use of solvent calibrants and the detection of considerably low concentrations of the analytes (e.g. from 0.1 to 3.8 μg/kg in wheat) (Tanaka et al. 2006). Similarly, mycotoxins could be reliably quantified from a purified cereal extract (using QuEChERS) with the UHPLC-ESI-TOF-MS (Zachariasova et al. 2010). On the other hand, the detection capacity of the instrument was not considered sufficient when unpurified extracts were injected, which was due to the high chemical noise and signal suppression encountered. The use of in-source CID to produce MS^n transitions was not found as a feasible way to improve the selectivity and sensitivity of the detection since adequate responses for the product ions were obtained only at relatively high concentrations, e.g. for DON at 500 μg/kg (Zachariasova et al. 2010).

The potential of coupling an Obritrap analyser to UHPLC-APCI was demonstrated by the same investigators (Zachariasova et al. 2010). This instrument could be used for the quantitative determination of the mycotoxins of interest from untreated cereal extracts using the single MS due the higher mass resolution and mass accuracy; the limits of detection (LODs) achieved were comparable to those obtained with the UHPLC-ESI-TOF-MS from the purified samples. Orbitrap technology has been used in two other interesting applications for mycotoxin determination from crude cereal extracts. Herebian et al. (2009) coupled ESI-Orbitrap-MS to a capillary-LC achieving a rapid and sensitive determination of 30 mycotoxins. The method performance was comparable to some HPLC-ESI-QqQ based applications, although the LODs were generally higher. A disadvantage of the method was the instable electrospray in the negative ionization mode, which hindered the inclusion of MON and CIT (Herebian et al. 2009). A multi-mycotoxin application without chromatographic separation was presented by Vaclavik et al. (2010), who were able to determine 11 mycotoxins quantitatively from cereal extracts with a DART-Orbitrap-MS. However, lower detection capability as compared to the published LC/MS based methods

was obtained, which was largely due to problems with the ionization and signal suppression in the DART interface. With technological advances, both micro-flow LC-MS and direct ionization techniques could hold potential in rapid multi-residue analysis in the future.

It is likely that the versatility of the most recent MS technology, such as the hybrid linear ion trap/Orbitrap and quadrupole-TOF (QqTOF) analysers will be implemented increasingly in the field of mycotoxin research even if applications are few at present. The feasibility of full scan accurate mass data obtained with nano-flow LC-QqTOF-MS was demonstrated recently in identifying OTA and OTB from wheat (Aqai et al. 2011). More comprehensive applications taking advantage of the modern MS technology can be found in pesticide multi-residue analysis. The potential of LC-QqTOF-MS has been demonstrated in the analysis of target pesticides and their unknown metabolites and degradation products that could be identified and finally confirmed using the accurate product ion mass spectra without reference standards (Pico et al. 2007; 2008). The feasibility of the hybrid function of a quadrupole linear ion trap instrument was utilized when SRM data was acquired with the quadrupole function for the quantitative determination of pesticides while producing confirmatory data with the MS^3 or the so called enhanced product ion (EPI) mode using the instrument as a linear ion trap during a single LC run (García-Reyes et al. 2007). The approach was useful in particular for the confirmation of those analytes that yielded only one product ion of sufficient abundance in SRM mode. In the future, coupling of the most modern analysers to UHPLC separation will probably enhance further the applicability of the latest MS techniques for the rapid screening and identification of both target and non-target analytes in the whole area of food safety (Malik et al. 2010).

2.3.4.3 Matrix effects

Matrix effects are a well-known phenomenon in mass spectrometry. These effects are caused by co-eluting sample matrix components that can induce either suppression or enhancement of the analyte signal (Boyd et al. 2008). Chemical background (or chemical noise) can also cause decrease of limits of quantification, but it is distinguished from matrix effect, as it is caused by contaminants originating from e.g. solvents, laboratory utensils or instrument tubing (Boyd et al. 2008).

Matrix effects can take place in all MS ionization techniques, although the problem is more serious with API techniques with ESI being particularly prone (Boyd et al. 2008). In the majority of the published multi-mycotoxin methods that employ ESI, significant matrix effects, mostly encountered as signal suppression, have been observed (Sulyok et al. 2006;2007b; Herebian et al. 2009; Romero-González et al.

2009; Desmarchelier et al. 2010). The effect of matrix components on an analyte response can be tested by monitoring the MS signal of an analyte standard that is infused via a T-piece connection inserted between the LC and the MS, while injecting the extracts of blank sample matrix on-column (Boyd et al. 2008). The comparison of the analyte signals of the standard solution and in the presence of matrix components reveals the presence of ionization suppression or enhancement. In this way, the efficiency of sample clean-up procedures on reducing matrix effects could also be evaluated (Boyd et al. 2008). This type of systematic testing approach has not been introduced in any of the published multi-mycotoxin methods but several researchers have adopted the signal suppression or enhancement (SSE) percentage to illustrate the extent of the matrix effect.

SSE is calculated as a relative difference of the slopes of calibration curves constructed from calibrants made in solvents and matrix-assisted standards at the corresponding concentrations (Sulyok et al. 2006). Matrix effects are particularly serious (i.e. SSE values deviate clearly from 100 %) when a food or feed extract is injected without purification (Sulyok et al. 2006;2007b; Spanjer et al. 2008). For example, the SSEs determined from various cereals ranged from 25 up to 196 % for the 39 mycotoxins determined by Sulyok et al. (2006;2007b). The matrix effect is dependent on both the analyte and the matrix. For example, the signals of AFs were not remarkably affected in wheat but significant suppression (SSE 17-68 % for the different AFs) occurred in maize (Sulyok et al. 2006). Variation in the extent of the matrix effect can be encountered between individual samples of the same commodity e.g. maize harvested in different years or between different types of beers (Sulyok et al. 2007b; Romero-González et al. 2009).

Matrix effects can be minimized by injecting samples containing less matrix impurities, which can be accomplished by more selective extraction or clean-up (Boyd et al. 2008). The effects can be reduced by diluting the extract, but at some point this may not compensate for the loss of detection limits (Sulyok et al. 2006; Herebian et al. 2009). Since matrix effects are practically unavoidable in MS, they need to be compensated to reduce the impact on the accuracy and precision of the results (Boyd et al. 2008). Internal standards (IS) may be used for compensating for both matrix effects and losses of analyte during sample preparation, and in that way improve the quality of the analytical results (Boyd et al. 2008; Rychlik and Asam 2008). A suitable IS is a compound that is chemically related and resembles the analyte as closely as possible in chromatographic and mass spectrometric behaviour but is not naturally present in real samples (Boyd et al. 2008). In multi-mycotoxin analysis, the use of IS has been rather limited. Some structurally related compounds have been added as IS including zearalanone (ZAN, a derivative of ZEN), verrucarol (hydrolysis product of macrocyclic TRs), de-epoxy-DON (a metabolite of DON), AF M1 and diclofenac (an anti-inflammatory drug) (Cavaliere et al. 2007; Ren et al. 2007; Monbaliu et al. 2009;2010b; Capriotti et al. 2010). In some cases, the use of

a particular IS has been limited to the structurally close analytes, e.g. only ZEN was quantified with using ZAN, whereas external matrix-matched calibration was used for the rest of the 17 mycotoxins (Ren et al. 2007). On the other hand, Monbaliu et al. (2009) quantified all the 23 mycotoxins of interest with ZAN, obtaining mostly comparable results with IS and external matrix-matched calibration.

The disadvantages of using structurally related IS (and not an analogue) are the systematic errors and imprecision of results that may occur due to different behaviour of the IS and the analyte during the analysis (Rychlik and Asam 2008). Therefore, the use of stable isotopes as IS is considered as the most optimal approach for compensating completely for analyte losses and matrix effects (Rychlik and Asam 2008; Boyd et al. 2008). Several deuterated and ^{13}C-labelled IS have been produced for mycotoxins (including AFs, TRs, ZEN, FBs, OTA, PAT, alternariol and alternariol methyl ether), and applied in analyte and group specific analysis (Cervino et al. 2008; Rychlik and Asam 2008; Asam et al. 2009). A selection of ^{13}C-labelled compounds is commercially available, e.g. a total of 21 are marketed currently by Biopure (Tulln, Austria). A few groups have investigated the applicability of stable isotopes for use as IS in multi-mycotoxin methods reporting improved method performance such as accuracy, repeatability and linearity of calibration (Herebian et al. 2009; Jin et al. 2010; Vaclavik et al. 2010; Zachariasova et al. 2010). Additionally, due to significant signal fluctuation and profound matrix related signal suppression, the use of IS was found to be necessary for quantitative work with DART-MS (Vaclavik et al. 2010). The most comprehensive sets of isotope labelled or other types of IS comprised of seven or nine standards that were used for determining a total of 32 or 11 toxins, respectively (Herebian et al. 2009; Zachariasova et al. 2010). It was demonstrated that both the accuracy (i.e. recovery) and repeatability (as relative standard deviation, RSD) of the method was enhanced when using properly selected ^{13}C-labelled IS with the recoveries obtained being from 96 to 108 % and RSD_r between 2.6 and 7.0 % instead of the corresponding ranges of 31 to 120 % (recovery) and 1.3 to 30 % (RSD_r) obtained with external matrix-assisted calibration (Zachariasova et al. 2010).

The main reasons limiting the use of the stable isotopes in the multi-residue methods are probably the still rather restricted availability and the high price of the commercial products. In addition, other types of IS can be challenging to find when a larger number of compounds need to be analysed. Therefore, the compensation for matrix effects can be achieved practically by preparing matrix-assisted (or matrix-matched) calibrants as has been the current practice in multi-residue methods of mycotoxins (e.g. Sulyok et al. 2006; Spanjer et al. 2008; Martos et al. 2010). This is performed by adding a standard solution in a blank sample extract before injection to the LC-MS instrument to create a calibration curve. The blank matrix used for this purpose should resemble the actual sample material as closely as possible due to the variations in matrices (Sulyok et al. 2007b).

2.3.5 QUALITY ASSURANCE OF MULTI-MYCOTOXIN METHODS

Every laboratory conducting chemical analysis should undertake quality assurance (QA) measures to ensure that it is competent to perform the analytical measurements and to provide analytical data of the required quality (Taverniers et al. 2004). These measures include using validated methods, performing internal control procedures, participating in proficiency testing schemes and becoming accredited, as defined in the guidelines of International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al. 2002). International Organization for Standardization (ISO), IUPAC and the Association of Official Analytical Chemists (AOAC) International have produced protocols and guidelines about the different aspects of quality assurance to aid single laboratories carry out this task (Thompson et al. 2002). However, it is each laboratory's decision on how to adopt these and to form its own QA policy. Nonetheless, the QA needs to be an on-going process with regular follow-up (Taverniers et al. 2004).

Validation is a process where method performance characteristics are systematically evaluated and proven to meet certain requirements, thereby demonstrating that the developed method is fit-for-purpose, i.e. applicable to a specified type of test material and to a defined concentration range of the analyte(s) (Thompson et al. 2002, Taverniers et al. 2004). Variations exist in the validation procedures between the published multi-mycotoxin methods, since there are no guides specifically dedicated for the validation of mycotoxin methods to the extent that they would have been established for the residues in animal products by the EU (European Union 2002a). Some groups have reported carrying out the validation tasks according to guides published by the European Committee for Standardization (CEN) or ISO (International Organization for Standardization 1994; The European Committee for Standardization 2010) or the European Commission decision 2002/657/EC (Sørensen and Elbæk 2005; Capriotti et al. 2010; Desmarchelier et al. 2010; Monbaliu et al. 2010a; Rasmussen et al. 2010). However, all of the methods have been in-house validated (in comparison to the methods with official or standard status that have undergone an inter-laboratory validation). At the minimum, limits of detection and quantification (LOD and LOQ), linearity of calibration, recovery (an estimate for trueness), precision (within and between days repeatability) and selectivity or specificity (often equivalent to matrix effect) have been reported for most of the mycotoxin multi-residue methods.

The validation results presented for multi-mycotoxin methods highlight the variation of performance of the methods in terms of recovery, repeatability and the LOQs (Table 4). Differences in methodology, validation protocols and calculation of results can be a cause of variation. The variation demonstrates clearly that the conditions have not been optimal for all the analytes, resulting in less satisfactory method performance. For example, recoveries of the range from

31 to 125 % and within-day repeatability from 1.6 up to 26 % had to be satisfied with for the 22 mycotoxins analysed from untreated cereal extracts (Martos et al. 2010). Furthermore, the detection capability of a multi-residue method may vary significantly between the analytes; e.g. LOQs of 0.5 to 800 μg/kg were reported for 32 mycotoxins by Herebian et al. (2009). Probably the most inclusive method validation regarding the multi-mycotoxin methods has been carried out for a feed matrix by Monbaliu et al (2010a), who performed the tasks according to guidelines laid down in the Commission Decision 2002/657/EC (European Union 2002a). Measurement uncertainty was also determined (i.e. 8.0 to 40.2 % for the 23 toxins) as has been recommended in validation guides (Eurachem 1998; Thompson et al. 2002). This parameter is required to be given together with the analytical result for the official control of mycotoxins (European Union 2006d). Finally, the method of Monbaliu et al (2010a) was accredited according to ISO 17025 (International Organization for Standardization 2005), which has not been reported for the other published multi-mycotoxin methods.

QA procedures should include the use of (certified) reference materials (CRM or RM), if possible to evaluate the method bias (Thompson et al. 2002). CRMs are available for certain mycotoxin-matrix combinations from the European Commission's Joint Research Centre/Institute for Reference Materials and Measurements (JRC/IRMM). RMs can be purchased from e.g. Food Analysis Performance Schemes (FAPAS) or Biopure (Tulln, Austria). These have been used to variable extent to evaluate the trueness of multi-mycotoxin methods as a part of validation process (e.g. Sulyok et al. 2006; Capriotti et al. 2010; Martos et al. 2010; Zachariasova et al. 2010). For example, Sulyok et al. (2006) demonstrated that the trueness of their multi-residue method was satisfactory, most of the concentrations measured for different cereal based (C)RMs containing DON and ZEN being within the assigned ranges. In addition, participation in FAPAS proficiency tests of different mycotoxin-matrix combinations has been reported achieving good performance with a multi-mycotoxin method as evaluated by the z-scores being $< \left| 2 \right|$ (Martos et al. 2010; Desmarchelier et al. 2010).

The fitness-for-purpose of the multi-mycotoxin methods has been evaluated according to various criteria, such as those described in 2002/657/EC (Capriotti et al. 2010; Monbaliu et al., 2010a). The recovery and precision of the methods have been assessed also on the basis of the requirements laid down in the CEN protocols or in the European Commission Regulation 401/2006 given for the methods intended for official control of mycotoxins (European Union 2006d). Furthermore, LOD and LOQ may be assessed on the basis whether they can measure the concentrations at MRLs set by EC or nationally (Spanjer et al. 2008; Monbaliu et al. 2009;2010a; Martos et al. 2010; Zachariasova et al. 2010). Typically, the validation parameters determined for multi-mycotoxin methods do not fulfill all the criteria established in

these documents (Rasmussen et al. 2010; Zachariasova et al. 2010). However, this is not an utmost prerequisite if the multi-residue method is not intended for official control purpose (Rasmussen et al. 2010). Sometimes it may be more relevant to evaluate the adequacy of LOD and LOQ on the basis of levels usually found in real samples (Cavaliere et al. 2007 ; Rasmussen et al. 2010). In addition, the method performance can be compared to an established procedure. For example, Spanjer et al. (2008) obtained comparable results when measuring the levels of AF B1 in peanuts with a multi-mycotoxin method and a standardized CEN method.

Table 4. A summary of the validation results of some multi-mycotoxin methods developed for cereal and feed analysis. (Description of the methods and analytes are presented in Table 2.)

No of toxins	Sample matrix	Recovery (%)	RSD_r (%)	LOQ (μg/kg) in matrix	Reference
13	Maize Wheat	81-133 71-132	2.3-4.7 2.1-5.2	0.1-4.9 0.1-3.8	Tanaka et al. (2006)
39	Wheat Maize Barley	43-104 57-110 59-117	0.4-44.8 0.3-40.4 1.5-13.1	0.08-170 0.03-220 not reported	Sulyok et al. (2006); Sulyok et al. (2007a)
17	Maize	83-99	5-11	3-125	Cavaliere et al. (2007)
11	Maize	79-104	0.2-13	0.3-4.2	Lattanzio et al. (2007)
13 24	Wheat Maize	35-115 13-109	9-57.5 6.5-105	0.5-50 0.5-200	Spanjer et al. (2008)
12	Maize	71.1-92.1	1.9-17.4	0.03-5.1	Garrido-Frenich et al. (2009)
11	Maize	58-120	3-23	0.3-150	Beltrán et al. (2009)
32	Wheat Maize	87-131 68-152	1.4-12.4 1.5-18	0.5-800 0.5-800	Herebian et al. (2009)
23	Feed	97-104.8	1.8-11.8	0.7-60.6[a]	Monbaliu et al. (2010a)
27	Maize silage	37-205	5-27	1.0-739[b]	Rasmussen et al. (2010)
11	Wheat Barley Maize	31-114 41-45 43-120	1.3-24.5 2.4-30 1.3-23.1	10-100[c]	Zachariasova et al. (2010)
9	Wheat Barley Maize	94-103[d] 95-108[d] 96-108[d]	3.3-6.9[d] 3.9-7.0[d] 2.6-5.9[d]	10-100[c]	Zachariasova et al. (2010)
22	Wheat Barley Oats	31-121 47-127 31-125	1.8-20 1.6-26.1 2.3-20	1-36 1-40 1-37	Martos et al. (2010)

RSD_r = relative standard deviation representing the within-day repeatability, LOQ=limit of quantification
a = decision limit
b = limit of detection
c = matrix not specified
d = quantification with internal standard calibration

3. AIMS OF THE STUDY

In this study, the potential of LC-MS/MS technique was explored in the field of mycotoxin research. The aim was to develop improved chemical methods capable of rapid determination of several, chemically diverse mycotoxins simultaneously. The objective was to apply the novel methods in food safety applications in order to gather information on mycotoxin co-occurrence in different commodities as well as on toxigenic potential of fungal species.

These tasks were accomplished by

Developing and validating multi-residue HPLC-MS/MS methods for determining mycotoxins in cheese, cereal based agar matrices and grains (**I, II, IV**)

Developing and validating a UHPLC-MS/MS method for determining ergot alkaloids in grains (**V**)

Utilizing the methods to analyse mycotoxin levels in mould cheeses and cereal grains (**I, V**)

Applying the methods to investigate mycotoxin production of *F. sporotrichioides* and *F. langsethiae* strains on agar substrates and grains (**III, IV**)

4. MATERIALS AND METHODS

4.1 REAGENTS AND STANDARDS

The solvents and reagents used were of HPLC or analytical grade and purchased from either J.T Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany). Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland). Diatomaceous earth (DE) drying material for ASE® extraction was from Dionex (Espoo, Finland).

Mycotoxin standards were purchased from Sigma Aldrich (St. Louis, MO, USA) or Biopure (Tulln, Austria). The standard for aflatoxin M1 was a BCR Reference Material 423 (IRMM, European Commission, Brussels, Belgium), and antibiotic Y was donated by Dr. Kristian Fog Nielsen (Technical University of Denmark, Lyngby, Denmark). The stock solutions and further dilutions of the standards were prepared as described in **I, II, IV, V**.

4.2 CALCULATION OF CHROMATOGRAPHIC PARAMETERS

For each chromatographic separation, retention factors (*k'*), resolution (*Rs*), peak asymmetry factors (*As*) at 10 % peak height and peak widths at half height ($w_{½}$) were calculated. The *k'*, *As* and $w_{½}$ were determined using Empower software (Waters, Milford, MA, USA), and *Rs* and the column dead time (t_0, used for calculating the *k'*) were calculated manually as described in Snyder et al. (1997) using the equations given below where: V_m=column void volume, F=flow rate, L=column length, d_c=inner diameter of the column, t_r=retention time of an analyte, t1 and t2=retention times of two adjacent peaks (t1 for the first and t2 for the second peak), $w_{½}1$ and $w_{½}2$=peak widths at half height for two adjacent peaks.

$$t_0 = V_m / F$$

$$V_m = 0.5\, L d_c^2$$

$$k' = \frac{tr - t0}{t0}$$

$$Rs = \frac{1.18\,(t2 - t1)}{w_{½}1 + w_{½}2}$$

4.3 MULTI-MYCOTOXIN HPLC-MS/MS DETERMINATION FROM CHEESE (I)

A method based on HPLC-MS/MS was developed for determining nine mycotoxins, i.e. aflatoxins (B1, B2, G1, G2 and M1), penicillic acid (PA), MPA, ochratoxin A and roquefortine C from cheese. The sample preparation procedure was modified from the method reported by Rundberget and Wilkins (2002). In brief, ten grams of cheese were weighed and 60 ml of ACN (containing 0.1 % formic acid) and 50 ml of hexane were added. The sample was homogenized and centrifuged at +10 °C. The ACN layer was filtered and a 10 ml portion of it was evaporated to dryness. The residue was dissolved in 0.2 ml of MeOH and filtered into an autosampler vial. Matrix-assisted calibration curves were used for quantification of the analytes. The calibrants were prepared in blank cheese matrix by adding standard solutions before the sample preparation procedure at concentrations of 5 to 1000 μg/kg, except for AF M1 for which the range was from 0.5 to 100 μg/kg. The blank material used was a model cheese tested not containing the mycotoxins being investigated. If a mycotoxin concentration of a sample exceeded that of the highest calibrant, the sample extract was diluted with the blank matrix extract to give a total volume of 10 ml.

The selected mycotoxins were separated and detected with HPLC/MS/MS consisting of Alliance 2960 Separations Module (Waters, Milford, MA, USA) which was coupled to MicroMass Quattro Micro triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an electrospray ionization probe. The analytes were separated on a Symmetry C18 column (100 x 2.1 mm, 3.5 μm particles) and a guard column (20 x 2.1 mm) of the same stationary phase (Waters, Milford, MA, USA). A total of 10 μl of the sample was injected on to the column. A gradient elution with a flow rate of 0.2 ml/min was applied. The initial mobile phase conditions were water:ACN both containing 0.1 % acetic acid (75:25, pH 3.5). These conditions were held for 16 minutes and then changed to water:ACN (10:90) during the next 24 minutes. The initial conditions were restored and the column conditioned with 25 % acetonitrile for nine minutes before the next injection. The MS/MS was performed with the MRM mode using positive polarity of ESI. Protonated molecular ions $[M+H]^+$ of the analytes were monitored and fragmented to their product ions in CID. These were used for identification and quantification. The optimization and the detailed MS parameters are described in **I**.

The method performance was evaluated by determining limit of detection (LOD), limit of quantification (LOQ), recovery and within-day repeatability (as RSD_r). Recoveries and repeatability were determined using spiked samples at levels of 50 and 200 μg/kg (n=5) except for aflatoxin M1 (at 5 and 20 μg/kg, n=5). LOD was calculated on the basis of signal-to-noise ratio of 3:1 by using matrix-assisted calibrants of the lowest concentration. LOQ was determined as two times LOD.

The linearity of the calibration was tested and proved within the concentration range used with the lack of fitness test (data not shown) as described in van Trijp and Roos (1991).

The method developed was applied for determining mycotoxins in ten blue and ten white mould cheese samples and one blue-white mould cheese purchased from Finnish supermarkets.

4.4 MULTI-MYCOTOXIN HPLC-MS/MS DETERMINATION FROM CEREAL AND RICE FLOUR AGARS (IV) AND GRAINS (II-III)

An HPLC-MS/MS method was developed for determining a total of 31 mycotoxins including TRs, FBs, MON, ENNs, antibiotic Y (ANT Y), AFs, GLIO, OTA, MPA, PA and ERGs. The method was applied for determining mycotoxins produced by two *Fusarium* species, *F. sporotrichioides* and *F. langsethiae*, grown on cereal and rice flour agars and whole grains.

4.4.1 HPLC-MS/MS PROCEDURE

The mycotoxins included in the method were analysed with an HPLC-MS/MS system consisting of Alliance 2960 Separations Module (Waters, Milford, MA, USA) and a MicroMass Quattro Micro triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an ESI probe. The separation of the selected analytes was performed by analysing the same sample in two chromatographic runs, both using a gradient elution. In both runs, 10 µl of the sample was injected onto an Inertsil ODS-EP column (150 mm x 2.1 mm, 5 µm) equipped with a guard column (5 mm x 2.1 mm, 5 µm) of the same stationary phase (GL Sciences, Torrance, CA, USA). The mobile phases used were: A) water containing 0.2 % formic acid, B) ACN containing 0.2 % formic acid, C) 1.0 mM ammonium acetate and D) 100 % ACN. In the first chromatographic run, a constant flow rate of 0.2 ml/min was used. The starting conditions were 65:35 (A:B, pH 2.8) and these were held during the first 3 min, after which a linear gradient was applied, reaching 10:90 (A:B) at 22 min. Then the initial conditions were restored and held until 35 min. In the second run, flow rates of 0.1 ml/min (0-2 min) and 0.2 ml/min (2-35 min) were applied. Isocratic conditions with 70:30 (C:D, pH 6.7) were held during the first 10 min, after which a linear gradient was applied reaching 10:90 (C:D) at 15 min. These conditions were held until 22 min before restoring the initial conditions and conditioning the column during the next 13 min.

The MS/MS was performed in the MRM mode using both positive and negative polarities of ESI. Depending on the analyte, either protonated or deprotonated molecular ions $[M+H]^+$ or $[M-H]^-$ or ammonium $[M+NH_4]^+$ or acetate $[M+CH_3COO]^-$ adducts were monitored and fragmented to their product ions in CID, and used for identification and quantification. The optimization and the final MS parameters are described in more detail in **II**.

4.4.2 AGARS: SAMPLE PREPARATION AND METHOD VALIDATION (IV)

The sample preparation procedure for the cereal and rice flour agar matrices was modified from that presented by Smedsgaard et al. (1997). Plugs of agar with a total mass of 3.5 g were transferred into a test tube. Mycotoxins were extracted in two steps: first, 5 ml of MeOH:DCM:EtOAc (1:2:3 + 4 % HCOOH, pH 3.0) was added to the test tube, shaken for 1 min and sonicated for 30 min. After centrifugation at + 10 °C, the supernatant was transferred into a new test tube and the extraction procedure was repeated for the remaining sample with 3 mL of MeOH:DCM:EtOAc (1:2:3 + 1% HCOOH, pH 3.9). The two supernatants were combined and evaporated to dryness, after which the sample was dissolved in 0.4 ml of 50 % ACN and filtered into an autosampler vial. Standards for the external calibration curves were prepared in 50 % ACN corresponding to concentrations from 1.1 to 570 µg/kg in agar matrix. The linearity of the calibration was evaluated and approved (data not shown) with the lack of fitness test (van Trijp and Roos 1991).

