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FACULTEIT FARMACEUTISCHE WETENSCHAPPEN

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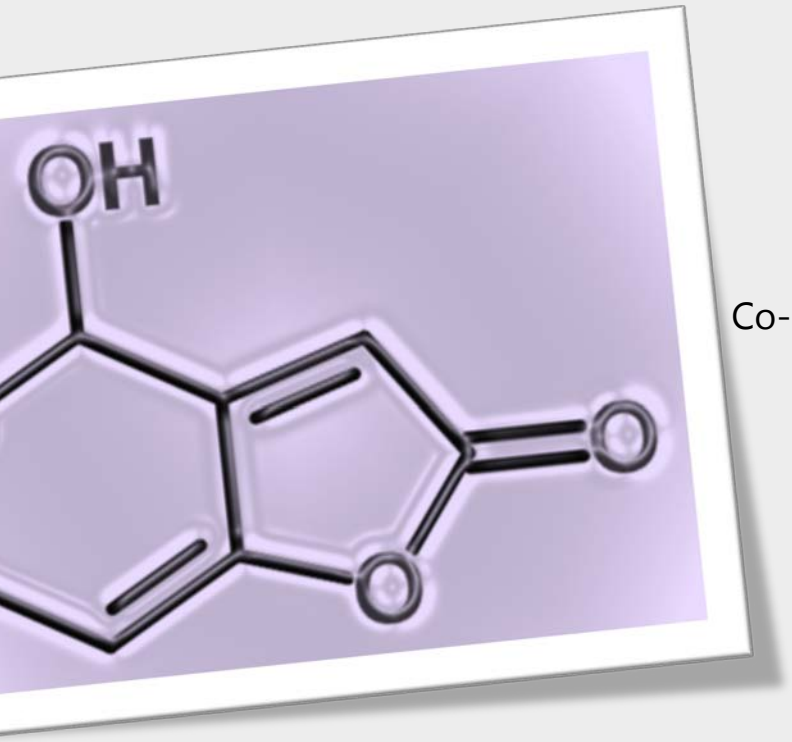

Svetlana V. Malysheva

Promoter: Prof. Dr. Sarah De Saeger

Co-promoters: Prof. Dr. Irina Yu. Goryacheva

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Promoter:

Prof. Sarah De Saeger *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Co-promoters:

Prof. Irina Yu. Goryacheva *(Chemistry Institute, Saratov State University, Russia)*

Dr. José Diana Di Mavungu *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Members of the reading committee:

Prof. Ann Van Schepdael *(Faculty of Pharmaceutical Sciences, KU Leuven, Belgium)*

Prof. Bart De Spiegeleer *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Prof. Patrice Chiap *(Institute of Pathology, Academic Hospital of Liège, Belgium)*

Dr. Christof Van Poucke *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Prof. Sarah De Saeger *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Members of the examination committee:

Dr. Els Daeseleire *(ILVO, Melle, Belgium)*

Prof. Irina Yu. Goryacheva *(Chemistry Institute, Saratov State University, Russia)*

Prof. Serge Van Calenbergh *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Prof. Em. Carlos Van Peteghem *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Dean:

Prof. Stefaan De Smedt *(Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium)*

Rector:

Prof. Paul Van Cauwenberghe *(Ghent University, Ghent, Belgium)*

Ghent, 10 June 2013

Author,

M.Chem. S. V. Malysheva

Promoters,

Prof. Dr. S. De Saeger

Prof. Dr. I. Yu. Goryacheva

Dr. J. Diana Di Mavungu

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Svetlana V. Malysheva

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GLOSSARY

Abbreviation/Symbol	Definition
a.u.	Arbitrary units
ACN	Acetonitrile
AdoMet	S-adenosyl-methionine
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
BSA	Bovine serum albumin
CCF	Central composite face-centered
CC_α	Decision limit
CC_β	Detection capability
CEL	Collision energy level
CID	Collisionally-induced dissociation
CoA	Coenzyme A
Da	Dalton
DA	Dopant-assisted
DAD	Diode array detection
DCM	Dichloromethane
DHEt	Dihydroergotamine
DHN	Dihydronaphthalene
DMAPP	Dimethylallyldiphosphate
DMK	Dimethyl ketone (= acetone)
DNA	Deoxyribonucleic acid
Eco	Ergocornine
Econ	Ergocorninine
Ecr	Ergocristine
Ecrn	Ergocristinine
EFSA	European Food Safety Authority
Ekr	Ergokryptine
Ekrn	Ergokryptinine
Em	Ergometrine
Emn	Ergometrinine
Es	Ergosine
ESI	Electrospray ionization
Esn	Ergosinine
Et	Ergotamine

Abbreviation/Symbol	Definition
Etn	Ergotaminine
EtOAc	Ethyl acetate
eV	Electron volt
FDA	Food and Drug Administration
FEFAC	European Feed Manufacturer's Federation
FT-ICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
GC	Gas chromatography
GMM	Glucose Minimal Medium
HCD	Higher energy collisional dissociation
HILIC	Hydrophilic interaction liquid chromatography
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
hv	Light
IARC	International Agency for Research on Cancer
IF	Impact factor
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
LTQ	Linear trap quadrupole
M	Molecular mass
m/z	Mass-to-charge ratio
MCP	Micro-channel plate
MEA	Malt extract agar
MeEm	Methylergometrine
MeErgi	Methysergide
MeOH	Methanol
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted polymer solid-phase extraction
MP	Mobile phase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry

Abbreviation/Symbol	Definition
MS^n	Multi-stage mass spectrometry
NMR	Nuclear magnetic resonance
NP	Normal phase
NRPS	Non-ribosomal peptide synthase
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PLS	Partial Least Squares
ppm	Parts per million
PSA	Primary secondary amine
PTR	Prenyltransferase
Q-TOF	Quadrupole time-of-flight
r	Regression coefficient
R^2	Coefficient of determination
RASFF	Rapid Alert System for Food and Feed
RP	Reversed phase
RSD	Relative standard deviation
RSD_r	Relative standard deviation under repeatability conditions
RSD_R	Relative standard deviation under reproducibility conditions
RSM	Response surface modelling
RT	Retention time
S/N	Signal-to-noise ratio
SBS	Sick Building Syndrome
SCX	Strong cation exchange
sMRM	Scheduled multiple reaction monitoring
SMURF	Secondary Metabolite Unknown Region Finder
sp.	Species (singular)
SPE	Solid-phase extraction
spp.	Species (plural)
SRM	Selected reaction monitoring
S_{RW}	Within-laboratory reproducibility
SSE	Signal suppression-enhancement
S_{yx}	Standard error of the estimate
TIC	Total ion chromatogram
TLC	Thin-layer chromatography

Abbreviation/Symbol	Definition
TOF	Time-of-flight
Trp	Tryptophan
U	Expanded measurement uncertainty
$U(C_{ref})$	Purity of standard
u_c	Combined measurement uncertainty
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
WATM-US	Wickerham medium supplemented with uracil and ammonium sulphate
WP	Wallpaper

AIM OF THE STUDY

Fungi, members of eukaryotic organisms, are distributed worldwide; they can grow on a wide range of substrates and under various conditions. Fungi not only play an important role in most ecosystems, but they have a direct impact on society due to production of secondary metabolites. **Secondary metabolites** are organic compounds which are, in contrast to primary metabolites, not vital for the fungal growth, development or reproduction.

From a big array of secondary metabolites produced by fungi, some compounds are important for the society (*e.g.*, antibiotics), while others (*e.g.*, mycotoxins) represent a threat to human and animal health and cause economic losses. Considering that about 30 % of prescribed medications are of fungal origin and that only about 5 % of fungi have been studied, fungi and their metabolites represent a great source of new, possibly bioactive compounds. In this respect, two research directions in the field of secondary fungal metabolites can be distinguished: *1)* discovery of novel compounds possibly possessing bioactivity; *2)* detection and monitoring of known secondary metabolites.

The ultimate aim of the study is to extend knowledge of secondary fungal metabolites by performing targeted and untargeted investigation of these compounds in selected samples. As an analytical tool, such a powerful analytical technique as **liquid chromatography - mass spectrometry** (LC-MS) will be utilized in the doctoral research. As the area of fungi accumulation is diverse, the set of samples for the study is broad, from foods and feeds to biological material and samples from mouldy indoor environments.

The main objectives of the **targeted part** of the PhD dissertation are development and validation of multi-analyte LC-MS methods and their further application for quantification of known toxic metabolites. In this regard, an LC-MS/MS method will be developed and applied to study occurrence of toxic metabolites of *Claviceps* spp., ergot alkaloids, in grain and grain products for human consumption and animal feeding. As epimerization is a specific feature of this group of compounds, a care will be taken throughout the whole analysis with a major concern being extraction procedure. Method parameters will be determined in a thorough validation study. Furthermore, in order to investigate prevention of digestive absorption of these feed-borne metabolites, the developed method will be adapted for quantification of ergot alkaloids in buffered solutions and used in an *in vitro* efficacy testing of a mycotoxin binder.

Through careful investigation of ionization conditions, another important contaminant, mycotoxin patulin, will be included in a single run multi-analyte LC-MS/MS method, which previously was not possible. The established method will be validated for fluids from transdermal penetration studies with the prospect of assessing exposure to mycotoxins through dermal route.

The goal of this part is to make available sensitive analytical tools for control of contamination and to extend the information on the toxin occurrence. Such data are of great importance for risk assessment and setting regulatory limits in order to promote human and animal health.

The **untargeted part** is focused on the development of suitable tools for detection of less studied (or novel) organic compounds produced by fungi. Considering the chemical diversity of secondary fungal metabolites and their broad abundance in the samples, development of a generic extraction procedure is the first objective of the untargeted part. For investigation of a wider range of metabolites, establishing a workflow for identification of untargeted compounds is another objective of the study.

Two approaches for executing untargeted analysis will be used, namely simple screening of mouldy samples and gene manipulation experiments to observe changes in metabolic profile of a fungus. Application of modern sophisticated instrumentation will be the key element. As concerns the first approach, untargeted analysis of ergot alkaloid derivatives will be performed in mouldy cereal samples. Untargeted metabolite screening will also be carried out in samples from mouldy buildings with the intention to gather more information on secondary fungal metabolite occurrence in indoor environments. Regarding the gene manipulating experiments, *Aspergillus flavus*, a prevalent contaminant of food and feed, will be investigated. Inactivation of the cluster 27 polyketide synthase (*pks*) gene in the species will be performed. A comparative metabolomics approach, based on high resolution MS, will be applied to identify the metabolites associated with the cluster 27 *pks* gene.

The obtained information will assist in achieving the research goals of this part of the dissertation such as completing some missing blocks in a scheme of fungal metabolism, investigating occurrence of less studied metabolites and discovery of novel organic compounds.

CHAPTER 1

Introduction to secondary fungal metabolites

1.1. General information

Secondary metabolites are low-molecular weight organic compounds (150-1,000 Da) produced by many organisms including fungi. Scientific attention to secondary fungal metabolites and understanding of their importance started to grow since the first half of the 20th century after Fleming's discovery of penicillin from *Penicillium* sp. Since that time, many other beneficial secondary metabolites were discovered (*e.g.*, antiviral drugs, anti-tumor drugs). Along with this, production of numerous toxic secondary metabolites (*e.g.*, aflatoxins, zearalenone) was revealed.

Although secondary metabolites are structurally diverse, they usually contain C, H, O and N, and less frequently S, P, Cl, Br and F, and basic functional groups such as hydroxyl, carboxyl, carbonyl, amino. The metabolites possess unusual chemical linkages, such as β-lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings.

Considering that about 1.5 million different species of fungi are known (Deacon, 2006), and that a single species is able to produce more than one secondary metabolite, the diversity of these products of fungal metabolism seems to be enormous. Though great number of secondary metabolites is yielded, they have no obvious role in the life cycle of fungi. Plausible reasons of their production include necessity of secondary metabolism, regardless the end compound, for removing the intermediates of the basic metabolic pathways of an organism, when growth is temporarily restricted. These intermediates could be taken into secondary metabolism and afterwards exported from the cell or accumulate as inactive compounds (Deacon, 2006). Another explanation could be the use of these metabolites by fungus as cell-signaling molecules for communication, self-defense or inhibition of competitor's growth (Brakhage and Schroeckh, 2011; Brakhage, 2013; Yim *et al*., 2007). For instance, antibiotics could be useful in defense of territory, mycotoxins as animal antifeedants, melanin for protection against UV damage, flavour or odour components for attracting insects for spore dispersal (Deacon, 2006).

1.2. Classification of secondary fungal metabolites

1.2.1. Classification challenges

Secondary fungal metabolites comprise an enormous group of chemicals. Due to diversity in chemical structures of the metabolites, their biosynthetic origins and biological effects, classification of these chemicals becomes a challenging task. Such groups as teratogens, mutagens, carcinogens, and allergens are identified by cell biologists, while clinicians simply classify secondary fungal metabolites by the organs they have effect on (*e.g*., hepatotoxins, nephrotoxins, neurotoxins, immunotoxins). From the point of view of organic chemists, division of the metabolites into groups is based on chemical structures (*e.g*., lactones). Mycologists list secondary metabolites by the fungi that produce them (*e.g*., *Aspergillus* metabolites, *Penicillium* metabolites, *Fusarium* metabolites). Considering the above, a single secondary metabolite can be assigned to different groups at the same time making all types of classifications insufficient and not uniform (Bennett and Klich, 2003; Bhatnagar *et al*., 2002).

Owing to their complexity and diversity, only exemplary classifications of secondary fungal metabolites will be discussed in this chapter.

1.2.2. Classification based on biosynthetic origin

Despite their structural diversity, secondary fungal metabolites can be grouped based on biosynthetic origin (Table 1.1). The major route for secondary metabolite production is the polyketide metabolic pathway. A polyketide is formed by polymerisation of acetate. Further cyclization, lactonization or formation of thioesters or amides can occur in the chain resulting in a range of products (Deacon, 2006). Polyketide metabolites include antibiotic griseofulvin (*Penicillium griseofulvum*), sterigmatocyctin, carcinogenic aflatoxins, ochratoxins (*Aspergillus* spp., *Penicillium* spp.), fumonisins (*Fusarium* spp.), antibiotic patulin (*Penicillium spp.*) (Abarca *et al*., 1994; Deacon, 2006; Schroeder and Kelton, 1975; Seydametova *et al*., 2010; Sommer *et al*., 1976; Torres *et al*., 1987).

The mevalonate pathway is intended for synthesis of sterols. Initially, mevalonic acid (a 6-carbon compound) is formed and then converted to 5-carbon isoprene units, which, in turn,

condense head-to-tail to form chains of various lengths. The chains further undergo cyclization and other modifications. Following this synthesis, the secondary metabolites of *Fusarium* spp. (trichothecenes) are produced among other compounds (Deacon, 2006).

Table 1.1. *Examples of secondary fungal metabolites derived from different metabolic pathways of fungi (summarized from Ballio et al., 1964; Deacon, 2006; Griffin, 1994; Hanson, 2008; Kozlovskii et al., 2000; Moore, 1998).*

Pathway	Secondary metabolite (or group of metabolites)	Representative producer	Bioactivity
Polyketide	Aflatoxins	*Aspergillus parasiticus, A. flavus*	Carcinogenic, mycotoxin
	Citrinin	*Penicillium citrinum*	Anti-bacterial, nephrotoxic, mycotoxin
	Cladosporin	*Cladosporium* spp.	Anti-fungal
	Fumonisins	*Fusarium* spp.	Mycotoxin
	Gladiolic acid	*Penicillium gladioli*	Anti-bacterial
	Griseofulvin	*Penicillium griseofulvum*	Anti-fungal, mycotoxin
	Mycophenolic acid	*Penicillium* spp.	Anti-fungal, anti-bacterial, anti-viral, mycotoxin
	Ochratoxins	*Aspergillus ochraceus, Penicillium spp.*	Nephrotoxic, mycotoxin
	Patulin	*Penicillium spp.*	Anti-bacterial, mycotoxin
	Penicillic acid	*Penicillium spp.*	Mycotoxin
	Sterigmatocystin	*Aspergillus* spp.	Mycotoxin
Mevalonate	Cephalosporins	*Cephalosporium* spp.	Anti-bacterial
	Fusicoccin	*Fusicoccum amygdali*	Phytotoxic
	Trichothecenes	*Fusarium* spp.	Mycotoxin

Continue

Table 1.1 (continued)

Pathway	Secondary metabolite (or group of metabolites)	Representative producer	Bioactivity
Amino acid	AM-toxin	*Alternaria alternata*	Phytotoxic
	Beauvericin	*Beauveria bassiana*	Insect pathogen, mycotoxin
	Cephalosporins	*Cephalosporium* spp.	Anti-bacterial
	Cyclopenin-viridicatin group	*Penicillium* spp.	Mycotoxin
	Cyclosporins	*Tolypocladium* spp.	Immunosupressive
	Ergot alkaloids	*Claviceps* spp.	Anti-migraine, mycotoxin
	Fusaric acid	*Fusarium* spp.	Phytotoxic
	Gliotoxin	*Gliocladum* spp., *Trichoderma* spp., *Aspergillus fumigatus*, *Penicillium terlikowski*	Anti-fungal, anti-bacterial, mycotoxin
	Lysergic acid	*Claviceps* spp.	Psychedelic drug precursor
	Penicillins	*Penicillium* spp.	Anti-bacterial
	Pulcheriminic acid	*Candida* spp.	Mycotoxin
	Roquefortine C	*Penicillium roqueforti*	Neurotoxic
Shikimate	Ergot alkaloids	*Claviceps* spp.	Anti-migraine, mycotoxin
	Gallic acid	*Penicillium* spp., *Alternaria* spp.	Anti-fungal, anti-viral
	Lysergic acid	*Claviceps* spp.	Psychedelic drug precursor

The shikimate pathway, used normally for production of primary metabolites, such as aromatic amino acids, also provides the precursors for hallucinogenic secondary metabolites, *e.g*., lysergic acid (Deacon, 2006; Griffin, 1994).

The precursors of metabolites formed through the amino acid pathway are amino acids. Among others, cyclic peptides and indole alkaloids are formed through this route.

Metabolites derived from this pathway include, *e.g*., penicillins, cephalosporins, gliotoxin and ergot alkaloids (Griffin, 1994).

It has to be mentioned that biosynthetic routes are interrelated, therefore a secondary metabolite can be derived from a combination of pathways, as in the case of ergot alkaloids. These compounds are mainly formed through the amino acid pathway. However, the shikimic acid pathway contributes to the formation of the indole ring structure of the precursor amino acid, tryptophan (Trp), whereas some other parts of the molecular structure of ergot alkaloids are formed through the mevalonic acid pathway (Griffin, 1994).

1.2.3. Classification based on structure

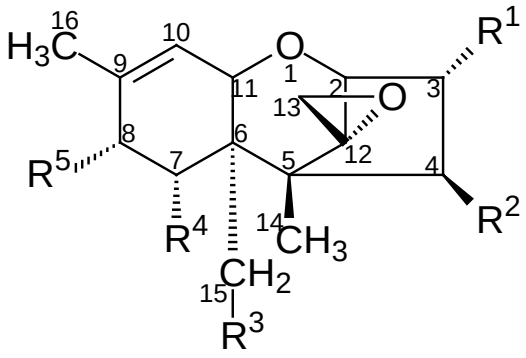
Though many classes of secondary fungal metabolites can be distinguished (Zhong and Xiao, 2009), only a part will be discussed in the present work.

1.2.3.1. Terpenoids

Numerous secondary fungal metabolites are referred to a class of terpenoids. Terpenoids are formed from C_5 isoprene units leading to their characteristic branched structure (Hanson, 2008). They are sub-divided into several groups depending on the number of isoprene units: monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}) and caroterpenoids (C_{40}). Additionally to these sub-groups, there are compounds containing the isoprene unit attached to a carbon skeleton which is derived from another biosynthetic route. For instance, the lysergic moiety of the ergot alkaloids bears an isoprene unit linked to the indole of Trp.

Among all terpenoids, sesquiterpenoids are the most common to fungal metabolism with trichothecenes representing a major group (Hanson, 2008; Moore, 1998). The trichothecenes are a family of more than sixty sesquiterpenoid metabolites produced by several fungi, including *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium* (Bennet and Klich, 2003). The name "*trichothecene*" is derived from trichothecin, which was one of the first members of the family identified (Bennett and Klich, 2003).

As a common feature, all trichothecenes contain a C9-C10 double bond, a C12-C13-epoxide ring, and various hydroxyl and acetoxy groups (Fig. 1.1). Trichothecenes are classified according to their characteristic functional groups; thus, there are four types (A-D) of trichothecenes. Type A trichothecenes have an oxygen function at C8, which is different from a keto-group. HT-2 toxin, T-2 toxin and diacetoxyscirpenol are representative members of this group. Nivalenol, deoxynivalenol, fusarenon X, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol belong to the type B trichothecenes, which have a keto-group at position C8. Type C trichothecenes are characterized by a second epoxide group. Type D trichothecenes are macrocyclic compounds which possess a macrocylic ester or an ester-ether bridge between C4 and C15 (*e.g.*, verrucarins, roridins, satratoxins) (Bennett and Klich, 2003; Berthiller *et al*., 2005a).



Trichothecene	R_1	R_2	R_3	R_4	R_5
Nivalenol	-OH	-OH	-OH	-OH	=O
Deoxynivalenol	-OH	-H	-OH	-OH	=O
Fusarenon X	-OH	$-OCOCH_3$	-OH	-OH	=O
3-Acetyldeoxynivalenol	$-OCOCH_3$	-H	-OH	-OH	=O
15-Acetyldeoxynivalenol	-OH	-H	$-OCOCH_3$	-OH	=O
Diacetoxyscirpenol	-OH	$-OCOCH_3$	$-OCOCH_3$	-H	-H
HT-2 toxin	-OH	-OH	$-OCOCH_3$	-H	$-OCOCH_2CH(CH_3)_2$
T-2 toxin	-OH	$-OCOCH_3$	$-OCOCH_3$	-H	$-OCOCH_2CH(CH_3)_2$
Macrocyclics	-H	-O-R´-O-		-H	-H

R´ - Macrocyclic ester or ester-ether bridge between C4 and C15

Figure 1.1*. Basic trichothecene structure with examples of most prevalent metabolites.*

1.2.3.2. Polyketides

Polyketides are a major class of fungal metabolites. They are formed through a condensation of acetyl-coenzyme A (acetyl-CoA) (as starter unit) with the appropriate number of malonyl-CoA units (as extending unit) followed by modification of the completed poly-β-ketone and release of the end product. Other building blocks, such as propionyl-CoA, butyryl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA, can also be used in the biosynthesis. These sequential reactions are catalysed by a collection of enzyme complexes called polyketide synthases (PKSs). The formed poly-β-ketones may undergo modification by alkylation, cyclization, oxidation or reduction (Herbert, 1989; Shen *et al*., 2000; Zhou *et al*., 2010). Aflatoxins, patulin, bikaverin and zearalenone, secondary metabolites of *Aspergillus*, *Penicillium* and *Fusarium* spp., respectively, are members of the aromatic polyketide class (Fig. 1.2).

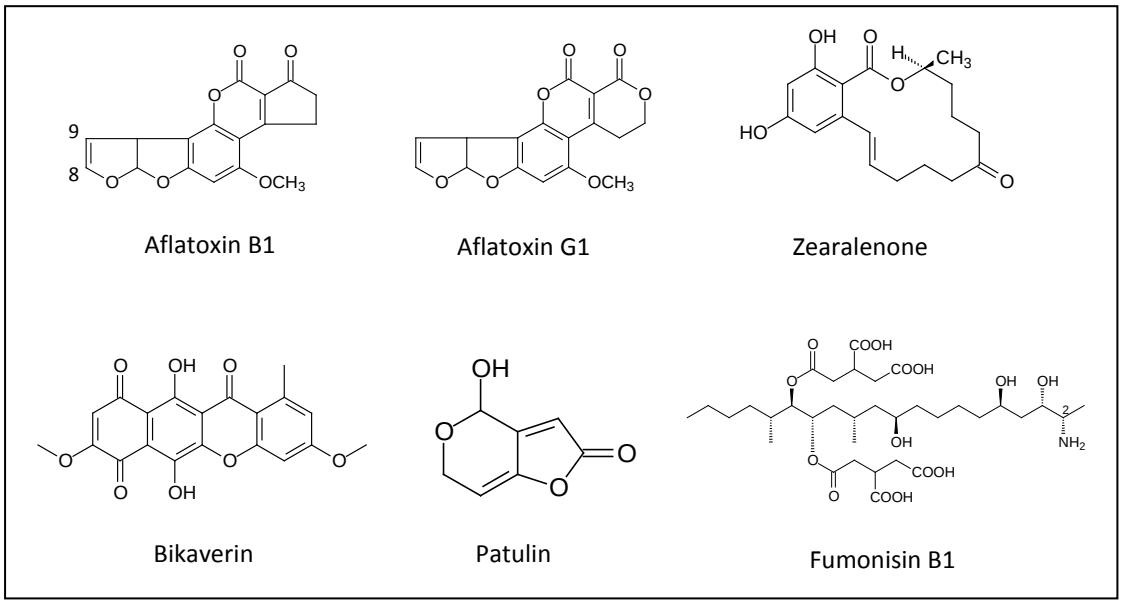


Figure 1.2. *Examples of polyketide fungal metabolites.*

Linear polyketides, fumonisins, are frequently produced by *Fusarium* species. Fumonisins consist of a linear 19- or 20-carbon backbone with hydroxyl, methyl, and tricarballylic acid moieties at various positions along the backbone. Fumonisins belonging to the B-series possess an amino group at the C2-position (Fig. 1.2), whereas in other series this amino

group can be acetylated or substituted with hydroxypyridine (Bezuidenhout *et al.,* 1988; Musser *et al.,* 1996; Musser and Plattner, 1997; Proctor *et al*., 1999).

1.2.3.3. Non-ribosomal peptides

Non-ribosomal peptides are polypeptides which are formed via a mechanism independent on mRNA, *i.e*., not through the traditional translation mechanism (Rohlfs *et al*., 2009). Non-ribosomal peptides usually have cyclic and/or branched structures, can contain non-proteogenic amino acids, carry modifications like N-methyl and N-formyl groups, or are glycosylated, acrylated, halogenated or hydroxylated. Non-ribosomal peptides are often dimers or trimers of identical sequences chained together, cyclized or branched and consist of two and up to 40 amino acids (Eisfeld, 2009). A great part of non-ribosomal peptides are cyclic peptides, including macrocyclic lactones (Hanson, 2008).

Cyclosporins are a group of cyclic peptides isolated from *Tolypocladium inflatum,* with cyclosporin A being a major compound (Fig. 1.3). This group contains unusual amino acids; however, amino residues such as L-leucine and L-valine are also present as their N-methyl derivatives. Beauverolides and beauvericin (Fig. 1.3) are cyclic depsipeptides (peptido-lactones) produced by *Beauveria bassiana* (Hanson, 2008). Examples of linear non-ribosomal peptides are integramide A (Fig. 1.3) and B produced by *Dendrodochium* sp. (De Zotti *et al*., 2010; Singh *et al*., 2002).

Penicillins (mainly produced by *Penicillium* spp.) also belong to the group of non-ribosomal peptides and consist of a thiazolidine ring fused to a β-lactam ring and variable substituents attached by a peptide bond. For instance, in benzylpenicillin (= penicillin G), this substituent is a benzyl group (Fig. 1.3) (Berg *et al*., 2002).

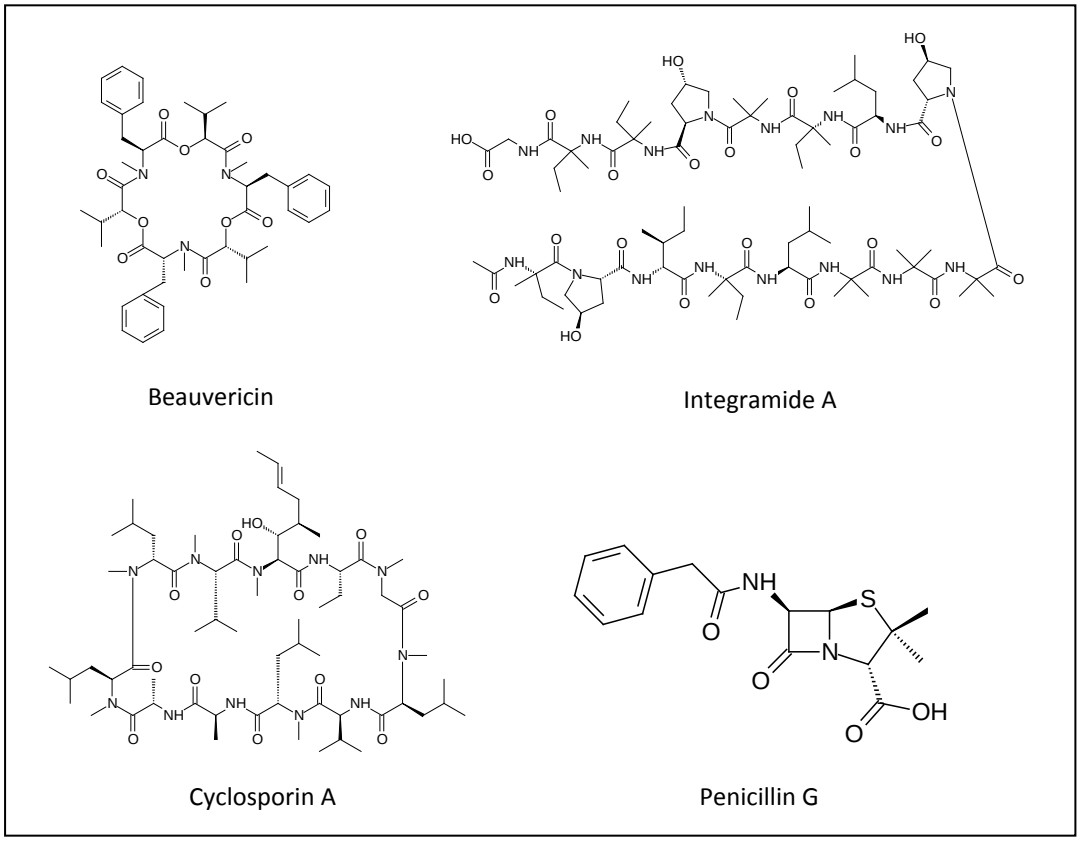


Figure 1.3. *Examples of non-ribosomal fungal peptides.*

1.2.3.4. Alkaloids

Alkaloids are mostly referred to as a group of basic organic compounds, containing at least one nitrogen atom in a ring structure of the molecule (Mahmood *et al*., 2010). However, neutral or weakly acidic substances can also be distinguished in the group. Due to the great structural diversity, the sub-classification of alkaloids is very complex, but yet not uniform. More than 20 alkaloid classes can be recognized including indole, tropane, imidazole and pyridine derivatives.

From the medical and toxicological point of view, the most important among indole alkaloids are ergot alkaloids produced by *Claviceps purpurea*, a fungus growing on rye and other cereals and grasses (Krska *et al*., 2008). More specifically, ergot alkaloids are hemiterpenoid indole alkaloids related to lysergic acid, which, in turn, is formed through multiple reactions involving Trp and dimethylallyldiphosphate (DMAPP). A characteristic feature of ergot alkaloids is the tetracyclic ergoline ring system or the tricyclic alkaloids open between N6 and C7 positions (according to ergoline numbering) (Fig. 1.4a). They can be divided into

several groups, namely, clavine alkaloids, lysergic acid-derivatives, peptide ergot alkaloids (ergopeptines and ergopeptams). Ergine and ergonovine (= ergometrine) (Fig. 1.5) are among the simpler lysergic acid amides. The most complex indole steroidal, terpenoid alkaloids of natural origin, the ergopeptines with a C17-amide substituent, have a tripeptide-derived cyclol-lactam structure (Fig. 1.4b) (Schardl *et al*., 2006).

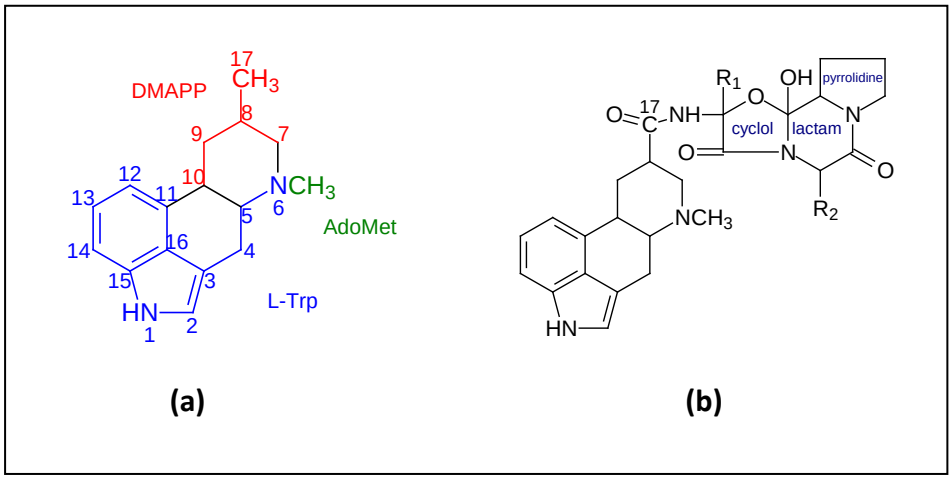


Figure 1.4. *Generic structure of the ergoline-derivatives (a) and the ergopeptines (b). Colour-code in (a) represents the substituent origin: DMAPP, dimethylallyldiphosphate (red); AdoMet, S-adenosyl-methionine (green), and L-Trp, L-tryptophan (blue) (Schardl et al., 2006).*

The most common and pharmacologically active alkaloids produced by *Fusarium* spp. include bostrycoidin, fusaric acid (Fig. 1.5) and lycomarasmine (Mahmood *et al*., 2010). Another alkaloid, roquefortine C (Fig. 1.5), is primarily detected in samples infected by *Penicillium* spp., especially *Penicillium roqueforti* (Polonsky *et al*., 1977; Tiwary *et al*., 2009). Along with 12 known metabolites, two new alkaloids, 9-deacetylfumigaclavine C (Fig. 1.5) and 9-deacetoxyfumigaclavine C, were recently isolated from the cultures of *Aspergillus fumigatus* (Ge *et al*., 2009).

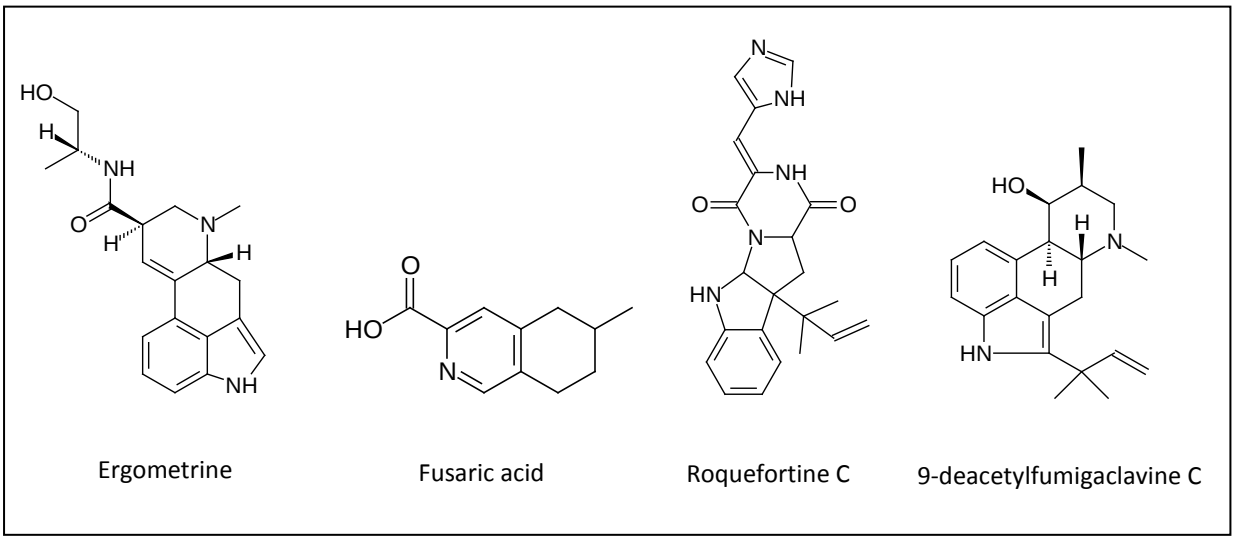


***Figure 1.5**. Examples of fungal alkaloids.*

1.2.4. Classification based on bioactivity

Secondary fungal metabolites possess a vast range of bioactivity. Although a great number of drugs and other beneficial substances of fungal origin have been discovered and put into use, for many other metabolites, already identified, biological activity is not yet revealed.

Physico-chemical properties, steric arrangements of the structure and presence of bioactive moieties are responsible for biological activity of a compound (Betina, 1989).

1.2.4.1. Anti-microbial activity

The anti-microbial activity of secondary fungal metabolites involves anti-bacterial, anti-fungal and/or anti-viral activity (Zhong *et al*., 2009).

Penicillins (a group of related compounds produced by *Penicillium* spp.) discovered in 1928 by Alexander Fleming, are a classical example of anti-bacterial fungal metabolites (Table 1.1) (Bud, 2007). It kills susceptible bacteria by interfering with the cell wall biosynthesis (Zhong *et al*., 2009). The common structural feature of penicillins, a four-membered β-lactam ring, is essential for their anti-bacterial activity (Wilke *et al*., 2005). Other secondary metabolites of *Penicillium* spp., such as funiculosin, also exhibit anti-bacterial activity, whereas fulvic acid,

8-O-methylaverufin and 1,8-O-dimethylaverantin have anti-fungal effects (Ando *et al*., 1969; Kurobane *et al*., 1981; Maskey *et al*., 2003).

A secondary metabolite of *Chaetomium* spp., chaetochalasin A, exhibited anti-bacterial activity against *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 25923) (Oh *et al*., 1998). Cytochalasin A was also effective against *B. subtilis* as well as against *Escherichia coli*, and, as cytochalasin D, demonstrated anti-fungal properties by inducing unusual morphological changes in *Botrytis cinerea* (Betina, 1989). Such activity can be explained by the macrocyclic lactone structure (Betina, 1989). Altomare *et al.* reported anti-fungal activity of two *Fusarium* metabolites, fusapyrone and deoxyfusapyrone. These compounds showed considerable anti-fungal activity against several plant pathogenic and/or mycotoxigenic filamentous fungi and good inhibitory activity toward agents of human mycoses (Altomare *et al*., 2000).

Recently, four novel naphtho-g-pyrones, namely, isochaetochromins B1, B2 and D1 and oxychaetochromin B, produced by *Fusarium* spp., were discovered. Evaluation of their bioactivity revealed that they can serve as inhibitors of HIV-1 integrase (Singh *et al*., 2003). In another study, wickerols A and B, diterpene derivatives isolated from *Trichoderma* sp., showed potent anti-viral activity against the A/H1N1 flu virus (Yamamoto *et al*., 2012). Polyketides from *Penicillium* sp., purpurquinones B and C, purpuresters A and TAN-931, also demonstrated anti-influenza activity (Wang *et al*., 2011).

A single compound can possess a large spectrum of biological activity. An outstanding case of miscellaneous bioactivity is patulin and mycophenolic acid, produced mainly by *Penicillium* spp., which were first isolated as anti-microbial agents. However, in the following years, it was discovered that, in addition to its anti-bacterial and anti-viral activity, patulin and mycophenolic acid were toxic to both plants and animals, and were later reclassified as mycotoxins (Table 1.1) (Bennet and Klich, 2003; Betina, 1989; Ciegler *et al*., 1971).

1.2.4.2. Mycotoxins

Fungi can produce a wide assortment of toxic chemicals, which can pose (even at low concentrations) health risk to humans and animals leading to mycotoxicoses. These compounds were combined into a group called *mycotoxins* [μύκης (mykes, mukos) -

"fungus"; toxicum - "poison"] (Bennett and Klich, 2003). Mycotoxins are known to occur in mycelium of filamentous fungi as well as in spores (D'Mello and Macdonald, 1997; Fox and Howlett, 2008). Since the introduction of the "*mycotoxin*" term in the 1960's when the turkey "X" disease was linked to a peanut meal contaminated with secondary metabolites of *Aspergillus flavus* (Blount, 1961), great scientific attention has been paid to collect data on toxic secondary metabolites produced by different fungi. At present, more than 400 compounds are referred to the group of mycotoxins (Bennett and Klich, 2003).