The method validation included the determination of LOD and LOQ, recovery and repeatability as (RSD_r). LOD and LOQ were calculated from the standards prepared in 50 % ACN. LOD was determined as the signal-to-noise ratio of 5:1 and LOQ as two times LOD (n=3-6). To evaluate recovery and repeatability, spiked samples at a concentration level of 200 µg/kg (n=6) were prepared in both agar matrices.

4.4.3 GRAINS: SAMPLE PREPARATION AND METHOD VALIDATION (II)

A 10 g portion of ground grain was weighed and mixed with three grams of DE drying matrix and extracted with an accelerated solvent extraction instrument ASE 200® (Dionex, Espoo, Finland). The extraction was carried out in two sequential steps performed with the static mode of ASE with 90 % ACN at a temperature of +100 °C and a pressure of 1500 psi. The extracts were kept overnight at +4 °C, filtered and filled to a volume of 50 ml. An aliquot of 5 ml was evaporated to

dryness, dissolved in 0.2 ml of 50 % ACN and filtered into an autosampler vial. Matrix-assisted calibration curves were used for quantification of the analytes. The calibrants were prepared in blank grain extracts by adding standard solutions before the evaporation step at concentrations from 10 to 1500 µg/kg, with the exception of ENNs that were added at following ranges: 1-45 µg/kg (ENN A), 2-300 µg/kg (ENN A1), 2-285 µg/kg (ENN B) and 5.4-810 µg/kg (ENN B1). If an analyte concentration of a sample exceeded that of the highest calibrant, the sample extract was diluted with blank cereal extract to give a total volume of 5 ml.

The method was validated for wheat, barley and oats and the validation included the determination of LOD, LOQ, selectivity, recovery, repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) and accuracy of the method. The matrix-assisted standards (n=3-6) were used for the determination of LOD and LOQ. When suitable blank material was not available (for ENN B in wheat and barley and BEA in wheat) standards dissolved in 50 % ACN were used. Signal to noise ratios of 3:1 were used as LOD and S/N of 15 as LOQ. The linearity of calibration was evaluated and approved (data not shown) with the lack of fitness test (van Trijp and Roos 1991). The selectivity of the method was determined by calculating the percentual difference of the slopes of calibration curves obtained with and without (i.e. standards in solvent) sample matrix (n=3). The signal suppression or enhancement (SSE) approach (Sulyok et al., 2006) was used. An SSE value of 100 % indicated no matrix effect; values below this signified a signal suppressive effect.

To determine the recovery and RSD_r, six replicates of spiked samples at concentration levels of 50, 200 and 500 µg/kg were prepared (the concentrations for ENNs are presented in **II**). The within-laboratory reproducibility (RSD_R) was evaluated by analysing a grain sample containing naturally DON, 3-AcDON, HT-2, T-2 and ENN B and prepared and analysed by different analysts over several months (n=13). The accuracy of the method was determined for DON (n=3), ZEN (n=3) and T-2 (n=2) by analysing reference materials (T2214, T2225 and T2234) purchased from FAPAS (Central Science Laboratory, Sand Hutton, UK). Measurement uncertainty was evaluated for the *Fusarium* toxins on the basis of spiked samples (at 500 µg/kg) analysed over several months (n=10-15). The expanded measurement uncertainty (*U*) was calculated by multiplying the combined measurement uncertainty u_c with a coverage factor of 2. The u_c was obtained from the square root of the sums of squares of u_1 of method bias (the average recovery percentage) and u_2 of method precision (the average RSD_r): $u_c = (u_1^2 + u_2^2)^{1/2}$.

4.4.4 TOXIN PRODUCTION OF *F. SPOROTRICHIOIDES* AND *F. LANGSETHIAE* (III-IV)

The methods developed were applied for investigating the mycotoxin production of *F. sporotrichioides* and *F. langsethiae* on cereal and rice flour agars and a whole grain mixture. In brief, three strains of each *Fusarium* species isolated from Finnish grains were inoculated as triplicates on two types of agar substrates (containing 4 % of cereal or rice flour, preparation of the media is described in **IV**) and a mixture of wheat, oats and barley grains (1:1:1, w/w). The agar cultures (a_w 0.99) were incubated for seven days at + 25 °C, and the grain cultures in a water activity/temperature combination of 0.994/25°C for four weeks. After the incubation period, the grain cultures were air dried and milled. The mycotoxins produced on the agar plates and grains were extracted, and the samples prepared and analysed with HPLC-MS/MS as described above.

4.5 ERGOT ALKALOID DETERMINATION WITH UHPLC-MS/MS FROM GRAINS (V)

A method based on UHPLC-MS/MS technique was developed for determining six ergot alkaloids: ergometrine (EMETR), ergosine (ESINE), ergocornine (ECORN), ergocristine (ECRIS), ergocryptine (ECRYPT) and ergotamine (EAMINE) and four of their respective (*S*)-epimers: ergocorninine (ECORN-INE), ergocristinine (ECRIS-INE), ergocryptinine (ECRYPT-INE) and ergotaminine (EAMININE) from grains.

The samples were prepared according to the instructions of Mycosep® 150 Ergot SPE clean-up columns (Römer Labs, Tulln, Austria) with minor deviations from the procedure. In brief, 20 g of ground grains were weighed and 100 ml of a mixture of ACN and 3.0 mM ammonium carbonate buffer (84:16, pH 8.5) was added. The samples were shaken for one hour and filtered. An aliquot of 4 ml was transferred to a glass tube and purified by passing it through the Mycosep column. A portion of 1 ml of the purified extract was transferred to a clean test tube and evaporated to dryness. The residue was dissolved in 0.5 ml of ACN:ammonium carbonate (50:50) and filtered into an autosampler vial. Matrix-assisted calibration curves were used for the quantification of the analytes. The calibrants were prepared in blank grain extract at concentrations from 1 to 500 μg/kg (10-500 μg/kg for ergometrine). If an analyte concentration in a sample exceeded that of the highest calibrant, the sample extract was diluted with blank cereal extract to give a total volume of 4 ml.

The selected ergot alkaloids were determined with a UHPLC-MS/MS system consisting of Acquity UPLC (Waters, Milford, MA, USA) connected to a MicroMass Xevo triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an ESI probe. Separation of the compounds was achieved by injecting

10 µL of the sample onto an Acquity BEH C18 column (2.1 x 100 mm, 1.7 µm particles) (Waters, Milford, MA, USA). A gradient elution with a flow rate of 0.4 ml/min was used. The initial mobile phase conditions were ACN and 3.0 mM ammonium carbonate buffer (50:50). The concentration of ACN was increased to 60 % during the first four minutes after which the initial conditions (50:50) were restored during the next 0.5 minutes and maintained for an additional 1.5 minutes. The MS/MS was performed in the MRM mode using the positive mode of ESI. Protonated molecular ions $[M+H]^+$ of the analytes were monitored and fragmented to their product ions which were used for identification and quantification. The optimization and the MS parameters are described in more detail in **V**.

The method was validated by determining the LOD and LOQ, the linearity of calibration, recovery, repeatability (RSD_r) and selectivity. The values of LOD and LOQ (n=3) were determined as the analyte concentration corresponding to a mean + 3 times standard deviation (SD) of the response measured for a blank sample (LOD) and a mean + 10 times SD of the response of a blank sample (LOQ) according to validation guide published by Eurachem (1998). The linearity of the calibration was calculated from the matrix-assisted calibration curves by using the least square method (van Trijp and Roos, 1991). The selectivity of the method was determined by using the SSE approach (Sulyok et al. 2006). The recovery and within-day repeatability were evaluated for rye, barley and wheat using spiked samples (n=6) at three concentration levels (10, 50, 200 µg/kg).

The method was applied for determining the concentrations of the selected 10 ergot alkaloids in Finnish cereals. A total of 42 samples (25 rye, 12 barley and 5 wheat) were analysed. Most of the barley samples were intended for animal feed and the majority of the wheat and rye samples for human consumption.

5. RESULTS

5.1 MULTI-MYCOTOXIN DETERMINATION FROM CHEESE

A quantitative method for determining nine mycotoxins from cheese was successfully developed. The analytes included toxins produced by fungi of the genus *Aspergillus* and *Penicillium*, i.e. five aflatoxins (B1, B2, G1, G2 and M1), penicillic acid, mycophenolic acid, ochratoxin A and roquefortine C could be analysed with the method (Table 5, Appendix 1).

5.1.1 METHOD PERFORMANCE

The extraction of the analytes was achieved with acidic ACN. Sample clean-up was kept to a minimum, and lipids could be removed with hexane partitioning with proteins being precipitated by centrifuging the extract in the cold. The separation of the analytes was accomplished in an HPLC with an RP C18 column and by applying a linear gradient starting from 25 % of acidic ACN. The total run-time was 40 min, with the first of the analytes (PA) eluting at 3.3 min and the last (OTA) at 31.9 min (Figure 2). The calculated retention factors (k') of these analytes were 2.0 and 27.9. Complete baseline resolution was not achieved for the analyte pairs of PA and ROQ C, ROQ C and AF M1, and AF B2 and AF G1. The corresponding resolution factors (*Rs*) calculated for these pairs were 0.3, 0.6 and 1.3, respectively. Peak asymmetry factors (*As*) varied from 0.9 to 1.3 with the exception of the peaks of ROQ C and OTA (*As* of 1.6 for both). The peaks of the AFs were broad, the peak widths at half height ($w_{1/2}$) being between 0.6 to 0.7 min in comparison with the calculated $w_{1/2}$ of 0.3 to 0.4 min for the rest of the compounds.

The MS detection of all the selected mycotoxins was accomplished in the positive mode of ESI as protonated molecular ions $[M+H]^+$ (Table 5, Appendix 1). The analytes were successfully fragmented in CID with the optimized MS parameter settings, and for all of them two ion transitions could be monitored for quantification and confirmation purposes in the MRM mode (Table 5, Appendix 1). A minimum of 12 data points across each peak could be collected by dividing the analytes into two MRM data acquisition windows.

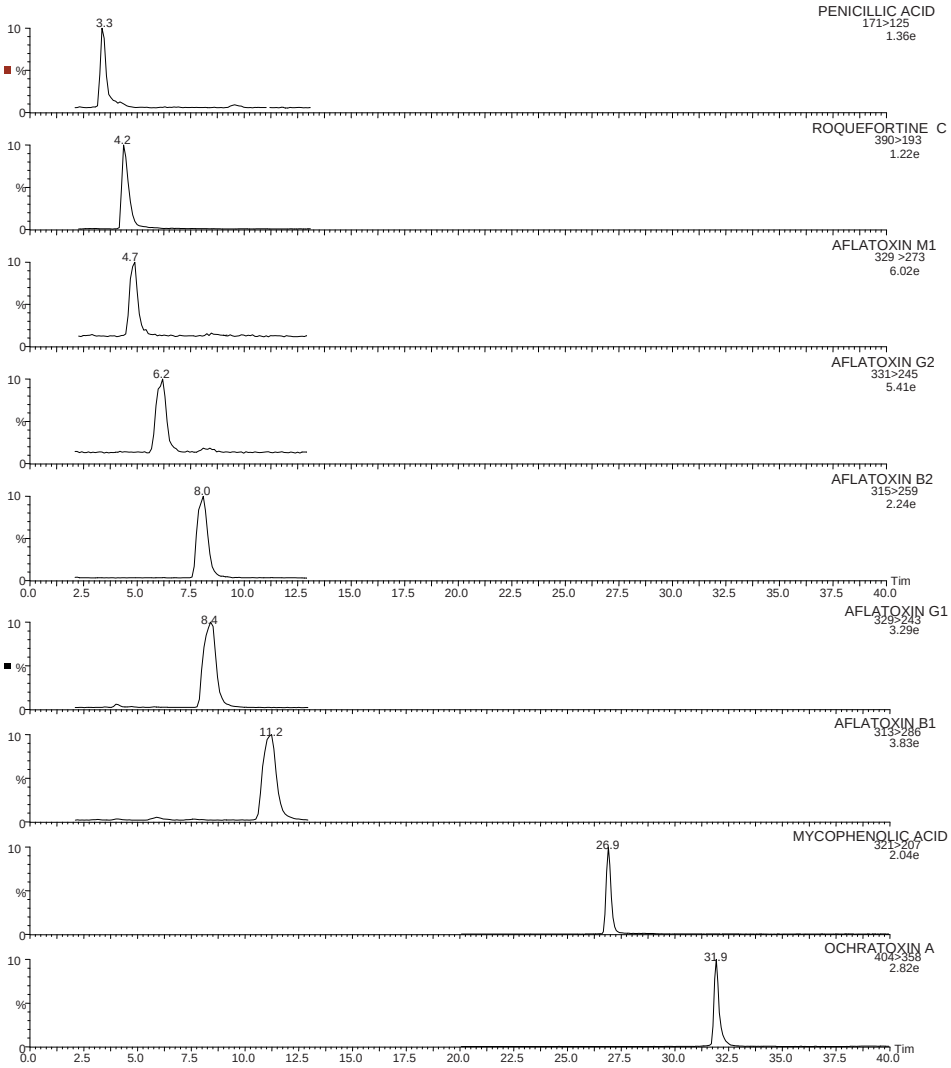


Figure 2. A chromatogram of a blank cheese spiked at 50 μg/kg (AF M1 at 5 μg/kg) analysed with HPLC-MS/MS.

The performance of the method was evaluated on the basis of the validation results (summarised in Table 6, Appendix II). The LODs varied from 0.3 μg/kg for AF M1 to 2.0 μg/kg for PA. LOQ for AF M1 was 0.6 μg/kg, whereas 5.0 μg/kg was used for the other analytes corresponding to the lowest concentration on the calibration curve. The recoveries of the method varied between 96 and 143 %, with the highest values being obtained for AFs and MPA (from 129 to 143 %). The within-day repeatability as RSD_r varied from 2.3 up to 12.1 %.

5.1.2 MYCOTOXINS DETECTED IN MOULD CHEESES

All eleven blue or blue-white mould cheeses analysed for mycotoxins contained ROQ C (from 800 to 12 000 μg/kg) and one of them contained MPA (300 μg/kg) (Table 7). A chromatogram of the blue cheese sample containing the two toxins is presented in Figure 3. None of the mycotoxins examined was detected in the white mould cheese samples.

Table 7. Mycotoxins detected in the mould cheese samples (mean and the range of concentration of the positive samples

Cheese type	ROQ C (μg/kg)	MPA (μg/kg)
Blue mould	3600 (800-12000)	300[a]
Blue-white mould	800[a]	n.d
White mould	n.d	n.d

ROQ C=roquefortine C, MPA=mycophenolic acid,
n.d=not detected, a=only one positive sample

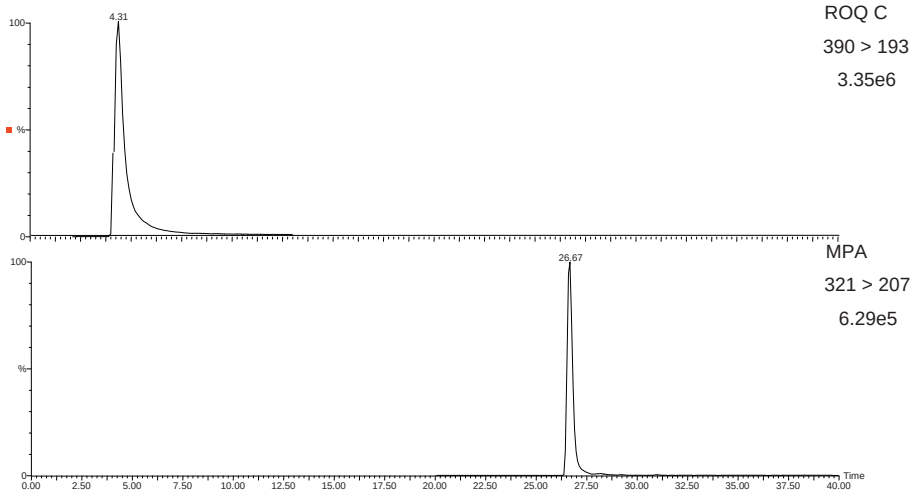


Figure 3. A chromatogram of a blue cheese sample containing roquefortine C (ROQ C) and mycophenolic acid (MPA).

5.2 MULTI-MYCOTOXIN DETERMINATION FROM CEREAL AND RICE FLOUR AGARS AND GRAINS

Methods were successfully developed for the determination of a total of 29 or 31 mycotoxins from cereal and rice flour agars or grains, respectively. The analytes included *Aspergillus*, *Fusarium*, *Penicillium* and *Claviceps* metabolites, such as TRs, FBs, MON, ENNs, ANT Y, AFs, GLIO, OTA, MPA, PA and ERGs (Table 5, Appendix 1). MON and NIV were excluded from the method developed for the agar matrices.

5.2.1 METHOD PERFORMANCE

The extraction of the analytes was accomplished with acidified MeOH:DCM:EtOAc mixture in conjunction with ultrasonic agitation (agar matrices) or with 90 % ACN with the aid of the ASE® technique (grains). A minimum amount of sample pre-treatment was carried out, and after the extraction, the precipitation of proteins was achieved by centrifuging the samples at cold temperature (agar samples) or keeping the extracts in a refridgerator overnight (grains). After these steps, only concentration and filtration steps were required.

The separation of the analytes was achieved by analysing the same sample in two HPLC runs both using a RP C18 column and a gradient elution. In the first run, the separation of 16 analytes was accomplished with a gradient starting from acidified water:ACN (65:35) in a total run-time of 35 min (Figure 4a). The first eluting analyte neosolaniol (NEO) had a retention time (t_R) of 3.0 min corresponding to a k' of 0.8. ENN A eluted last at t_R of 26 min (k' of 14.7). Complete baseline resolution was not achieved for four analyte pairs: NEO and FB1 (*Rs* 1.8), AF B2 and AF G1 (*Rs* 0.4), AF B1 and FB2 (*Rs* 0.7) and ENN A1 and BEA (*Rs* 0.2). The peak asymmetry factors varied from 0.7 to 1.3 and the half heights from 0.2 to 0.4 min with the exception of FB2 ($w_{1/2}$ =0.6 min). In the second chromatographic run, separation of 15 analytes was accomplished in a run-time of 35 min with a gradient starting from ammonium acetate:ACN (70:30) (Figure 4b). MON eluted first at t_R 2.6 min (k'=0.6) and ECRIS last at 20.8-21.5 min as two peaks representing the two epimers (k' was 12 for the last peak). Complete baseline separation was not obtained for five analyte pairs: MON and NIV (*Rs* 1.7), NIV and DON (*Rs* 1.0), DON and PA (*Rs* 0), PA and fusarenon X (F-X) (*Rs* 0.9), and EMETR and diacetoxyscirpenol (DAS) (*Rs* 0.9). Peak asymmetry factors varied from 1.1. to 1.5 with the exception of the peaks of MON and EMETR (*As* 2.7 and 2.9, respectively). The calculated $w_{1/2}$ varied between 0.2 and 0.4 min. Wider peaks were obtained for EMETR and HT-2 ($w_{1/2}$ of 0.6 and 0.5 min, respectively). Ergot alkaloids eluted as two separate peaks with the exception of EMETR.

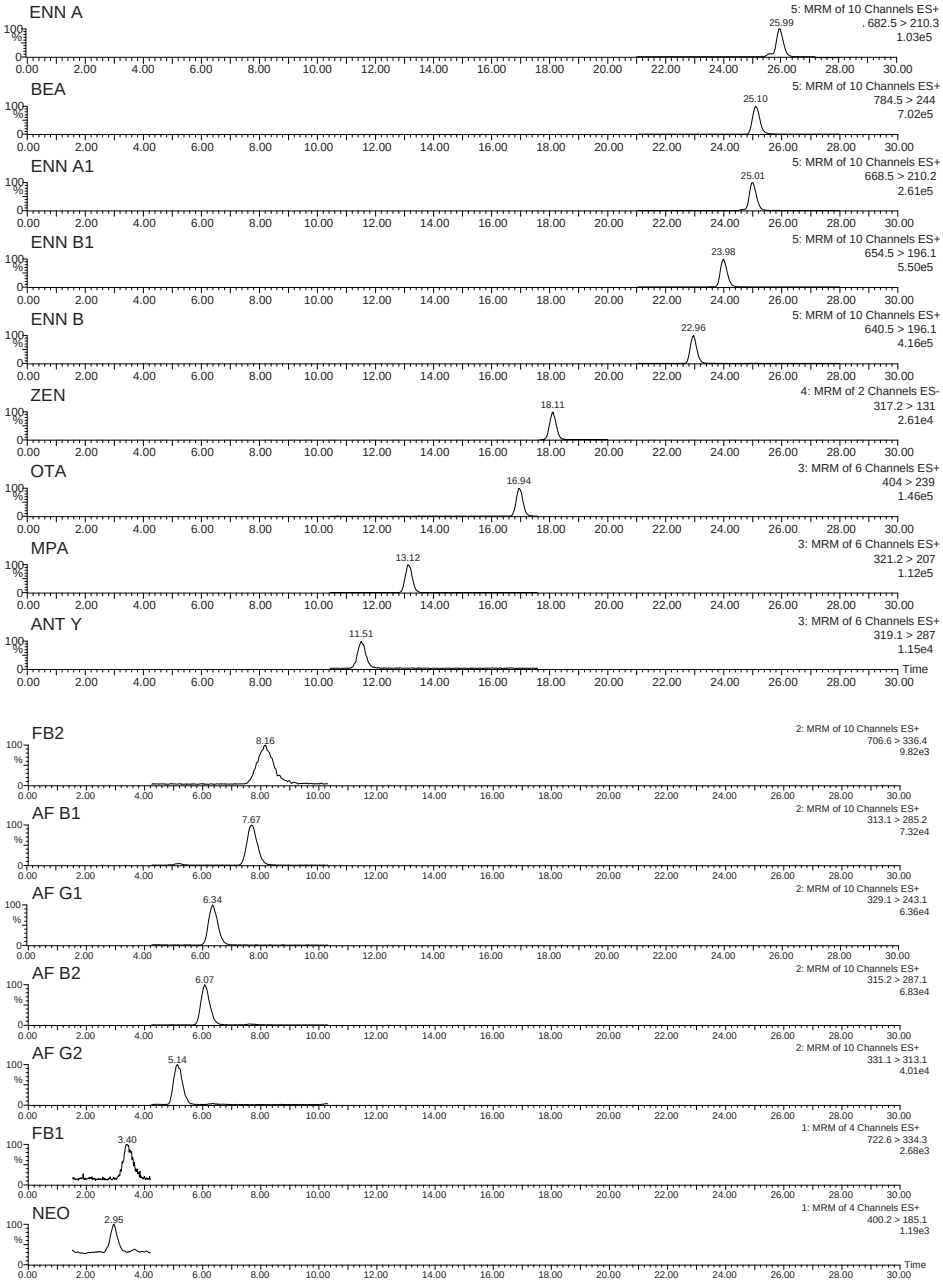


Figure 4a. A chromatogram (mobile phase ACN:water:acetic acid) of a blank wheat sample spiked at 500 µg/kg analysed with the HPLC-MS/MS method developed for grains.

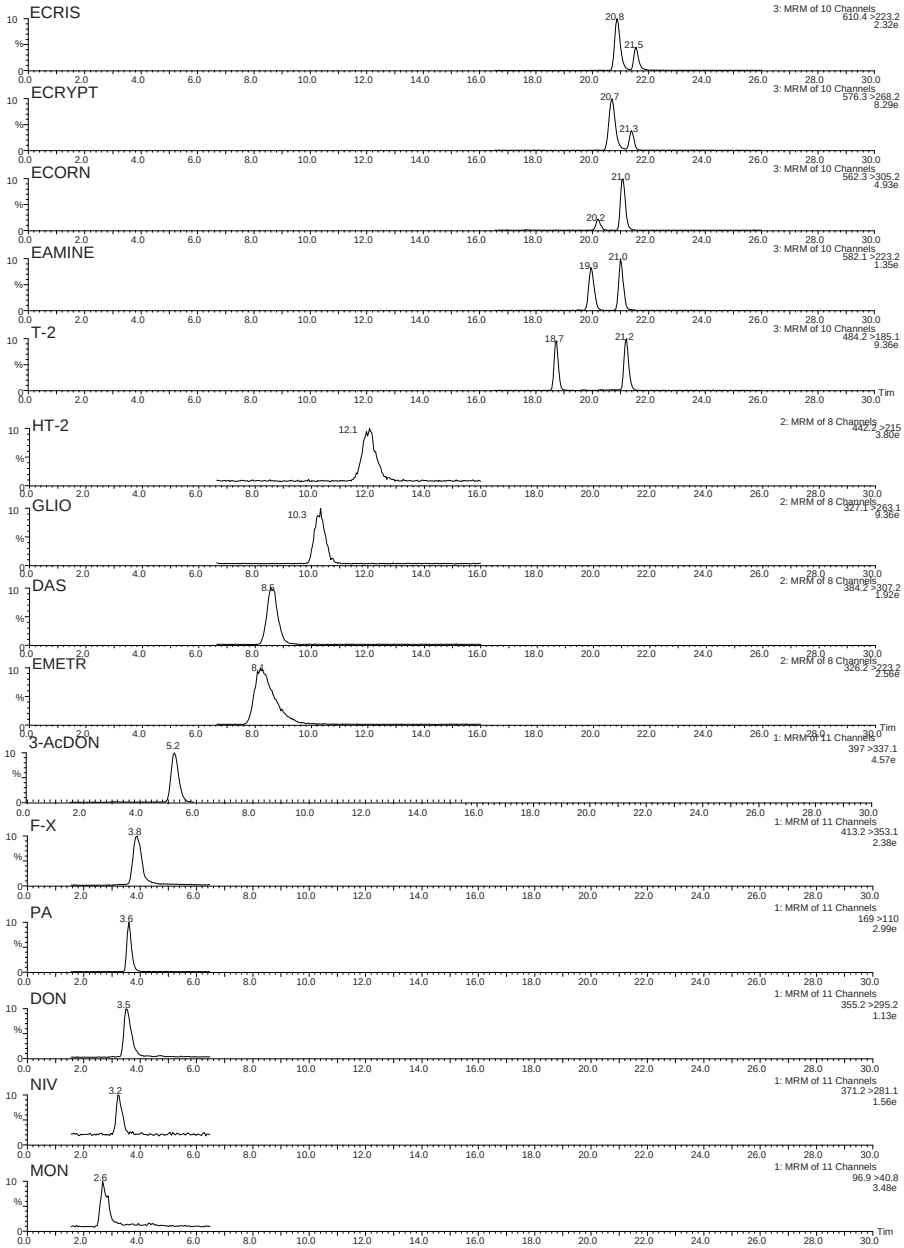


Figure 4b. A chromatogram (mobile phase ACN:water:ammonium acetate) of a blank wheat sample spiked at 500 μg/kg analysed with the HPLC-MS/MS method developed for grains.

The MS detection of the analytes was achieved using either positive or negative mode of ESI. The majority of the analytes could be detected as protonated molecular ions $[M+H]^+$ (Table 5, Appendix 1). Deprotonated molecular ions $[M-H]^-$ were

chosen as precursors for MON, ZEN and PA. The type-A and type-B trichothecenes were detected as ammonium adducts $[M+NH_4]^+$ or acetate adducts $[M+CH_3COO]^-$, respectively, with the exception of F-X which was monitored as $[M-H]^-$. The chosen precursors were successfully fragmented in CID with the optimized MS parameter settings, and two product ion transitions could be chosen (Table 5, Appendix 1). The only exception was MON, for which only one mass transition could be detected. The monitoring of the analytes in five MRM data acquisition windows allowed for ESI polarity switching and collection of enough data points across each peak.

5.2.1.1 Validation results for agar matrices

The LOQs determined for the method of agar matrices varied from 0.1 to 86 µg/kg. The recoveries determined at 200 µg/kg overall ranged between 19 and 163 % from cereal flour agar and from 8 up to 134 % from rice flour agar. The lowest recoveries were determined for FB1 and FB2 on rice flour agar (8-22 %) and for EMETR on cereal flour agar (19 %), whereas the highest values were obtained for 3-AcDON, ENN A1, BEA and ECRYPT on cereal flour agar (from 144 to 165 %). The within-day RSD_r ranged from 2.8 to 62 % for cereal agar and from 5.1 to 53 % for rice agar. Rather high RSD_r values (≥38 %) were obtained for DON, F-X and fumonisins. The validation results are summarised in Table 6, Appendix II.

5.2.1.2 Validation results for grains

With the HPLC-MS/MS multi-mycotoxin method developed for grains, the LOQs varied overall from 1 to 1250 µg/kg, with the highest values being calculated for NEO (370–800 µg/kg), MON (400–1250 µg/kg), NIV (400–620 µg/kg) and HT-2 (280–500 µg/kg). The recoveries from wheat, barley and oats at the three spiking levels (50, 200, 500 µg/kg) mostly ranged from 51 to 122 %. Lower values were obtained for ECORN (48 % in barley at 50 µg/kg). With respect to the fumonisins, recoveries from 7 to 36 % were measured in wheat and oats (at levels of 200 and 500 µg/kg). The within-day RSD_r varied from 2 to 26 %. The within-laboratory reproducibility was calculated for DON (16 %), 3-AcDON (14 %), HT-2 (19 %), T-2 (16 %) and ENN B (28 %). A summary of the validation results is presented in Table 6, Appendix II. The matrix effect in MS was mostly suppressive with the calculated SSE values ranging from 5.6 to 95 % (Table 8). Only signals of AF G1 and AF G2 were enhanced in wheat (SSE 105 and 106 %). SSE values of ≤ 10 %

(indicating remarkable signal suppression) were measured for MON, NIV, ZEN and some of the ENNs and ERGs. Most of the low SSE values were calculated for oats.

Table 8. Signal suppression or enhancement (SSE %) calculated for the analytes included in the HPLC-MS/MS multi-mycotoxin method for grains.

Mycotoxin	SSE % in wheat	SSE % in barley	SSE % in oats
NEO	17	-	-
FB 1	74	50	48
FB 2	95	57	72
ZEN	20	21	8.9
ANT Y	48	43	18
ENN A	49	37	5.6
ENN A1	71	47	8.5
ENN B	57	49	14
ENN B1	63	48	13
BEA	89	45	8.1
AF B1	86	60	67
AF B2	95	62	64
AF G1	105	68	71
AF G2	106	64	69
MPA	47	32	27
OTA	36	20	18
MON	6.9	-	7.0
NIV	-	-	9.4
DON	30	23	20
3-AcDON	50	52	33
F-X	39	27	21
DAS	11	28	34
HT-2	28	44	26
T-2	27	26	19
PA	55	25	38
GLIO	21	33	24
EAMINE	26	51	10
ECRIS	22	48	17
ECRYPT	13	59	16
ECORN	26	37	16
EMETR	8.0	15	20
Median	43	45	20

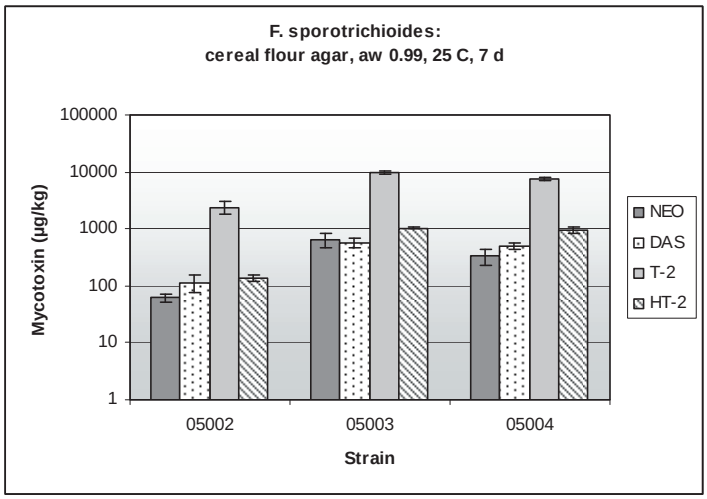
The accuracy of the method was evaluated on the basis of RM measurements. The mean concentrations measured were 899 µg/kg (DON), 107 µg/kg (T-2) and 201 µg/kg (ZEN), corresponding to 100, 75 and 129 % of the concentrations assigned for the corresponding RMs. Expanded measurement uncertainty determined for the *Fusarium* toxins varied from 26 % for 3-AcDON up to 160 % for both FBs (Table 9).

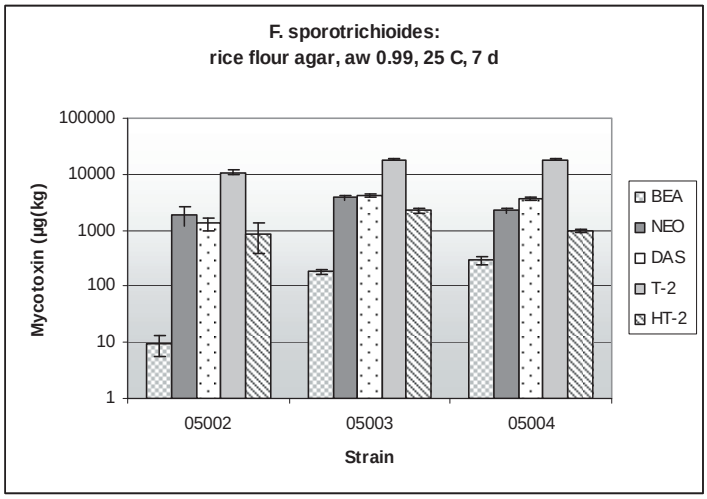
Table 9. The expanded measurement uncertainty *U* determined for the *Fusarium* toxins at 500 µg/kg, except for ENNs A (at 15 µg/kg), A1 (at 100 µg/kg), B (at 95 µg/kg) and B1 (at 270 µg/kg).

Mycotoxin	Expanded uncertainty *U* (%)
NEO	73
FB1	160
FB2	160
ZEN	57
ANTY	81
ENN A	65
ENN A1	74
ENN B	68
ENN B1	50
BEA	67
MON	67
NIV	40
DON	28
3-AcDON	26
F-X	33
DAS	34
HT-2	44
T-2	52

5.2.2 MYCOTOXINS DETECTED FROM *F. SPOROTRICHIOIDES* AND *F. LANGSETHIAE* CULTURES

Type-A trichothecenes (T-2, HT-2, DAS and NEO) and BEA were detected from the cultures of *F. sporotrichioides* and *F. langsethiae* strains (Figure 5). The three *F. sporotrichioides* strains produced the trichothecenes and BEA on all three substrates. The concentrations of BEA were < LOQ on the cereal flour agar. The *F. langsethiae* strains also produced T-2, HT-2, DAS, and NEO on the three substrates. Additionally, BEA production could be verified on the cereal flour (levels < LOQ) and rice flour agars.





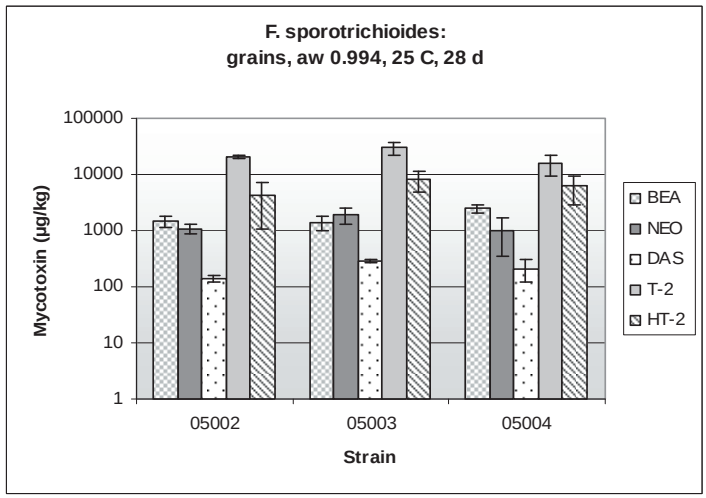
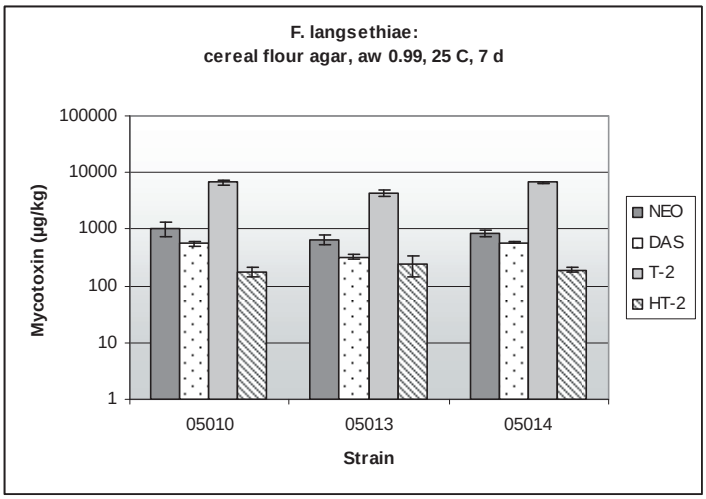
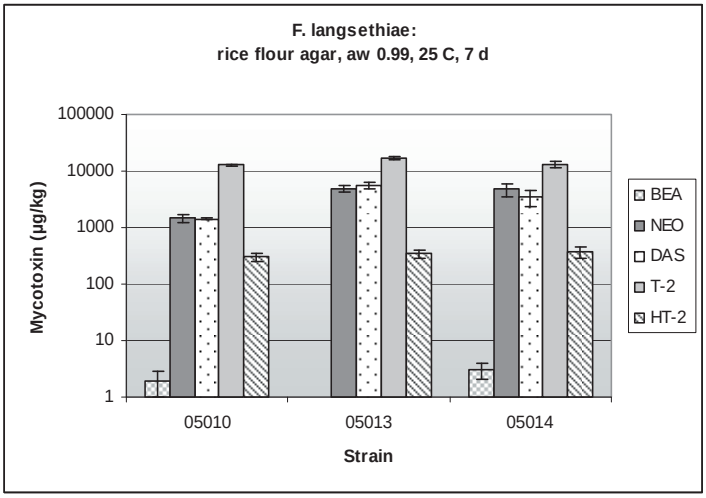


Figure 5. Trichothecene and beauvericin (BEA) production of *F. sporotrichioides* and *F. langsethiae* strains on cereal and rice flour agars and grain mixture (concentration of three replicate inoculations as mean±SD).





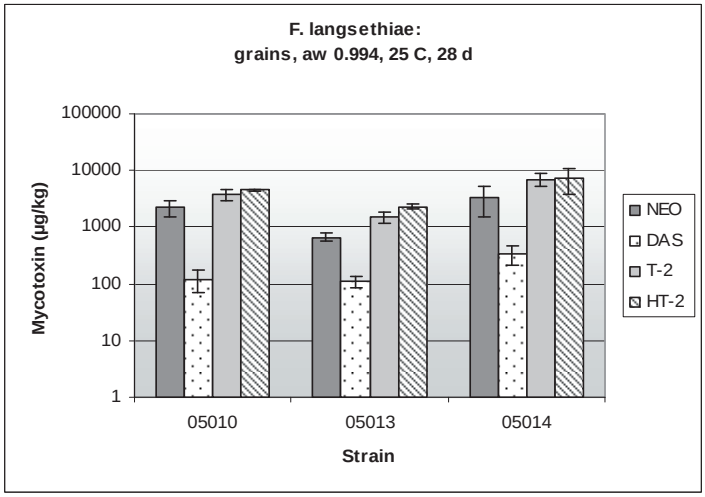


Figure 5.
(continued).

The average mycotoxin levels produced by each species during the incubation periods used are presented in Table 10. The total concentrations varied overall from n.d (BEA from the *F. langsethiae* grain cultures) to 22000 μg/kg (T-2 produced by *F. sporotrichioides* on grain cultures).

Table 10. Total concentration levels (mean of three strains cultivated as triplicates±SD) of type-A trichothecenes (NEO, DAS, T-2 and HT-2) and beauvericin measured from the *Fusarium sporotrichioides* and *F. langsethiae* cultures on cereal flour and rice flour agars and on a grain mixture.

	BEA (μg/kg)	NEO (μg/kg)	DAS (μg/kg)	T-2 (μg/kg)	HT-2 (μg/kg)
F. sporotrichioides					
Cereal flour agar[a]	< 1.0	350±280	400±230	6600±3300	700±430
Rice flour agar[b]	160±130	2700±960	3000±1400	16000±3900	1400±730
Grains[c]	1800±630	1400±640	210±79	22000±12000	6200±3300
F. langsethiae					
Cereal flour agar[a]	< 1.0	850±230	490±130	6000±1300	210±63
Rice flour agar[b]	2.5±1.0	3700±1900	3500±1900	14000±2400	340±67
Grains[c]	n.d	2100±1500	190±130	4100±2700	4600±2700

BEA=beauvericin, NEO=neosolaniol, DAS=diacetoxyscirpenol, T-2=T-2 toxin, HT-2=HT-2 toxin, n.d = not detected
[a] a_w 0.99, 25 °C, 7 d incubation
[b] a_w 0.99, 25 °C, 7 d incubation
[c] a_w 0.994, 25 °C, 28 d incubation

5.3 ERGOT ALKALOID DETERMINATION FROM GRAINS

A method based on UHPLC-MS/MS was developed for determining selectively a total of 10 ergot alkaloids (EMETR, ESINE, EAMINE, ECORN, ECRYPT, ECRIS, EAMININE, ECORN-INE, ECRYPT-INE, ECRIS-INE) from grains (Table 5, Appendix 1).

5.3.1 METHOD PERFORMANCE

The extraction of the investigated ergot alkaloids was achieved by using an ACN:ammonium carbonate solution. A rapid sample pre-treatment procedure for routine use was developed and after filtration of the extract, commercial Mycosep

SPE push-through columns intended for selective isolation of ergot alkaloids were successfully applied to obtain visually clear extracts. The separation of the analytes was accomplished in a total run time of 6 min with UHPLC in a RP C18 column and applying a linear gradient elution starting from ACN:ammonium carbonate (50:50) (Figure 6). The first eluting compound EMETR had a t_R of 0.65 min (k'=0.2) and the last eluting ECRIS-INE 4.2 min (k'=6.7). A complete baseline separation was achieved for all the other compounds except for ESINE and EAMINE, and ECRYPT and ECRIS (*Rs* of 1.9 for both analyte pairs). All the (*R*)- and (*S*)- epimer pairs could be separated under these conditions (*Rs* factors were between 18 and 29).

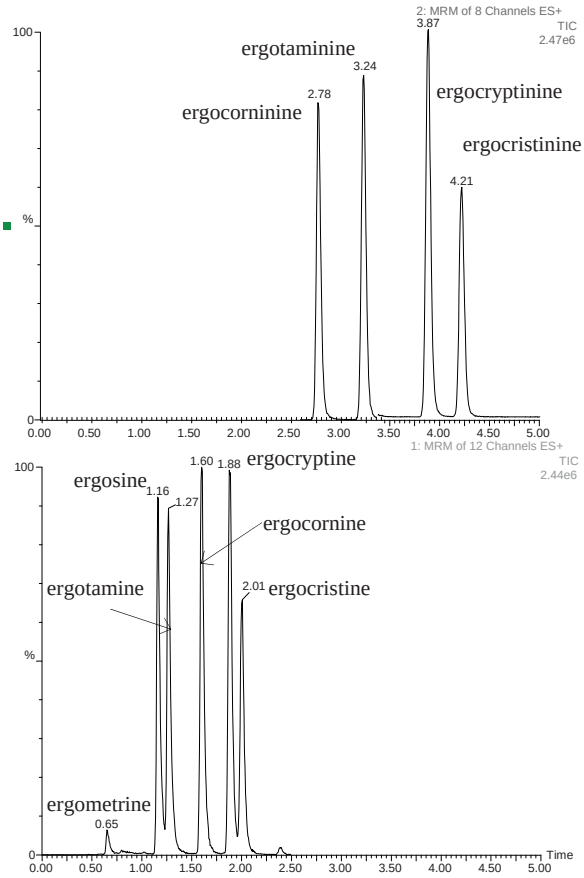


Figure 6. A total ion chromatogram obtained with UHPLC-MS/MS of a blank rye sample spiked at 50 µg/kg.

The MS detection of all the selected ergot alkaloids was accomplished in the positive mode of ESI as protonated molecular ions $[M+H]^+$ (Table 5, Appendix 1.). These precursors were successfully fragmented in CID with the optimized MS parameter settings. Two ion transitions for the quantification and confirmation purposes could be monitored for each precursor ion in the MRM mode. The division of the analytes in two MRM data acquisition windows ensured collection of enough data points across the peaks.

With the developed method, LOQs ranged from 0.01 to 0.8 μg/kg with the exception that higher limits of 1.0 to 10.0 μg/kg were determined for EMETR. The recoveries from wheat, rye and barley at the three spiking levels (10, 50, 200 μg/kg) varied overall from 51 up to 139 %. A tendency was observed for recoveries to be below 100 % for the (*R*)-epimers and above 100 % for the (*S*)-epimers. The RSD_r was between 0.6 and 13.9 % (Table 6, Appendix II). A matrix effect was observed in MS. Most of the SSE percentages were within the range of 63 and 113 % (Table 11). The response of EMETR was suppressed most remarkably (SSE from 9.8 to 29 %), whereas significant signal enhancement was observed for ECRIS, ECORN and ECORN-INE (SSE between 152 and 698 %).

Table 11. Signal suppression or enhancement (SSE %) calculated for ergot alkaloids from grains.

Mycotoxin	SSE % in wheat	SSE % in barley	SSE % in rye
EMETR	15	29	9.8
ESINE	73	62	63
ECORN	450	698	367
ECORN-INE	227	104	156
ECRYPT	99	59	99
ECRYPT-INE	88	85	88
EAMINE	72	188	73
EAMININE	93	82	113
ECRIS	81	84	71
ECRIS-INE	172	168	152
Median	90	85	94

5.3.2 ERGOT ALKALOIDS DETECTED IN FINNISH GRAINS

A total of 55 % (i.e. 18 rye and 5 barley) of the samples were found to be contaminated with one or more ergot alkaloids at a level exceeding the LOD. In wheat, detectable levels of the ERGs were not present. The mean concentrations of total ERGs were 717 µg/kg in rye and 59 µg/kg in barley. The amounts of total ergot alkaloids measured in the individual samples ranged from n.d to 6200 µg/kg in rye and from n.d to 420 µg/kg in barley. Each of the ten ergot alkaloids analysed could be detected at variable concentration levels in both rye and barley samples (Table 12). With respect to the individual alkaloids, ECORN and ECRYPT were measured at the highest concentrations and most often (in 20 out of 42 samples). The highest amount of an individual ergot alkaloid was 2200 µg/kg of ECORN in a rye sample. A chromatogram of a contaminated rye sample is featured in Figure 7.

Table 12. The levels of ergot alkaloids determined in Finnish grains (as a mean and range of the concentrations found in the samples).

Ergot alkaloid	Rye (µg/kg), n=25		Barley (µg/kg), n=12		Wheat (µg/kg), n=5
Ergometrine	8.0	(n.d – 61)	< 2.6	(n.d – <2.6)	n.d
Ergosine	96	(n.d – 760)	14	(n.d – 130)	n.d
Ergocornine	230	(n.d – 2200)	12	(n.d – 71)	n.d
Ergocorninine	70	(n.d – 590)	3.7	(n.d – 22)	n.d
Ergocryptine	75	(n.d – 890)	8.8	(n.d – 62)	n.d
Ergocryptinine	62	(n.d – 790)	2.2	(n.d – 14)	n.d
Ergocristine	110	(n.d – 1200)	11	(n.d – 130)	n.d
Ergocristinine	51	(n.d – 460)	1.9	(n.d – 22)	n.d
Ergotamine	7.5	(n.d – 150)	3.5	(n.d – 39)	n.d
Ergotaminine	6.8	(n.d – 130)	0.8	(n.d – 8.4)	n.d

n.d = not detected

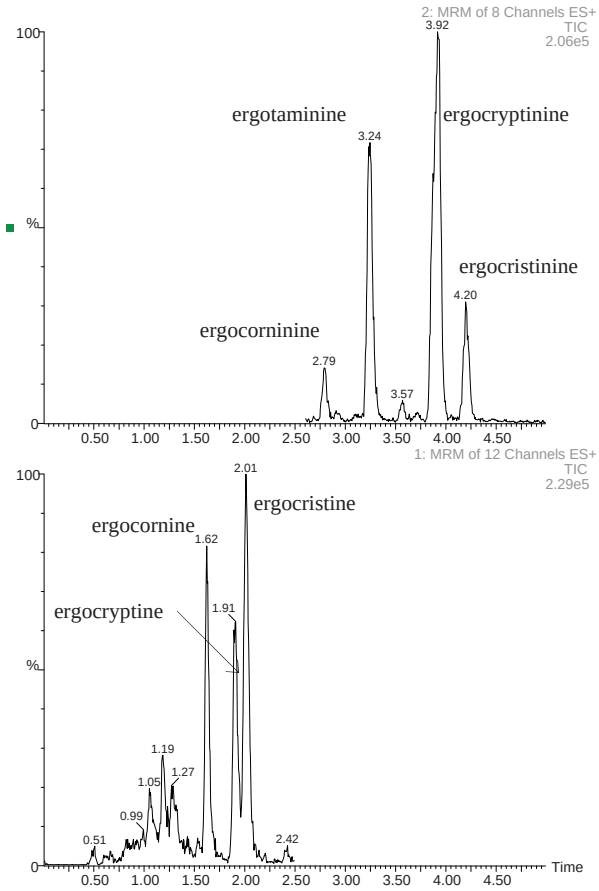


Figure 7. A total ion chromatogram obtained with UHPLC-MS/MS of a rye sample naturally containing ergot alkaloids.

6. DISCUSSION

6.1 EVALUATION OF THE SIMPLIFIED SAMPLE PREPARATION PROCEDURES

In modern analytical chemistry, simplicity and speed of analysis are important goals to achieve high throughput of samples (Anastassiades et al. 2003; Mol et al. 2008). Since sample pre-treatment is often the most time-consuming step, several investigators have looked for ways to facilitate this step by reducing clean-up procedures (Anastassiades et al. 2003; Suloyk et al 2006; Mol et al. 2008). Another way has been to reduce sample size, which allows the procedure to be carried out in smaller scale and may also result in decreased consumption of solvents (Anastassiades et al. 2003; Suloyk et al 2006). In this study, relatively large sample sizes (from 10 to 20 g) of cheese or grains were taken in comparison to several other multi-mycotoxin methods which have utilized about ten fold smaller sizes (i.e. from 0.25 g to 3 g) for similar sample matrices (Cavaliere et al. 2005; Sulyok et al. 2006; Herebian et al. 2009; Monbaliu et al. 2009). Bearing in mind that mycotoxins are frequently present at low concentrations and can be heterogeneously distributed in various commodities (Whitaker 2006), large sample sizes were considered necessary to ensure the representativeness of samples. There are no published guidelines for the size of a laboratory sample for mycotoxin analysis. However, in several official and standardized methods a portion of 10 up to 50 g of sample is taken (e.g. AOAC International 2006; International Organization for Standardization 2007; The European Committee for Standardization 2009a). A total of 3.5 g of agar was taken for the toxin production studies to obtain sample plugs around the whole area where the fungus had grown and thereby to guarantee repeatable quantitative analysis. In this case also, the sample size was large in comparison to those of 0.5 and 0.75 g agar taken in other studies examining mycotoxin production (Medina and Magan 2011; Van Pamel et al. 2011).