Mycotoxicoses are categorized as acute or chronic. Acute toxicity manifests within a relatively short time period (few hours to several days) and has obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time period (greater than 3 months), resulting in cancers and other generally irreversible effects (Bennett and Klich, 2003; Williams *et al*., 2003). Chronic exposure (*e.g*., cancer, kidney toxicity, immune suppression) is considered as the main human and veterinary health problem related to mycotoxins. However, acute effects (*e.g*., turkey "X" disease, ergotism, stachybotryotoxicosis) are the most noticeable cases of mycotoxin exposure (Bennett and Klich, 2003).

Aflatoxins ("*A. flavus* toxin") are acutely toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds (Table 1.1) (Blount, 1961; Kensler *et al*., 2011; Peraica *et al*., 1999). Aflatoxins mainly affect liver, and several studies have shown that the development of liver cancer can be induced by aflatoxins in the diet, particularly in individuals already exposed to hepatitis B (Li *et al*., 2001; Peers and Linsell, 1973; Van Rensburg *et al*., 1985). This group of toxins was classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens, except for aflatoxin M1, which is possibly carcinogenic to humans (Group 2B) (IARC, 1987). It has been demonstrated that epoxidation of the unsaturated bonds at the position 8, 9 of the terminal furan ring is critical for the carcinogenic potency of aflatoxin B1 (Fig. 1.2) (Groopman and Kensler, 2005; Kensler *et al*., 2011).

Adverse effects of ergot alkaloids (produced by *Claviceps purpurea*) (Table 1.1) have been known since centuries; in the Middle Ages, outbreaks of ergotism (also known as St. Anthony's Fire) became epidemic and led to death of thousands of people in Europe. It was caused by eating rye bread contaminated with *C. purpurea* (Van Dongen and De Groot, 1995). Toxic effects of ergot alkaloids are due to their structural similarity to several

neurotransmitters (Mulac and Humpf, 2011; Schiff, 2006). Two forms of ergotism have been identified, namely, gangrenous ergotism, which lately occurred in Ethiopia in 1977-78 (King, 1979), and convulsive ergotism characterized by gastrointestinal symptoms (nausea, vomiting and giddiness) and effects on the central nervous system (drowsiness, prolonged sleepiness, twitching, convulsions, blindness and paralysis), last seen in India in 1975 (Peraica *et al.*, 1999). In addition to the above-mentioned symptoms, hallucinogenic properties were recorded for lysergic acid diethylamide, known as LSD (Van Dongen and De Groot, 1995). Owing to improved grain cleaning and milling processes, ergotism is extremely rare in present days (Peraica *et al*., 1999).

The most frequently-occurring ochratoxin from *Aspergillus* and *Penicillium* strains, ochratoxin A, has shown to be nephrotoxic, immunosuppressive, carcinogenic and teratogenic in experimental animals (Peraica *et al*., 1999) and was proposed as a causative agent of endemic nephropathy (Table 1.1) (Goliński *et al*., 1984; Peraica *et al*., 1999). IARC classified ochratoxin A as a substance possibly carcinogenic to humans (Group 2B) (IARC, 1987). The toxic activity of ochratoxin A (Fig. 1.6) has been attributed to its isocoumarin moiety, the lactone carbonyl group and presence of the chlorine atom (Betina, 1989; Xiao *et al*., 1996).

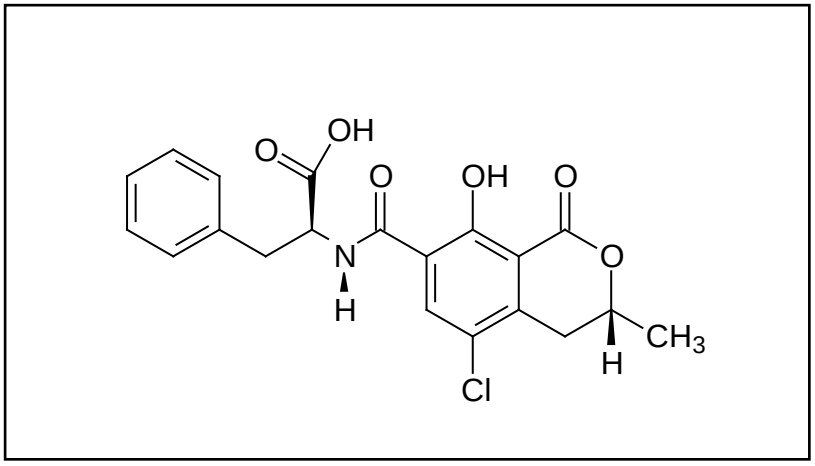


Figure 1.6. *Structure of ochratoxin A.*

The first recognized trichothecene mycotoxicosis was recorded in the USSR in 1932. Common manifestations of trichothecene toxicity are depression of immune responses and

nausea, rarely vomiting (Table 1.1) (Peraica *et al*., 1999). In experimental animals, trichothecenes showed higher toxicity when inhaled compared to oral intake (Smoragiewicz *et al*., 1993). Furthermore, trichothecenes were associated with the Sick Building Syndrome (Croft *et al*., 1986; Smoragiewicz *et al*., 1993); the symptoms of airborne toxicosis disappeared when the buildings and ventilation systems were thoroughly cleaned (Croft *et al*., 1986). There is some evidence that trichothecenes have been used as chemical warfare agents in South-East Asia (Mirocha *et al*., 1983; Wannemacher and Wiener, 1997). A sesquiterpenoid ring structure is responsible for the toxicological activity of the trichothecenes (Lattanzio *et al*., 2009).

Zearalenone is a non-steroidal oestrogenic mycotoxin resembling 17-estradiol, the principal hormone produced by the human ovary (Kuiper-Goodman *et al*., 1987). Zearalenone and its derivatives showed estrogenic effects in various animal species (infertility, vulval oedema, vaginal prolapse and mammary hypertrophy in females and feminization of males) (Kuiper-Goodman *et al*., 1987; Peraica, 1999). IARC has categorized zearalenone as a class 3 carcinogen (IARC, 1993). Interestingly, a derivative of zearalenone, α-zearalanol, is used as a growth promotor in cattle in the USA and other countries (Metzler, 2011).

The group of fumonisins comprises of several structure-related metabolites, of which fumonisin B1 and fumonisin B2 are of toxicological importance, while the others (fumonisins B3, B4, A1 and A2) are less toxic (Table 1.1) (Peraica *et al*., 1999). The toxic effect of fumonisins (as 1-deoxy-analogs of sphinganine) is due to their structural analogy to sphingoid bases (Humpf *et al*., 1998). Symptoms caused by consumption of fumonisin B1 (from food) include transient abdominal pain, borborygmus and diarrhoea (Bhat *et al*., 1997). A high incidence of oesophageal cancer was attributed to the presence of fumonisin B1 in food (in particular, maize) (Peraica *et al*., 1999). IARC classified fumonisins as possibly carcinogenic to humans (Group 2B) (IARC, 1987).

Other mycotoxins can also cause severe health problems, including nephropathy, human tremor, liver damage, vomiting, nausea (Bennet and Klich, 2003; D'Mello and Macdonald, 1997).

1.3. Occurrence

Exposure to secondary fungal metabolites occurs through ingestion of mouldy food or feed as well as through the dermal and inhalation routes (Zain, 2011).

1.3.1. Feed

Feed contamination with fungi and, as a consequence, with their secondary metabolites, is of great concern in feed safety. This issue of contamination has a considerable impact not only on animal welfare, but can potentially lead to toxin transfer into animal products (eggs, meat and milk) for human consumption. As the majority of mycotoxins are very stable compounds, once formed in a feed ingredient, they can contaminate mixed feeds manufactured from it.

A global survey on the incidence of mycotoxins (aflatoxin B1, deoxynivalenol, fumonisins B1, B2 and B3, ochratoxin A, T-2 toxin and zearalenone) was conducted in animal feedstuffs (Binder *et al.*, 2007; Monbaliu *et al*., 2010a; Streit *et al*., 2012). Low contamination levels were found throughout the world, however, significant differences were noted on a regional scale (tropical, temperate areas). Deoxynivalenol, T-2 toxin and zearalenone were the major contaminants in European feeds, whereas aflatoxins, deoxynivalenol, fumonisins and zearalenone were dominant in samples from Asia and the Pacific (Binder *et al.*, 2007). Although aflatoxin production was found to occur primarily in tropical or subtropical climates, from a European prospect, imported feed (peanut cake, palm kernel, copra, corn gluten meal) is considered as the most common source of feed contamination (EFSA, 2004). Despite this, the European Food Safety Authority (EFSA) reported incidence of aflatoxin B1 contamination in European feed. This toxin was recently detected in maize originating from Italy where the growth period was characterized by high temperatures, drought and strong insect damage (EFSA, 2004). The Rapid Alert System for Food and Feed (RASFF) of the European Commission has reported 10 notifications on aflatoxin B1 in maize of European origin since autumn 2012. In 2013, feed originating from Serbia and imported in the Netherlands and Germany was found to be contaminated with aflatoxins (RASFF, http://ec.europa.eu). It was stated that the mycotoxin pattern in Europe might change due to a general global rising of average temperatures (Miraglia *et al*., 2009).

Southern Europe, where the importance of deoxynivalenol diminishes and, at the same time, *Aspergillus flavus* infection and aflatoxin contamination become more common, can be a good example of this phenomenon. Regarding trichothecene mycotoxins, T-2 toxin and HT-2 toxin, EFSA estimated exposure to these particular contaminants as low (EFSA, 2011).

Co-contamination of feed with several mycotoxins is frequently reported. A UK study of maize feed products revealed that all 67 samples were co-contaminated with up to 12 *Fusarium* mycotoxins (Scudamore *et al*., 1998; Streit *et al*., 2012). In 75 % of samples, fumonisins occurred together with deoxynivalenol; and 15-acetyldeoxynivalenol, moniliformin and zearalenone were also frequent co-contaminants. Co-occurrence of deoxynivalenol and zearalenone was reported in 44 % of feed (including silage and compound feed) for dairy cattle in the Netherlands (Driehuis *et al.*, 2008).

Ergot alkaloids were reported in 91 % of the grain and mixed feed samples in Germany. A maximum of 4,883 µg/kg was observed for mixed feed for pigs and the largest observation for rye feed was 1,236 µg/kg (Ruhland and Tischer, 2008). About half of Dutch feed samples contained ergot alkaloids (Mulder *et al*., 2012). Unexpectedly, compound feeds, containing grains as only one of the ingredients, had a relatively higher average ergot alkaloid content compared to cereal feeds. A maximum level of 1,000 mg/kg of rye ergot (*Claviceps purpurea*) has been established for feed containing unground cereals in the European Union (Directive 2002/32/EC, 2002). Many countries have set limits for ergot in grains, but only a few have regulation for the individual and total ergot alkaloid content; for instance, the current limit for the total ergot alkaloid content in animal feed in Uruguay is 450 µg/kg (EFSA, 2005).

In recent times, the problem of masked mycotoxins has begun to emerge. These compounds can be formed from their parent forms as a result of plant metabolism (Berthiller *et al*., 2007), but are not detected with analytical methods, targeted only to parent compounds. It has been shown that the conjugated mycotoxins zearalenone-4-glucoside and deoxynivalenol-3-glucoside can constitute up to 46 % of the total content of the precursor mycotoxin in feedstuffs (De Boevre *et al*., 2012; Berthiller *et al*., 2005b, Berthiller *et al*., 2006). Reports on conjugated forms of ochratoxin A, fumonisins, T-2 toxin and HT-2 toxin are also available (Humpf and Voss, 2004; Lattanzio *et al*., 2012; Ruhland *et al*., 1996; Seefelder *et al*., 2003). It is believed that these conjugates can increase exposure to (the parent) mycotoxins (Gareis *et al*., 1990).

1.3.2. Food

Food safety is a complex issue that has an impact on all segments of society. Fungi are extremely common, being able to grow on a variety of substrates under a wide range of environmental conditions. Secondary fungal metabolites have been detected in agricultural products all around the world. These compounds can enter the food chain at different stages, *e.g.*, in the field, during storage (Fig. 1.7). Detection, removal and diversion are considered to be preventive approaches against the entry of fungal metabolites into the food chains. The severity of the contamination problem is more apparent considering that about 25 % of the world's crops are contaminated to some extent with mycotoxins (Fink-Gremmels, 1999).

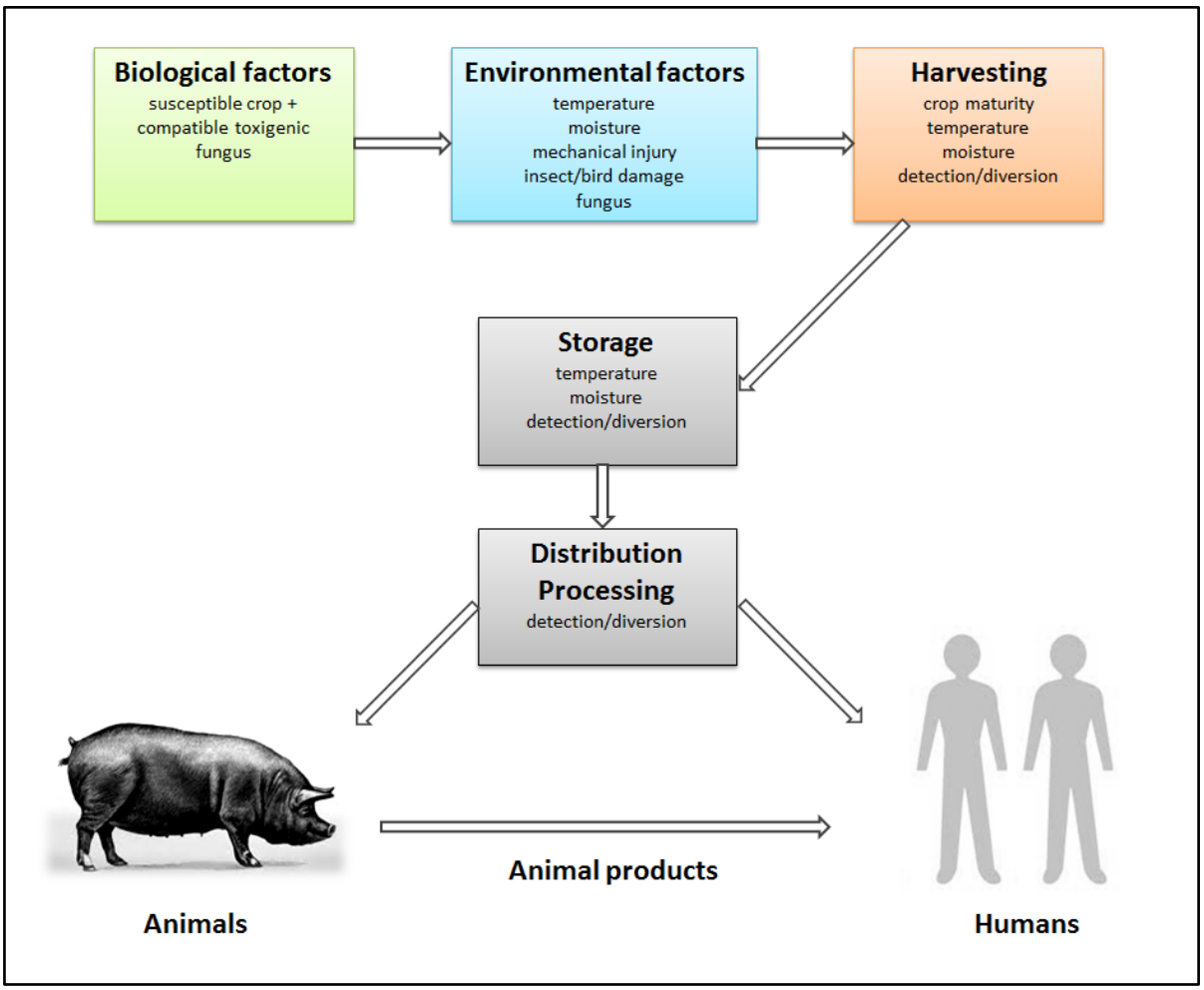


Figure 1.7. *Factors influencing secondary fungal metabolite occurrence in the food chain (adapted from Paterson and Lima, 2010).*

The toxigenic fungi involved in the food chains belong mainly to five genera: *Aspergillus, Fusarium, Claviceps, Alternaria* and *Penicillium* (Lasztity, 2009; Sweeney and Dobson, 1998).

Secondary fungal metabolites can enter the human food chain directly (food consumption) and indirectly (consumption of animal-derived food).

Cereal crops worldwide including corn, wheat, barley, oats and rice were found to be contaminated with one or more fungal metabolites. Zearalenone was recently detected in corn and corn products in the USA (Hewitt *et al*., 2012), Spain (Cano-Sancho, 2012) and Mexico (Briones-Roeys, 2007). Assessment of the natural occurrence of trichothecenes and zearalenone in Argentina was carried out in six different crops during harvest (Quiroga, 1995) revealing contamination of about 50 % of collected samples. All the contaminated samples contained deoxynivalenol. Zearalenone, 3-acetyldeoxynivalenol, T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol were present as well.

Ergot alkaloids are frequent contaminants of cereals. About 90 % of rye-based cereal products were contaminated to some extent with ergot alkaloids (Crews *et al*., 2009). Ergotamine, ergocristine and ergosine were the predominant ergot alkaloids in terms of level and frequency of occurrence.

Fumonisins are a frequently occurring group of mycotoxins in maize. Ediage *et al*. (Ediage *et al*., 2011) detected fumonisins at a maximum concentration of 836 µg/kg accompanied by beauvericin in maize samples from Benin. Maximum levels of fumonisins in maize from Burkina Faso reached 16,040 µg/kg which exceeded the tolerable daily intake by five times (Nikièma *et al*., 2004). As maize is one of the widely grown staple foods in tropical countries, consumption of this commodity can pose risks to public health related to chronic exposure. Additionally, a study in Germany revealed the presence of type A and B trichothecenes in maize with up to twelve metabolites co-occurring in a single sample (Schollenberger *et al*., 2012).

Moroccan 68 cereal products were found to contain fumonisins (B1, B2 and B3); frequency of contamination was 29 % of total samples. The highest value was found in breakfast cereals with 228 µg/kg of total fumonisins (Mahnine *et al*., 2012).

Different spices (including pepper, chilli, curry powder, cayenne, paprika, cinnamon, coriander, turmeric and cumin) were contaminated with aflatoxins. Aflatoxin B1 had the

highest incidence of contamination in spice preparations and was found in 20 of 130 samples. In five samples (4 %), consisting of chilli, cayenne pepper and turmeric pepper, aflatoxin B1 was above the regulatory limits of the European Union (*i.e*., level of 5 µg/kg) (O'Riordan and Wilkinson, 2008). Aflatoxins can be found in other commodities such as cereals, nuts, peanuts, fruits, oilseeds, olives, dried fruits, cocoa, beer (Cavaliere *et al*., 2007; Copetti *et al*., 2011; Daradimos *et al*., 2000; Fernández-Cruz *et al*., 2010; Ghitakou *et al*., 2006; Leszczynska *et al*., 2000; Tam *et al*., 2006; Ventura *et al*., 2004; Ventura *et al*., 2006). These metabolites were even detected in smoked-dried fishes in Nigeria, however, the food was still acceptable for consumption (aflatoxin levels were lower than the FDA prescribed maximum concentration of 20 µg/kg) (Adetayo, 2011).

Alternariol, alternariol monomethyl ether and tenuazonic acid were frequently detected in apples, apple products, apple juice concentrates, mandarins, olives, pepper, red pepper, tomatoes, tomato products, oilseed rape meal, sunflower seeds, sorghum, wheat and edible oils (olive oil, rapeseed oil, sesame oil, sunflower oil) (Ostry, 2008; Scott, 2001). Moreover, alternariol and its derivative, alternariol monomethyl ether, were found in raspberries, red currant, barley, oats and carrots (Scott, 2001; Solfrizzo *et al*., 2004a).