Sample pre-treatment was kept to a minimum in the developed methods, which permitted both simple and rapid analytical procedures for routine use. The extraction of the analytes was achieved with liquid extraction either with ACN based solvents (grains and cheese) or with a mixture of MeOH, EtOAc and DCM (agars). In the case of grain samples intended for multi-mycotoxin determination, the extraction was enhanced and automated by using pressurized liquid extraction instrument (ASE®), which conferred an additional benefit of reduced occupational exposure to hazardous solvents. Ultrasonic agitation was utilized to enhance the extraction of

analytes from agar matrices. However, the enhanced extraction methods were not compared to any conventional procedure to demonstrate experimentally improved extraction efficiency. After the extraction, the developed methods consisted of only centrifuging (cheese and agars), filtering and concentration steps. Low temperatures were used in each method to enhance precipitation of proteinaceous sample components, i.e. the sample extracts were either centrifuged at low temperature (cheese, agar) or kept in cold storage overnight (grains). Since cheese has a high fat content, hexane partitioning was performed simultaneously with the extraction step in order to remove lipid compounds. In the specific analysis of ergot alkaloids, a sample clean-up step was introduced for the grain extracts in order to achieve the best possible detection capability of the method. A commercially available Mycosep Ergot push-through SPE cartridge (Römer labs, Tulln, Austria) provided a simple and robust clean-up option for routine use.

Sample purification has been considered as an essential part of an analytical method but modern mass spectrometric instruments allow selective and sensitive determination of low analyte concentrations also from unpurified food and feed sample extracts (Malik et al. 2010). There are some factors limiting the applicability of clean-up procedures in multi-analyte applications. Generally, selective clean-up options are not available when chemically diverse compounds need to be evaluated. For example, the IA clean-up column Myco6in1™ manufactured by Vicam (Waters corp., Manchester, UK) is restricted to the eleven EU regulated toxins and T-2 and HT-2, thus offering only limited use in multi-mycotoxin analysis. Additionally, due to the restricted number of active binding sites, the loading capacity of IA columns can be limited (Lattanzio et al. 2007). The use of more general clean-up methods, such as SPE could provide a feasible option. However, this type of approach involves a degree of optimization and may require employing multiple columns with different chemistries and thus, the final method may prove tedious for routine use (Rasmussen et al. 2010; Monbaliu et al. 2010). Mixed mode SPE might have a wider applicability and applications utilizing graphitized carbon black sorbents and a laboratory-made column have been described in multi-mycotoxin analysis (Cavaliere et al. 2005;2007; Jin et al. 2010). However, challenges in optimizing this approach could arise when both acidic and basic compounds need to be analysed. In addition, a QuEChERS procedure without the dispersive-SPE step has been tested in multi-mycotoxin analysis (Rasmussen et al. 2010; Zachariasova et al. 2010). However, it seems that only a limited clean-up effect can be achieved in this way as profound matrix effects in MS have been reported (Rasmussen et al. 2010; Zachariasova et al. 2010). In summary, the sample preparation procedures were kept simple in order to allow rapid analytical procedures and to avoid loss of analytes during the analysis. This approach also allows the application or adaptation of the methods to include new analytes and matrices later. It has been stated that the only way to accomplish a true multi-analyte method for a wide range of compounds

enabling fast sample throughput and an easy inclusion of new analytes and matrices is to devise a simplified sample preparation procedure prior to direct injection of the sample extract (Sulyok et al. 2007a;2007b).

6.2 CHALLENGES AND SOLUTIONS OF LC SEPARATION IN MULTI-MYCOTOXIN ANALYSIS

The challenge of developing an LC method for multi-analyte separation arise from adjusting the conditions for chemically diverse compounds in order to achieve good chromatographic performance in a reasonable analysis time. The separation of the mycotoxins investigated was accomplished with reversed phase liquid chromatography. Generally, RPLC is considered a suitable choice for separating mixtures of neutral and ionic low molecular weight compounds (Snyder et al. 1997), and it has been used successfully in most of the other multi-mycotoxin methods developed for food and feed (e.g. Sulyok et al. 2006; Spanjer et al. 2008; Martos et al. 2010). Chromatographic separation can be characterized in terms of various parameters, including retention and resolution of the analytes as well as width and symmetry of peaks (Snyder et al. 1997). In this study, satisfactory chromatographic performance with respect to retention, resolution and peak shapes was obtained using C18 stationary phases, although the conditions could not be fully optimized for all of the compounds. Mobile phases compatible with MS were chosen, and aqueous mixtures of ACN with volatile modifiers were used. Similar solvents have been utilized previously for separating a large number of mycotoxins (Spanjer et al. 2008; Sørensen et al. 2009; Rasmussen et al. 2010), although mixtures of MeOH have been more common (e.g. Sulyok et al. 2006; Beltrán et al. 2009; Martos et al. 2010). In the present study, suitable compromises in terms of chromatography and MS responses were achieved by using ACN after careful optimization of the operation parameters of LC-MS/MS. Gradient elution was necessary to achieve the elution of the most strongly retained compounds in a reasonable run-time but still achieving good peak shapes. A gradient elution is usually a prerequisite when analytes with a wide range of polarity are separated (Snyder et al. 1997), and it has been applied practically in all the multi-mycotoxin methods published to date (e.g. Sulyok et al. 2006; Spanjer et al. 2008).

The nine mycotoxins analysed from cheese could be separated in a run-time of 40 min with a linear gradient starting from ACN:water (25:75+0.1 % acetic acid). The retention factors ($2.0 < k' < 27.9$) showed that adequate retention ($k' > 0.5$) was achieved for the first eluting compound penicillic acid but the last eluting OTA had a relatively high retention factor since ideally, a gradient can be adjusted so that $0.5 < k' < 20$ (Snyder et al. 1997). The resolution factors of 0.3 to 1.3 for three

analyte pairs indicated incomplete baseline separation if *Rs* of ≥ 2.0 is used to define baseline resolution (Snyder et al. 1997). In multi-residue methods, at least some degree of co-elution is usually unavoidable (Sulyok et al. 2007a). However in the method developed, incomplete resolution was not a problem since different precursor ions were used for the MS detection of the analytes with overlapping peaks. Furthermore, enough data points across each peak could be acquired in the MS despite the partial co-elution. The chromatographic conditions could not be optimized to achieve fully symmetric peak shapes for all the mycotoxins investigated from cheese, and slight tailing was observed for OTA and ROQ C. With regard to ROQ C, this could be explained by basic functionalities present in this compound, which undergo interactions with the free silanol groups of the column stationary phase. Cyclopiazonic acid and citrinin, initially intended to be included in the method, were omitted due to their very broad peaks indicating inadequate retention of these very polar mycotoxins. Similar results have been reported for these two compounds in RP multi-mycotoxin separations (Sulyok et al. 2007b; Spanjer et al. 2008; Rasmussen et al. 2010). Broad peaks were obtained for AFs compared to the other analytes, which was possibly due to the injection of the samples in a higher content of organic solvent (i.e. 100 % MeOH) than that present at the initial LC conditions (ACN:water, 25:75). This practice usually leads to the distortion or broadening of early eluting compounds (Snyder et al. 1997). Since lipids or emulsifying agents originating from the cheese matrix were present in the samples at the final stage, dissolving the sample into an aqueous or pure ACN lead to partition of two phases. When using MeOH, a homogenous sample was achieved.

The HPLC separation of the 29 or 31 mycotoxins extracted from cereal based agars or grains was achieved in two chromatographic runs, each requiring 35 min. The use of several analytical runs is a common practice in mycotoxin multi-residue methods and previously, there are reports of the HPLC separation of 27 or more toxins achieved in two separate runs each taking 21 to 44 min (e.g. Sulyok et al. 2006;2007a; Herebian et al. 2009; Rasmussen et al. 2010). A complete baseline separation was not achieved for all the analytes, and *Rs* of 0.2 to 1.8 were obtained for a total of nine analyte pairs in the two LC runs. DON and PA practically co-eluted (*Rs*=0). These co-eluting analytes produced different precursor ions upon MS ionization, enabling their specific detection. The collection of enough data points across peaks was accomplished by dividing the analytes into three or five MRM acquisition windows.

The use of two separate chromatographic runs was necessary because some of the co-eluting compounds were ionizable only with either negative or positive polarity of ESI, thus preventing the polarity switching within a single run. In several other multi-mycotoxin LC-MS/MS methods, the analytes have been divided into two LC analyses utilizing either the ESI+ or ESI- solely (Sulyok et al. 2006; Herebian et al. 2009; Rasmussen et al. 2010). However in this study, the use of polarity switching

in each of the two runs provided more options when selecting the chromatographic conditions (achieving the best possible peak shape, retention and resolution) and the more optimal of the two ESI modes. Although the analytes were divided into two runs allowing flexibility in the method development, compromises were needed, i.e. some mycotoxins had to be analysed in the less favourable chromatographic conditions or with the less optimal ESI mode. For example, NEO and MON co-eluted but required different ionization polarities. Therefore, NEO was transferred to the less favourable acidic LC conditions although it was analysed as an ammonium adduct. Furthermore, interfering matrix peaks caused problems with the detection of ZEN and PA and they both had to be ionized in the less advantageous negative mode of ESI. Additionally, PA had to be analysed in the ACN:ammonium acetate run even if it produced a higher MS response when formic acid was present.

In the final methods, 15 mycotoxins were separated using ACN:water (+ 0.2 % formic acid) and 16 analytes using ACN:1.0 mM ammonium acetate. The retention factors of $0.8 < k' < 14.7$ (for NEO and ENN A) and $0.6 < k' < 12$ (for MON and ECRIS, respectively in the second run) demonstrated that the gradients were adjusted well to achieve retention of the first eluting compounds and avoiding unnecessarily long run-times. It should be noted that the k' of 0.6 for MON was calculated using a flow rate of 0.2 ml/min although a flow of 0.1 ml/min was applied during the first two minutes of the run. Therefore, it is possible that less retention was achieved for MON in practice. However, it was assumed that retention of $k' > t_0$ occurred since the retention time of MON was reproducible. MON is a very polar compound which is difficult to retain on an RP column without resorting to ion pairing agents (Jestoi et al. 2003). On the contrary, good retention has been reported for this mycotoxin occasionally in RP columns without any such modifiers (Sulyok et al. 2006; Sørensen et al. 2009). Nevertheless, the retention was hypothesized as being either due to ion-paring or ionic interactions occurring unintentionally (Sulyok et al. 2006) or due to some property of the particle material of the column used (i.e. Gemini®, Phenomenex, Torrance, CA, United States) rather than the actual RP stationary phase (Sørensen et al. 2009).

The peak shapes were mostly acceptable although peaks of FB2 and HT-2 were broad and asymmetrical peaks were obtained for MON and EMETR (*As* of 2.7 and 2.9, respectively). The peak of MON was distorted due to improper retention or because the injection solvent had a higher ACN content compared to the prevalent LC conditions. Tailing of EMETR could be explained by the amine group interacting with free silanols of the stationary phase. It is also possible that small amounts of the (*S*)-diastereomer of EMETR were present, and this was not resolved but could be seen as a shoulder in the chromatogram. The other ergot alkaloids eluted as two peaks which originated most probably from the (*R*)- and *(S*)-epimers. Earlier studies have demonstrated that the two epimers can be separated in a RP C18 column under both acidic and basic conditions (Sulyok et al. 2006; Müller et al.

2009; Krska et al. 2008b). Despite the separation obtained, ergot alkaloids were quantified as a sum of the two epimers since standards of the individual epimers were not incorporated into this method.

The use of UHPLC allowed rapid separation of six ergot alkaloids and four of their respective *(S)*-epimers. The recently introduced UHPLC technique utilizes columns packed with sub-2 μm particles and high flow rates, which results in improved efficiency and speed of separation (Swartz 2005). In the present application, the UHPLC ensured the separation of the ten ERGs as sharp, narrow peaks in a total run-time of 6 min, using a linear gradient with ACN:ammonium carbonate and a flow rate of 0.4 ml/min. In comparison, the separation of 12 ergot alkaloids with similar mobile phase took 21 minutes with RP-HPLC (Krska et al. 2008b). The good resolution achieved highlights the efficiency of the UHPLC technique; only the peaks of two analyte pairs were not separated completely until the baseline (*Rs* = 1.9). The slightly basic mobile phase (pH 8.4) was favourable for the peak shapes of the ergot alkaloids which are neutral at these conditions improving the retention and diminishing the undesired interactions with free silanols of the stationary phase. Despite the fact that the peaks were narrow and seemed symmetric, the asymmetry factors of 1.5 to 2.3 for some of the peaks were indicative of tailing. The calculated retention factors ($0.2 < k' < 6.7$) revealed that the gradient was adjusted well but adequate retention of the first eluting EMETR was not achieved. The improper retention may be only theoretical, as reproducible retention times and peak intensities were obtained for this compound over several months.

The (*R*)- and (*S*)-epimers of each ergot alkaloid could be resolved with the chosen column and conditions, which was an important objective for this method, enabling the gathering of occurrence data on the individual epimers. It has been proven that epimerization of the ergot alkaloids at the C-8 position, i.e. transformation of (*R*)-diastereomers to (*S*)-diastereomers, occurs readily in aqueous acidic and alkaline solutions and in protic solvents (Komarova and Tolkachev 2001; Hafner et al. 2008; Storm et al. 2008). Conversion at a rate ranging from 4 to 6 % can take place at room temperature in ACN:ammonium carbonate mixture (84:16) during an LC analysis lasting 18 hours (Hafner et al. 2008). However, it was assumed that during the usually shorter UHPLC runs, the conversion was controllable, especially as the sample vials were kept at + 10 °C during the UHPLC analysis.

In conclusion, suitable liquid chromatographic conditions were found in the multi-mycotoxin methods developed by using reversed phase chromatography performed with gradient elution utilizing ACN based solvents. However, the conditions could not be fully optimized for all the analytes in terms of retention, resolution and peak shapes since occasionally, the peaks overlapped and broad or tailing peaks were obtained. Challenges were encountered with polar analytes in particular. The use of UHPLC ensured rapid separation and good peak appearance for ergot alkaloids, demonstrating its potential in multi-residue analysis.

6.3 THE POTENTIAL OF MS IN MULTI-MYCOTOXIN DETECTION

The MS analysis was performed by utilizing electrospray ionization and tandem MS accomplished with triple quadrupole analysers. Ionization with the electrospray interface was a feasible choice for the multi-residue LC-MS/MS methods considering the diverse chemical nature of the analytes. Since a number of acidic and basic compounds were included in addition to neutral ones, ESI was chosen, as it is generally considered to be more sensitive for polar and ionic compounds in comparison to APCI (Boyd et al. 2008). No comparative study was conducted between these two interfaces. However, the applicability of ESI technique has been demonstrated in a number of the multi-mycotoxin methods published to date (e.g. Sulyok et al. 2006; Spanjer et al. 2008; Desmarchelier et al. 2010; Martos et al. 2010). The ionization polarity mode (i.e. ESI+ or ESI-) was chosen on the basis of the preliminary tests performed with direct injection, although in some cases, the less optimal mode had to be used ultimately because of co-eluting analytes requiring different ionization polarities or matrix components interfering with the detection. For most of the analytes, protonated molecular ions were formed in ESI. This was in accordance with the studies of Sulyok et al. (2006;2007a) who demonstrated that generally, the known mycotoxins are ionizable in the ESI+ as $[M+H]^+$.

The triple quadrupole instruments were considered as an appropriate choice for the quantitative determination of the mycotoxins of interest. The QqQ mass analysers are utilized widely in this type of targeted trace analysis as they can provide the required selectivity and detection capacity (Boyd et al. 2008). The precursor ions selected in the preliminary experiments could be fragmented in CID, and for most of the analytes, two mass transitions could be monitored for quantification and confirmation purposes. In general, structure specific mass transitions were selected. The use of MRM data of QqQ ensured a total of four identification points (i.e. one precursor and two product ions) as required for example from the official control methods for residues in animal products (European Union 2002a). Data acquisition speed of the instruments employed was adequate to produce good and reproducible peak shapes especially, when the analytes were divided in several acquisition windows. The drawback of employing QqQ instruments was the possibility of gathering data on the selected analytes only and thus, information on non-targeted mycotoxins possibly present in samples could not be obtained.

In the HPLC-MS/MS method developed for the cheese matrix, the mycotoxins included (AFs, OTA, MPA, PA and ROQ C) were ionizable in the positive mode of ESI. Hence, polarity switching was not necessary, simplifying the method development. All the toxins produced good responses as protonated molecular ions, which is in agreement with earlier reports (e.g. Rundberget and Wilkins 2002; Sulyok et al. 2007a;2010; Spanjer et al. 2008). However, OTA, MPA and ROQ C have been observed to produce the optimal sensitivity also as $[M-H]^-$ (Desmarhcelier et al.

2010; Rasmussen et al. 2010), which indicates that these compounds have ability to both acquire and lose a proton upon ionization depending on the conditions. Although there did not seem to be any significant amounts of other type of adducts observable in the spectra of the analytes, acetic acid was added to the mobile phase to enhance the proportion of the $[M+H]^+$. Two structure-specific product ions could be monitored for each analyte with the exception of MPA, for which a non-specific transition produced from the loss of water was used as a confirmatory ion. This was a justified choice since other mass transitions observed for MPA did not yield a sufficient response upon CID.

The MS ionization of the 29 or 31 mycotoxins from cereal based agar or grain extracts was accomplished utilizing both polarity modes of ESI, as well as adduct formation with the aid of mobile phase modifiers. The analytes ionizing readily in ESI+ as protonated molecular ions (i.e. ANT Y, AFs, FBs, ENNs, BEA, MPA and OTA) were analysed in the chromatographic run using the acidic mobile phase containing formic acid. On the basis of earlier studies, all of these compounds have a high tendency to form $[M+H]^+$ (e.g. Sulyok et al. 2007a; Spanjer et al. 2008). However, exceptions do exist, and ENNs, BEA, MPA as well as AF B1 and AF B2 have been reported as forming also ammonium adducts in the presence of ammonium acetate in the mobile phase (Sulyok et al. 2007a; Herebian et al. 2009; Monbaliu et al. 2009). In addition, ZEN was analysed under acidic conditions. In the initial tests, a better response was obtained for ZEN as $[M+H]^+$ but due to co-eluting matrix interferences, it was monitored as $[M-H]^-$ in the negative mode of ESI. A good response was obtained since ZEN is readily ionisable with both polarities, as has also been demonstrated in previous studies (Sulyok et al. 2006; Herebian et al. 2009; Monbaliu et al. 2009; Martos et al. 2010).

Ammonium acetate was added in the mobile phase of the second run of the multi-mycotoxin analysis from grains. This was principally to ensure ionization of the trichothecenes which did not produce an adequate signal as protonated or deprotonated molecular ions but formed readily ammonium adducts (the type-A TRs) or acetate adducts (the type-B TRs). There are similar observations in the literature of the poor ionization of TRs in LC/MS with ESI and the feasibility of monitoring the adducts (Berthiller et al. 2005 ; Sulyok et al. 2006), even if a response for type-A and B trichothecenes has been obtained also as $[M+H]^+$ (Spanjer et al. 2008; Garrido-Frenich et al. 2009; Desmarchelier et al. 2010). The addition of ammonium acetate inhibited the formation of undesired sodium adducts that were observed for TRs. Stable Na-adducts, which are not easily fragmented in CID and do not produce abundant product ions have been demonstrated to occur frequently for TRs and AFs (Sulyok et al. 2006; Garrido-Frenich et al. 2009). MON, PA, GLIO and ergot alkaloids were also analysed in the presence of ammonium acetate; the first two being monitored as $[M-H]^-$ and GLIO and ERGs as $[M+H]^+$. With respect to PA, the negative ionization was needed to avoid problems of the co-eluting matrix

interferences, whereas MON is generally ionisable only in the negative mode of ESI as a deprotonated molecular ion (Jestoi et al. 2003). On the other hand, GLIO can be ionized at both polarities and $[M-H]^-$ and $[M+H]^+$ have produced a satisfactory response (Sulyok et al. 2007a; Rasmussen et al. 2010).

Two structure specific mass transitions per analyte were chosen, with some exceptions. MON as a small molecule (molecular weight 98 Da) yields only one ion transition upon CID (Jestoi et al. 2003). Thus, this non-specific product ion arising from the loss of carbon monoxide was used for the quantification as has been done also in previous studies (Jestoi et al. 2003, Sulyok et al. 2006). In addition, the type-B TRs fragmented very easily in CID at low collision energies and produced few fragments which could be selected for monitoring. Therefore, the mass transitions corresponding to the deprotonated molecular ions $[M-H]^-$ and the acetic ion (m/z 59) producing the most intense response were used for DON, 3-AcDON and F-X. Non-specific mass transition ions formed from the loss of water were regarded as appropriate confirmatory ions for ERGs and MPA since these provided enhanced MS response in comparison to other product ions formed upon CID. Initially, both isomeric mycotoxins, 3-AcDON and 15-AcDON, were intended to be included in the multi-residue method. However, chromatographic resolution was not obtained, and the fragments produced in CID were not specific for reliably distinguishing between these two forms. Therefore, 15-AcDON was excluded and the MS parameters were optimized for 3-AcDON. This was considered as the more relevant isomer in Finnish crop as the producer organisms i.e. *F. graminearum* and *F. culmorum*, which are encountered in northern Europe, are of the 3-AcDON producing chemotype (Langseth et al. 2001; Jestoi et al. 2008).

Ergot alkaloids produced the best response as $[M+H]^+$ upon ionization in ESI in both the HPLC-MS/MS multi-mycotoxin method and the UHPLC-MS/MS method. These compounds did not display any tendency to form ammonium adduct in the conditions applied despite the presence of ammonium, which is in agreement with earlier studies (Sulyok et al. 2007a; Krska et al. 2008b). In the UHPLC-MS/MS based method for ERGs, structure specific product ions were produced in CID, corresponding to the transitions presented by Lehner et al. (2005).

In summary, in the methods developed, mass spectrometry employing electrospray ionization and the MRM function of the triple quadrupole analyser enabled the detection of the analytes. The mycotoxins of interest produced readily either protonated or deprotonated molecular ions in ESI. However, the addition of ammonium acetate was required to achieve sufficient response for trichothecenes as adduct ions. Generally, two specific product ions could be detected for each analyte resulting in selective identification also in those cases that chromatographic peaks overlapped. However, sometimes unspecific mass transitions had to be used for quantification and confirmation of analytes in order to achieve adequate response.

6.4 OVERCOMING THE MATRIX EFFECTS

The matrix components are known to affect the MS ionization process, and ESI is particularly prone to these effects with mostly signal suppression being encountered (Boyd et al. 2008). Matrix effects, evaluated as the signal suppression or enhancement (SSE %), were observed for most of the analytes determined from the grain matrices with either the HPLC-MS/MS multi-mycotoxin method or the UHPLC-MS/MS method for ergot alkaloids. Especially in the HPLC-MS/MS multi-toxin method, profound matrix effects were observed, with the calculated SSEs being between 5.6 and 106 %. For most of the analytes, significant signal suppression was observed, but its extent varied between the matrices. The suppression was generally stronger in oats compared to barley and wheat when evaluated on the basis of the median SSE percentages in these matrices (20, 43 and 45 %, respectively). The matrix effect was not unexpected as crude cereal extracts without any further clean-up were being injected into the MS instrument.

Extensive matrix effects seem to be unavoidable in multi-analyte methods which utilize direct injection in LC-ESI-MS/MS as several investigators have reported SSE values deviating clearly from 100 % (Sulyok et al. 2007b; Spanjer et al. 2008; Beltran et al. 2009). The SSEs for 39 mycotoxins in wheat (85-129 %), barley (47-196 %) and maize (25-148 %) presented by Sulyok et al. (2006;2007b) indicate clear analyte and matrix dependent effect and are comparable to the observations in this study. However, the signal suppression of the analytes in wheat (SSEs as low as 6.9 %) was more profound as compared to that reported by Sulyok et al. (2006). The discrepancy in data between the two studies can be explained by the differences in the sample preparation procedures leading to different amount of sample components being present in the injected sample. In the method devised here, the sample extract was concentrated with evaporation and theoretically, one injection contained extracts of 50 mg of cereal flour, which was about 100 times more than the approximately 0.6 mg injected by Sulyok et al. (2006). Dilution of the sample can reduce matrix effects but may also result in higher detection limits (Sulyok et al. 2007; Beltran et al. 2009). In the method developed here, the sample preparation procedure was regarded as representing a reasonable compromise between the matrix effects and the achievable LODs. Extensive matrix effects observed could be also attributed to the ASE extraction which was performed at an elevated pressure and temperature. It is possible that ASE enhances the co-extraction of matrix components e.g. in comparison to conventional extraction with shaking. In attempt to reduce the amount of co-extracted matrix components, ASE has been operated at room temperature when extracting multiple mycotoxins from cereals (Desmarchelier et al. 2010). Despite the low temperature, a QuEChERS clean-up was considered necessary after the extraction to avoid further the matrix

effects in ESI-MS. Unfortunately, SSEs or other numerical data attributable to the matrix effects were not presented in that study and the impact of the implemented actions cannot be evaluated.

Clear analyte-specific trends regarding the matrix effect were not observed. However, the effect was particularly dramatic (≤ 10 %) for MON, NIV, BEA, ENN A and A1, ZEN and EAMINE in oats, as well as EMETR and MON in wheat. The matrix effect is a complex phenomenon and therefore it is difficult to explain unequivocally why certain compounds were more affected than others. In some cases (e.g. MON, NIV and NEO), the suppressive effect was associated with the early elution of the analytes simultaneously with polar matrix interferences. The signal suppression was reflected as increased LOQs of these compounds. On the other hand, the compounds eluting late in the gradient (BEA, ENNs, ERGs) were possibly affected by the strongly retained matrix components causing signal suppression (Boyd et al. 2008). It is difficult to compare the analyte-specific SSEs to those described in the literature since the matrix effects are caused by various method and instrument dependent factors (Boyd et al. 2008). For example, in contrast to these results, a clear enhancement of the signal of MON has been observed with different cereal matrices (Sulyok et al. 2007b). In addition, AFs have been listed as critical compounds for signal suppression (Sulyok et al. 2007b; Spanjer et al. 2008, Beltran et al. 2009) whereas in the present study, these were among the least affected, and even slight signal enhancement was observed.

Considerable signal suppression or enhancement was observed also in the ergot alkaloid-specific UHPLC-MS/MS method with the SSEs varying overall between 9.8 and 700 %. In most cases, the effect was of the same type (either suppressive or enhancing) for a given analyte in each matrix. The median SSE values of 85, 90 and 94 % for barley, wheat and rye, respectively did not reveal any great differences between the matrices although barley did evoke somewhat more suppression than the other two cereals. The suppressive effect was especially severe for EMETR and was reflected in the LOQs of this compound being higher compared to the other ergot alkaloids. The signal suppression was assumed to be due to the early elution of this compound simultaneously with matrix interferences. A clear signal enhancement (SSE 152-700 %) of ECORN, ECORN-INE and ECRIS was observed in all three cereals. This kind of finding has not been reported previously, and therefore the reason remains unclear at the moment. The matrix effects could be investigated further e.g. by infusing standard compounds with the aid of a T-piece connection between the LC and the MS and simultaneously injecting blank cereal matrix on-column (Boyd et al. 2008). Subsequently, the chromatography could be adjusted better in order to avoid such extensive matrix effects.

The intense matrix effects were unexpected since the selective SPE clean-up with the Mycosep column was used to remove matrix components. Additionally, the improved resolution and narrower peaks in UHPLC-MS/MS has been demonstrated

to result in decreased matrix effects compared to HPLC-MS/MS as analytes co-elute less with interferences during ionization (Van De Steene and Lambert 2008). On the other hand, one can hypothesize that if matrix interferences co-elute, their concentration in the narrow peaks can be higher, thus leading occasionally to more drastic matrix effects in UHPLC/MS methods. The results obtained were not consistent with the data of Krska et al. (2008b) who reported the SSEs of ergot alkaloids deviating only slightly from 100 % in wheat and malted-milk biscuits determined with HPLC-ESI-MS/MS. In that study, the role of the dispersive-SPE with PSA was emphasized for avoiding severe matrix effects (Krska et al. 2008b). It is possible that the purifying effect of that procedure was more efficient compared to that achieved with the Mycosep cartridge clean-up, but verifying this would need careful comparison of the procedures. The theoretical amounts of the cereal matrix present in the injected samples can also explain the differences in the matrix effect observed. Krska et al. (2008b) injected theoretically extracts of 2 mg of sample whereas the amount was double in the present method possibly leading to more profound SSE.

In the methods developed, compensation was made for the matrix effects in MS by using matrix-assisted standards in the quantification of multiple mycotoxins from cheese and grain matrices as well as in the ergot alkaloid-specific method. The use of matrix-assisted calibration is well established when working with LC-ESI-MS/MS (Boyd et al. 2008) and has been adopted in the majority of the multi-residue methods developed for mycotoxins from food and feed (e.g. Sulyok et al. 2007b; Spanjer et al. 2008; Martos et al. 2010). Isotope labelled or other types of internal standards have been used for quantification in some multi-mycotoxin applications (Herebian et al. 2009; Monbaliu et al. 2009; Zachariasova et al. 2010). In particular, stable isotope diluted calibration is regarded as the ideal way to compensate for analyte losses and matrix effects especially when used in conjunction with matrix-assisted calibrants (Boyd et al. 2008; Rychlik and Asam 2008). Improved linearity of calibration, repeatability and accuracy (as recovery) of the results have been reported when using isotope analogues in multi-mycotoxin determinations (Vaclavik et al. 2010; Zachariasova et al. 2010). On the other hand, with other types of IS (i.e. not analogues), improvement of quantification is not necessarily achieved. For example, Monbaliu et al. (2009) quantified the 23 mycotoxins of interest with ZAN as IS, obtaining mostly comparable results with the internal calibration and external matrix-assisted calibration.

IS were not incorporated into the methods developed due to some drawbacks associated with their use in multi-residue methods. First of all, suitable internal standards, i.e. compounds not present in the real samples and resembling closely the chromatographic and mass spectrometric behaviour of the analytes, are not available for all the mycotoxins of interest. For example, isotope labelled IS were commercially available for only a few of the analytes at the time of method

development. Additionally, multi-mycotoxin analysis would require the inclusion of a large number of IS increasing the cost of analysis. However, when isotope labelled compounds become more easily available, the use of internal calibration should be examined as it could improve the quality of results. Another possibility to reduce matrix effects in MS would be to increase sample purification. In order to maintain the simplicity of the analytical procedures, this was not considered a practical option. With the emergence of feasible approaches, such as QuEChERS, option of clean-up should be considered. However, even with extensive sample clean-up, matrix effects may not be avoided completely (Desmarchelier et al. 2010).

It can be concluded that matrix effects frequently occur in ESI-MS based methods. The analyte signals were affected by cereal matrix components both in the multi-mycotoxin method where unpurified extracts were injected as well as in the ergot alkaloid-specific method utilizing SPE clean-up. The matrix effects were mostly suppressive but varied between the analytes and the cereals. Although the effects observed could not be explained unequivocally, signal suppression was frequently associated with early elution of analytes simultaneously with polar matrix interferences and was reflected as increased LOQs of these compounds. Matrix-assisted calibration was considered a practical approach to compensate for the matrix effects, although the use of internal standards and clean-up options could be examined in the future.

6.5 EVALUATION OF THE METHOD PERFORMANCE

Validation of analytical methods should be carried out in order to demonstrate that they are fit-for-purpose (Taverniers et al. 2004). The methods developed in this study were in-house validated. Since there are no guides specifically dedicated for the validation of mycotoxin methods, the degree of validation was evaluated on the basis of the intended use of the methods, which was for research purposes rather than official control of occurrence.

6.5.1 MULTI-MYCOTOXIN METHOD FOR CHEESE

The performance of the HPLC/MS/MS based method for cheese was defined by recovery, repeatability and LOQs. The recoveries for the nine mycotoxins analysed were between 96 and 143 % demonstrating that the acidic mixture of ACN ensured satisfactory extraction efficiency and that the analytes were not transferred to the hexane phase during the partitioning step. Formic acid supposedly enhanced the

extraction of acidic compounds PA and MPA, although the effect of the acid addition was not evaluated specifically. The recoveries above 100 % obtained mostly at the lower spiking level (5 or 50 μg/kg) could not be explained unequivocally. A similar solvent composition (i.e. acidified 90 % ACN) and hexane defatting were reported feasible in the analysis of *Penicillium* metabolites from a complex sample matrix (food mixture), reaching recoveries of 75 to 116 % (at 10 to 1000 μg/kg) with an HPLC-MS/MS method (Rundberget and Wilkins, 2002). In disagreement to the present results, low recoveries were obtained for OTA (i.e. ≤30 %) which was omitted from the quantitative analysis. There are no official guidelines for mycotoxin methods for cheese or related matrices hindering the evaluation of the compliance of the recoveries. However, considering the strictest range of 80 to 110 % set for the recovery of a method for official control of aflatoxins at > 10 μg/kg (EC/401/2006), all the values obtained would not fulfil these particular criteria. The within-day repeatability for all the analytes was ≤ 12.1 % which is comparable to the RSDs of ≤23.5 % presented by Rundberget and Wilkins (2002). The repeatability would be compliant also with the most stringent RSD_r of 15 % set in the EC/401/2006 (for patulin at > 50 μg/kg level). However, if the precision criteria are calculated on the basis of Horwitz equation as recommended for AFs in EC/401/2006 (European Union 2006d), the RSD_rs would not meet the values of 5.9 and 4.6 % corresponding to the spiking levels used in this study (50 and 200 μg/kg).

The toxins could be quantified from cheese at levels of 0.6 to 5.0 μg/kg, which was satisfactory considering the complexity of the sample matrix and the multi-analyte nature of the method. In comparison, the LOQs between 5.0 and 20 μg/kg were accomplished from a food mixture by Rundberget and Wilkins (2002). The LOQs cannot be evaluated on the basis of regulatory limits, as these do not exist for cheese. The EU has set an MRL of 0.05 μg/kg for AF M1 in milk, but this level was not achievable. However, the method developed was capable of quantifying the mycotoxins investigated at the levels which they have been detected in cheeses previously (discussed in 6.6.1).

6.5.2 MULTI-MYCOTOXIN METHOD FOR CEREAL AND RICE FLOUR AGARS

Values for recovery, repeatability and LOQs were determined for the method developed for cereal based agar matrices. The recovery varied extensively (from 8 to 165 %) between the 29 analytes of interest. The values below 100 % could be related to incomplete extraction of the compounds, in particular when low recoveries were obtained for the FBs (8-44 %) and for EMETR, ZEN, NEO and DON (≤ 55 %). Furthermore, MON and NIV were excluded from the method because of inappropriate extraction. It was hypothesized that part of the low molecular weight

compounds would be retained physically or chemically within the starch components of the agar matrix with similar interactions as have been demonstrated for flavour ingredients (Escher et al. 2000). However, this was a theoretical assumption and could not be confirmed on the basis of the literature. An acidified mixture of MeOH:DCM:EtOAc (1:2:3) was used for the extraction according to a procedure introduced for extracting *Penicillium* metabolites from Czapek yeast autolysate agar and yeast extract sucrose (YES) agar for qualitative determination (Smedsgaard 1997). However, it was demonstrated recently that the use of these three solvents separately could be more feasible for the quantitative extraction of mycotoxins from agar (Van Pamel et al. 2011). With this procedure, improved recoveries for FB1, DON, NIV and PAT from YES agar were achieved compared to those obtained with a mixture of MeOH:DCM:EtOAc (1:2:3 + 1 % formic acid). The overall recoveries ranged between 56 and 125 % for the 21 fungal metabolites (Van Pamel et al. 2011) being more consistent in comparison to the recoveries of our study. However, it should be noted, that the spiking levels used (up to 2000 and 6000 μg/kg) were also much higher.

In addition to the extraction efficiency, the recoveries might have been affected by the fact that possible matrix effects were not taken into account in the quantification which was performed using calibrants in solvent. For example, some of the recoveries deviating from 100 % (e.g. 163 and 165 % for 3-AcDON and BEA) could be attributable to this fact. The evaluation of the matrix effects would have been advisable since even simple matrices like agar based growth media consisting of mostly water can affect the MS signal (Van Pamel et al. 2011). Therefore, it is possible that the agar matrix had an effect on the responses also in the present study and the lack of matrix-assisted calibration decreased the accuracy of the quantification. The use of calibrants in solvent was considered acceptable since the repeatability of the results was generally good and the purpose of the method was the determination and comparison of toxin production of fungi during the same experiment. Additionally, the aim was to keep the procedure as simple as possible to allow a rapid screening of a large number of strains. However, comparison of toxin production on different matrices has to be conducted with caution.

The within-day repeatability of the method was satisfactory as the RSDs were mostly ≤ 12 %. However, for DON, F-X and FBs quite high RSDs (≥ 31 %) were encountered, which was associated with poorly repeatable extraction. In general, the repeatability is in accordance with RSD_r values from 1 to 39 % presented for mycotoxins from YES agar (Van Pamel et al. 2011). The LOQs determined from the standards in solvent varied between 0.1 and 86 μg/kg for the 29 analytes. However, the limits might have been quite different if they had been determined from the agar matrix. For example, Van Pamel et al. (2011) obtained very high LOQs (between 400 and 10000 μg/kg) for several toxins including DON, HT-2, NIV and ZEN from spiked agar samples. These values were possibly related to the

suppressive matrix effects but also to the difficulty of extracting lower concentrations. Although the method could not be validated for MON and NIV, qualitative data could be obtained at high concentrations since the compounds were included in the HPLC-MS/MS method developed. The studies conducted on the toxin production of fungi on solid agar have demonstrated that the toxin levels produced during 7 days' incubation are usually at the mg/kg level (Medina and Magan 2011; Van Pamel et al. 2011). Thus, the detection capability of the novel method was adequate for the intended purpose i.e. screening of toxin production of fungal species. The method allows quantification of the analytes of interest although for some compounds, such as DON, F-X and FBs, semi-quantitative or qualitative work is advisable due to inadequate recovery or repeatability.

6.5.3 MULTI-MYCOTOXIN METHOD FOR GRAINS

The values for the recovery, repeatability and LOQ determined for the HPLC-MS/MS method for grains varied greatly between the individual mycotoxins, which was not unexpected considering that the method could not be fully optimized for all 31 analytes. Generally, the recoveries were between 51 and 122 % indicating that the ASE extraction with 90 % ACN ensured satisfactory extraction for most of the mycotoxins of interest. The lower recoveries obtained for ECORN (48 %) and fumonisins (7 to 36 %) were probably related to the poor extraction efficiency. In particular, the efficient and reproducible extraction of the polar FBs would have required a low pH and/or high water content as has been reported earlier (Sulyok et al. 2006; Spanjer et al. 2008; Beltran et al. 2009; Zachariasova et al. 2010). Exceptionally high recoveries obtained for ANT Y (i.e. up to 196 %) are in line with the data of Sulyok et al. (2007a) reporting a recovery of 285 % for this compound from bread-crumbs. It was stated that the result could not be explained by matrix effects or a blank containing the analyte, as was found also in this study. Considering the inconsistent recoveries of FBs and the exceptionally high recoveries obtained for ANT Y, only semi-quantitative or qualitative determination of these compounds would be appropriate.

The extraction solvent composition was chosen on the basis of preliminary studies where ACN and MeOH based mixtures were compared. The use of ACN provided a better overall extraction of the mycotoxins of interest (data not shown). It has been reported previously that the use of ACN, MeOH and acetone can provide equally efficient extraction of chemically diverse contaminants, including mycotoxins (Mol et al. 2008). However, the use of ACN has resulted in improvements in the extraction efficiency in some multi-mycotoxin methods (Sulyok et al. 2006; Beltrán et al. 2009; Garrido Frenich et al. 2009). The reason could be the higher selectivity of ACN

towards most of the mycotoxins due to its tendency of interacting through dipole rather than acidic/basic functionalities as compared to MeOH (Snyder et al. 1997). In the light of the results of Mol et al. (2008), ACN can also be advantageous to reduce the co-extraction of sample matrix components, thus leading to less profound matrix effect in MS as compared to MeOH. However, any experimental comparison of the solvents was not made in this sense within the present study. A high percentage of MeOH also increased the back pressure of ASE hindering occasionally its operation, which favoured the choice of ACN. The extraction with ASE was not compared with any alternative extraction method, such as horizontal shaking.

It has been demonstrated that an adequate extraction efficiency from cereal based matrices can be achieved with ASE at room temperatures (compared to 100 °C used in the present method) (Desmarchelier et al. 2010). The absolute recoveries describing the efficiency of the extraction varied from 49 to 126 % for the 17 analytes being comparable to the overall method recoveries obtained in this study. The higher recoveries obtained for FBs (49 to 55 %) from wheat by Desmarchelier et al. (2010) might be explained by the higher water content and the acid in the extraction solvent (i.e. ACN:water:acetic acid, 80:19.5:0.5). A broad examination of the recoveries achieved with other published multi-mycotoxin methods (Table 4) demonstrates that they all have variations in their results, highlighting the challenges of optimizing a multi-residue method. For example, recoveries between 31 and 127 % and between 43 and 117 % have been presented for 22 and 39 mycotoxins, respectively from wheat, barley and oats (Sulyok et al. 2006;2007b; Martos et al. 2010). Even more variable recoveries from 37 to 205 % were reported for 27 toxins analysed from maize silage (Rasmussen et al. 2010).

The within-day repeatability (RSD_r) for the individual mycotoxins ranged from 2 to 26 %, which is satisfactory for a multi-residue method. These values are in accordance with other published multi-mycotoxin LC-MS/MS methods utilizing direct injection of cereal and feed extracts and reporting RSD_r of ≤ 27 % (e.g. Herebian et al. 2010; Martos et al. 2010; Rasmussen et al. 2010). The between-day RSD_R of 14 to 28 % measured for DON, 3-AcDON, T-2, HT-2 and ENN B from the naturally contaminated grain flour also indicate that the day-to-day variation with this method is acceptable. Due to the lack of CRMs with suitable mycotoxin-matrix combinations at relevant concentration levels, the trueness of the method was determined by analysing the FAPAS reference materials available for DON, T-2 and ZEN. The concentration levels determined were 100, 75 and 129 % of the assigned values of the RMs, showing that the bias of the method was acceptable despite the fact that there was some underestimation of T-2 and overestimation of ZEN could occur.

The expanded measurement uncertainty (*U*) calculated for the *Fusarium* toxins demonstrates that the results include relatively high uncertainties, as 11 out of 18 values were ≥ 50 %. The highest *U* was obtained for the fumonisins, which can be

explained by the large variation of the results of the spiking experiments used for the calculation of *U*. Additionally, possible outlying values were not discarded. The measurement uncertainty has not been commonly presented in the literature on multi-mycotoxin methods. Monbaliu et al. (2010a) reported *U* of 8 to 40.2 % for 23 mycotoxins determined from spiked feed samples. These values also indicate that measurement uncertainty can be considerable. The better overall *U* compared to that in the present study is probably due to the extensive sample clean-up performed, leading to more repeatable results (e.g. RSD_rs of 1.8 to 11.8% and RSD_Rs of 2.2 to 13.2 % were reported).

The LOQs determined were matrix and analyte dependent ranging overall from 1 to 1250 μg/kg. The relatively high LOQs of NEO, NIV, MON and HT-2 (≥ 280 μg/kg) were attributable largely to mass spectrometric factors, i.e. signal suppression caused by matrix components and/or poor ionization in ESI. Signal suppression especially affected the quantification of the early eluting analytes (e.g. NEO, NIV and MON). It is also likely that the evaporation of solvent and the ionization process in ESI were not optimal in the high water content (Boyd et al. 2008), which affected preferentially these compounds eluting at the beginning of the run. The high LOQ determined for HT-2 could not be solely due to matrix suppression, since the SSE values were within the same range as those of the structurally close T-2 which however, had much lower LOQs. Similarly to the other validation parameters, the LOQs obtained demonstrate the variation that is inherent when working with multi-residue direct injection analysis. Previously, LOQs of the same range (i.e. from 0.5 to 800 μg/kg) for 32 mycotoxins have been reported from unpurified wheat and maize extracts (Herebian et al. 2009). Somewhat lower values (from 0.03 to 200 μg/kg) from the same commodities were reported by Sulyok et al. (2006). The discrepancy could be due to the dilution of the extract leading to less severe matrix effects and consequently improved LOQs as compared to the method used in the present study. In addition, the lower LOQs achieved for the type-B trichothecenes might be explained by the use of acetate ion (m/z 59) for quantification (Sulyok et al. 2006). In the present study, acetate gave the highest response of the product ions produced in CID for the type-B TRs. Nonetheless, acetate was used only as a confirmatory ion due to its unspecific nature.

It is not relevant to make a close comparison of the method performance with the published multi-mycotoxin methods because the validation parameters are dependent on the ways that they have been calculated as well as on the actual analytical procedure and the technique used. Furthermore, the poor performance obtained for certain analytes seems to be application dependent and generalizations are difficult to make. The overall performance of the developed method reveals rather large variation between the analytes in terms of recoveries, repeatability and detection capability, similarly to several other multi-mycotoxin methods especially those utilizing the direct injection approach (as summarized in Table

4). Considering the method performance criteria laid down in the Commission Regulation EC/401/2006, precision (i.e. RSD_r and RSD_R) and recoveries were in accordance for DON, T-2 and HT-2 at the corresponding concentration levels. With regard to OTA and ZEN, within-day repeatability and most of the recovery values were within the recommended range. On the contrary, for AFs and FBs, several values were outside the approved range. Regarding the LOQs, the present method was compliant with the existing and proposed MRLs for mycotoxins in animal feed (European Union 2002b;2006a). The only exception was AF B1, the LOQ of which was too high to meet the recommended limits in some specific cases (European Union 2002b). With respect to the regulatory limits in cereals intended for human consumption, the method performance was satisfactory for measuring adequately low levels of DON, FBs and ZEN (except for oats intended for direct human consumption) (European Union 2006c). It should be noted that the compliancy of the multi-mycotoxin method with the regulations was not a prerequisite since the method was not developed for official control purposes.

6.5.4 SELECTIVE METHOD FOR ERGOT ALKALOIDS FROM GRAINS

The recoveries obtained for the ergot alkaloids with the selective UHPLC-MS/MS method were generally good as most of the values were between 70 and 120 %. Lower recoveries were obtained for ECRIS, ECRYPT, ECORN and ESINE, and higher values for ECRIS-INE, ECRYPT-INE, ECORN-INE and EAMININE. It seemed that the recovery percentages were affected by the epimerisation process since recoveries below 100 % were obtained for the (*R*)-diastereomers (–ines) and recoveries above 100 % for all the respective (*S*)-isomers. Hence, it was hypothesized that a portion of the (*R*)-epimers had been converted to the respective (*S*)-forms during the analysis or sample storage. It has been demonstrated that some isomerisation can occur during storage of six weeks at + 4 and + 20 °C in a mixture of ACN:ammonium carbonate mixture (80:20) comparable to the extraction solvent used in the present study (Hafner et al. 2008). Although the samples were never stored for such a long time, it is likely that the conversion of (*R*)-diastereomers to the (*S*)-isomers took place as epimerization may occur also during a shorter period like LC analysis of 18 hours (Hafner et al. 2008). Attention needs to be paid to protect the samples from excess isomerisation during the sample preparation and analysis, and to avoid long-term storage to obtain repeatable and reliable data for the individual ergot alkaloid epimers. In addition, isomerisation is one of the factors that needs to be taken into account when calculating the measurement uncertainty of this method in the future.

The recoveries of the sum of the two epimers were approximately 200 % showing that the extraction efficiency with the chosen solvent as well as the recovery from the Mycosep clean-up cartridge were satisfactory. According to earlier studies, ergot alkaloids can be extracted from cereal matrices with both aqueous acidic and basic mixtures of organic solvents (Scott and Lawrence 1980; Bürk et al. 2006; Storm et al. 2008). However, an ACN:ammonium carbonate mixture (84:16 v/v) has been demonstrated to achieve improved extraction efficiency from naturally contaminated rye and barley in comparison to acidic (MeOH:0.25% phosphoric acid, 60:40) and neutral (ACN:10 mM ammonium acetate, 1:2) solvents (Krska et al. 2008a) that have been used in some earlier studies (Ware et al. 2000; Mohamed et al. 2006). The result could be explained by the fact that at neutral or basic pH, the N-6 of ergot alkaloids, the pKa values of which are between 4.8 and 6.2 depending on the compound, exists in the non-ionized form (Krska and Crews 2008), decreasing the interaction with charged groups of matrix components and enhancing the extraction efficiency with organic solvents. Exact recovery percentages were not presented but in another study applying the same analytical procedure, recoveries ranged mostly between 70 and 110 % from various cereal matrices for six ergot alkaloids and their epimers (Krska et al. 2008b), thus being comparable to the recoveries of this study. In disagreement with the results of the present study, values < 65 % were measured for EMETR and ECRIS-INE. The possible impact of epimerisation on the recoveries was not discussed by Krska et al. (2008b).

The developed method was repeatable with the RSD_r of the individual ergot alkaloids being from 0.61 to 14 %, which is in good agreement with the RSDs of 9 to 18 % or 4.1 to 9.3 % presented for other LC-MS/MS methods (Crews et al. 2009; Mohamed et al. 2006). Low concentrations could be measured, and the LOQs between 0.01 and 10 μg/kg were achieved. The highest values were determined for EMETR (i.e. 1.2-10 μg/kg) the probable reason being the severe signal suppression encountered in the MS. The LOQs were calculated from the response obtained from blank cereals according to the Eurachem validation guide (Eurachem 1998). Considering the possible variation between cereals used as blanks, the LOQs may not be practical and hence, the lowest concentration in the calibration curve (i.e. 0.1 μg/kg) might be a more suitable limit for routine work (for other compounds than EMETR). The LOQs of the developed UHPLC-MS/MS method were comparable to those reported for other LC-MS/MS based methods. For example, LOQs of 0.17 to 2.78 μg/kg have been presented for (*R*)- and (*S*)-diastereomers of ergot alkaloids from cereal based matrices (Krska et al. 2008b). Bürk et al. (2006) obtained LODs between 0.1 and 1.0 μg/kg whereas clearly higher values (from 23 to 37 μg/kg) were reported by Mohamed et al. (2006). Both of these studies analysed only the (*R*)-forms of five or six ergot alkaloids, respectively.

At present, few investigators have presented validation (or occurrence) data for the individual epimers of ergot alkaloids, which is possibly due to the poor

availability of the standards. In some cases, difficulties have been encountered in stabilizing the conditions which would have hindered the epimerization processes and thereby the reliable quantification of the two epimers. In this case it would be reasonable to express the results as a sum of the two epimers (Storm et al. 2008). However, methods capable of quantifying both epimers are of major importance in order to gather information on their occurrence for risk assessment and legislative work (Battilani et al. 2009). Legal limits for ergot alkaloids do not currently exist but values from 100 to 600 μg/kg for the individual ergot alkaloids have been discussed, based on their estimated average content in 1 mg of ergot sclerotia (Battilani et al. 2009; European Union 2010). Considering the suggested values, the developed UHPLC-MS/MS method is capable of quantifying the selected 10 ergot alkaloids at sufficiently low levels.

In conclusion, the validation results demonstrated that a multi-residue method cannot be fully optimized for all the analytes of interest. Generally, the recovery, repeatability and limits of quantification determined for the methods were acceptable but the values varied greatly between individual analytes. Low recoveries were mostly associated with insufficient extraction with the solvents used, a typical example being the fumonisins. In the case of the specific method of ergot alkaloids, the epimerisation affected the recovery percentages. Some of the high LOQs determined were attributed to poor ionization and extensive signal suppression evoked by matrix components. In some cases, the method performance would not permit reliable quantitative analysis and thus, only semi-quantitative or qualitative determination would be appropriate. The multi-mycotoxin method for grains could be used for official control in the cases where the method performance fulfils the established regulatory criteria. The UHPLC method for ergot alkaloids would enable quantification at the concentration levels proposed as legal limits.

6.6. MYCOTOXIN OCCURRENCE AND TOXIN PRODUCTION

6.6.1 MYCOTOXINS IN MOULD CHEESES

Roquefortine C and mycophenolic acid were detected in the blue cheese samples and these compounds originated most probably from the metabolism of the starter organisms used in the cheese production. *P. roqueforti* is a common starter in blue cheeses and it is known to produce these two metabolites (Lafont et al. 1979; Frisvad et al. 2004). The concentrations of ROQ C (from 800 to 12000 μg/kg) and

MPA (300 mg/kg) found were generally in accordance with earlier studies. ROQ C has been reported at levels from 50 to 2290 μg/kg in various blue cheeses (Schoch et al. 1984; Finoli et al. 2001). Compared to these results, the level of 12000 μg/kg of ROQ C in one of the samples was rather high. MPA has been reported from different types of blue mould cheeses at levels of 100 to 500 μg/kg (Zambonin et al. 2002), although levels of up to 15000 μg/kg have also been reported (Lafont et al. 1990). In the present study, only one blue cheese sample contained a detectable amount of MPA, which indicates that this toxin can be avoided with the proper selection of the starter strain (Lafont et al. 1990).