The use of mouldy raw materials (*e.g*., grapes, coffee beans) in beverage production can lead to contamination of the final product with fungal metabolites, since the majority of these substances are heat-resistant. Natural occurrence of alternariol has been reported in apple juice, cranberry juice, grape juice, prune nectar, raspberry juice, red wine (Lau *et al*., 2003; Scott, 2001; Scott *et al*., 2006). The highest level of this toxin in apples was up to 58,800 µg/kg. Chaetoglobosins, communesins and patulin were detected in juices as well (Andersen *et al*., 2004; Cho *et al*., 2010). A survey on ochratoxin A in wine originating from European (*e.g*., Greece, Italy, France, Portugal) and other countries (*e.g*., Morocco) revealed maximum concentrations of ochratoxin A up to 7.6 µg/L (Magan and Olsen, 2004). High concentrations of ochratoxin A (max. 2,340 µg/L), aflatoxins (max. 400 µg/L) and zearalenone (max. 426 µg/L) were detected in home-made South African beers, for which maize and barley were used in brewing. The mycotoxin levels were much higher than the recommended values for safe consumption (Odhav and Naicker, 2002). Several surveys on tea and herbal infusions revealed presence of fumonisin B1 at concentrations up to 1,487 µg/kg (Martins *et al*., 2001;

Monbaliu *et al*., 2010b; Omurtag and Yazicioglu, 2004). Ochratoxin A and aflatoxin B1 were natural contaminants of coffee (Bokhari, 2007).

To determine transmission of fungal metabolites from mouldy feed to animal tissue, carry-over studies in livestock have been conducted, however, only for major mycotoxins. Ochratoxin A in pig feed can accumulate in porcine tissues (Milićević *et al*., 2009), while some amounts of aflatoxins, citrinin and deoxynivalenol can be carried over into eggs (Völkel *et al*., 2011). Aflatoxin B1 in feed can be metabolized to a hydroxylated derivative, aflatoxin M1, by cows which is then secreted in milk. This metabolite could be therefore detected in dairy products (cheese) (Anfossi *et al*., 2012; Yaroglu *et al*., 2005).

To prevent formation of secondary fungal metabolites (specifically mycotoxins), the food industry has established internal monitoring methods. Furthermore, government regulatory agencies constantly initiate surveys on occurrence of mycotoxins in foods and feeds and establish regulatory limits.

1.3.3. Indoor environment

Secondary fungal metabolites can also be associated with mouldy indoor environments (damp buildings). Building-associated fungi, such as *Stachybotrys chartarum*, *Aspergillus versicolor*, *A. sydowii*, *Penicillium* spp., *Chaetomium* spp., comprise a limited group of species that can grow on nutrients available from building materials and adapt to particular amount of available water (Jarvis and Miller, 2005).

In the 1980s the term Sick Building Syndrome (SBS) was introduced describing a group of symptoms of unclear aetiology comprised of mucous membrane symptoms related to eyes, nose, and throat, dry skin, and general symptoms of headache and lethargy. The symptoms were temporally and related to leaving or working in a particular building (Burge, 2004; Murphy, 2006). Mould growth and production of secondary metabolites are considered as one of the causes of the syndrome (Bennett and Klich, 2003). As mycotoxins are not volatile, respiratory exposure is related to inhalation of mould spores, hyphal fragments and contaminated dust (Bennett and Klich, 2003).

A proof of natural fungal metabolite presence indoor was provided by Bloom *et al.* by analyzing more than 100 building samples (settled dust, gypsum board, wallpaper and other)

(Bloom *et al*., 2007; Bloom *et al*., 2009). Several mycotoxins, namely sterigmatocystin, gliotoxin, satratoxins G and H, verrucarol and trichodermol, could be detected in water-damaged buildings. Interestingly, in most cases the natural producer of a certain metabolite was identified in the sample. Polizzi *et al*. were able to detect roquefortine C, chaetoglobosin A, sterigmatocystin, roridin E, ochratoxin A, aflatoxins B1 and B2 in 69 % of building materials, and dust and air samples (Polizzi *et al*., 2009). As in the study of Bloom *et al*., there was a good concordance between identified fungi and detected metabolites. Mouldy interiors of Finnish damp buildings also contained sterigmatocystin, satratoxins G and H as well as diacetoxyscirpenol, deoxynivalenol, 3-acetyldeoxynivalenol, verrucarol, T-2-tetraol and citrinin. *Aspergillus versicolor* was identified in most sterigmatocystin-containing samples, and, similarly, *Stachybotrys* spp. were present in the samples with satratoxins. However, in many other cases, the presence of fungi was not in accordance with the found metabolites (Tuomi *et al*., 2000).

An extensive study of secondary fungal metabolites in moisture-damaged indoor environments was performed by Täubel *et al.* (Täubel *et al*., 2011). 186 fungal metabolites were included in the LC-MS-based method which revealed presence of 28 compounds with emodin, enniatin B and beauvericin being the most prevalent analytes and frequently detected in dust.

1.3.4. Biological material

The link between the observed toxic effect (or disease) and a certain dietary contaminant is represented by biomarkers. Biomarkers are divided into two categories, namely direct (biomarkers of exposure) and indirect (biomarkers of effect). A biomarker of exposure refers to the quantification of a specific compound, its metabolite(s) or interaction products in a body compartment or fluid. These biomarkers can give an indication of the presence and magnitude of exposure to the contaminant. A biomarker of effect indicates the presence and magnitude of a certain biological response following exposure to the contaminant (Dragusel, 2011; Kensler *et al*., 2011). The most common parameters in quantifying exposure or effect are available from urine, serum and milk, but also feces and hair (Dragusel, 2011).

As aflatoxin B1 is among the most harmful mycotoxins and can be detected in numerous commodities, it was one of the first included in biomarker studies to human exposure.

Serum aflatoxin B1-albumin adducts, pointing to chronic exposure, and urinary aflatoxin B1-N7-guanine adduct, revealing actual genetic damage, are validated biomarkers of aflatoxin exposure (Wild *et al*., 1990; Groopman *et al*., 1992; Jarvis and Miller, 2005). Metabolic products of aflatoxin B1, namely aflatoxin M1 and aflatoxin B1-mercapturic acid, in urine can be good indicators of recent exposure (Jarvis and Miller, 2005). Assessment of fumonisin B1 exposure to animals can be estimated through changes in ratio of sphinganine to sphingosine in urine and blood (Cai *et al*., 2007; Kim *et al*., 2006; Tran *et al*., 2006). However, the use of these biomarkers to human exposure was less promising (Abnet *et al*., 2001; Jarvis and Miller, 2005; Nikiema *et al*., 2008; Solfrizzo *et al*., 2004b). A strong correlation was found between the sum of deoxynivalenol and its urinary metabolite deoxynivalenol-3-glucuronide and cereal intake of the population (Turner *et al*., 2008). Therefore, the sum of these two metabolites in human urine has been recommended for monitoring of deoxynivalenol exposure (Ediage *et al*., 2012). Presence of ochratoxin A and its metabolites ochratoxin α and the hydroxylated form, 4-hydroxyochratoxin A, was reported in urine samples (Jonsyn-Ellis, 2000; Muñoz *et al*., 2010). Zearalenone can be metabolized through hydroxylation, producing the metabolites α-zearalenol and β-zearalenol, and subsequent conjugation with glucuronic acid (Zinedine *et al*., 2007). To date, presence of α-zearalenol-glucuronide and β-zearalenol-glucuronide in human urine has not been reported.

Secondary fungal metabolites (specifically mycotoxins) can also adversely affect human and animal health through a dermal route. Two methodologies (*in vivo* and *in vitro*) to study mycotoxin absorption via skin have been described (Kemppainen, 1988-1989). The skin permeation studies of mycotoxins were generally focused on a few compounds. It was shown that aflatoxin B1 and trichothecenes were able to penetrate skin (Kemppainen, 1988-1989). Aflatoxins applied to the skin of rabbits in an *in vivo* experiment penetrated the stratum corneum and caused changes in epidermis and dermis (Joffe and Ungar, 1969). An *in vitro* study, intended to simulate the conditions which occur when agricultural workers are exposed to corn dust contaminated with T-2 toxin, demonstrated that T-2 toxin can penetrate through the skin (Kemppainen, 1984).

1.4. References

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CHAPTER 2

Liquid chromatography – mass spectrometry

2.1. General information

Investigation of secondary fungal metabolites is a complicated task which involves dedicated analysts and specialized equipment. From the analytical point of view, samples (*e.g.*, mouldy feed, mouldy building materials) used for analysis of fungal metabolites are considered as complex matrices. They can contain not only the analytes of interest, but also an unlimited number of matrix components which can disturb the analysis; therefore, sensitive and selective analytical methods are vital.

Separation methods for secondary fungal metabolite analysis include thin-layer chromatography (TLC), capillary electrophoresis, high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Cigić and Prosen, 2009). For the detection of the metabolite response, UV, fluorescence detector and mass spectrometer (MS) are frequently used. Each technique has its limitations. For instance, the disadvantage of GC technique compared to LC is the necessity of prior derivatization of the analyte which limits the use of this method in non-targeted analysis (Villas-Bôas *et al*., 2005). UV detection has lesser sensitivity and specificity in the analysis of complex matrices as compared to MS. Matrix components can interfere with the targeted analyte and exhibit similar UV spectra. Additionally, it has been observed that, depending on the compound, metabolized molecules may have the same retention time and UV spectral characteristic as the parent molecule (Anonymous, 2012).

Nowadays, LC-MS is the most frequently employed hyphenated technique in qualitative and quantitative analysis of secondary fungal metabolites owing to its high sensitivity, high-throughput and possibility to confirm the identity of the components present in complex samples (Songsermsakul and Razzazi-Fazeli, 2008; Villas-Bôas *et al*., 2005).

LC is a separation technique that is based on distributing the components of a mixture between two phases, a stationary phase and a mobile phase, depending on the relative affinity of the molecules to the phases (Scott, 2012). Due to differences in physico-chemical properties, each compound has a characteristic affinity to the phases. The separation of analytes in multi-analyte methods is achieved with HPLC and the more recently emerged technique, ultra high performance liquid chromatography (UHPLC or UPLC). By using smaller particles of packing material and higher flow rates, UPLC becomes more popular in

secondary fungal metabolite analysis due to the increased speed of analysis, higher separation efficiency and resolution as compared to HPLC (Kokkonen and Jestoi, 2010; Swartz, 2005). The two main types of LC are normal-phase (NP) and reversed-phase (RP) LC. Of these two types, RP-LC is most widely used in the separation of fungal metabolites due to the essentially inert stationary phase allowing exploitation of a diverse range of solvent mixtures (addition of salts, pH alteration) (Fallon *et al*., 1987; Lattanzio *et al*., 2007; Spanjer *et al*., 2008; Wilson and Walker, 2010; Zhang *et al*., 2013). Recently, a new particle technology called Fused-Core has been introduced in separation science. Such particles are manufactured by "fusing" a porous silica layer onto a solid silica particle. Due to shorter diffusion path, columns based on this technology can be used at higher flow rates still providing good peak resolution (DeStefano *et al*., 2008; HALO Brochure, http://www.gc-lc.com/HALO_Brochure(2).pdf).

An alternative LC mode for separation of polar compounds, namely hydrophilic interaction LC (HILIC), has also been used for the analysis of secondary fungal metabolites (Fæste *et al*., 2012; Sørensen *et al*., 2007). HILIC is a variant of NP-LC, and it employs polar stationary phases such as silica or amino; however, the mobile phase used is similar to those employed in the RP-LC mode (Buszewski and Noga, 2012).

The principle of MS is based on ionization of molecules or atoms in a sample and their separation according to mass-to-charge (*m/z*) ratio and further detection by the respective *m/z* and abundance (Barker and Ando, 1999; Gross, 2011). A variety of ionization modes and types of mass analyzers is currently available making MS useful in routinely control of targeted analytes as well as in the identification of less studied or unknown secondary fungal metabolites.

In the atmospheric pressure ionization (API) LC-MS techniques, ions are formed at atmospheric pressure and transferred into the vacuum of the mass analyzer (Bruins, 1994; Nielsen *et al*., 2004). The API includes electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) (De Hoffmann and Stroobant, 2007).

In ESI, ionization occurs in the liquid phase (Kokkonen, 2011). The eluent from the column is sprayed through a narrow bore capillary to which a high voltage is applied; and a spray of highly charged droplets is produced (Fig. 2.1a).

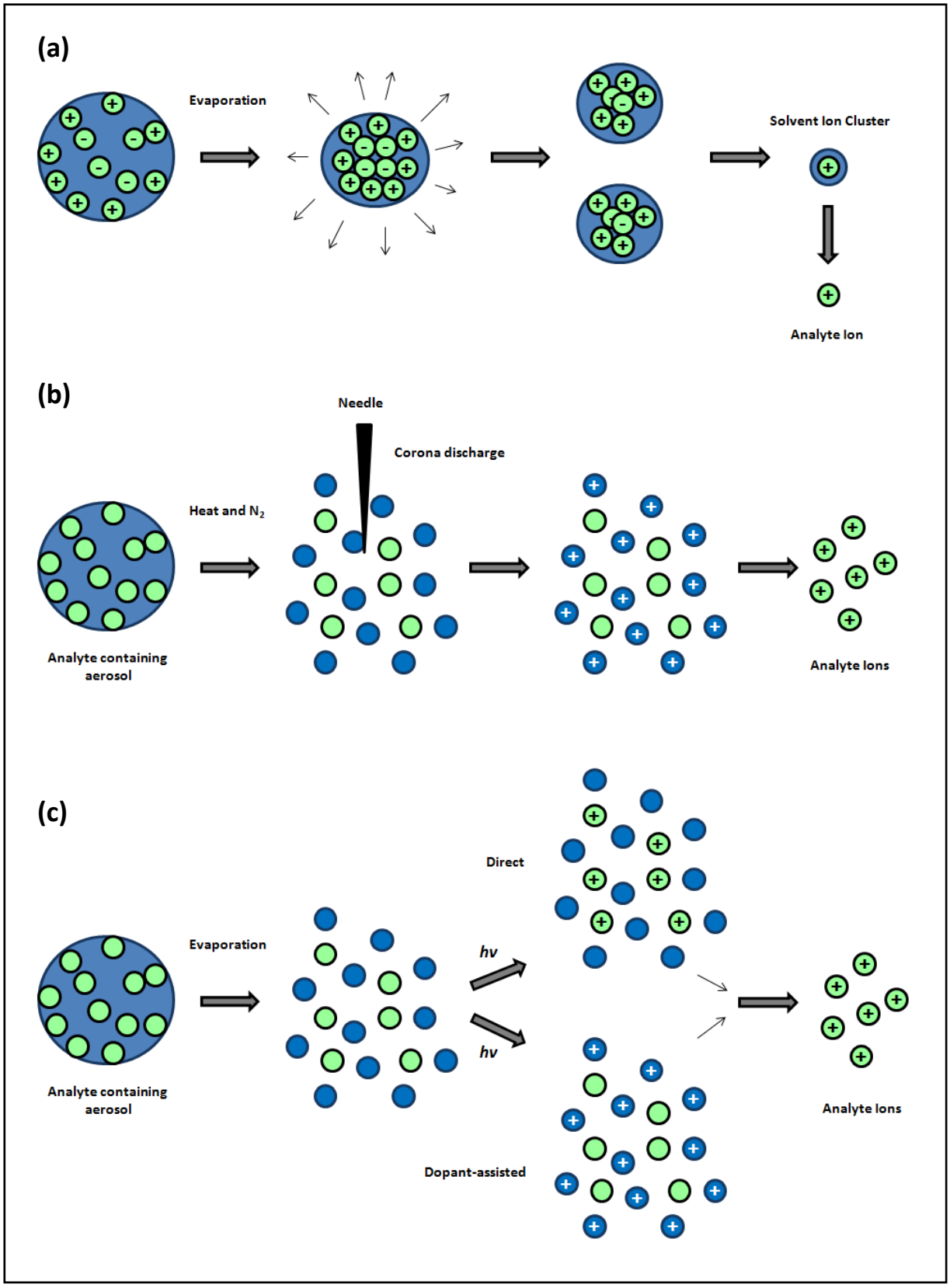

Figure 2.1. *ESI (a), APCI (b) and APPI (c) ionization process (adapted from Downard, 2004; Nieckarz, 2010).*

The solvent is evaporated from the droplets by a heated gas, leading to shrinkage and disintegration to charged species through a complex process. The ions are formed either in the solvent before spraying or during the spray droplet shrinkage. The charged species are then sampled into the vacuum of the mass analyzer (Nielsen *et al*., 2004). In APCI the eluent from the HPLC column is sprayed through a co-axial capillary with a heated gas to evaporate the solvent (Fig. 2.1b). Evaporated solvent molecules are ionized by a corona discharge from a needle that is usually placed across the sampling orifice. Analyte molecules are ionized by chemical reaction in the gas phase at atmospheric pressure (Nielsen *et al*., 2004).

Unlike ESI, APCI does not produce multiply charged ions, therefore, it cannot be applied in the analysis of compounds with high molecular mass (Ashcroft, 1997). In APPI the liquid eluent is vaporized in a heated nebulizer to form a cloud of gas-phase analytes (Fig. 2.1c). Ionization is initiated by a beam of photons emitted from a gas discharge lamp (UV lamp; potential 10 eV). There are two ways of APPI, namely direct and dopant-assisted APPI (DA-APPI). In the first format, an analyte molecule absorbs the photon directly. It occurs when the ionization energy of the analyte molecule is lower than the photon energy. In the DA-APPI, a dopant (*e.g*., toluene, acetone, phenol) is infused into the ionization region. The dopant absorbs photons and ionizes either the sample molecules by charge exchange or the solvent molecules which, in turn, ionize sample molecules. APPI is used to ionize non-polar and polar low-mass compounds (Dass, 2007; Wanner and Höfner, 2007).

Though ESI is the dominating ionization method in LC-MS analysis of secondary fungal metabolites (Åberg *et al*., 2013; Diana Di Mavungu *et al*., 2012; Ediage *et al*., 2011; Krska *et al*., 2008; Şenyuva *et al*., 2008), APCI and APPI are also utilized (Biselli *et al*., 2005; Razzazi-Fazeli *et al*., 2002; Takino *et al*., 2003).

A wealth of mass analyzers is currently available for the analyst. Mass analyzers use electric and magnetic fields (combined or alone) to separate the ions according to *m/z*; and the main difference between them is the way in which these fields are used to achieve the separation (De Hoffmann and Stroobant, 2007). The most common mass analyzers used for a variety of analytes including secondary fungal metabolites, are quadrupole, ion trap, time-of-flight (TOF), Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR) (Bartók *et al*., 2006; Diana Di Mavungu *et al*., 2012; Şenyuva *et al*., 2008; Siegel *et al*., 2009; Zachariášová *et al*., 2011). Hybrid combinations and variants of these analyzers are also employed for structural

characterization (Herebian *et al*., 2009). In triple quadrupole, ions are separated based on the stability of their trajectories in the oscillating electric fields that are applied to the rods (Barker and Ando, 1999; De Hoffmann and Stroobant, 2007). In ion trap, ions are trapped and stored within the trap using an oscillating electric or magnetic field where collisionally-induced dissociation (CID) can be performed to produce MS/MS or multiple stage MS data. Traps that make use of magnetic fields are called ICR. TOF MS separates the ions and measures *m/z* based on the time they take to pass ('fly') from the ion source to the detector (Downard, 2004).

Mass resolution and accuracy are the two most important factors for the identification of compounds whereas scan speed is of most chromatographic importance (Nielsen *et al*., 2004). The performance of common mass analyzers is shown in Table 2.1.