No other mycotoxins investigated were found in the cheese samples. AF M1 could be present in cheeses due to its occurrence in milk, and some recent studies have reported concentrations from 0.03 to 2.52 μg/kg in different types of cheeses (Montagna et al. 2008; Virdis et al. 2008; Er et al. 2010; Hampikyan et al. 2010). Unfortunately, cyclopiazonic acid and citrinin could not be detected with the method applied. CPA has been detected in white mould cheeses as a consequence of the production by the commonly used starter organism, *P. camemberti* (Le Bars 1990; Zambonin et al. 2001). On the other hand, CIT producing wild strains of *Penicillium* have been isolated from cheeses and cheese manufacturing sites (Vázquez et al. 1997). Generally, reports of the occurrence of mycotoxins in cheese other than those produced by starter fungi or AF M1 are scarce. This is likely to be attributable to good manufacturing practices applied in the modern cheese production where the raw materials, manufacturing processes and storage conditions are controlled to avoid fungal contamination and mycotoxin formation (Bullerman 1981; Engel and Teuber 1989). Additionally, toxigenic moulds are believed to represent only a minor proportion of the total flora in cheese and cheese factory environments (Bullerman 1980). Furthermore, cheese is not necessarily a good substrate for mould growth and mycotoxin formation, and some mycotoxins, such as patulin, penicillic acid and PR-toxin, have been shown to be unstable in cheese matrix (Lieu and Bullerman 1977; Scott and Kanhere 1979).

The levels of ROQ C and MPA found in the blue cheeses examined are safe for consumers considering the presumably low daily consumption of this type of products and on the basis of the current knowledge on the toxicity of these compounds. The existing data indicates that the acute toxicities of ROQ C and MPA are low (Wilson 1971; Arnold et al. 1978; Kopp and Rehm 1979; Bentley 2000). For example, LD50 values of 450 to 550 mg/kg per bodyweight (with intravenous administration) have been presented for MPA in mice and rats (Wilson 1971), and LD50 values between 169 and 189 mg/kg (with intraperitoneal administration to mice) were reported for ROQ C (Arnold et al. 1978). Furthermore, the neurotoxic properties associated to ROQ C (Wagener et al. 1980) have been questioned as blue mould cheese has been consumed for centuries without any reports of harmful effects on humans (Bullerman 1981).

6.6.2 TOXIN PRODUCTION OF *F. SPOROTRICHIOIDES* AND *F. LANGSETHIAE*

Beauvericin and type-A trichothecenes (i.e. NEO, DAS, T-2 and HT-2) were detected on both *F. sporotrichioides* and *F. langsethiae* cultures demonstrating that the general toxin profiles of these two fungal species are similar. The toxin profiles obtained were to be expected on the basis of reports presented in the literature. The type-A trichothecene synthesis of *F. sporotrichioides* and *F. langsethiae* has been demonstrated in several studies (e.g. Langseth et al. 1998; Mateo et al. 2002; Thrane et al. 2004), and *F. sporotrichioides* is a known producer of BEA (Thrane et al. 2004; Jestoi et al. 2008). However, the BEA production of the *F. langsethiae* strains was unexpected because this species has not been demonstrated unequivocally as a BEA producer. The production was modest, but the findings indicate that *F. langsethiae* possesses toxin producing capability. On the basis of the amount of the toxins detected, T-2 was the main metabolite of both *F. sporotrichioides* and *F. langsethiae*, which is in accordance with the earlier studies conducted on the trichothecene production of *F. sporotrichioides* (Marasas et al. 1987; Mateo et al. 2002). Generally, it seems that the species examined favour the type-A TR production over that of BEA. However, the biosynthetic pathways of trichothecenes and BEA are independent (Desjardins and Proctor 2007) and therefore their concentrations cannot be expected to correlate. These toxins might also have very different functions for the fungus.

Both species possess a very high potential of producing mycotoxins under *in vitro* conditions. The average levels of T-2 produced by *F. sporotrichioides* and *F. langsethiae* on the cereal flour agar (6600 and 6000 μg/kg, respectively) and of HT-2 (704 and 210 μg/kg) are in agreement with those presented previously for *F. langsethiae* on oats based agar at similar conditions (i.e. a_w 0.99, 25 °C, 10 d incubation) (Medina and Magan 2011). The levels of around 3000 and 13000 μg/kg of T-2 and around 1000 to 3000 μg/kg of HT-2 (exact concentrations were not presented) were reported for strains isolated from UK and the Nordic countries (including two isolates from Finland that were investigated also in the present study). The somewhat higher toxin levels measured are presumably due to the longer incubation period used by Medina and Magan (2011).

The mycotoxin production was abundant also on the rice based agar. T-2 was produced on average at levels of 14400 and 16000 μg/kg by *F. sporotrichioides* and *F. langsethiae*, respectively. In addition, the other type-A TRs were measured on rice agar at high levels, ranging overall from 340 to 3700 μg/kg. Furthermore, BEA production was quantifiable on the rice cultures of all three *F. sporotrichioides* and two of the *F. langsethiae* strains. Sometimes the toxin levels measured on the rice cultures after seven days were even higher compared to those produced on the grain mixture after 28 days. For example, *F. langsethiae* produced higher amounts of T-2, DAS and NEO on the rice substrate compared to the grains. Rice has been stated as being a suitable substrate for fungal growth and toxin production (Jestoi et al. 2008),

which is postulated to be because of the readily available carbohydrates as the excess of carbon promotes the production of fungal secondary metabolites (Carlile et al. 2001). The rice substrate used contained added sucrose which presumably enhanced also the mycotoxin production. It has been demonstrated previously that sucrose promoted trichothecene (i.e. DON and 3-AcDON) synthesis of *F. graminearum* (Jiao et al. 2008).

The *F. sporotrichioides* and *F. langsethiae* strains produced high amounts of mycotoxins also on the grain mixture at the conditions applied. Average T-2 toxin levels of 4100 and 22000 µg/kg were measured on the *F. sporotrichioides* and *F. langsethiae* grain cultures, respectively, with the levels of other TRs varying from 190 to 6200 µg/kg. Additionally, grain mixture was favourable for the BEA production of *F. sporotrichioides*, and 1800 µg/kg were detected on average. The type-A TR production of *F. sporotrichioides* has been investigated earlier on maize, rice and wheat grains (Mateo et al. 2002). As the incubation conditions (a_w from 0.990 to 0.999, + 26 °C, 21 d) were similar to those in the present study, toxin production of the same magnitude could have been expected. However, the concentrations of T-2, HT-2, DAS and NEO presented were clearly lower, varying from 34 to 243 µg/kg in wheat grains (Mateo et al. 2002). Even the highest concentration of T-2 toxin measured on maize (i.e. 1083 µg/kg) was low. The differences in data can be explained by the shorter incubation time used in the study of Mateo et al. (2002) as well as by the differences in the toxin production capacity of the isolates examined in the two studies.

The toxin production observed on the grain mixture, in comparison to the two agars, might best resemble the situation in the natural habitat of these fungi. The toxin profiles of *F. sporotrichioides* and *F. langsethiae* reflect quite well the mycotoxin occurrence data in Finnish cereals. Frequent contamination with low levels of BEA (mostly < 10 µg/kg) has been reported, and T-2 and HT-2 toxins have been detected at occasionally high concentrations, e.g. average levels of 300 to 500 µg/kg have been measured in oats (Eskola et al. 2000;2001; Jestoi et al. 2004c; Yli-Mattila et al. 2008). On the other hand, the occurrence of DAS or NEO has not been described although these were produced readily *in vitro*. However, prediction on the situation in the field cannot be made on the basis of these results, since in the *in situ* conditions, various environmental and agricultural factors as well as the presence of other microbiota affect the toxin production of fungi (Doohan et al. 2003; Edwards 2004; Xu et al. 2007) . The information on the toxin production capacity of these two species is of great importance as the recent studies indicate that *F. langsethiae* in particular is becoming more prevalent in northern Europe (Parikka et al. 2008; Edwards et al. 2009). At the same time, the increased occurrence of T-2 and HT-2 toxins has been observed (Edwards et al. 2009; van der Fels-Klerx 2010).

6.6.3 ERGOT ALKALOID OCCURRENCE IN FINNISH GRAINS

All ten ergot alkaloids examined were found in the grain samples at variable concentration levels. Ergot alkaloids were most prevalent in the rye samples (72 % positive) compared to barley (42 % positive), or wheat where these compounds were not detected. The result was expected as the most important producer organism in grains, *Claviceps purpurea*, is most commonly encountered in rye (Battilani et al. 2009). Furthermore, data compiled recently from Europe and Canada has demonstrated the frequent occurrence of ergot alkaloids in rye (Battilani et al. 2009). For example, some studies conducted on European rye, rye flour or rye bread have reported that 89 to 100 % of the investigated samples (n=28-51) did contain ergot alkaloids (Lauber 2006; Storm et al. 2008; Crews et al. 2009).

Relatively high levels of ergot alkaloids were present in rye, i.e. on average 720 µg/kg was measured, with the highest level in a single sample being 6200 µg/kg. The levels were in line with those presented previously. For example, a mean concentration of 818 µg/kg in rye samples (n=30) collected from Germany has been reported (Lauber 2006). Furthermore, maximum values of 1063 and 3280 µg/kg of total ergot alkaloids have been found in German rye samples (Lauber 2006; Reinhold and Reinhardt 2011), while in mixed feed, a total amount of 4883 µg/kg has been measured (Ruhland and Tischler 2008). According to the present results, also barley can contain ergot alkaloids, and a mean concentration of 59 µg/kg was determined. Little is known about the occurrence of ergot alkaloids in cereals other than rye. A study conducted on infant foods indicated that these compounds may be found in wheat based biscuits and oat, barley and multi-grain cereals with the highest incidence (56 %) and highest average concentration (18 µg/kg) determined in barley products (Lombaert et al. 2003). In the present study, the wheat samples were not contaminated. However, other researchers have detected a median value of 29 µg/kg (with a maximum level as high as 1236 µg/kg) of ergot alkaloids in wheat intended for use as animal feed (Ruhland and Tischler 2008).

The levels of individual ergot alkaloids presented in the literature are lower than in this study (from n.d. to 2200 µg/kg in rye). For example, mean concentrations from 0.7 to 24.4 µg/kg of individual alkaloids in rye and rye flour were reported by Müller et al. (2009), who examined both the (*R*)- and (*S*) –epimers. In addition, levels ranging from 0 to 19 µg/kg and from 42.4 to 94.5 µg/kg (as a sum of the two ergot alkaloid epimers) have been reported (Storm et al. 2008; Reinhold and Reinhardt 2011). The ergot alkaloid patterns detected in both ergot sclerotia and in rye and rye products have been demonstrated to vary considerably (Young 1981a;1981b; Storm et al. 2008; Appelt and Ellner 2009; Crews et al. 2009). According to the studies reporting both (*R*)- and (*S*) –epimer concentrations, the physiologically active (*R*)-forms have been more prevalent, although the ratios of the two epimers can vary significantly (Crews et al. 2009; Müller et al. 2009). In

the samples examined in this study, the (*R*)-epimers of ergocornine and ergocristine were the major alkaloids followed by ergocryptine. Other studies have reported EAMINE and ECRIS together with ESINE as being predominant in rye and related products (Storm et al. 2008; Crews et al. 2009; Reinhold and Reinhardt 2011). EAMINE and ECRYPT were reported as the main alkaloids by Storm et al. (2008). Furthermore, ECRIS, EAMINE and ECORN were the major alkaloids in rye and triticale ergot sclerotia in the study of Appelt and Ellner (2009). According to these reports, EAMINE is common in rye whereas in the present study, EAMINE and EMETR were the least prevalent alkaloids. The variations of ergot alkaloid patterns between the studies may be due to different rye cultivars and agricultural practises used as well as different climate conditions prevailing. In addition, the prevalence of *Claviceps* species and variations in their ability to produce toxins could have affected.

The results indicate that rye frequently contains ergot alkaloids. Although ergot sclerotia should be separated from grains in the milling processes, smaller ergots and their fractions can end up in flour (Lauber 2006). Ergot alkaloids may also survive food processing to some extent although they are unstable if heated and thus, they can be present in the final cereal products (Crews et al. 2009; Müller et al. 2009). Currently, no legal maximum limits for ergot alkaloids have been established in the EU. A value of 1000 µg/kg has been presented as it corresponds to a content of 0.2 % ergot alkaloids (w/w) in ergot sclerotia at a contamination level of 0.05 % (MRL for ergot in intervention grain). However, lower values from 100 to 500 µg/kg of total ergot alkaloids have been proposed (Battilani et al. 2009). In addition, MRLs between 100 and 600 µg/kg have been discussed for individual ergot alkaloids (European Union 2010). Considering the values proposed, rye samples exceeding these concentration levels would be encountered in Finland even if the MRL were to be 1000 µg/kg. It should be noted that the samples were raw cereals that had not been processed, and therefore the content of ergot alkaloids might not represent completely that of the final products intended for human consumption. However, the results obtained do provide an estimate of the ergot alkaloid contamination levels in Finnish grains. Risk assessment and legislation for ergot alkaloids are of great importance due to their frequent occurrence. There is agreement that the content of ergot alkaloids cannot be estimated simply on the basis of the presence of sclerotia due to the high variation of patterns and concentrations of individual ergot alkaloids (Battilani et al. 2009). Therefore, more data is needed about the prevalence as well as on toxicological effects of all individual ergot alkaloids and their combinations in grains and related products (Appelt and Ellner 2009).

In summary, co-occurring, chemically different mycotoxins could be determined from food and agar samples with the LC-MS/MS methods developed. Roquefortine C and mycophenolic acid were detected in blue cheeses but not at levels considered as being harmful. In addition, the production of beauvericin and type-A trichothecenes

by *F. sporotrichioides* and *F. langsethiae* could be verified from cereal based agar and whole grain cultures. T-2 toxin was the main metabolite of these species with the average concentrations measured being as high as 22000 µg/kg. Furthermore, occurrence of several ergot alkaloids was demonstrated in Finnish barley and rye samples at average levels of 59 and 720 µg/kg.

CONCLUSIONS

Novel methods were successfully developed for simultaneous determination of several, chemically diverse mycotoxins from food and agar matrices. Liquid chromatography coupled to tandem mass spectrometry was proved to be a feasible technique for this type of targeted multi-residue analysis. HPLC-MS/MS based methods were devised for determining nine mycotoxins from cheese and 29 to 31 mycotoxins from cereal based agar matrices and grains. UHPLC-MS/MS was demonstrated to be an effective tool in the selective determination of ten ergot alkaloids from grains.

Straightforward analytical procedures ensured rapid determination of the mycotoxins of interest. The extraction of the analytes from the sample matrices was accomplished with liquid extraction after which only minimal sample clean-up was required before the extracts were injected to the LC-MS/MS. Reversed phase chromatography in C18 columns ensured the separation of the analytes in reasonable run-times by using gradient elution with ACN based mobile phases. The ionization of the mycotoxins was achieved by utilizing ESI interface, and reliable confirmation and quantification were performed by monitoring the product ions formed in the collision-induced dissociation of triple quadrupole-MS.

Several compromises were needed during the method development, as the extraction, chromatographic and mass spectrometric conditions could not be optimized completely for all the analytes. Additionally, the co-extracted matrix components led to profound signal suppression, or in some cases enhancement, in ESI. Therefore, the matrix effects were compensated by using matrix-assisted calibration. The validation showed that the performance of the methods was generally good for this type of multi-residue analysis, allowing reliable, repeatable and selective determination of the mycotoxins of interest at sufficiently low concentrations. Occasionally, one had to be satisfied with the high limits of quantification and reduced recovery and repeatability and therefore, it is recommended that either semi-quantitative or qualitative determination would be advisable for certain compounds. The efficiency of selective SPE sample clean-up in combination with the high separation and detection capacity of UHPLC-MS/MS was demonstrated in the ergot alkaloid analysis. Low LOQs and good recoveries and within-day repeatability were obtained.

The methods were suitable for applications where several mycotoxins needed to be detected simultaneously. In the mould cheese samples analysed, co-occurring roquefortine C and mycophenolic acid could be detected. The concentration levels were not at a harmful level considering the normal consumption of this type of cheese products. The method developed for cereal based agar matrices and grains

was applicable for the screening of toxin production of fungal strains. Chemically diverse metabolites, i.e. type-A trichothecenes (T-2, HT-2, DAS and NEO) and beauvericin, of *F. sporotrichioides* and *F. langsethiae* could be determined with a single method. The results provided new knowledge on the toxigenic potential of these important fungal species, which is especially valuable for the less extensively investigated *F. langsethiae*. The production of BEA by *F. langsethiae* has not been confirmed unequivocally, but was demonstrated in this study. Contamination levels and patterns of ergot alkaloids could be determined with the developed UHPLC-MS/MS method from grain samples. The high prevalence and variety of ergot alkaloids were demonstrated for the first time from Finnish rye and barley. This occurrence data will be valuable for future risk assessment purposes.

In the future, the LC-MS/MS methods devised in this study can be used in other food safety applications. The simple procedures allow the modification of the methods in order to include new analytes and sample matrices. Nonetheless, possibilities to improve the present methods should be investigated. For example, the use of internal standard calibration as well as feasible ways to purify samples to reduce matrix effects and to improve quantification could be examined. Additionally, wider use of UHPLC/MS in multi-mycotoxin analysis should be considered.

APPENDIX 1. Table 5. Mycotoxins analysed with the HPLC-MS/MS or UHPLC-MS/MS methods developed.

Analyte	Matrices where analysed			Molecular structure	Precursor ion	Precursor ion (m/z)	Primary product ion (m/z)	Secondary product ion (m/z)
	Cheese	Grains	Agar					
Fusarium toxins								
NEO		x		$C_{19}H_{26}O_8$	$[M+NH_4]^+$	400.2	185.1	305.2
DAS		x	x	$C_{19}H_{26}O_7$	$[M+NH_4]^+$	384.2	307.2	247.2
HT-2		x	x	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2	215.0	263.1
T-2		x	x	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2	185.1	305.2
NIV		x	x	$C_{15}H_{20}O_7$	$[M+CH_3COO]^-$	371.2	281.1	311.1
DON		x	x	$C_{15}H_{20}O_6$	$[M+CH_3COO]^-$	355.2	295.2	58.8
3-AcDON		x	x	$C_{17}H_{22}O_7$	$[M+CH_3COO]^-$	397.0	337.1	58.8
FX		x	x	$C_{17}H_{22}O_8$	$[M+CH_3COO]^-$	413.2	353.1	58.8
ZEN		x	x	$C_{18}H_{24}O_5$	$[M-H]^-$	317.2	131.0	175.1
FB1		x	x	$C_{34}H_{59}NO_{15}$	$[M+H]^+$	722.6	334.3	352.4
FB2		x	x	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706.6	336.4	318.4
MON		x		$C_4H_2O_3$	$[M-H]^-$	96.9	40.8	-
ENN A		x	x	$C_{36}H_{63}N_3O_9$	$[M+H]^+$	682.5	210.3	228.3
ENN A1		x	x	$C_{35}H_{61}N_3O_9$	$[M+H]^+$	668.5	210.2	228.2
ENN B		x	x	$C_{33}H_{57}N_3O_9$	$[M+H]^+$	640.5	196.1	214.1
ENN B1		x	x	$C_{34}H_{59}N_3O_9$	$[M+H]^+$	654.5	196.1	214.2
BEA		x	x	$C_{45}H_{57}N_3O_9$	$[M+H]^+$	784.5	244.0	262.4
ANT Y		x	x	$C_{15}H_{10}O_8$	$[M+H]^+$	319.1	287.0	175.0
Claviceps toxins								
EAMINE		x	x	$C_{33}H_{35}N_5O_5$	$[M+H]^+$	582.1	223.2/208.0[b]	564.1/223.1[b]
ECRIS		x	x	$C_{35}H_{39}N_5O_5$	$[M+H]^+$	610.4	223.2/208.0[b]	592.3/223.0[b]
ECRYPT		x	x	$C_{32}H_{41}N_5O_5$	$[M+H]^+$	576.3	268.2/208.0[b]	558.2/268.1[b]
ECORN		x	x	$C_{31}H_{39}N_5O_5$	$[M+H]^+$	562.3	305.2/208.0[b]	545.3/223.0[b]
EMETR		x	x	$C_{19}H_{23}N_3O_2$	$[M+H]^+$	326.2	223.2	208.2
ESINE		x[b]		$C_{30}H_{37}N_5O_5$	$[M+H]^+$	548.4	208.0	268.1
EAMININE		x[b]		$C_{33}H_{35}N_5O_5$	$[M+H]^+$	582.4	223.2	297.1
ECRIS-INE		x[b]		$C_{35}H_{39}N_5O_5$	$[M+H]^+$	610.4	305.1	223.1
ECRYPT-INE		x[b]		$C_{32}H_{41}N_5O_5$	$[M+H]^+$	576.4	223.1	305.1
ECORN-INE		x[b]		$C_{31}H_{39}N_5O_5$	$[M+H]^+$	562.4	277.1	223.1
Aspergillus toxins								
AF B1	x	x	x	$C_{17}H_{12}O_6$	$[M+H]^+$	313.1	285.2/286.0 [a]	241.2/270.0 [a]
AF B2	x	x	x	$C_{17}H_{14}O_6$	$[M+H]^+$	315.2	287.1/259.0 [a]	259.1/286.0 [a]
AF G1	x	x	x	$C_{17}H_{12}O_7$	$[M+H]^+$	329.1	243.1	311.1
AF G2	x	x	x	$C_{17}H_{14}O_7$	$[M+H]^+$	331.1	313.1/245.0 [a]	189.0/313.0 [a]
AF MI	x			$C_{17}H_{12}O_7$	$[M+H]^+$	329.0	273.0	301.0
OTA	x	x	x	$C_{20}H_{18}NO_6Cl$	$[M+H]^+$	404.0	239.0/358.0 [a]	358.1/239.0 [a]
GLIO		x	x	$C_{13}H_{14}O_4N_2S_2$	$[M+H]^+$	327.1	263.1	245.1
Penicillium toxins								
MPA	x	x	x	$C_{17}H_{20}O_6$	$[M+H]^+$	321.2	207.0	303.1/304.0[a]
PA	x	x	x	$C_8H_{10}O_4$	$[M-H]^-/[M+H]^+$[a]	169.0/171.0[a]	110.0/125.0[a]	92.9/97.0[a]
ROQ C	x			$C_{22}H_{23}N_5O_2$	$[M+H]^+$	390.0	193.0	322.0

a=in cheese, b=with UHPLC-MS/MS method

APPENDIX 2. Table 6. A summary of the validation results for the mycotoxins analysed with HPLC-MS/MS or UHPLC-MS/MS.

Mycotoxin	Cheese at 50 to 200 μg/kg (with multi-mycotoxin LC-MS/MS)			Grains at 50 to 500 μg/kg[c] (with multi-mycotoxin LC-MS/MS)			Agar 200 μg/kg (with multi-mycotoxin LC-MS/MS)			Grains at 10 to 200 μg/kg (with UHPLC-MS/MS)		
	Recovery (%)	RSD (%) within-day	LOQ (μg/kg)	Recovery (%)	RSD (%) within-day	LOQ (μg/kg)	Recovery (%)	RSD (%) within-day	LOQ (μg/kg)	Recovery (%)	RSD (%) within-day	LOQ (μg/kg)
AF B1	103-132	2.3-10.7	1.6	61-95	4-13	20-45	72-113	3.4-7.9	2.7	-	-	-
AF B2	110-143	3.5-9.6	1.6	63-99	4-17	20-50	94-110	2.8-6.9	2.7	-	-	-
AF G1	104-130	3.6-10.9	1.6	62-97	4-16	25-50	94-121	3.8-5.1	3.9	-	-	-
AF G2	101-129	3.1-12.1	1.6	61-90	5-18	45-65	84-121	4.2-11	5.3	-	-	-
AF MI	96-129	3.6-6.7	0.6	-	-	-	-	-	-	-	-	-
OTA	100-105	2.5-5.9	0.6	60-94	4-17	20-35	96-100	4.7-12.1	6.3	-	-	-
GLIO	-	-	-	58-79	7-13	115-275	90-104	4.7-8.8	38	-	-	-
MPA	96-135	3.1-6.5	0.6	55-98	3-14	30-50	85-89	6.2-12.2	17	-	-	-
PA	109	5.4-8.2	4.0	70-108	2-15	35-70	91	4.4-8.8	86	-	-	-
ROQ C	102-119	3.0-11	0.8	-	-	-	-	-	-	-	-	-
EAMINE	-	-	-	63-105	4-10	35-50	69-80	5.3-23	7.1	72-104	1.9-8.6	0.04-0.5
ECRIS	-	-	-	51-122	4-16	20-50	115-120	7.7-8.3	5.7	59-106	0.61-9.8	0.01-0.34
ECRYPT	-	-	-	57-140	6-12	20-40	125-144	15-25	5.7	65-105	1.8-12	0.03-0.04
ECORN	-	-	-	48-99	4-15	50-65	98-115	14-16.5	10	61-100	1.3-11	0.01-0.05
EMETR	-	-	-	54-77	3-8	40-100	19-81	20-25	7.1	85-120	2.2-14	1.2-10.2
ESINE	-	-	-	-	-	-	-	-	-	51-98	2.2-9.6	0.17-0.2
EAMININE	-	-	-	-	-	-	-	-	-	103-123	1.4-11	0.02-0.43
ECRIS-INE	-	-	-	-	-	-	-	-	-	100-133	1.8-11	0.06-0.8
ECRYPT-INE	-	-	-	-	-	-	-	-	-	93-139	1.3-11	0.04-0.1
ECORN-INE	-	-	-	-	-	-	-	-	-	105-129	1.7-12	0.02-0.08
NEO[a]	-	-	-	87[a]	16[a]	370-800	44-52	9.4-15	8.0	-	-	-
DAS	-	-	-	64-82	3-9	50-75	99-113	4.7-8.3	5.7	-	-	-
HT-2	-	-	-	74-85	4-9	280-500	117-125	4.5-7.9	80	-	-	-
T-2	-	-	-	77-103	3-10	35-40	63-104	4.5-15	4.8	-	-	-
NIV[b]	-	-	-	77[b]	5[b]	400-620	-	-	-	-	-	-
DON	-	-	-	67-108	2-18	65-85	44-51	53-62	63	-	-	-
3-AcDON	-	-	-	81-100	4-13	45-75	134-163	15-18	57	-	-	-
FX	-	-	-	87-107	4-8	70-125	55-76	38-41	57	-	-	-
ZEN	-	-	-	61-97	2-11	40-110	51-54	5.1-8.6	19	-	-	-
FB1	-	-	-	7-114	2-18	30-80	8-44	19-46	48	-	-	-
FB2	-	-	-	13-90	2-15	25-65	22-42	12-31	17	-	-	-
MON	-	-	-	68-116	5-9	400-1250	-	-	-	-	-	-
ENN A	-	-	-	53-107	5-24	1-9	98-116	8.4-16	0.1	-	-	-
ENN A1	-	-	-	57-119	5-20	2-10	113-146	13-15	0.3	-	-	-
ENN B	-	-	-	56-93	5-17	2-8	98-116	4.4-11	0.3	-	-	-
ENN B1	-	-	-	54-116	6-18	3-8	120-133	7.3-16	0.8	-	-	-
BEA	-	-	-	61-122	4-26	6-10	101-165	17-20	1.1	-	-	-
ANT Y	-	-	-	64-196	4-14	80-250	114-139	5.2-12	6.8	-	-	-

RSD=relative standard deviation, a=measured in wheat at 500 μg/kg, otherwise spiking levels < LOQ, b=measured in oats at 500 μg/kg, otherwise spiking levels < LOQ, c=see concentrations for ENNs from the original article (II)

8. REFERENCES

Ali,N., Sardjono, Yamashita,A., and Yoshizawa,T. 1998. Natural co-occurrence of aflatoxins and *Fusarium* mycotoxins (fumonisins, deoxynivalenol, nivalenol and zearalenone) in corn from Indonesia. Food Additives and Contaminants 15: 377-384.