Table 2.1. *Performance of common mass analyzers (summarized from De Hoffmann and Stroobant, 2007; Holčapek et al., 2010; Hu et al., 2005; Kazakevich and LoBrutto, 2007; Lu et al., 2008; Nielsen et al., 2004).*

Mass analyzer	Resolving power (FWHM)[a]	Mass accuracy (ppm)	Scan speed[b]	Dynamic range[c]
Quadrupole	1000-2000	100-300	high	$>10^4$
Ion trap	1000	100-300	high	$>10^3$
Time-of-flight	>10,000	2-5	very high	$>10^3$
Orbitrap	>100,000	<1-5	high	$>10^3$
Fourier transform ion cyclotron resonance	>500,000	0.05-0.1	very high	$>10^4$

[a] FWHM: full width at half-maximum

[b] scan speed defined as the rate at which analyte measures over a particular mass range

[c] dynamic range is defined as the range of ion counts over which a linear response is obtained

Mass resolution usually refers to the ability of separating two narrow mass spectral peaks (Metabolomics Fiehn Lab, http://fiehnlab.ucdavis.edu/). The group of low resolution mass

analyzers typically includes quadrupole and ion trap analyzers with resolving power in the range of few thousands using the full width at half-maximum (FWHM) definition and without the possibility of accurate determination of *m/z* values (Holčapek *et al*., 2010). High resolution (resolving power in the range of tens of thousands; TOF) and ultra-high resolution (>100,000 FWHM; Orbitrap, ICR) analyzers give the highest confidence in the spectra interpretation and analyte identification (Holčapek *et al*., 2010).

Simply stated, mass accuracy is the ability to measure or calibrate the instrument response against a known entity. Commonly expressed in parts per million (ppm), the mass accuracy indicates the deviation of the instrument response from a known monoisotopic calculated mass (Balogh, 2004). High mass accuracy (<5 ppm; TOF) or even better ultra-high mass accuracy (<1-5 ppm; Orbitrap, ICR) significantly reduces the number of possible elemental compositions for the ion within a given mass tolerance (Holčapek *et al*., 2010).

As a conclusion, quadrupole and ion trap mass analyzers possess sufficient features to serve in targeted metabolite analysis, whereas TOF, Orbitrap and FT-ICR MS have a great potential for untargeted full MS screening of secondary fungal metabolites due to superior full-scan sensitivity, mass accuracy and resolution. Owing to the ability of consequential fragmentation, the ion trap MS can also be helpful in the establishment of fragmentation pathways of an analyte and its structure elucidation. Having outstanding accuracy and resolution, the FT-ICR analyzer is however rarely used for the LC-MS analysis of secondary fungal metabolites which is likely to be attributed to its high costs.

2.2. Targeted LC-MS analysis: strategy and methods

Targeted LC-MS analysis comprises of detection of a pre-selected metabolite in a sample and determination of its concentration (if reference standard is available). To unambiguously confirm the targeted analyte, a mass spectrometer capable of fragmentation is used. Tandem mass spectrometry, abbreviated as MS/MS, is commonly accomplished with triple quadrupole. In this two-stage mass analysis, a combination of a specific precursor mass and (a) unique product ion(s) is used to selectively monitor and unambiguously confirm the analyte to be quantified (De Hoffmann and Stroobant, 2007; Songsermsakul and Razzazi-Fazeli, 2008).

For increased sensitivity and selectivity, data acquisition is performed in the selected reaction monitoring (SRM) mode. Generally, two SRM transitions are selected for each of the analytes. As stated in Commission Decision 2002/657/EC (Commission Decision, 2002), for positive identification of an analyte in a sample, three criteria should be met:

1) A peak with a signal-to-noise (S/N) ratio of at least 3 should be identified for each of SRM transitions;

2) The deviations of relative ion intensities for the SRM transitions should not be greater than the maximum permitted tolerances according to the Commission Decision. The relative ion intensities expressed as a percentage of the intensity of the most abundant ion should correspond to those of the ions in the solutions of standards, with a maximum permitted deviation of 20 % (relative ion intensity > 50 %), 25 % (relative ion intensity: 21-50 %), 30 % (relative ion intensity: 11-20 %), 50 % (relative ion intensity < 10 %);

3) The relative retention times with regard to the internal standard should be below the maximum permitted deviation of 2.5 %.

Thus, LC-MS/MS methods can discriminate different analytes by a combination of chromatographic, precursor ion and product ion(s) data. However, it is not always possible to obtain two specific product ions. A good example is moniliformin which exhibited only one abundant product ion (Sulyok *et al*., 2006). The MS spectra of type B trichothecenes showed $[M+CH_3COO]^-$ adducts as the main precursor ion, which produced either the acetate ion (*m/z* 59) or the $[M-H]^-$ ion as the most abundant product ions. Despite being non-specific, these product ions were used for quantification, while a third transition (less abundant) was added for identification (Sulyok *et al*., 2006).

If required, the number of fragmentation steps can be increased; this is referred to as multistage MS and labelled as MS^n. For this purpose, ion trap is used allowing a maximum number of fragmentation steps of seven to eight (De Hoffmann and Stroobant, 2007).

A number of single- or multi-analyte LC-MS/MS methods has been developed in recent years; the majority of them focuses on quantification of mycotoxins (Ediage *et al*., 2011; Freitas-Silva *et al*., 2011, Kataoka *et al.*, 2009; Kokkonen and Jestoi, 2009; Krska *et al*., 2008; Lattanzio *et al*., 2007; Lau *et al*., 2003; Reinsch *et al*., 2007). Only few studies reported LC-MS

methods which included secondary fungal metabolites other than mycotoxins (Abia *et al*., 2012; Lehner *et al*., 2011; Vishwanath *et al*., 2009).

Separation of the targeted analytes prior to MS analysis is mostly done utilizing HPLC (Berthiller *et al*., 2005; Diana Di Mavungu *et al*., 2012; Ediage *et al*., 2011; Lattanzio *et al*., 2011; Sewram *et al*., 2000; Sulyok *et al*., 2006); however, in the last years, the popularity of UPLC in secondary fungal metabolite analysis is rising (Beltrán *et al*., 2006; Kokkonen and Jestoi, 2010; Monbaliu *et al*., 2010; Romero-González *et al*., 2009; Ventura *et al*., 2006). As an example, UPLC-MS/MS analysis of 10 ergot alkaloids could be finished in only 6 minutes (Kokkonen and Jestoi, 2010). In another study, although the advantages of a fast UPLC were not fully exploited, it was possible to separate the isomers 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol, and fumonisin B2 and fumonisin B3 (Monbaliu *et al*., 2010). Only a few studies reported a base-line separation of these compounds (Kotal and Radová, 2002), others relied on the difference of characteristic product ions under the SRM mode (Ren *et al*., 2007).

Regarding the MS part of analysis, an important factor which can influence the sensitivity of a targeted LC-MS method is the production of different types of ions in the ionization processes of the API techniques. Rundberget and Wilkins (Rundberget and Wilkins, 2002) compared the spectra of some mycotoxins in APCI and ESI ionization modes using a mixture of methanol and water containing ammonium acetate as mobile phase. APCI (positive) as well as ESI (positive and negative) inlets resulted in mainly (de)protonated molecules, however, some analytes demonstrated loss of water or formation of adducts, such as $[M+NH_4]^+$, $[M+Na]^+$. Mycophenolic acid and chaetoglobosin A also formed higher mass addition ions ($[2M+H]^+$, $[2M+Na]^+$) which reduced the ion current of the protonated molecule by 50-60 % of the total ion current. In negative ESI, $[M+HCOO]^-$ and $[M+CH_3COO]^-$ ions were often observed along with deprotonated molecule. Adduct formation was also experienced by other researchers (Beltrán *et al*., 2009; Rasmussen *et al*., 2010; Sulyok *et al*., 2006). Careful selection of mobile phase composition and ion source parameters can help to reduce/eliminate undesirable adducts (Nielsen and Smedsgaard, 2003; Rundberget and Wilkins, 2002).

The most common mass analyzer for quantitative analysis of secondary fungal metabolites (especially, mycotoxins) is triple quadrupole MS (Ediage *et al*., 2011; Martos *et al*., 2010;

Monbaliu *et al*., 2009; Sulyok *et al*., 2006; Rasmussen *et al*., 2010). It is cost-effective and easy to operate. The triple quadrupole mass analyzer has adequately high data acquisition rates for quantitative multi-analyte analysis to provide enough data points across the chromatographic peak, even if co-elution of some compounds is unavoidable (Kokkonen, 2011).

Besides triple quadrupole, other mass analyzers or hybrid mass spectrometers are utilized in targeted secondary fungal metabolite analysis. A hybrid triple quadrupole/linear ion trap mass spectrometer was used for quantification of 159 fungal metabolites in indoor matrices (Vishwanath *et al*., 2009) and 340 fungal metabolites in cereals, nuts and their products (Abia *et al*., 2012). The data acquisition was done in positive and negative polarities in two separate chromatographic runs. In the analysis of such a wide range of compounds, the main challenge is the time available for data acquisition of each of the transitions. To ensure enough time for acquisition, a scheduled multiple reaction monitoring (sMRM) mode was used. In this mode a separate retention time window is defined for each analyte, thus, each MRM transition is monitored only around the expected retention time (Schreiber and Pace, 2010).

Takino *et al.* (Takino *et al*., 2008) performed analysis of selected toxic macrocyclic trichothecenes using LC-MS/MS and LC-TOF-MS. Detection of two product ions and a mass error for the precursor ion of less than 2 ppm enabled confirmation and quantification of roridin L2, satratoxin G, satratoxin H and iso-satratoxin F in the spores of *Stachybotrys chartarum*. Although it is claimed that less noise is observed using high-resolution instruments due to high mass resolving power and therefore narrow mass windows (Lehner *et al*., 2011), limits if detection (LODs) of the LC-TOF-MS method were 6 to 60 times higher than those of the LC-MS/MS (Takino *et al*., 2008).

Lehner *et al.* established a sensitive LC-Orbitrap-MS method for quantification of 20 relevant mycotoxins in food (Lehner *et al*., 2011). Simultaneously, a suspects screening of 200 other fungal metabolites was performed. Suspects screening can be defined as a kind of targeted analysis where full-scan data are examined for a list of analytes of interest (Krauss *et al*., 2010). Working in such a way allowed the detection of 13 additional metabolites. Orbitrap-MS used for quantitative analysis of regulated mycotoxins in cereal grains and grain foods

showed similar performance in terms of detection limit, recovery, repeatability and matrix effect as well-established triple quadrupole-based approaches (Lattanzio *et al*., 2011).

2.3. Untargeted LC-MS analysis: strategy and methods

Detection of less studied or novel compounds promotes further developments in science and, as a consequence, gives rise to new targeted methods. In case of secondary fungal metabolite analysis, discovery of such compounds is crucial since they might represent a danger for human or animal health.

Strictly defining, untargeted analysis presumes no a priori information on the compounds to be detected. However, some studies are designed in such a way that an unknown peak can correspond to a structure having a close relationship with the parent (known) compound and an adequate control sample is available (Cary *et al*., 2006; Cirlini *et al*., 2012). Independently on the approach, identification of untargeted analytes is challenging and requires sophisticated instrumentation and several data processing steps (Krauss *et al*., 2010). The initial step in the workflow of identification of untargeted compounds (Fig. 2.2) is full-scan data acquisition followed by (automated) peak detection.

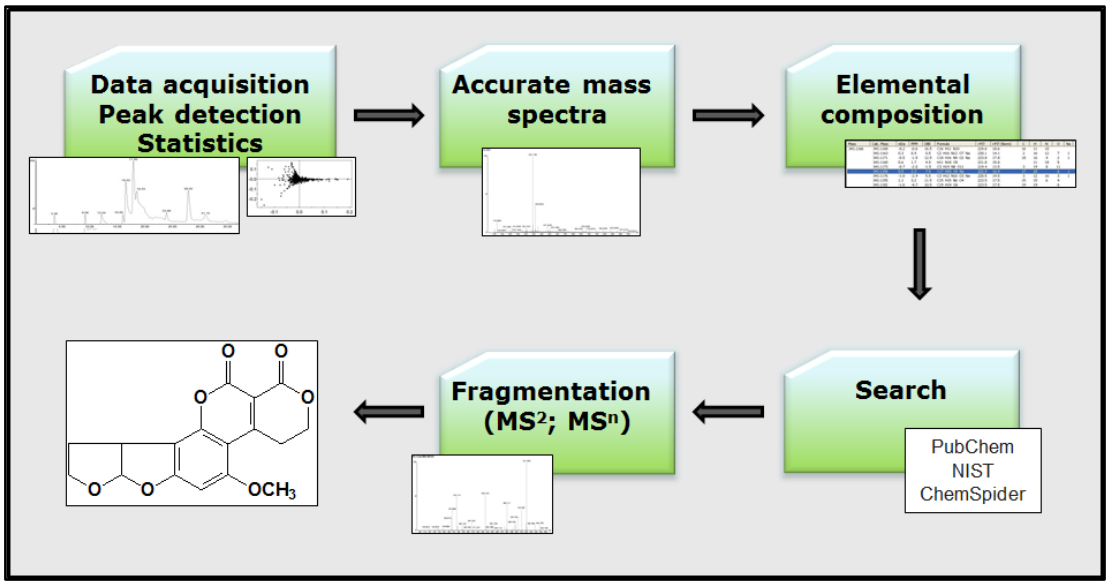

Figure 2.2. *General workflow for identification of untargeted compounds.*

The advantage of accurate mass instruments at this step is that they can provide high selectivity by performing the exact mass chromatograms with a narrow mass window. An example illustrating such potentiality is shown in Fig. 2.3. A peak of roquefortine C was extracted from the total ion chromatogram using the nominal mass (Fig. 2.3a) and the exact mass of the metabolite (Fig. 2.3b). The obtained S/N ratios of these peaks differ considerably. It is clear that a narrow mass window helps to reduce interferences in the chromatogram. This is of great importance especially for low-intensity metabolites. Due to the complexity of the spectral data, the step of peak detection is often performed automatically by a software using different peak detection algorithms (*e.g.*, SIEVE™, MarkerLynx™, MZmine™) (Katajamaa, 2006; Krauss *et al.*, 2010; Stumpf and Goshawk, 2004; Sutton *et al.*, unknown; Vilas-Bôas *et al.*, 2005).

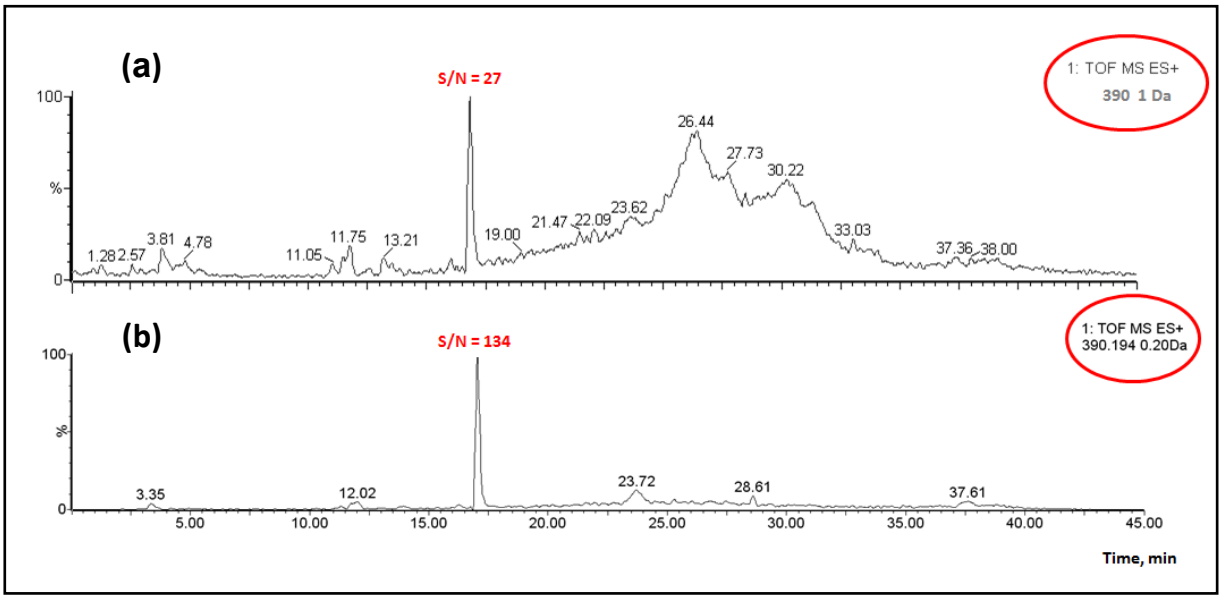

Figure 2.3. *Signal-to-noise ratio (S/N) of a peak of roquefortine C (extracted from Penicillium sp. grown on wallpaper) in the nominal mass chromatogram (a) and the exact mass chromatogram (b).*

Statistical analysis can be applied to a data matrix produced from raw data to allow identification of variables (metabolites) that explain most of variation between datasets. Multivariate classification methods, such as, Principal Component Analysis (PCA), Partial

Least Squares Analysis (PLS) or cluster analysis, are widely-used statistical tools for this purpose (Sugimoto *et al*., 2012). PCA converts high dimensional data into fewer dimensions, by projecting the data into a reduced dimensional sub-space, while maintaining as much variance from the original data as possible. The procedure is repeated until the datasets can be presented usually within two or three dimensions (Nyamundanda *et al*., 2010; Sugimoto *et al*., 2012). Clustering analysis divides observed datasets into several sub-classes or clusters based on a selected statistical distance function; in this way datasets with similar metabolomic profiles are placed in each sub-group (Blekherman *et al*., 2010). PLS, a regression-based method, builds a low-dimensional sub-space based on linear combinations of the original X variables. It makes use of additional Y information by adjusting the model to capture the (Y)-related variation into the original X variables. The goal of PLS regression is to predict Y from X and to describe their common structure (Abdi, 2003; Sugimoto *et al*., 2012).

For the exact mass of interest, an elemental formula is assigned within a set mass tolerance (Fig. 2.2). It is unnecessary to set up high element limits for formula generation. For instance, it has been shown that for 47 carbon atoms the maximum hydrogen number does not exceed 150 using the most common six elements (C, H, N, S, O, P and Si) (Kind and Fiehn, 2007). Software (*e.g*., XCalibur™) generates a list of formulas corresponding to the given exact mass and disregards the physical existence of chemical structures. Thus, some formulas can be either wrong or contain an unlikely high or low number of elements. These unreasonable elemental compositions can mislead the analyst and should be manually or automatically (Kind and Fiehn, 2007) filtered. Analyzing a large set of molecular formulas available from mass spectral databases, it has been observed that the hydrogen/carbon ratio (H/C) does not exceed 3 with a few exceptions (*e.g*., methylhydrazine CH_6N_2). For the majority of formulas this parameter was in the range from 2.0 to 0.5 (Kind and Fiehn, 2007). However, this rule has to be handled with caution as there are different chemical classes which can fall out of this range.

For a successful compound elucidation, information on the isotopic pattern should also be taken into consideration. The abundance of the isotope ions (M+1, M+2, M+3) is dependent on the actual elemental composition and can therefore serve as a powerful filter in calculating unique elemental compositions. It has been shown that applying an isotopic abundance pattern can remove most of the false candidates from the list of elemental

compositions (Kind and Fiehn, 2006). Modern mass spectrometers such as TOF-MS, report isotopic abundance patterns with a low relative error of around 5 % (Anonymous, 2009). The isotopic pattern is especially useful for detecting the presence of bromine or chlorine-containing compounds as these atoms have highly characteristic isotopic distributions (Ipsen *et al*., 2010). However, for monoisotopic elements (F, Na, P, I) this feature is of no value (Kind and Fiehn, 2007).

To calculate the elemental composition, the nitrogen rule can be applied (Thermo XCalibur User Guide, 2009). The rule states that if the molecular mass of an unknown compound to the nearest integer value is an odd number, the compound contains an odd number of nitrogens in its molecular formula. If the mass is even, the number of nitrogens in the molecule is also even (including 0). However, the nitrogen rule is only reliable in a mass range not higher than 500 Da (Kind and Fiehn, 2007).

An important consideration in the examination of spectral data is formation of adducts (Table 2.2). In contrast to the targeted analysis (see section 2.2.), adducts and/or fragments can represent a diagnostic value and are used to validate the identity of the (de)protonated molecule (Nielsen and Smedsgaard, 2003).