Altenbach,B. and Giger,W. 1995. Determination of benzene- and naphthalenesulfonates in wastewater by solid-phase extraction with graphitized carbon black and ion-pair liquid chromatography with UV detection. Analytical Chemistry 67: 2325-2333.

Anastassiades,M., Lehotay,S.J., Stajnbaher,D., and Schenck,F.J. 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of AOAC International 86: 412-431.

Andreolini,F., Borra,C., Caccamo,F., Di Corcia,A., and Samperi,R. 1987. Estrogen conjugates in late-pregnancy fluids: Extraction and group separation by a graphitized carbon black cartridge and quantification by high-performance liquid chromatography. Analytical Chemistry 59: 1720-1725.

AOAC International 2006. Natural toxins. *In* Official methods of analysis of AOAC International. 18 ed. AOAC International, Gaithersburg. pp. 1-89.

Appelt,M. and Ellner,F.M. 2009. Investigations into the occurrence of alkaloids in ergot and single sclerotia from the 2007 and 2008 harvests. Mycotoxin Research 25: 95-101.

ApSimon,J.W. 1994. The biosynthetic diversity of secondary metabolites. *In* Mycotoxins in grains. Compounds other than aflatoxin. Eagan Press, St. Paul. pp. 3-18.

Aqai,P., Peters,J., Gerssen,A., Haasnoot,W., and Nielen,M.W.F. 2011. Immunomagnetic microbeads for screening with flow cytometry and identification with nano-liquid chromatography mass spectrometry of ochratoxins in wheat and cereal. Analytical and Bioanalytical Chemistry 400: 3085-3096.

Arnold,D.L., Scott,P.M., McGuire,P.F., Harwig,J., and Nera,E.A. 1978. Acute toxicity studies on roquefortine and pr toxin, metabolites of *Penicillium roqueforti*, in the mouse. Food and Cosmetics Toxicology 16: 369-371.

Asam,S., Konitzer,K., and Rychlik,M. 2010. Precise determination of the *Alternaria* mycotoxins alternariol and alternariol monomethyl ether in cereal, fruit and vegetable products using stable isotope dilution assays. Mycotoxin Research 27: 23-28.

Asam,S., Konitzer,K., Schieberle,P., and Rychlik,M. 2009. Stable isotope dilution assays of alternariol and alternariol monomethyl ether in beverages. Journal of Agricultural and Food Chemistry 57: 5152-5160.

Battilani,P., Costa,L.G., Dossena,A., Gullino,M.L., Marchelli,R., Galaverna,G., Pietri,A., Dall'Asta,C., Giorni,P., and Gualla,A. 2009. Ergot alkaloids in food. Scientific information on mycotoxins and natural plant toxicants, CFP/EFSA/CONTAM/2008/01: pp. 53-125.

Beltrán,E., Ibañez,M., Sancho,J.V., and Hernández,F. 2009. Determination of mycotoxins in different food commodities by ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. Rapid Commununications in Mass Spectrometry 23: 1801-1809.

Bentley,R. 2000. Mycophenolic acid: A one hundred year Odyssey from antibiotic to immunosuppressant. Chemical Reviews 100: 3801-3826.

Berde,B. and Stürmer,E. 1978. Introduction to the pharmacology of ergot alkaloids and related compounds. *In* Ergot alkaloids and related compounds. Springer Verlag, Berlin. pp. 1-28.

Berthiller,F., Schuhmacher,R., Adam,G., and Krska,R. 2009. Formation, determination and significance of masked and other conjugated mycotoxins. Analytical and Bioanalytical Chemistry 395: 1243-1252.

Berthiller,F., Schuhmacher,R., Buttinger,G., and Krska,R. 2005. Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1062: 209-216.

Bhat,R., Rai,R.V., and Karim,A.A. 2010. Mycotoxins in food and feed: present status and future concerns. Comprehensive Reviews in Food Science and Food Safety 9: 57-81.

Binder,E.M., Tan,L.M., Chin,L.J., Handl,J., and Richard,J. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Animal Feed Science and Technology 137: 265-282.

Bircan,C. 2009. Incidence of ochratoxin A in dried fruits and co-occurrence with aflatoxins in dried figs. Food and Chemical Toxicology 47: 1996-2001.

Biselli,S., Hartig,L., Wegner,H., and Hummert,C. 2004. Analysis of *Fusarium* toxins using LC/MS-MS: Application to various food and feed matrices. LC-GC Europe 25-31: 1-7.

Blesa,J., Berrada,H., Soriano,J.M., Moltó,J.C., and Mañes,J. 2004. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. Journal of Chromatography A 1046: 127-131.

Blesa,J., Soriano,J.M., Moltó,J.C., and Mañes,J. 2006. Factors affecting the presence of ochratoxin A in wines. Critical Reviews in Food Science and Nutrition 46: 473-478.

Bogialli,S. and Di Corcia,A. 2009. Recent applications of liquid chromatography-mass spectrometry to residue analysis of antimicrobials in food of animal origin. Analytical and Bioanalytical Chemistry 395: 947-966.

Boonzaaijer,G., van Osenbruggen,W., Kleinnijenhuis,A., and van Dongen,W. 2008. An exploratory investigation of several mycotoxins and their natural occurrence in flavour ingredients and spices, using a multi-mycotoxin LC-MS/MS method. World Mycotoxin Journal 1: 167-174.

Borchers,A., Teuber,S.S., Keen,C.L., and Gershwin,M.E. 2010. Food safety. Clinical Reviews in Allergy and Immunology 39: 95-141.

Boudra,H., Barnouin,J., Dragacci,S., and Morgavi,D.P. 2007. Aflatoxin M1 and ochratoxin A in raw bulk milk from french dairy herds. Journal of Dairy Science 90: 3197-3201.

Boyd,R.K., Basic,C., and Bethem,R.A. 2008. Trace Quantitative Analysis by Mass Spectrometry. John Wiley & Sons Ltd., Chichester.

Bullerman,L.B. 1980. Incidence of mycotoxic molds in domestic and imported cheeses. Journal of Food Safety 2: 47-58.

Bullerman,L.B. 1981. Public health significance of molds and mycotoxins in fermented dairy products. Journal of Dairy Science 64: 2439-2452.

Bürk,G., Höbel,W., and Richt,A. 2006. Ergot alkaloids in cereal products. Results from the Bavarian Health and Food Safety Authority. Molecular Nutrition & Food Research 50: 437-442.

Capriotti,A.L., Foglia,P., Gubbiotti,R., Roccia,C., Samperi,R., and Laganà,A. 2010. Development and validation of a liquid chromatography/atmospheric pressure photoionization-tandem mass spectrometric method for the analysis of mycotoxins subjected to commission regulation (EC) No. 1881/2006 in cereals. Journal of Chromatography A 1217: 6044-6051.

Carabias-Martínez,R., Rodríguez-Gonzalo,E., Revilla-Ruiz,P., and Hernández-Méndez,J. 2005. Pressurized liquid extraction in the analysis of food and biological samples. Journal of Chromatography A 1089: 1-17.

Carlile,M.J., Watkinson,S.C., and Gooday,G.W. 2001. The Fungi. Second edition. Academic Press, London.

Cavaliere,C., Foglia,P., Pastorini,E., Samperi,R., and Laganà,A. 2005. Development of a multiresidue method for analysis of major *Fusarium* mycotoxins in corn meal using liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 19: 2085-2093.

Cavaliere,C., Foglia,P., Guarino,C., Motto,M., Nazzari,M., Samperi,R., Lagana,A., and Berardo,N. 2007. Mycotoxins produced by *Fusarium* genus in maize: determination by screening and confirmatory methods based on liquid chromatography tandem mass spectrometry. Food Chemistry 105: 700-710.

Cervino,C., Asam,S., Knopp,D., Rychlik,M., and Niessner,R. 2008. Use of isotope-labeled aflatoxins for LC-MS/MS stable isotope dilution analysis of foods. Journal of Agricultural and Food Chemistry 56: 1873-1879.

Chan,D., Macdonald,S.J., Boughtflower,V., and Brereton,P. 2004. Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column clean-up and liquid chromatography-fluorescence detection. Journal of Chromatography A 1059: 13-16.

Crews,C., Anderson,W.A.C., Rees,G., and Krska,R. 2009. Ergot alkaloids in some rye-based UK cereal products. Food Additives and Contaminants - Part B 2: 79-85.

D'Mello,J.P.F., Placinta,C.M., and Macdonald,A.M.C. 1999. *Fusarium* mycotoxins: A review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology 80: 183-205.

Desjardins,A.E. and Proctor,R.H. 2007. Molecular biology of *Fusarium* mycotoxins. International Journal of Food Microbiology 119: 47-50.

Desmarchelier,A., Oberson,J.M., Tella,P., Gremaud,E., Seefelder,W., and Mother,P. 2010. Development and comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. Journal of Agricultural and Food Chemistry 58: 7510-7519.

di Mavungu,J.D., Monbaliu,S., Scippo,M.L., Maghuin-Rogister,G., Schneider,Y.J., Larondelle,Y., Callebaut,A., Robbens,J., van Peteghem,C., and de Saeger,S. 2009. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Additives and Contaminants - Part A 26: 885-895.

Doohan,F.M., Brennan,J., and Cooke,B.M. 2003. Influence of climatic factors on *Fusarium* species pathogenic to cereals. European Journal of Plant Pathology 109: 755-768.

Driehuis,F., Spanjer,M.C., Scholten,J.M., and te Giffel,M.C. 2008. Occurrence of mycotoxins in feedstuffs of dairy cows and estimation of total dietary intakes. Journal of Dairy Science 91: 4261-4271.

Driffield,M., Hird,S.J., and Macdonald,S.J. 2003. The occurrence of a range of mycotoxins in animal offal food products by HPLC-MS/MS. Aspects of Applied Biology 68: 205-210.

Edwards,S.G. 2004. Influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicology Letters 153: 29-35.

Edwards,S.G. 2009. *Fusarium* mycotoxin content of UK organic and conventional oats. Food Additives and Contaminants - Part A 26: 1063-1069.

Edwards,S., Barrier-Guillot,B., Clasen,P.E., Hietaniemi,V., and Pettersson,H. 2009. Emerging issues of HT-2 and T-2 toxins in European cereal production. World Mycotoxin Journal 2: 173-179.

EFSA 2001. Opinion of the Scientific Committee on Food on *Fusarium* toxins. Part 5: T-2 and HT-2. SCF/CS/CNTM/MYC/25 Rev 6 Final.

EFSA 2004a. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Deoxynivalenol (DON) as undesirable substance in animal feed. Question N° EFSA-Q-2003-036.

EFSA 2004b. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] related to Aflatoxin B1 as undesirable substance in animal feed.

EFSA 2005. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] related to fumonisins as undesirable substances in animal feed.

Engel,G. and Teuber,M. 1989. Toxic metabolites from fungal cheese starter cultures (*Penicillium camemberti* and *Penicillium roqueforti*). *In* Mycotoxins in dairy products. Elsevier Applied Science, London. pp. 163-192.

Er,B., Demirhan,B., Onurdag,F.K., and Yentur,G. 2010. Determination of aflatoxin M1 level in milk and white cheese consumed in Ankara region, Turkey. Journal of Animal and Veterinary Advances 9: 1780-1784.

Escher,F.E., Nuessli,J., and Conde-Petit,B. 2000. Interactions of flavor compounds with starch in food processing. ACS Symposium Series 763: 230-245.

Eskola,M., Rizzo,A., and Soupas,L. 2000. Occurrence and amounts of some *Fusarium* toxins in Finnish cereal samples in 1998. Acta Agriculturae Scandinavica, Section B - Plant Soil Science 50: 183-186.

Eskola,M., Parikka,P., and Rizzo,A. 2001. Trichothecenes, ochratoxin A and zearalenone contamination and *Fusarium* infection in Finnish cereal samples in 1998. Food Additives and Contaminants 18: 707-718.

Eurachem 1998. The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. http://www.eurachem.org/guides/pdf/valid.pdf. Last accessed in September, 2011.

European Union 2002a. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities L221: 8-36.

European Union 2002b. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Official Journal of the European Communities L140: 10-22.

European Union 2006a. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). Official Journal of the European Communities L229: 7-9.

European Union 2006b. Commission Regulation (EC) No 1572/2006 of 18 October 2006 amending Regulation (EC) No 824/2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals. Official Journal of the European Communities L290: 29-34.

European Union 2006c. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Communities L364: 5-24.

European Union 2006d. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union L70: 12-34.

European Union 2007. Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. Official Journal of the European Communities L255: 14-17.

European Union 2010. SANCO/10226/2010. Draft. Commission Recommendation on the monitoring of the presence of ergot alkaloids in feed and food.

Finoli,C., Vecchio,A., Galli,A., and Dragoni,I. 2001. Roquefortine C occurrence in blue cheese. Journal of Food Protection 64: 246-251.

Floss,H. and Anderson,J. 1980. Biosynthesis of ergot toxins. *In* The biosynthesis of mycotoxins. Academic Press, Orlando. pp. 17-67.

Frisvad,J.C., Smedsgaard,J., Larsen,T.O., and Samson,R.A. 2004. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Studies in Mycology 49: 201-241.

García-Reyes,J.F., Hernando,M.D., Ferrer,C., Molina-Díaz,A., and Fernández-Alba,A.R. 2007. Large scale pesticide multiresidue methods in food combining liquid chromatography–time-of-flight mass spectrometry and tandem mass spectrometry. Analytical Chemistry 79: 7308-7323.

Gareis,M., Zimmermann,C., Schotthorst,R., Paulsch,W., Vidnes,A., Bergsten,C., Paulsen,B., Brera,C., Miraglia,M., Grossi,S., and Debegnach,F. 2003. Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary exposure of the population of EU Member States. Reports on tasks for scientific cooperation. Task 3.2.10.

Garrido Frenich,A., Vidal Martínez,J.L., Romero-González,R., and Aguilera-Luiz,M. 2009. Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography/tandem mass spectrometry. Food Chemistry 117: 705-712.

Garrido Frenich,A., Romero-González,R., Gómez-Pérez,M.L., and Vidal Martínez,J.L. 2011. Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. Journal of Chromatography A 1218:4349-4356.

Gazzotti,T., Lugoboni,B., Zironi,E., Barbarossa,A., Serraino,A., and Pagliuca,G. 2009. Determination of fumonisin B1 in bovine milk by LC-MS/MS. Food Control 20: 1171-1174.

Ghali,R., Hmaissia-Khlifa,K., Ghorbel,H., Maaroufi,K., and Hedili,A. 2008. Incidence of aflatoxins, ochratoxin A and zearalenone in Tunisian foods. Food Control 19: 921-924.

Göbel,R. and Lusky,K. 2004. Simultaneous determination of aflatoxins, ochratoxin A, and zearalenone in grains by new immunoaffinity column/liquid chromatography. Journal of AOAC International 87: 411-416.

Grimes,J.L., Koci,M.D., Stark,C.R., Smith,D.P., Nighot,P.K., and Middleton,T. 2010. Biological effect of naturally occurring mycotoxins fed to poults reared to 21 days of age. International Journal of Poultry Science 9: 871-874.

Hafner,M., Sulyok,M., Schuhmacher,R., Crews,C., and Krska,R. 2008. Stability and epimerisation behaviour of ergot alkaloids in various solvents. World Mycotoxin Journal 1: 67-78.

Hampikyan,H., Bingol,E.B., Cetin,O., and Colak,H. 2010. Determination of aflatoxin M1 levels in Turkish white, kashar and tulum cheeses. Journal of Food, Agriculture and Environment 8: 13-15.

Herebian,D., Zühlke,S., Lamshöft,M., and Spiteller,M. 2009. Multi-mycotoxin analysis in complex biological matrices using LC-ESI/MS: Experimental study using triple stage quadrupole and LTQ-Orbitrap. Journal of Separation Science 32: 939-948.

Heussner,A.H., Dietrich,D.R., and O'Brien,E. 2006. *In vitro* investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. Toxicology in Vitro 20: 332-341.

Huffman,J., Gerber,R., and Du,L. 2010. Recent advancements in the biosynthetic mechanisms for polyketide-derived mycotoxins. Biopolymers 93: 764-776.

Hussein,H.S. and Brasel,J.M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167: 101-134.

IARC 1993. IARC Monographs on the evaluation of carcinogenic risks to humans. Volume 56. Some naturally occurring substances: food items and constitutents, heterocyclic aromatic amines and mycotoxins. IARC Press, Lyon.

IARC 2002. IARC Monographs on the evaluation of carcinogenic risks to humans. Volume 82. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Press, Lyon.

Ibañez-Vea,M., Corcuera,L.A., Remiro,R., Murillo-Arbizu,M.T., González-Perés,E., and Lizarraga,E. 2011. Validation of a UHPLC-FLD method for the simultaneous quantification of aflatoxins, ochratoxin A and zearalenone in barley. Food Chemistry 127: 351-358.

International Organization for Standardization. 1994. ISO 5725-2:1994. Accuracy (trueness and precision) of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

International Organization for Standardization. 2005. ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories.

International Organization for Standardization. 2007. ISO 14501:2007. Milk and milk powder -- Determination of aflatoxin M1 content -- Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography.

Ito,R., Yamazaki,H., Inoue,K., Yoshimura,Y., Kawaguchi,M., and Nakazawa,H. 2004. Development of liquid chromatography-electrospray mass spectrometry for the determination of patulin in apple juice: Investigation of its contamination in Japan. Journal of Agricultural and Food Chemistry 52: 7464-7468.

Jestoi,M. 2008. Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin - A review. Critical Reviews in Food Science and Nutrition 48: 21-49.

Jestoi,M., Rokka,M., Rizzo,A., and Peltonen,K. 2003. Moniliformin in Finnish grains: analysis with LC-MS/MS. Aspects of Applied Biology 68: 211-216.

Jestoi,M., Rokka,M., Yli-Mattila,T., Parikka,P., Rizzo,A., and Peltonen,K. 2004a. Presence and concentrations of the *Fusarium*-related mycotoxins beauvericin, enniatins and moniliformin in Finnish grain samples. Food Additives and Contaminants 21: 794-802.

Jestoi,M., Somma,M.C., Kouva,M., Veijalainen,P., Rizzo,A., Ritieni,A., and Peltonen,K. 2004b. Levels of mycotoxins and sample cytotoxicity of selected organic and conventional grain-based products purchased from Finnish and Italian markets. Molecular Nutrition & Food Research 48: 299-307.

Jestoi,M., Ritieni,A., and Rizzo,A. 2004c. Analysis of the *Fusarium* mycotoxins fusaproliferin and trichothecenes in grains using gas chromatography–mass spectrometry. Journal of Agricultural and Food Chemistry 52: 1464-1469.

Jestoi,M., Rokka,M., Rizzo,A., Peltonen,K., and Aurasaari,S. 2005. Determination of *Fusarium*-mycotoxins beauvericin and enniatins with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Journal of Liquid Chromatography & Related Technologies 28: 369-381.

Jestoi,M.N., Paavanen-Huhtala,S., Parikka,P., and Yli-Mattila,T. 2008. *In vitro* and *in vivo* mycotoxin production of *Fusarium* species isolated from Finnish grains. Archives of Phytopathology and Plant Protection 41: 545-558.

Jestoi,M., Rokka,M., Järvenpää,E., and Peltonen,K. 2009. Determination of *Fusarium* mycotoxins beauvericin and enniatins (A, A1, B, B1) in eggs of laying hens using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Food Chemistry 115: 1120-1127.

Jiao,F., Kawakami,A., and Nakajima,T. 2008. Effects of different carbon sources on trichothecene production and Tri gene expression by *Fusarium graminearum* in liquid culture. FEMS Microbiology Letters 285: 212-219.

Jin,P.G., Han,Z., Cai,Z.X., Wu,Y.J., and Ren,Y.P. 2010. Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography-tandem mass spectrometry using $^{13}C_{15}$ Deoxynivalenol as internal standard. Food Additives and Contaminants - Part A 27: 1701-1713.

Kanisawa,M. 1984. Synergistic effect of citrinin on hepatorenal carcinogenesis of OA in mice. *In* Toxicogenic fungi: their toxins and health hazard. Elsevier, Tokyo. pp. 245-254.

Kimura,M., Tokai,T., Takahashi-Ando,N., Ohsato,S., and Fujimura,M. 2007. Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: Pathways, genes, and evolution. Bioscience, Biotechnology and Biochemistry 71: 2105-2123.

Klötzel,M., Gutsche,B., Lauber,U., and Humpf,H.U. 2005. Determination of 12 type A and B trichothecenes in cereals by liquid chromatography-electrospray ionization tandem mass spectrometry. Journal of Agricultural and Food Chemistry 53: 8904-8910.

Klötzel,M., Lauber,U., and Humpf,H.U. 2006. A new solid phase extraction clean-up method for the determination of 12 type A and B trichothecenes in cereals and cereal-based food by LC-MS/MS. Molecular Nutrition & Food Research 50: 261-269.

Kmellár,B., Fodor,P., Pareja,L., Ferrer,C., Martínez-Uroz,M.A., Valverde,A., and Fernandez-Alba,A.R. 2008. Validation and uncertainty study of a comprehensive list of 160 pesticide residues in multi-class vegetables by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1215: 37-50.

Komarova,E.L. and Tolkachev,O.N. 2001. The chemistry of peptide ergot alkaloids. Part 1. Classification and chemistry of ergot peptides. Pharmaceutical Chemistry Journal 35: 504-513.

Kopp,B. and Rehm,H.J. 1979. Antimicrobial action of roquefortine. Applied Microbiology and Biotechnology 6: 397-401.

Köppen,R., Koch,M., Siegel,D., Merkel,S., Maul,R., and Nehls,I. 2010. Determination of mycotoxins in foods: Current state of analytical methods and limitations. Applied Microbiology and Biotechnology 86: 1595-1612.

Kovalczuk,T., Jech,M., Poustka,J., and Hajslova,J. 2006. Ultra-performance liquid chromatography-tandem mass spectrometry: A novel challenge in multiresidue pesticide analysis in food. Analytica Chimica Acta 577: 8-17.

Krska,R. and Crews,C. 2008. Significance, chemistry and determination of ergot alkaloids: A review. Food Additives and Contaminants - Part A 25: 722-731.

Krska,R., Berthiller,F., Schuhmacher,R., Nielsen,K.F., and Crews,C. 2008a. Determination of ergot alkaloids: purity and stability assessment of standards and optimization of extraction conditions for cereal samples. Journal of AOAC International 91: 1363-1371.

Krska,R., Stubbings,G., Macarthur,R., and Crews,C. 2008b. Simultaneous determination of six major ergot alkaloids and their epimers in cereals and foodstuffs by LC-MS-MS. Analytical and Bioanalytical Chemistry 391: 563-576.

Lafont,P., Debeaupuis,J.P., Gaillardin,M., and Payen,J. 1979. Production of mycophenolic acid by *Penicillium roqueforti* strains. Applied and Environmental Microbiology 37: 365-368.

Lafont,P., Siriwardana,M.G., and DeBoer,E. 1990. Contamination of dairy products by fungal metabolites. Journal of Environmental Pathology, Toxicology and Oncology 10: 99-102.

Langseth,W., Bernhoft,A., Rundberget,T., Kosiak,B., and Gareis,M. 1998. Mycotoxin production and cytotoxicity of *Fusarium* strains isolated from Norwegian cereals. Mycopathologia 144: 103-113.

Langseth,W., Ghebremeskel,M., Kosiak,B., Kolsaker,P., and Miller,D. 2001. Production of culmorin compounds and other secondary metabolites by *Fusarium culmorum* and *F. graminearum* strains isolated from Norwegian cereals. Mycopathologia 152: 23-34.

Lattanzio,V.M.T., Solfrizzo,M., Powers,S., and Visconti,A. 2007. Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. Rapid Communications in Mass Spectrometry 21: 3253-3261.

Lauber,U. 2006. Ergot alkaloids in foods: V. Analysis of ergot alkaloids. Journal für Verbraucherschutz und Lebensmittelsicherheit 1: 160-162.

Le Bars,J. 1990. Detection and occurrence of cyclopiazonic acid in cheeses. Journal of Environmental Pathology, Toxicology and Oncology 10: 136-137.

Lehner,A.F., Craig,M., Fannin,N., Bush,L., and Tobin,T. 2005. Electrospray[+] tandem quadrupole mass spectrometry in the elucidation of ergot alkaloids chromatographed by HPLC: screening of grass or forage samples for novel toxic compounds. Journal of Mass Spectrometry 40: 1484-1502.