Once elemental compositions are known, a database annotation can be performed (Fig. 2.2) to find out whether the unknown was ever patented, studied or commercialized. Searching in large compound databases (*e.g*., Pubchem, ChemSpider) for plausible structures might result in numerous propositions which need to be further ranked by MS/MS (or MS^n) data. A helpful tool is an *in silico* fragment simulation (*e.g*., Mass Frontier, Mass Fragment). However, a prediction of product mass spectra yields a large number of possible fragments, of which a small number are actually observed (Hill *et al*., 2008). It has to be also considered that multiple structures can be constructed for the same monoisotopic molecular weight (Hill *et al*., 2008). If the 'unknown' compound is not reported in databases, a thorough fragmentation study combined with a history of the sample (extraction, typical metabolites observed for the type of sample investigated) is essential; however, in most complicated cases, metabolite isolation and use of Nuclear Magnetic Resonance (NMR) are inevitable for structure elucidation (Holčapek *et al*., 2010).

Table 2.2. *Common adducts and fragments in ESI and APCI ionization modes (summarized from Boyd et al., 2008; Holčapek et al., 2010; Nielsen and Smedsgaard, 2003).*

Adduct/fragment	Formula	*m/z* shift[a]	Ionization mode	Cause of adduct formation
Alkali metal ions	$[M+Me]^+$	+ 22 (Na) + 38 (K)	positive	Alkali metal salts
Ammonia	$[M+NH_4]^+$	+17	positive	Ammonium salts and/or ammonia
Water	$[M\pm H+H_2O]^\pm$	+18	positive/negative	Aqueous solvent or water impurity
Chloride	$[M+Cl]^-$	+34	negative	Chlorinated solvents; chloride salts
Methanol	$[M\pm H+CH_3OH]^\pm$	+32	positive/negative	Methanol in solvent
Acetonitrile	$[M\pm H+CH_3CN]^\pm$	+41	positive/negative	Acetonitrile in solvent
Formate	$[M+HCOO]^-$	+46	negative	Formic acid and/or formates
Acetate	$[M+CH_3COO]^-$	+60	negative	Acetic acid and/or acetates
Trifluoroacetate	$[M+CF_3COO]^-$	+114	negative	Trifluoroacetic acid
Water loss	$[M\pm H-H_2O]^\pm$	-18	positive/negative	NA
CO loss	$[M\pm H-CO]^\pm$	-28	positive/negative	NA
CO_2 loss	$[M\pm H-CO_2]^\pm$	-42	positive/negative	NA

[a] the *m/z* shift is calculated with regards to $[M+H]^+$ (positive) or $[M-H]^-$ (negative); M – molecular monoisotopic mass; NA – not applicable

LC-MS for untargeted analysis has become a well-established technique in the investigation of secondary fungal metabolites. The beneficial properties of untargeted metabolite screening by LC-MS include classification of fungal types based on their metabolic profiles and additional knowledge on a wider range of metabolites (Şenyuva *et al*., 2008). Şenyuva *et al.* (Şenyuva *et al*., 2008) performed a metabolite profiling in fungi grown on different media and on figs by means of LC-TOF-MS. The minimum identification criteria were fixed, namely, a mass tolerance of 5 ppm for the protonated molecule and predicted adducts and a minimum peak height of 1,000 counts (or S/N of 10). In addition to the known metabolites expected to be produced by the fungi (*Aspergillus flavus* and *A. parasiticus*), roquefortine A,

fumagillin, fumigaclavine B, malformins, aspergillic acid, nigragillin, terrein, terrestric acid and penicillic acid were also identified.

High-resolution MS is an excellent tool for revealing new or less studied masked mycotoxins. A new *Fusarium* masked mycotoxin, fusarenon X-glucoside, was reported for the first time in wheat grain together with previously detected nivalenol-glucoside. The new glucoside was identified using LC-Orbitrap MS on the basis of accurate mass measurement of characteristic ions and MS/MS fragmentation patterns. However, structural isomers of the same elemental composition are difficult to identify or distinguish without their chemical standards. Therefore, the absolute structure, such as the position of glucosylated OH residue in nivalenol and fusarenon X molecules, remained to be clarified. Considering the fragment profile, 3-OH glucosylation seemed to be the most probable structure (Nakagawa *et al*., 2011). Another study provided data on oligoglycosylated deoxynivalenols which were present in cereal products along with the known parent metabolite, deoxynivalenol, and deoxynivalenol-3-glucoside. The structure elucidation was supported by two complementary approaches, namely, UHPLC-Orbitrap MS with monitoring of specific fragments formed upon in-source fragmentation and specific enzymatic hydrolysis reactions. Moreover, literature information on MS fragmentation patterns of various deoxynivalenol glycosides was helpful in the generic interpretation of obtained mass spectra (Zachariasova *et al*., 2012). Presence of glucoside derivatives of T-2 and HT-2 toxins (type A trichothecene mycotoxins) was also identified with a mass accuracy lower than 2.8 ppm and characterized using high-resolution mass spectrometry based on Orbitrap technology (Lattanzio *et al*., 2012).

Overall, though the instrumentation has advanced, the number of untargeted LC-MS methods for secondary fungal metabolite detection and characterization is scarce possibly due to time-consuming data interpretation.

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CHAPTER 3

Untargeted screening of secondary fungal metabolites in crude extracts and samples from mouldy indoor environments by time-of-flight mass spectrometry

The content of this chapter was partially derived from:

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3.1. Introduction

In recent years, the indoor environment has gained increasing attention due to health complaints of inhabitants. A term called SBS (see Chapter 1) was introduced to describe a set of health problems associated with poor indoor air quality. Indoor-air problems include insufficient ventilation, excess temperature and dry air, emission of chemicals from construction materials, mites and fungal growth caused by water damage in a building (Husman, 1996). Serious consideration has been given to indoor moulds and their metabolites (mycotoxins), as one of the causative agents of SBS (Abbott, 2002). As a result, numerous studies were dedicated to the investigation of selected mycotoxins in mouldy buildings. Several LC-MS/MS methods for mycotoxin detection in samples from mouldy indoor environments confirmed the presence of satratoxins and other trichothecenes, and sterigmatocystin (Bloom *et al*., 2007; Bloom *et al*., 2009; Delmulle *et al*., 2006; Hintikka *et al*., 2009; Polizzi *et al*., 2009; Tuomi *et al*., 2000). But, when working only in MS/MS mode, the instrument is tuned to detect a limited set of the most relevant targeted compounds; other compounds, which can be present in complex matrices, are not detected. Those metabolites should be taken in consideration since they may also represent a danger for human health. Therefore, there is a need for untargeted screening.

Nowadays, LC-TOF-MS is one of the most common techniques for untargeted analysis. Quadrupole TOF (QTOF) mass analyzers are characterized by high accuracy and sensitivity in full scan mode compared to tandem quadrupole instruments. A number of TOF-MS-based methods have been applied for secondary fungal metabolite analysis. An LC-TOF-MS method for fungal metabolite profiling in dried figs was established by Senyuva *et al*. (Senyuva *et al*., 2008). Metabolites of *Aspergillus flavus* and *A. parasiticus* (*e.g*., aflatoxins, kojic acid, 5-methoxysterigmatocystin, roquefortine A, penicillic acid, aspergillic acid) could be identified by automated database searching. This fast and simple method was also used to daily monitor levels of aflatoxins, kojic acid and 5-methoxysterigmatocystin over a period of 14 days. Tanaka *et al.* (Tanaka *et al*., 2006) developed an LC-TOF-MS method for the screening of 13 *Fusarium* and *Aspergillus* mycotoxins (including trichothecenes and aflatoxins) in foodstuffs. Larsen *et al.* (Larsen *et al*., 2007) investigated six species in *Aspergillus* section *Fumigati* for secondary metabolite production by direct infusion mass spectrometry on a QTOF mass spectrometer. Twenty five secondary metabolites were identified, of which 14

(*e.g.*, gliotoxin, cyclopiazonic acid, trypacidin, helvolic acid) were additionally confirmed by comparison with available standards. Takino *et al*. (Takino *et al*., 2008) developed a method for the determination of four macrocyclic trichothecenes in water-damaged houses by LC-TOF-MS and LC-MS/MS. Both instruments were operated in ESI^+. Satratoxins G and H, iso-satratoxin F and roridine E were found in *Stachybotrys chartarum* which was isolated from a mouldy house. The accuracy of mass measurement ranged from 0.2 to 2.1 ppm.

In the present work, to establish an untargeted LC-QTOF-MS method and identification strategy, fungi, namely, *Penicillium brevicompactum* and *Chaetomium murorum*, isolated from water-damaged buildings, were grown on malt extract agar (MEA) and wallpaper for 7-21 days. Inoculated samples were extracted and analyzed by LC-QTOF-MS. As the recorded spectra of untargeted analysis are complex, containing metabolite fragment and isotope ions, matrix-associated ions and instrument noise, a statistical approach represented by PCA was applied to extract potential markers from the LC-MS data. Furthermore, untargeted metabolite screening was carried out in samples from mouldy buildings with the intention to gather more information on secondary fungal metabolite occurrence in indoor environments. This can help to better understand the relationship between SBS and fungal metabolites.

3.2. Experimental

3.2.1. Chemicals and material

Methanol (MeOH) of LC-MS grade was obtained from Biosolve (Valkenswaard, The Netherlands), whereas HPLC-grade MeOH was from VWR International (Zaventem, Belgium). Ethyl acetate (EtOAc) and dichloromethane (DCM) were purchased from Acros Organics (Geel, Belgium). Formic acid (HCOOH), acetic acid (CH_3COOH), ammonium acetate (CH_3COONH_4) and orthophosphoric acid (H_3PO_4) (85 %) from Merck (Darmstadt, Germany) were used. Ultrapure water (H_2O) was produced by a Milli-Q Gradient System (Millipore, Brussels, Belgium). Sodium chloride (NaCl) and sodium acetate (CH_3COONa) were purchased from VWR International. Sigma-Aldrich (Bornem, Belgium) was the supplier of Tween 80. Ultrafree®-MC centrifugal filter units (0.22 µm) from Millipore (Bedford, MA, USA) were used.

Standards of ochratoxin A, aflatoxins B1, B2, G1 and G2, T-2 toxin, zearalenone, neosolaniol, diacetoxyscirpenol, sterigmatocystin, nivalenol, deoxynivalenol, verrucarin A, verrucarol,

roridin A, deepoxy-deoxynivalenol, reserpine and leucine-enkephalin were purchased from Sigma-Aldrich. Standards of chaetoglobosin A and roquefortine C were delivered from Enzo Life Sciences (Antwerpen, Belgium). Fermentek (Jerusalem, Israel) was the supplier of mycophenolic acid. Ready-made solid portions of commercial standards were dissolved in MeOH to reach a concentration of 1 mg/mL, while deepoxy-deoxynivalenol was supplied as a 50 µg/mL solution in acetonitrile (ACN). Stock solutions were stored at - 20 °C.

MEA was prepared as follows: 2 % malt extract (Oxoid, Basingstoke, UK), 0.1 % bacteriological peptone (Oxoid), 2 % bacteriological agar (Oxoid) and 2 % α-D-(+)-glucose anhydrous 99 % (Acros Organics) were dissolved in water. Sterile Petri dishes, spreaders and disposable pipettes (1 mL, 10 mL) were purchased from VWR International. For sterilisation, a pressure cooker canner (Presto, USA) was used (121 °C, 1.2 bar, 20 min).

Wallpaper (WP) was bought in a local retail shop and cut into pieces of 5 x 5 cm.

3.2.2. Samples from mouldy buildings

Seven samples from two water-damaged buildings in Belgium were collected. Surface sampling was done by scraping the spots of fungal growth and/or by gently pressing a contact plate containing growth medium. Subsequently, the contact plates were kept at 25 °C for 10 days to allow development of fungal growth.

A non-extensive mould distribution was noticed in House #1. Contact plate sampling was performed in the living room under the window (1 sample) and in the cellar (1 sample), while mould scraping could only be done in the cellar (1 sample). Inhabitants of this building complained of allergy. In House #2, high humidity, low temperature and extensive mould growth were observed. Most of mould accumulation was found under the window. Contact plate sampling (1 sample) and wall scraping (3 samples) was done in the building. The inhabitants of House #2 also experienced allergic responses.

3.2.3. Inoculation

The fungal species (from a home-made collection of fungi sampled in water-damaged buildings) were cultivated in sterile Petri dishes filled with 10 mL MEA and on WP in sterile

glass containers filled with some amount of water. A spore suspension of each investigated fungal species was prepared in physiological solution (aqueous solution of 0.85 % NaCl and 0.5 % Tween 80) to a concentration of 10^6 colony forming units/mL. 0.5 mL of the spore suspension was put on a solid MEA (in Petri dishes) and WP (in a glass container). Prior to inoculation, all materials and solutions were sterilized. To homogenously distribute the spores over the media, a sterile spreader was used. The samples were incubated at 25 °C for 7-21 days. Non-inoculated samples were also prepared to provide a reference. A content of the whole plate (containing medium) and pieces of WP were extracted and analyzed by LC-QTOF-MS.

3.2.4. Sample preparation

Before extraction the samples were spiked with two internal standards – deepoxy-deoxynivalenol (*m/z* 281.1389) and reserpine (*m/z* 609.2812).

Sample preparation was based on the procedure described by Delmulle *et al.* (Delmulle *et al*., 2006; Polizzi *et al*., 2009) which was initially developed by our research group for the multi-mycotoxin LC-MS/MS analysis of samples from indoor mouldy environments. Extraction of the fungi inoculated on MEA was performed by adding 1.5 mL EtOAc to the samples and shaking for 15 min on a horizontal shaker (J. Toulemonde & Cie, Paris, France). The EtOAc layer was removed and kept separate. The next step was the addition of 1.5 mL DCM to the samples followed by shaking for 15 min. EtOAc and DCM layers were then combined. The extract was filtered through a folded paper filter (grade 3 hw, 65 g/m^2; Egilabo NV, Kontich, Belgium). The filtrate was evaporated to dryness at 40 °C under a stream of nitrogen. The dry residue was redissolved in 200 µL MeOH:H_2O (30:70, v/v) and injected into the LC-QTOF-MS system. The same sample preparation procedure was applied to the inoculated wallpaper, but the extraction solvent was 15 mL of MeOH:H_2O (90:10, v/v) which was added twice.

3.2.5. LC-MS conditions

An Alliance 2595 LC system (Waters, Milford, MA, USA) was used for the liquid chromatography part. The column used was a SunFire C18 column 3.0 x 150 mm, 3.5 µm

(Waters). The mobile phase consisted of (A) H_2O containing 0.1 % (v/v) HCOOH and (B) MeOH containing 0.1 % (v/v) HCOOH pumping at a flow rate of 0.2 mL/min. The gradient elution program was as follows: 0-11 min: 30-60 % B; 11-19 min: 60-85 % B; 19-30 min: 85 % B; 30-35 min: 85-30 % B; 35-45 min: 30 % B. The injection volume was 20 µL.

MS experiments were performed using an orthogonal acceleration QTOF micro MS (Waters) equipped with a dual sprayer orthogonal electrospray source (Z-spray, LockSpray). The instrument was operated in ESI^+ mode. Desolvation gas was set at 450 L/h, cone gas 60 L/h, desolvation temperature 300 °C and source temperature 120 °C. ESI capillary was set at 2500 V and cone voltage at 30 V. TOF flight tube voltage was set at 5630 V, reflectron voltage 1780 V, pusher voltage 798 V, MCP (micro-channel plate) detector 2200 V. Full scan spectra were acquired over a *m/z* 100-950 range at a resolving power of 5000 FWHM with a scan accumulation time of 1.0 sec and interscan delay of 0.1 sec. Leucine-enkephalin was used as a lock mass compound (*m/z* 556.2771; 200 ng/mL in MeOH:H_2O (50:50, v/v)) and infused at a constant flow rate of 0.1 mL/min using a Kontron pump IRIS 325 (Kontron Instruments, Milan, Italy). Real-time mass correction was used. With this technique leucine-enkephalin is introduced into the source at the same time as the samples and the instrument software constantly corrects the measured mass of the unknown compounds using the know mass of the reference compound. The reference compound was sampled every 8 s and data were averaged over 5 spectra/min. Daily calibration of the instrument was carried out infusing 0.1 % H_3PO_4 in ACN:H_2O (50:50, v/v) at a flow rate of 5 µL/min.

For optimization of MS conditions, selected mycotoxin standards were infused into the QTOF-MS at a flow rate of 10 µL/min using a syringe pump.

The MS/MS experiments were performed using variable collision energies in the range from 10 to 60 eV to provide the most informative spectra. The LC-MS system was controlled by MassLynx software 4.0 (Waters).

3.2.6. Data treatment

Raw LC-MS data were processed using MarkerLynx 4.1 (Waters). The track peak parameters were set as follows: peak width at 5 % height 20 s, automatic peak-to-peak baseline noise, intensity threshold 10, mass window 0.05, retention time window 0.2 min, noise elimination

level 4.0 and mass tolerance 0.05 Da. All acquired raw data were subjected to mean-centering and pareto scaling before multivariate analysis. Data from MarkerLynx were imported into SIMCA-P 11.0 software (Umetrics, Umeå, Sweden) for multivariate analysis (PCA).

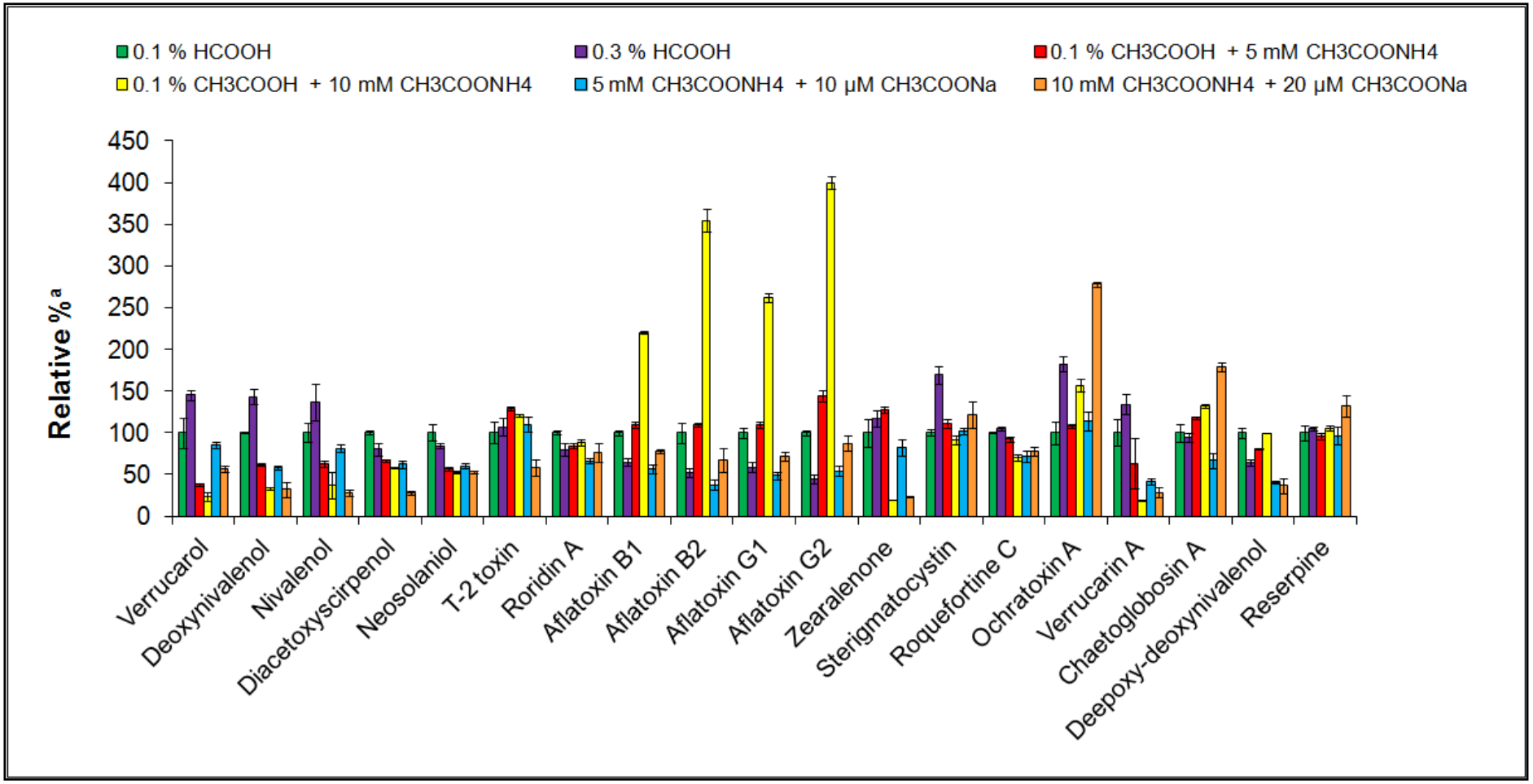
3.3. Results and discussion

3.3.1. Mobile phase selection and optimization of MS parameters

Mobile phase composition affects not only the chromatographic separation of compounds, but also their ionization in MS. Mixtures of H_2O and organic solvent (MeOH or ACN) are commonly used for secondary fungal metabolite analysis and can be additionally modified to enhance separation and/or ionization. In general, the effect of eluent has been considered as a more decisive factor for the MS response compared to separation efficiency (Kokkonen, 2011).

Mobile phases consisting of H_2O, MeOH, HCOOH, CH_3COOH or CH_3COONH_4 were previously used for the LC-MS/MS analysis of a wide range of mycotoxins achieving good separation (Diana Di Mavungu *et al*., 2009; Ediage *et al*., 2011; Martos *et al*., 2010; Sulyok *et al*., 2006). Therefore, a set of mobile phases containing different modifiers, such as HCOOH, CH_3COOH, CH_3COONH_4 and CH_3COONa, was prepared for the MS ionization study. MeOH was preferred to ACN, because ionization was significantly reduced when ACN was used as mobile phase component in previous studies (Frenich *et al*., 2009; Ren *et al*., 2007). A number of mycotoxin standards, selected as model compounds, were redissolved in mobile phase and infused into QTOF-MS.

All mobile phase combinations could be used for the analysis showing better results for some mycotoxins while being less suitable for the others (Fig. 3.1).



[a] Percentage was calculated for signal intensity in each mobile phase relative to the signal intensity recorded for mobile phase containing 0.1 % HCOOH

Figure 3.1. *Comparison of different mobile phase modifiers. Mobile phase composition consisted of H_2O:MeOH (70:30, v/v) containing modifiers mentioned in the figure legend.*

For example, aflatoxins demonstrated much higher signal intensity in H_2O:MeOH (30:70, v/v) containing 0.1 % CH_3COOH and 10 mM CH_3COONH_4, whereas ochratoxin A and chaetoglobosin A provided a higher MS signal in H_2O:MeOH (30:70, v/v) containing 10 mM CH_3COONH_4 and 20 µM CH_3COONa. Along with the protonated ions, sodium adducts were formed. This was not considered as a drawback, since adducts can be used for additional confirmation of compound identification in untargeted analysis. Finally, as a compromise, the mobile phase containing 0.1 % HCOOH was chosen.

A further step included the optimization of the MS parameters, such as desolvation and cone gas flow, source and desolvation temperature, capillary and cone voltage, and MCP detector voltage. Most of the parameters were set at common values used in QTOF-MS analysis, as their effect on the analyte MS signal was less pronounced. A change in MCP detector settings had a significant impact on the MS signal (Fig. 3.2). A dramatic increase of signal magnitude was noted for ochratoxin A when the MCP detector voltage was set at 2200 V instead of 2100 V. Similar observations were made for the other model analytes. The cone gas flow rate was also optimized. Although a lower cone gas flow rate provided a higher MS signal for all analytes, by increasing the cone gas, adduct formation was slightly reduced. Eventually, the MCP detector voltage and cone gas flow were set at 2200 V and 60 L/h, respectively.

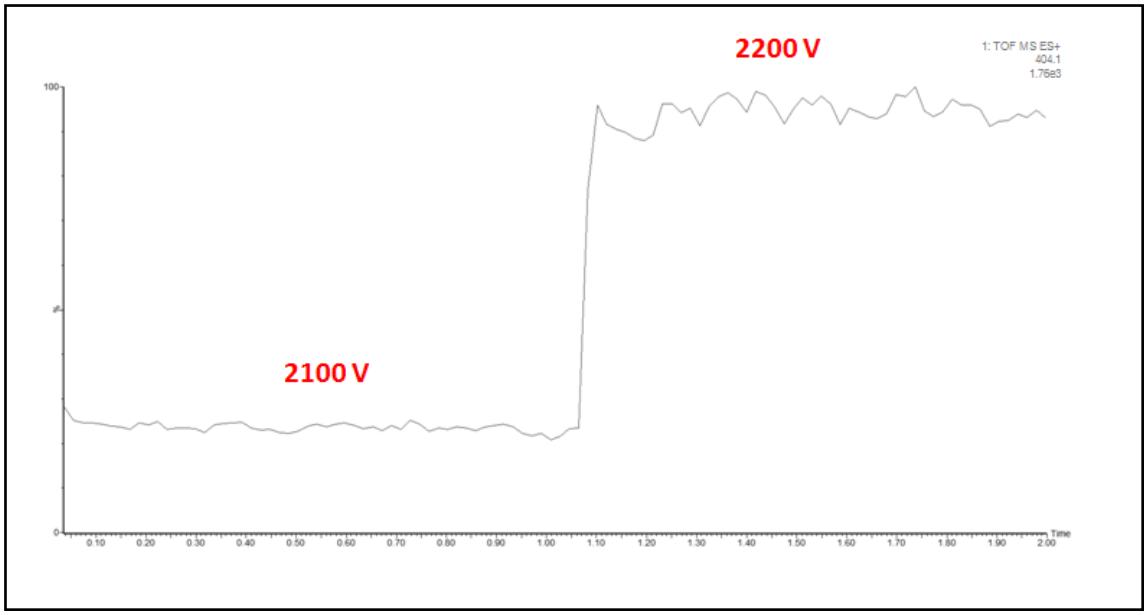


Figure 3.2. *Optimization of MCP detector voltage by direct infusion (example of ochratoxin A).*

3.3.2. Evaluation of experimental mass error in QTOF-MS analysis

Effectiveness and validity of establishing an elemental composition for a compound from its measured mass depend on correct determination of the mass error limits used for the composition generation. Setting a very narrow mass error limit might lead to elimination of a possibly correct elemental composition, and oppositely, setting a very wide limit results in many formulas and makes the identification procedure complicated (Blom, 2001). Therefore, the experimental mass error was established performing multiple injections of mycotoxin standard solutions, namely tenuazonic acid (*m/z* 198.1130), roquefortine C (*m/z* 390.1930) and fumonisin B1 (*m/z* 722.3963) (Fig. 3.3).

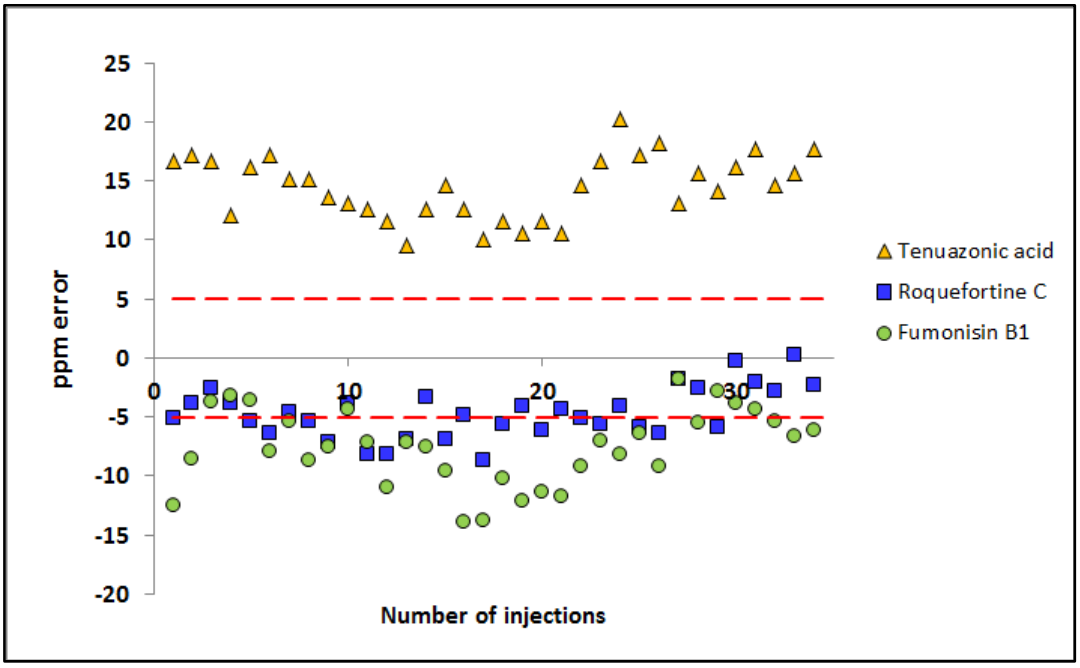


Figure 3.3. *Experimental mass error of QTOF-MS. The dashed lines represent the typical mass error of 5 ppm.*

Although the common mass error of QTOF-MS is declared to be 5 ppm (Want *et al*., 2007; Werner *et al*., 2008), deviations up to 20 ppm were noted. Several other reports also mentioned similar deviations in mass accuracy (Blom, 2001; Bristow and Webb, 2003). Significant changes in mass error over time (equal to 35 injections or about 26 hours of operation) were not observed.

Furthermore, two internal standards, namely deepoxy-deoxynivalenol and reserpine, were added to the samples and used to monitor the experimental mass errors during each analysis on QTOF-MS.

3.3.3. Screening of fungal cultures

The untargeted screening strategy, suggested in Chapter 2, was first evaluated analyzing known fungal cultures inoculated on MEA and WP.

At the first stage, fungal cultures, namely *P. brevicompactum* and *C. murorum*, isolated from mouldy houses were inoculated on MEA and WP and grown for 1-3 weeks at 25 °C. The cultures were solvent-extracted and analysed by LC-QTOF-MS. The steps of identification are presented in detail for *Penicillium* sp. as an example.

In order to identify specific metabolites produced by fungal species and to consider the matrix impact, a comparison between blank matrix and culture extracts should be made. In this case PCA can be a helpful statistical tool. In PCA the data matrix is examined to find markers that best describe the maximum variance in the data. Prior to PCA, a mean centering and pareto scaling were applied to the data. In mean centering the average value of each variable is calculated and subtracted from the data set (Cloarec *et al*., 2005). The mean of the transformed data equals zero. In scaling methods each variable is divided by a factor which is different for each variable (van den Berg *et al*., 2006). Pareto scaling uses the square root of variable's standard deviation as a factor. The advantage of this scaling is that it enhances the contribution of lower concentration metabolites without amplifying the noise (Cloarec *et al*., 2005). After data processing, a list of intensities of the peaks detected was generated for the samples using retention time and *m/z* data pairs as identifier of each peak. The score plot displays the sample relationships in a multi-dimentional space, with similar samples clustered together and dissimilar samples separated. The score plot obtained after PCA showed separate clustering of cases (inoculated MEA or WP) and blanks (non-inoculated MEA or WP) meaning that some characteristic markers for cases can be expected (Fig. 3.4a). To find out precisely what markers significantly contribute to the variance observed within the dataset, the loadings plot was constructed (Fig. 3.4b). The loadings plot describes the relationship between the variables. Variables contributing most to the variance are positioned further from the origin of the plot. Finally, markers with *m/z*

321, 343, 366, 388 and 390 were selected for assignment (Fig. 3.4b). These markers were positioned further from the plot origin and were present only in inoculated matrix.

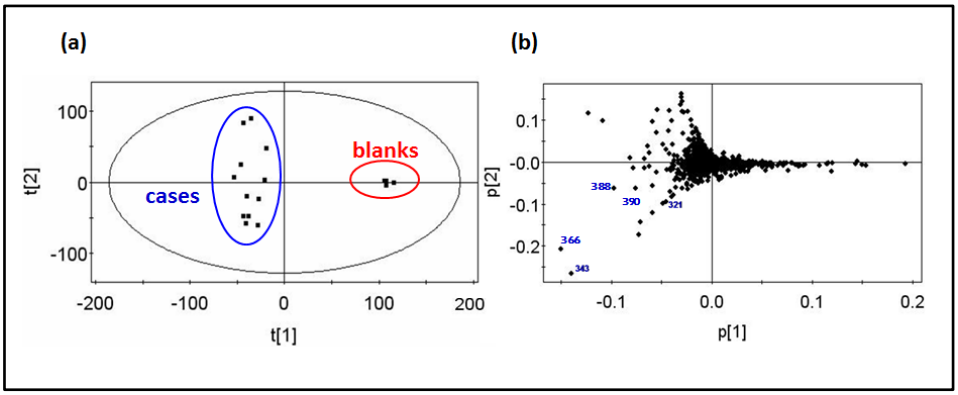
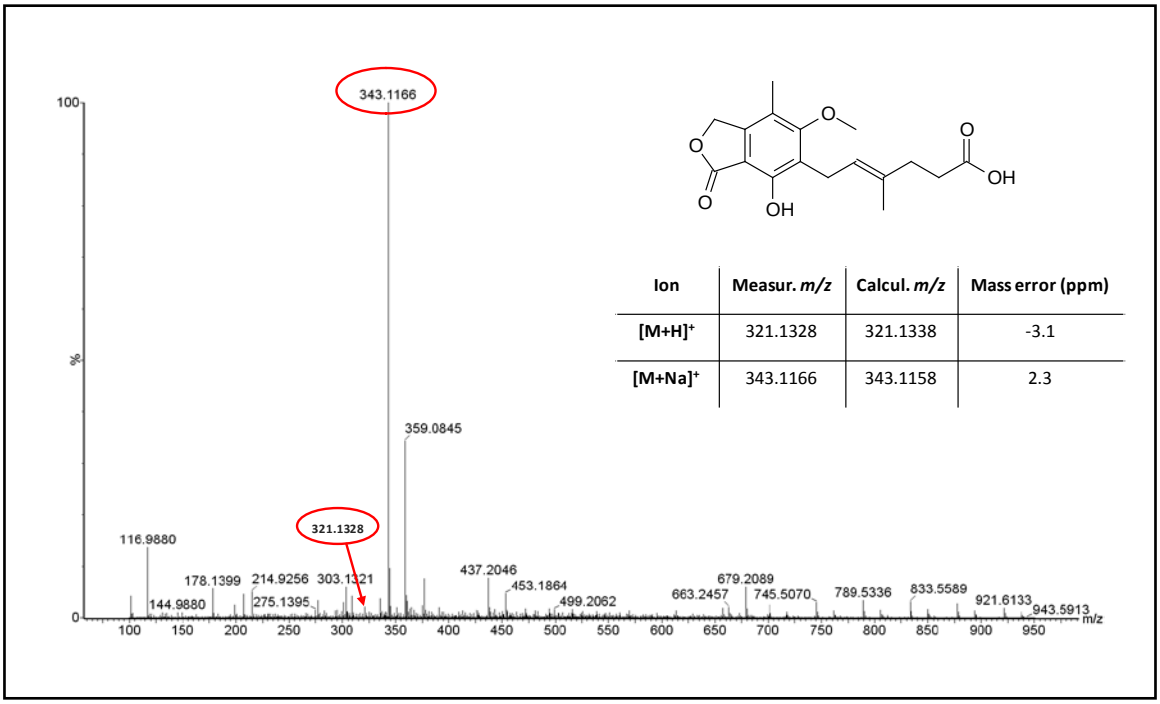


Figure 3.4. *PCA score plot (a) and loadings plot (b) of Penicillium brevicompactum. m/z values of the metabolites of interest are presented in (b).*

The spectrum of a peak eluted at 27.5 min was characterized by an abundant ion at *m/z* 343.1166 (Fig 3.5). An ion at *m/z* 321.1328, which appeared on the loadings plot, was also present in the spectrum. Owing to the mass difference of 22 Da, it was speculated that *m/z* 343 could be a sodium adduct of the protonated ion at *m/z* 321.



Ion	Measur. *m/z*	Calcul. *m/z*	Mass error (ppm)
$[M+H]^+$	321.1328	321.1338	-3.1
$[M+Na]^+$	343.1166	343.1158	2.3

Figure 3.5. *Full MS spectrum of mycophenolic acid produced by Penicillium brevicompactum.*

The exact masses were transferred into the Molecular Formula Generator, incorporated into MassLynx software, which simulated possible elemental compositions (Fig 3.6). Within a 15 ppm mass tolerance, 17 elemental compositions were generated for *m/z* 321.1328 and 25 compositions for *m/z* 343.1166. A subsequent database search assisted in the tentative identification of the compound as mycophenolic acid (formula: $C_{17}H_{20}O_6$; molecular weight (MW): 320 Da) with a mass error of -3.1 ppm for the protonated ion and 2.3 ppm for its sodium adduct. A trend plot, showing marker levels across the samples, confirmed presence of mycophenolic acid only in MEA and WP inoculated with *P. brevicompactum* (Fig. 3.7). Non-inoculated matrix did not contain this marker.

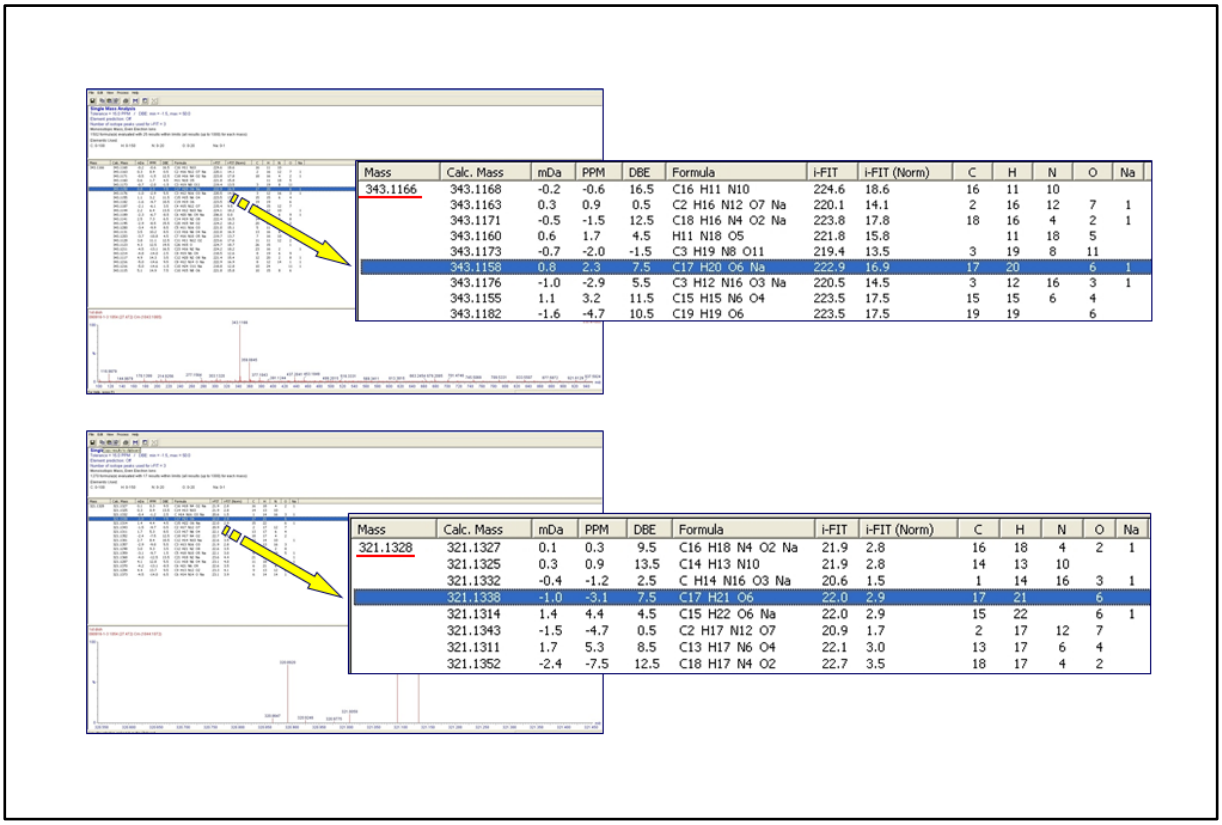
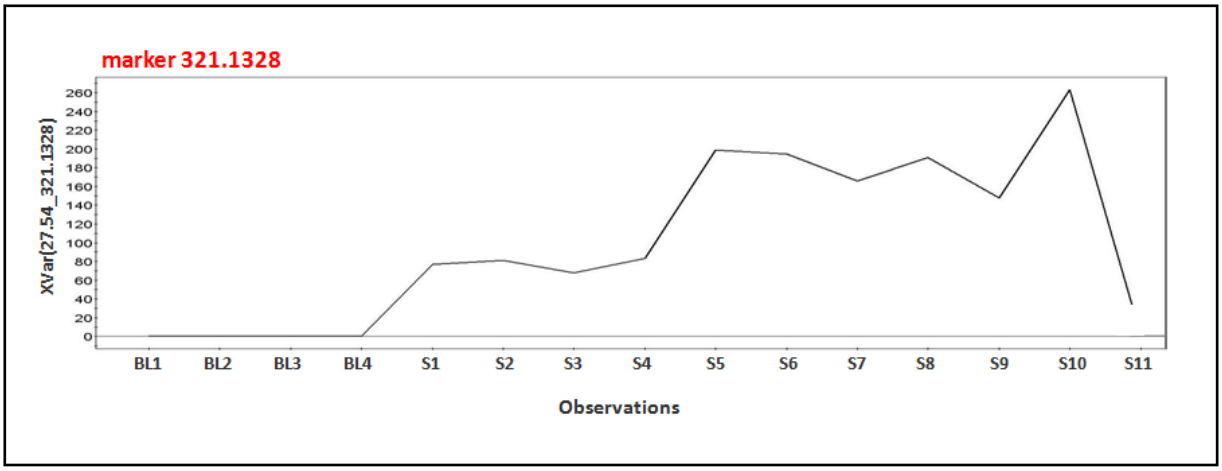