Lehotay,S.J., De Kok,A., Hiemstra,M., and Van Bodegraven,P. 2005. Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection. Journal of AOAC International 88: 595-614.

Lehotay,S.J., Son,K.A., Kwon,H., Koesukwiwat,U., Fu,W., Mastovska,K., Hoh,E., and Leepipatpiboon,N. 2010. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. Journal of Chromatography A 1217: 2548-2560.

Lieu,F.Y. and Bullerman,L.B. 1977. Production and stability of aflatoxins, penicillic acid and patulin in several substrates. Journal of Food Science 42: 1222-1224.

Logrieco,A., Moretti,A., Ritieni,A., Chelkowski,J., Altomare,C., Bottalico,A., and Randazzo,G. 1993. Natural occurrence of beauvericin in preharvest *Fusarium subglutinans* infected corn ears in Poland. Journal of Agricultural and Food Chemistry 41: 2149-2152.

Logrieco,A., Moretti,A., and Solfrizzo,M. 2009. *Alternaria* toxins and plant diseases: An overview of origin, occurrence and risks. World Mycotoxin Journal 2: 129-140.

Lombaert,G.A., Pellaers,P., Roscoe,V., Mankotia,M., Neil,R., and Scott,P.M. 2003. Mycotoxins in infant cereal foods from the Canadian retail market. Food Additives and Contaminants 20: 494–504.

Magan,N. and Aldred,D. 2007. Post-harvest control strategies: Minimizing mycotoxins in the food chain. International Journal of Food Microbiology 119: 131-139.

Malik,A.K., Blasco,C., and Picó,Y. 2010. Liquid chromatography-mass spectrometry in food safety. Journal of Chromatography A 1217: 4018-4040.

Marasas,W.F., Yagen,B., Sydenham,E., Combrinck,S., and Thiel,P.G. 1987. Comparative yields of T-2 toxin and related trichothecenes from five toxicologically important strains of *Fusarium sporotrichioides*. Applied and Environmental Microbiology 53: 693-696.

Martos,P.A., Thompson,W., and Diaz,G.J. 2010. Multiresidue mycotoxin analysis in wheat, barley, oats, rye and maize grain by high-performance liquid chromatography-tandem mass spectrometry. World Mycotoxin Journal 3: 205-223.

Mateo,J.J., Mateo,R., and Jimenez,M. 2002. Accumulation of type A trichothecenes in maize, wheat and rice by *Fusarium sporotrichioides* isolates under diverse culture conditions. International Journal of Food Microbiology 72: 115-123.

Medina,A. and Magan,N. 2011. Temperature and water activity effects on production of T-2 and HT-2 by *Fusarium langsethiae* strains from north European countries. Food Microbiology 28: 392-398.

Mezcua,M., Malato,O., García-Reyes,J.F., Molina-Díaz,A., and Fernández-Alba,A.R. 2009. Accurate-mass databases for comprehensive screening of pesticide residues in food by fast liquid chromatography time-of-flight mass spectrometry. Analytical Chemistry 81: 913-929.

Milicevic,D.R., Juric,V.B., Vukovic,D.Z., Mandic,M.M., and Baltic,T.M. 2009. Residue of ochratoxin A in swine tissues - Risk assessment. Archive of Oncology 17: 56-60.

Miraglia,M., Brera,C., Pazzaglini,B., and Grossi,S. 2002. Assessment of dietary intake of Ochratoxin A by the population of EU Member States. Reports on tasks for scientific cooperation. SCOOP Task 3.2.7.

Mohamed,R., Gremaud,E., Tabet,J.C., and Guy,P.A. 2006. Mass spectral characterization of ergot alkaloids by electrospray ionization, hydrogen/deuterium exchange, and multiple stage mass spectrometry: Usefulness of precursor ion scan experiments. Rapid Communications in Mass Spectrometry 20: 2787-2799.

Mol,H.G.J., Plaza-Bolaños,P., Zomer,P., De Rijk,T.C., Stolker,A.A.M., and Mulder,P.P.J. 2008. Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes. Analytical Chemistry 80: 9450-9459.

Monbaliu,S., Van Poucke,C., van Peteghem,C., Van Poucke,K., Heungens,K., and de Saeger,S. 2009. Development of a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for sweet pepper analysis. Rapid Communications in Mass Spectrometry 23: 3-11.

Monbaliu,S., Van Poucke,C., Detavernier,C., Dumoulin,F., Van De Velde,M., Schoeters, E., Van Dyck,S., Averkieva,O., van Peteghem,C., and de Saeger,S. 2010a. Occurrence of mycotoxins in feed as analyzed by a multimycotoxin LC-MS/MS method. Journal of Agricultural and Food Chemistry 58: 66–71.

Monbaliu,S., Wu,A., Zhang,D., Van Peteghem,C., and De Saeger,S. 2010b. Multimycotoxin UPLC-MS/MS for tea, herbal infusions and the derived drinkable products. Journal of Agricultural and Food Chemistry 58: 12664-12671.

Montagna,M.T., Napoli,C., De Giglio,O., Iatta,R., and Barbuti,G. 2008. Occurrence of aflatoxin M1 in dairy products in Southern Italy. International Journal of Molecular Sciences 9: 2614-2621.

Morrison,E., Kosiak,B., Ritieni,A., Aastveit,A.H., Uhlig,S., and Bernhoft,A. 2002. Mycotoxin production by *Fusarium avenaceum* strains isolated from Norwegian grain and the cytotoxicity of rice culture extracts to porcine kidney epithelial cells. Journal of Agricultural and Food Chemistry 50: 3070-3075.

Månsson,M., Phipps,R.K, Gram,L., Munro, H.G.M., Larsen,T.O. and Nielsen,K.F. 2010. Explorative solid-phase extraction (E-SPE) for accelerated microbial natural product discovery, dereplication, and purification. Journal of Natural Products 73: 1126–1132.

Müller,C., Kemmlein,S., Klaffke,H., Krauthause,W., Preiß-Weigert,A., and Wittkowski,R. 2009. A basic tool for risk assessment: A new method for the analysis of ergot alkaloids in rye and selected rye products. Molecular Nutrition & Food Research 53: 500-507.

Munkvold,G., Stahr,H.M., Logrieco,A., Moretti,A., and Ritieni,A. 1998. Occurrence of fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. Applied and Environmental Microbiology 64: 3923-3926.

Mwanza,M., Kametler,L., Bonai,A., Rajli,V., Kovacs,M., and Dutton,M.F. 2009. The cytotoxic effect of fumonisin B1 and ochratoxin A on human and pig lymphocytes using the Methyl Thiazol Tetrazolium (MTT) assay. Mycotoxin Research 25: 233-238.

Njobeh,P., Dutton,M., Koch,S., Chuturgoon,A., Stoev,S., and Mosonik,J. 2010. Simultaneous occurrence of mycotoxins in human food commodities from Cameroon. Mycotoxin Research 26: 47-57.

Ortelli,D., Cognard,E., Jan,P., and Edder,P. 2009. Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography B 877: 2363-2374.

Parikka,P., Rämö,S., and Hietaniemi,V. 2008. *Fusarium* infection and mycotoxins in Finnish cereals in 2005-2006. Journal of Plant Pathology 90: 56.

Peltonen,K., Jestoi,M., and Eriksen,G.S. 2010. Health effects of moniliformin: A poorly understood *Fusarium* mycotoxin. World Mycotoxin Journal 3: 403-414.

Peters,R.J.B., Bolck,Y.J.C., Rutgers,P., Stolker,A.A.M., and Nielen,M.W.F. 2009. Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry. Journal of Chromatography A 1216: 8206-8216.

Pfohl-Leszkowicz,A. and Manderville,R.A. 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Molecular Nutrition & Food Research 51: 61-99.

Pico,Y., la Farre,M., Soler,C., and Barcelo,D. 2007. Identification of unknown pesticides in fruits using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry imazalil as a case study of quantification. Journal of Chromatography A 1176: 123-134.

Pico,Y., la Farre,M., Tokman,N., and Barcelo,D. 2008. Rapid and sensitive ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry for the quantification of amitraz and identification of its degradation products in fruits. Journal of Chromatography A 1203: 36-46.

Placinta,C.M., D'Mello,J.P.F., and Macdonald,A.M.C. 1999. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Animal Feed Science and Technology 78: 21-37.

Plattner,R.D. 1999. HPLC/MS analysis of *Fusarium* mycotoxins fumonisins and deoxynivalenol. Natural Toxins 7: 365-370.

Prelusky,D.B., Rotter,B.A., and Rotter,R.G. 1993. Toxicology of mycotoxins. *In* Mycotoxins in grains, compounds other than aflatoxin. Eagan Press, St. Paul, Minnesota. pp. 359-404.

Rasmussen,R.R., Storm,I.M.L.D., Rasmussen,P.H., Smedsgaard,J., and Nielsen,K.F. 2010. Multi-mycotoxin analysis of maize silage by LC-MS/MS. Analytical and Bioanalytical Chemistry 397: 765-776.

Razzazi-Fazeli,E., Rabus,B., Cecon,B., and Bohm,J. 2002. Simultaneous quantification of A-trichothecene mycotoxins in grains using liquid chromatography atmospheric pressure chemical ionisation mass spectrometry. Journal of Chromatography A 968: 129-142.

Reinhold,L. and Reinhardt,K. 2011 Mycotoxins in foods in Lower Saxony (Germany): results of official control analyses performed in 2009. Mycotoxin Research 27:137-143.

Ren,Y.P., Zhang,Y., Shao,S.L., Cai,Z.X., Feng,L., Pan,H.F., and Wang,Z.G. 2007. Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. Journal of Chromatography A 1143: 48-64.

Richard,E., Heutte,N., Sage,L., Pottier,D., Bouchart,V., Lebailly,P., and Garon,D. 2007. Toxigenic fungi and mycotoxins in mature corn silage. Food Chemistry Toxicology 45: 2420-2425.

Romero-González,R., Vidal,J.L.M., Aguilera-Luiz,M.M., and Frenich,A.G. 2009. Application of conventional solid-phase extraction for multimycotoxin analysis in beers by ultrahigh-performance liquid chromatography-tandem mass spectrometry. Journal of Agricultural and Food Chemistry 57: 9385-9392.

Romero-González,R., Garrido Frenich,A., Martínez Vidal,J.L., Prestes,O.D., and Grio,S.L. 2011. Simultaneous determination of pesticides, biopesticides and mycotoxins in organic products applying a quick, easy, cheap, effective, rugged and safe extraction procedure and ultra-high performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1218: 1477-1485.

Ruhland,M. and Tischler,J. 2008. Determination of ergot alkaloids in feed by HPLC. Mycotoxin Research 24: 73-79.

Rundberget,T. and Wilkins,A.L. 2002. Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography-mass spectrometry. Journal of Chromatography A 964: 189-197.

Rychlik,M. and Asam,S. 2008. Stable isotope dilution assays in mycotoxin analysis. Analytical and Bioanalytical Chemistry 390: 617-628.

Sanchis,V. and Magan,N. 2004. Environmental profiles for growth and mycotoxin production. *In* Mycotoxins in food: detection and control. Woodhead Publishing Ltd., Cambridge.

Sangare-Tigori,B., Moukha,S., Kouadio,H.J., Betbeder,A.M., Dano,D.S., and Creppy,E.E. 2006. Co-occurrence of aflatoxin B1, fumonisin B1, ochratoxin A and zearalenone in cereals and peanuts from Côte d'Ivoire. Food Additives and Contaminants 23: 1000-1007.

Schneweis,I., Meyer,K., Engelhardt,G., and Bauer,J. 2002. Occurrence of zearalenone-4-ß-D-glucopyranoside in wheat. Journal of Agricultural and Food Chemistry 50: 1736-1738.

Schoch,U., Luthy,J., and Schlatter,C. 1984. Mycotoxins in *P. roqueforti* and *P. camemberti* in cheese. Milchwissenschaft 39: 76-80.

Schollenberger,M., Müller,H.M., Rüfle,M., Suchy,S., Planck,S., and Drochner,W. 2005. Survey of *Fusarium* toxins in foodstuffs of plant origin marketed in Germany. International Journal of Food Microbiology 97: 317-326.

Schollenberger,M., Müller,H.M., Rüfle,M., Suchy,S., Plank,S., and Drochner,W. 2006. Natural occurrence of 16 *Fusarium* toxins in grains and feedstuffs of plant origin from Germany. Mycopathologia 161: 43-52.

Schollenberger,M., Müller,H.M., Rüfle,M., Terry-Jara,H., Suchy,S., Plank,S., and Drochner,W. 2007. Natural occurrence of *Fusarium* toxins in soy food marketed in Germany. International Journal of Food Microbiology 113: 142-146.

Scott,P.M. and Kanhere,S.R. 1979. Instability of PR toxin in blue cheese. Journal of Association of Official Analytical Chemists 62: 141-147.

Scott,P.M. and Lawrence,G.A. 1980. Analysis of ergot alkaloids in flour. Journal of Agricultural and Food Chemistry 28: 1258-1261.

Scudamore,K.A. and Patel,S. 2009. Occurrence of *Fusarium* mycotoxins in maize imported into the UK, 2004-2007. Food Additives and Contaminants - Part A 26: 363-371.

Senyuva,H.Z., Gilbert,J. and Öztürkoğlu,S. 2008. Rapid analysis of fungal cultures and dried figs for secondary metabolites by LC/TOF-MS. Analytica Chimica Acta 617:97-106.

Senyuva,H.Z. and Gilbert,J. 2010. Immunoaffinity column clean-up techniques in food analysis: A review. Journal of Chromatography B 878: 115-132.

Sewram,V., Mshicileli,N., Shephard,G.S., Vismer,H.F., Rheeder,J.P., Lee,Y.W., Leslie,J.F., and Marasas,W.F.O. 2005. Production of fumonisin B and C analogues by several *Fusarium* species. Journal of Agricultural and Food Chemistry 53: 4861-4866.

Shwab,E.K. and Keller,N.P. 2008. Regulation of secondary metabolite production in filamentous ascomycetes. Mycological Research 112: 225-230.

Smedsgaard,J. 1997. Micro-scale extraction procedure for standardization screening of fungal metabolite production in cultures. Journal of Chromatography A 760: 264-270.

Smith,T.K., McMillan,E.G., and Castillo,J.B. 1997. Effect of feeding blends of *Fusarium* mycotoxin-contaminated grains containing deoxynivalenol and fusaric acid on growth and feed consumption of immature swine. Journal of Animal Science 75: 2184-2191.

Snyder,L.R., Kirkland,J.J., and Glajch,J.L. 1997. Practical HPLC Method Development. Second edition. John Wiley and sons, Inc., New York.

Sørensen,J.L., Phipps,R.K., Nielsen,K.F., Schroers,H.J., Frank,J., and Thrane,U. 2009. Analysis of *Fusarium avenaceum* metabolites produced during wet apple core rot. Journal of Agricultural and Food Chemistry 57: 1632-1639.

Sørensen,L.K. and Elbæk,T.H. 2005. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. Journal of Chromatography B 820: 183-196.

Spanjer,M.C., Rensen,P.M., and Scholten,J.M. 2008. LC-MS/MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. Food Additives and Contaminants - Part A 25: 472-489.

Speijers,G.J.A. and Speijers,M.H.M. 2004. Combined toxic effects of mycotoxins. Toxicology Letters 153: 91-98.

Storm,I.D., Rasmussen,P.H., Strobel,B.W., and Hansen,H.C.B. 2008. Ergot alkaloids in rye flour determined by solid-phase cation-exchange and high-pressure liquid chromatography with fluorescence detection. Food Additives and Contaminants - Part A 25: 338-346.

Sugiyama,K.I., Kawakami,H., Kamata,Y., and Sugita-Konishi,Y. 2011. Effect of a combination of deoxynivalenol and nivalenol on lipopolisaccharide-induced nitric oxide production by mouse macrophages. Mycotoxin Research 27: 57-62.

Sulyok,M., Berthiller,F., Krska,R., and Schuhmacher,R. 2006. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Communications in Mass Spectrometry 20: 2649-2659.

Sulyok,M., Krska,R., and Schuhmacher,R. 2007a. A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. Analytical and Bioanalytical Chemistry 389: 1505-1523.

Sulyok,M., Krska,R., and Schuhmacher,R. 2007b. Application of a liquid chromatography-tandem mass spectrometric method to multi-mycotoxin determination in raw cereals and evaluation of matrix effects. Food Additives and Contaminants 24: 1184-1195.

Sulyok,M., Krska,R., and Schuhmacher,R. 2010. Application of an LC-MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. Food Chemistry 119: 408-416.

Swartz,M.E. 2005. UPLC™ : An introduction and review. Journal of Liquid Chromatography & Related Technologies 28: 1253-1263.

Sypecka,Z., Kelly,M., and Brereton,P. 2004. Deoxynivalenol and zearalenone residues in eggs of laying hens fed with a naturally contaminated diet: Effects on egg production and estimation of transmission rates from feed to eggs. Journal of Agricultural and Food Chemistry 52: 5463-5471.

Tajima,O., Schoen,E.D., Feron,V.J., and Groten,J.P. 2002. Statistically designed experiments in a tiered approach to screen mixtures of *Fusarium* mycotoxins for possible interactions. Food and Chemical Toxicology 40: 685-695.

Takino,M., Tanaka,T., Yamaguchi,K., and Nakahara,T. 2004. Atmospheric pressure photo-ionization liquid chromatography/mass spectrometric determination of aflatoxins in food. Food Additives and Contaminants 21: 76-84.

Tamm,C. and Breitenstein,W. 1980. The biosynthesis of trichothecene mycotoxins. *In* The biosynthesis of mycotoxins. Academic Press, Orlando. pp. 69-104.

Tammer,B., Lehmann,I., Nieber,K., and Altenburger,R. 2007. Combined effects of mycotoxin mixtures on human T cell function. Toxicology Letters 170: 124-133.

Tan,D.C., Flematti,G.R., Ghisalberti,E.L., Sivasithamparam,K., and Barbetti,M.J. 2011. Toxigenicity of enniatins from Western Australian *Fusarium* species to brine shrimp (Artemia franciscana). Toxicon 57: 817-825.

Tanaka,H., Takino,M., Sugita-Konishi,Y., and Tanaka,T. 2006. Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs. Rapid Communications in Mass Spectrometry 20: 1422-1428.

Tanaka,H., Takino,M., Sugita-Konishi,Y., Tanaka,T., Toriba,A., and Hayakawa,K. 2009. Determination of nivalenol and deoxynivalenol by liquid chromatography/atmospheric pressure photoionization mass spectrometry. Rapid Communications in Mass Spectrometry 23: 3119-3124.

Taverniers,I., De Loose,M., and Van Bockstaele,E. 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. TrAC - Trends in Analytical Chemistry 23: 535-552.

The European Committee for Standardization 2009a. EN 14132:2009. Animal feeding stuffs - Determination of zearalenone in animal feed - High performance liquid chromatographic method with fluorescence detection and immunoaffnity column clean-up.

The European Committee for Standardization 2009b. EN 15792:2009. Foodstuffs - Determination of ochratoxin A in barley and roasted coffee - HPLC method with immunoaffinity column clean-up.

The European Committee for Standardization 2010. Technical report. CEN/TR 16059. Food analysis - Performance criteria for single laboratory validated methods of analysis for the determination of mycotoxins.

Theumer,M.G., Lopez,A.G., Masih,D.T., Chulze,S.N., and Rubinstein,H.R. 2003. Immunobiological effects of AFB1 and AFB1-FB1 mixture in experimental subchronic mycotoxicoses in rats. Toxicology 186: 159-170.

Thompson,M., Ellison,S.L.R., and Wood,R. 2002. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure and Applied Chemistry 74: 835-855.

Thrane,U., Adler,A., Clasen,P.E., Galvano,F., Langseth,W., Lew,H., Logrieco,A., Nielsen,K.F., and Ritieni,A. 2004. Diversity in metabolite production by *Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*. International Journal of Food Microbiology 95: 257-266.

Thuvander,A., Wikman,C., and Gadhasson,I. 1999. *In vitro* exposure of human lymphocytes to trichothecenes: Individual variation in sensitivity and effects of combined exposure on lymphocyte function. Food and Chemical Toxicology 37: 639-648.

Uhlig,S., Jestoi,M., Knutsen,A.K., and Heier,B.T. 2006. Multiple regression analysis as a tool for the identification of relations between semi-quantitative LC-MS data and cytotoxicity of extracts of the fungus *Fusarium avenaceum* (syn. *F.arthrosporioides*). Toxicon 48: 567-579.

Uhlig,S., Jestoi,M., and Parikka,P. 2007. *Fusarium avenaceum* - The North European situation. International Journal of Food Microbiology 119: 17-24.

Vaclavik,L., Zachariasova,M., Hrbek,V., and Hajslova,J. 2010. Analysis of multiple mycotoxins in cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to high resolution mass spectrometry. Talanta 82: 1950-1957.

Vahl,M. and Jørgensen,K. 1998. Determination of aflatoxins in food using LC/MS/MS. European Food Research and Technology 206: 243-245.

Valenta,H. and Dänicke,S. 2005. Study on the transmission of deoxynivalenol and deepoxy-deoxynivalenol into eggs of laying hens using a high-performance liquid chromatography-ultraviolet method with clean-up by immunoaffinity columns. Molecular Nutrition & Food Research 49: 779-785.

Valero,A., Marín,S., Ramos,A.J., and Sanchis,V. 2008. Survey: Ochratoxin A in European special wines. Food Chemistry 108: 593-599.

Van De Steene,J.C. and Lambert,W.E. 2008. Comparison of matrix effects in HPLC-MS/MS and UPLC-MS/MS analysis of nine basic pharmaceuticals in surface waters. Journal of the American Society for Mass Spectrometry 19: 713-718.

van der Fels-Klerx,H.J. 2010. External scientific report: Occurrence data of trichothecene mycotoxins T-2 toxin and HT-2 toxin in food and feed. Rep. EFSA-Q-2010-00949.

van der Westhuizen,L., Shephard,G.S., Rheeder,J.P., and Burger,H.M. 2010. Individual fumonisin exposure and sphingoid base levels in rural populations consuming maize in South Africa. Food and Chemical Toxicology 48: 1698-1703.

van Egmond,H.P. 1989. Aflatoxin M1: Occurrence, toxicity, regulation. *In* Mycotoxins in dairy products. Elsevier Applied Science, London. pp. 11-55.

van Egmond,H.P., Schothorst,R.C., and Jonker,M.A. 2007. Regulations relating to mycotoxins in food. Analytical and Bioanalytical Chemistry 389: 147-157.

Van Pamel,E., Vlaemynck,G., Heyndrickx,M., Herman,L., Verbeken,A., and Daeseleire,E. 2011. Mycotoxin production by pure fungal isolates analysed by means of an uhplc-ms/ms multi-mycotoxin method with possible pitfalls and solutions for patulin-producing isolates. Mycotoxin Research 27: 37-47.

van Trijp,J.M.P. and Roos,A.H. 1991. Model for the calculation of calibration curves. RIKILT Report 91.02.

Vázquez,B.I., Fente,C., Franco,C.M., Quinto,E., Cepeda,A., and Prognon,P. 1997. Rapid semi-quantitative fluorimetric determination of citrinin in fungal cultures isolated from cheese and cheese factories. Letters in Applied Microbiology 24: 397-400.

Vesela,D., Vesely,D., and Jelinek,R. 1983. Toxic effects of ochratoxin A and citrinin, alone and in combination, on chicken embryos. Applied and Environmental Microbiology 45: 91-93.

Virdis,S., Corgiolu,G., Scarano,C., Pilo,A.L., and De Santis,E.P.L. 2008. Occurrence of aflatoxin M1 in tank bulk goat milk and ripened goat cheese. Food Control 19: 44-49.

Wagener,R.E., Davis,N.D., and Diener,U.L. 1980. Penitrem A and roquefortine production by *Penicillium commune*. Applied and Environmental Microbiology 39: 882-887.

Wangikar,P.B., Dwivedi,P., and Sinha,N. 2004. Effect in rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. I. Maternal toxicity and fetal malformations. Birth Defects Research Part B 71: 343-351.

Ware,G.M., Price,G., Carter, L. Jr., and Eitenmiller,R.R. 2000. Liquid chromatographic preparative method for isolating ergot alkaloids, using a particle-loaded membrane extracting disk. Journal of AOAC International 83: 1395-1399.

Whitaker,T.B. 2006. Sampling foods for mycotoxins. Food Additives and Contaminants 23: 50-61.

Wilson,B.J. 1971. Miscellaneous *Penicillium* toxins. *In* Microbial toxins. Vol. VI. Fungal toxins. Sixth edition. Academic Press, New York. pp. 459-521.

Xu,X.M., Nicholson,P., and Ritieni,A. 2007. Effects of fungal interactions among *Fusarium* head blight pathogens on disease development and mycotoxin accumulation. International Journal of Food Microbiology 119: 67-71.

Yli-Mattila,T., Parikka,P., Lahtinen,T., Rämö,S., Kokkonen,M., Rizzo,A., Jestoi,M., and Hietaniemi,V. 2008. *Fusarium* DNA levels in Finnish cereal grains. *In* Current advances in molecular mycology. Nova Science Publishers, New York. pp. 107-138.

Young,J.C. 1981a. Variability in the content and composition of alkaloids found in Canadian ergot. I. Rye. Journal of Environmental Science and Health - Part B 16: 83-111.

Young,J.C. 1981b. Variability in the content and composition of alkaloids found in Canadian ergot. II. Wheat. Journal of Environmental Science and Health - Part B 16: 381-393.

Zachariasova,M., Lacina,O., Malachova,A., Kostelanska,M., Poustka,J., Godula,M., and Hajslova,J. 2010. Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. Analytica Chimica Acta 662: 51-61.

Zambonin,C.G., Monaci,L., and Aresta,A. 2001. Determination of cyclopiazonic acid in cheese samples using solid-phase microextraction and high performance liquid chromatography. Food Chemistry 75: 249-254.

Zambonin,C.G., Monaci,L., and Aresta,A. 2002. Solid-phase microextraction-high performance liquid chromatography and diode array detection for the determination of mycophenolic acid in cheese. Food Chemistry 78: 249-254.

Zöllner,P. and Mayer-Helm,B. 2006. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. Journal of Chromatography A 1136: 123-169.