Figure 3.6. *Elemental compositions generated for m/z 343.1166 and 321.1328.*

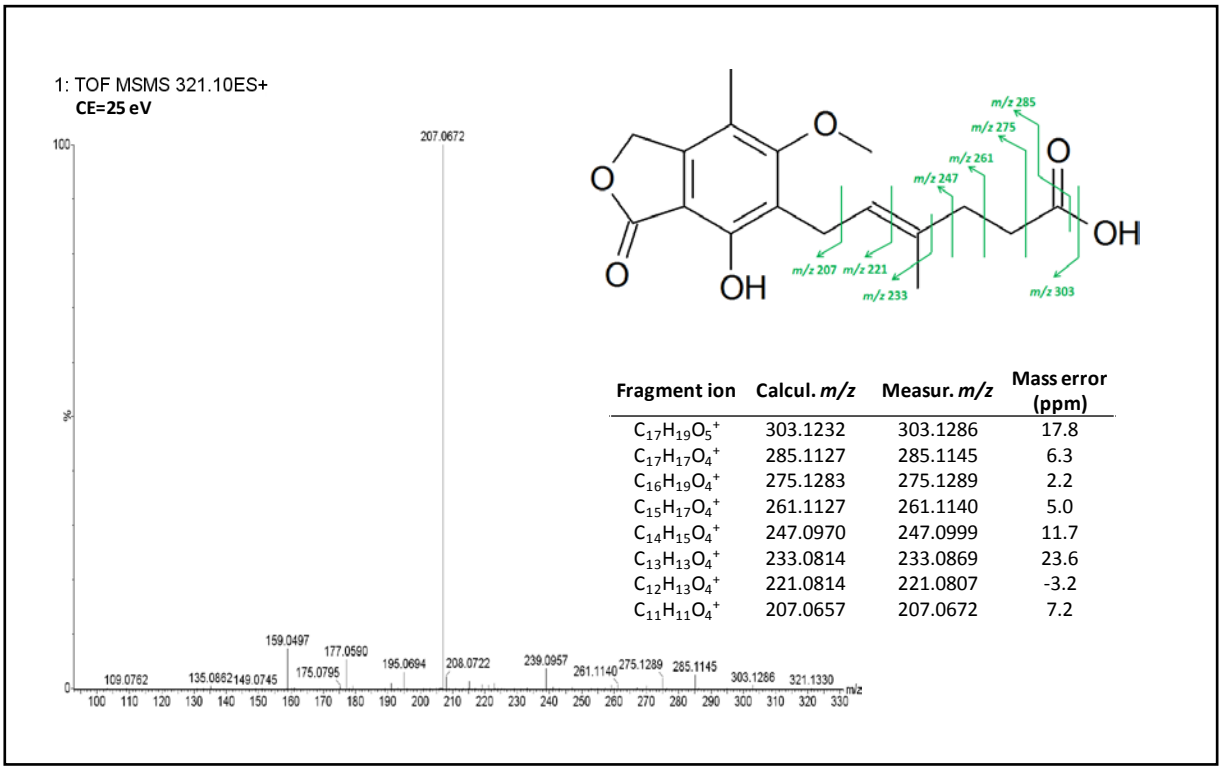


BL: refers to as blank and represents non-inoculated matrix, S: refers to as sample and represents inoculated matrix.

Figure 3.7. *Trend plot for marker at m/z 321.1328.*

To confirm the authenticity of the compound, MS/MS spectra were recorded (Fig. 3.8). The accurate mass of the observed fragment ions matched well with the calculated *m/z* values. Remarkably, the fragment ions did not provide the same mass accuracy as the protonated ions recorded in MS mode. In some cases, the mass error reached 24 ppm. This could be explained by energy differences between ions coming from the second quadrupole. It becomes more difficult to focus the kinetic energy of all the ions before each pulse in the TOF; also there are no calibration ions for continuous, on-line, accurate mass measurement corrections (García-Reyes *et al*., 2007).

In the same way, two other metabolites of *Penicillium* sp. grown on MEA and WP were identified, namely brevianamide A and roquefortine C; chaetoglobosin A was produced by *C. murorum* grown on MEA and WP (Table 3.1). Structures of detected metabolites are presented in Fig. 3.9, except for roquefortine C which structure was given in Chapter 1.



Fragment ion	Calcul. *m/z*	Measur. *m/z*	Mass error (ppm)
$C_{17}H_{19}O_5^+$	303.1232	303.1286	17.8
$C_{17}H_{17}O_4^+$	285.1127	285.1145	6.3
$C_{16}H_{19}O_4^+$	275.1283	275.1289	2.2
$C_{15}H_{17}O_4^+$	261.1127	261.1140	5.0
$C_{14}H_{15}O_4^+$	247.0970	247.0999	11.7
$C_{13}H_{13}O_4^+$	233.0814	233.0869	23.6
$C_{12}H_{13}O_4^+$	221.0814	221.0807	-3.2
$C_{11}H_{11}O_4^+$	207.0657	207.0672	7.2

Figure 3.8. *MS/MS spectrum of mycophenolic acid produced by Penicillium brevicompactum and assignment of some fragment ions.*

Table 3.1. *Metabolites produced by fungi inoculated on MEA and WP.*

Metabolite	Fungi	RT (min)	Ion	Measur. *m/z*	Calcul. *m/z*	Mass error (ppm)
Mycophenolic acid	*Penicillium brevicompactum*	27.5	$[M+H]^+$	321.1328	321.1338	-3.1
			$[M+Na]^+$	343.1166	343.1158	2.3
Roquefortine C	*Penicillium brevicompactum*	16.3	$[M+H]^+$	390.1920	390.1930	-2.6
Brevianamide A	*Penicillium brevicompactum*	17.7	$[M+H]^+$	366.1806	366.1818	-3.3
			$[M+Na]^+$	388.1637	388.1634	-0.8
Chaetoglobosin A	*Chaetomium murorum*	26.1	$[M+H]^+$	529.2720	529.2702	3.4
			$[M+Na]^+$	551.2551	551.2522	5.3

Brevianamide A and mycophenolic acid have been earlier reported as dominant metabolites of *P. brevicompactum* (Frisvad *et al*., 2004; Nielsen, 2003). Moreover, mycophenolic acid was previously found in *P. brevicompactum* inoculated on building materials (Nielsen *et al*., 1999). The alkaloid, roquefortine C, was detected in several other *Penicillium* species (Frisvad *et al*., 2004; Nielsen, 2003). As for chaetoglobosin A, it was produced as main metabolite by several *Chaetomium* species (De Vries *et al*, 2002; Nielsen *et al*., 1999; Oh *et al*., 1998). However, it was not reported being specifically produced by *C. murorum*.

The three metabolites detected in cultures of *Penicillium* sp. have been reported to cause inflammatory responses (Rand *et al*., 2005), while chaetoglobosin A from *Chaetomium* sp. has been found to be cytotoxic (Ohtsubo *et al*., 1978).

3.3.4. Screening of samples from mouldy environments

The developed LC-QTOF-MS method was applied for secondary fungal metabolite screening in mouldy buildings, where inhabitants experienced some health problems. In three contact plate samples (Houses #1 and #2) and in a wall scraping sample (House #2) a peak at 10.9 min was observed. The abundant ions in the MS spectrum were *m/z* 434.1830 and *m/z* 403.1628. Using generated elemental compositions, a database search revealed a compound meleagrin (formula: $C_{23}H_{23}N_5O_4$; MW: 433 Da). The observed ions at *m/z* 434.1830 and *m/z* 403.1628 correspond to $[M+H]^+$ (mass error 1.6 ppm) and $[M\text{-}CH_3O+H]^+$ (-2.7 ppm) ions of meleagrin, respectively. The structure of meleagrin is shown in Fig. 3.9. A number of peaks could not be identified, some of which might not necessarily belong to secondary fungal metabolites, since a good control sample was not always available.

It has been reported that meleagrin was mostly produced by *Penicillium* spp. (Frisvad *et al*., 2004; García-Estrada *et al*., 2011). As *Penicillium* spp. were identified in the sampled buildings, the meleagrin detection was in good agreement with the abundant presence of these fungi. Previous studies in water-damaged buildings disclosed occurrence of meleagrin in naturally-infested wallpaper (Nielsen *et al*., 1999) and other building materials from mouldy indoor environment (Täubel *et al*., 2011).

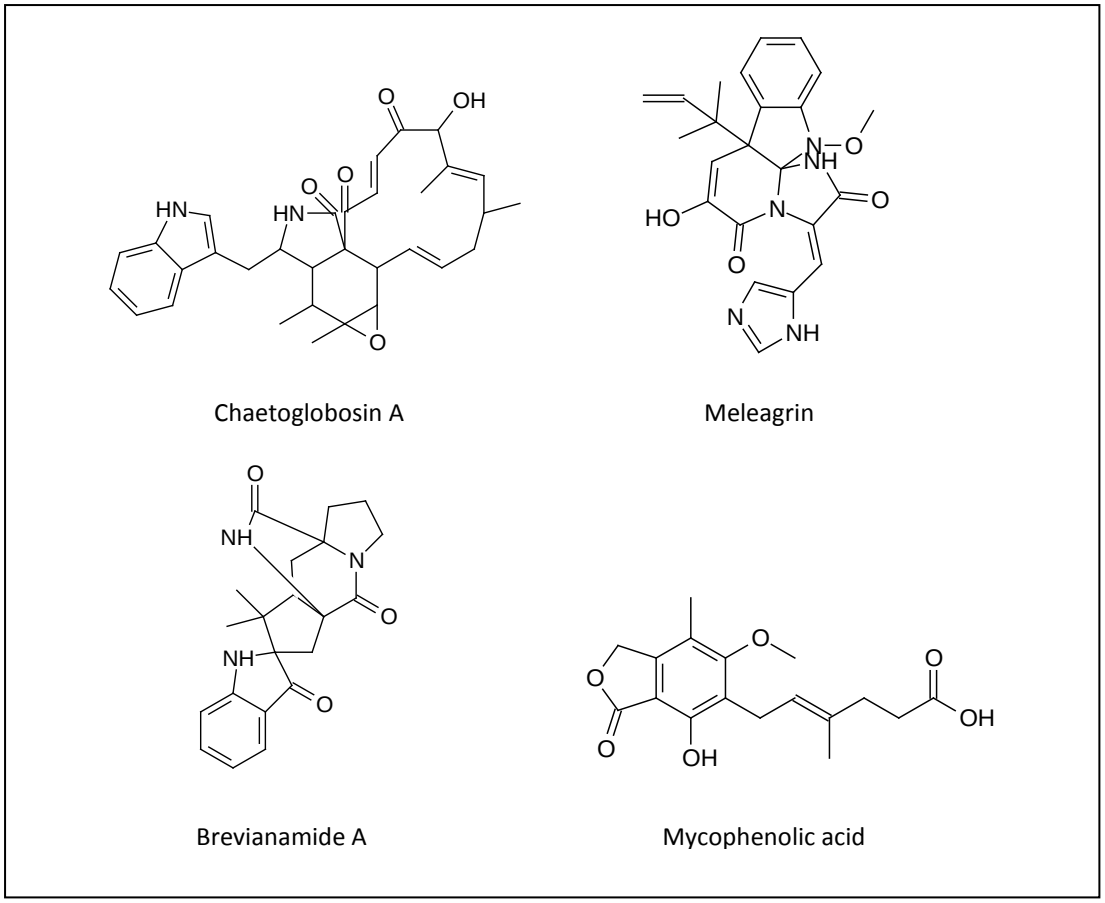


Figure 3.9. *Structures of secondary fungal metabolites detected in fungal cultures and mouldy houses.*

Meleagrin is defined as non-toxic, although it has to be considered that another common *Penicillium* metabolite, neurotoxic roquefortine C, is a precursor of meleagrin (Nielsen *et al*., 1999); however, roquefortine C was not detected in the mouldy houses under investigation. Although meleagrin was yielded by fungi collected from the mouldy environments and grown at laboratory conditions, that does not necessarily mean its production in the indoor environment, as the metabolite production is dependent on the substrate, temperature and water content (Polizzi *et al*., 2011; Van Lancker *et al*., 2008).

3.4. Conclusions

The present study was focused on the untargeted screening of secondary fungal metabolites in cultures isolated from water-damaged buildings and naturally-infested samples from mouldy environments. As a detection technique, QTOF-MS was preferred since it provides

enhanced sensitivity in full scan mode compared to triple quadrupole MS, and accurate mass measurement which is beneficial for structure elucidation. Post-acquisition data treatment was conferred to appropriate statistical tools, in particular PCA. The markers (metabolites), best describing variation in the LC-MS dataset, were extracted and identified by searching in freely available databases.

However, some limitations could be experienced with respect to the application of the method to the samples from mouldy houses. In the 'real-sample' analysis, a number of metabolites could not be identified manifesting the need for libraries of secondary fungal metabolites. Regarding the existing public libraries, spectral information is often missing or unstandardized. In addition, some metabolites, which were isolated and identified many years ago, have not been included in the databases.

Another obstacle lies in the availability of control samples which are used as reference in defining suspect metabolites in infected samples. Generally, the indoor environment profile (*e.g.*, type of building material) differs from one building or room to another making it difficult to characterize the matrix with appropriate non-infected control samples.

Although a wide range of MS detectors is available, none of them on its own can totally characterize the infected sample. A combination of several MS techniques can be more efficient for metabolite identification. When database search shows no result, the metabolite is considered as unknown and the study requires additional techniques for structure elucidation such as NMR. Still, in the case of samples from mouldy buildings it is not possible to isolate the metabolite of interest for further NMR analysis due to the limited amount of sample.

To conclude, a 'real-sample' analysis of fungal metabolites remains a complicated and time-consuming task, though the instrumentation and software for data processing have greatly advanced. If one intends to perform an untargeted screening, the restrictions mentioned above, should be taken into consideration. Apparently, more fundamental research is needed for the characterization of secondary fungal metabolites.

3.5. Acknowledgements

Dr. Anne Vancauwenberge and Nathalie Popovic (*Hainaut Vigilance Sanitaire*, Mons) are acknowledged for providing access to mouldy buildings and performing fungal identification. Prof. Jan Van Boxclaer and prof. Dieter Deforce are thanked for offering the opportunity to work on QTOF-MS. Dr. Ruben 't Kindt and Sofie Vande Casteele are acknowledged for technical assistance with the QTOF-MS. Viviana Polizzi is thanked for help with inoculation of the fungi.

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CHAPTER 4

Metabolic profiling in *Aspergillus flavus*: identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multiple stage mass spectrometry

The content of this chapter was partially derived from:

Malysheva SV, Arroyo-Manzanares N, Cary JW, Ehrlich KC, Vanden Bussche J, Vanhaecke L, Bhatnagar D, Diana Di Mavungu J, De Saeger S. Identification of *Aspergillus flavus* cluster 27 polyketide synthase metabolites by high resolution and multiple stage mass spectrometry. *To be submitted in Analytica Chimica Acta*

4.1. Introduction

Secondary fungal metabolites have long been used as drugs and still serve as structural inspiration for new compounds. To date, the genomes of several filamentous fungi have been sequenced which led to extensive investigation of biosynthetic metabolic pathways and identification of a number of secondary metabolites.

Members of the genus *Aspergillus* produce beneficial secondary metabolites such as the antibiotic penicillin (Brakhage *et al*., 2004) and the cholesterol-lowering drug, lovastatin (Bizukojc and Ledakowicz, 2007); however, they also yield secondary metabolites that are toxic to humans and animals (Bennett and Klich, 2003). *Aspergillus flavus* is one of the most important species in the *Aspergillus* genus and is distributed world-wide infecting food and feed (Bennett and Klich, 2003; Gourama and Bullerman, 1995; Mehl *et al*., 2012). *A. flavus* is known to produce the toxic and carcinogenic polyketide-derived secondary metabolites, aflatoxins, during growth on crops such as cereals, nuts and oilseeds (Bennett and Klich, 2003; Duran *et al*., 2007; Schroerder and Boller, 1973), and is the second (after *A. fumigatus*) leading cause of invasive aspergillosis (Hedayati *et al*., 2007). In addition to the health risks associated with aflatoxins, there are also significant adverse economic impacts to producers due to market rejections of contaminated crops and livestock losses as well as costs associated with monitoring for aflatoxin contamination (Wu, 2006). Besides aflatoxins, *A. flavus* produces a number of other toxic secondary metabolites, such as sterigmatocystin, aflatrem, cyclopiazonic acid and gliotoxin (Bennett and Klich, 2003; Duran *et al*., 2007; Lewis *et al*., 2005; Schroeder and Kelton, 1975).

The development of software programs for the systematic analyses of genome sequences has allowed investigators to identify putative secondary metabolic gene clusters based on sequence similarity to established genes such as PKSs, non-ribosomal peptide synthetases (NRPSs), hybrid PKS-NRPS and prenyltransferases (PTRs). One of these web-based programs, the Secondary Metabolite Unknown Regions Finder (SMURF; www.jcvi.org/smurf), designed specifically for analysis of fungal genomes, predicted the presence of 55 gene clusters associated with secondary metabolism in *A. flavus* (Georgianna *et al*., 2010; Khaldi *et al*., 2010). To date, many of these clusters remain uncharacterized with metabolites being assigned to only six clusters (Forseth *et al.,* 2012).

Studies have shown that production of a number of secondary metabolites in *A. flavus* and other filamentous fungi are controlled by the global regulator, VeA (Calvo *et al*., 2004; Calvo, 2008; Chettri *et al*., 2012; Dhingra *et al*., 2012; Kato *et al*., 2003; Merhej *et al*., 2012). Whole genome microarray transcript profiling of *A. flavus* wild-type and a *ΔveA* mutant demonstrated significant differential expression of a number of genes associated with putative secondary metabolic gene clusters (personal communication, Dr. Cary JC). One of the genes significantly downregulated in the *ΔveA* mutant was identified as the cluster 27 *pks* as designated by Georgianna *et al.* (Georgianna *et al*., 2010).

In this study, a comparative metabolomics approach, based on UHPLC-HRMS, was applied to identify the metabolites associated with *A. flavus* cluster 27 *pks* gene. Inactivation of the cluster 27 *pks* gene resulted in production of greyish-yellow sclerotia compared to the dark pigment normally observed for *A. flavus* sclerotia. The sclerotial metabolites of the wild-type strain and *Δpks27* mutant were compared using differential statistical analysis to disclose metabolites that were only present in the wild-type strain. Identification of the metabolites was based on accurate mass and supported by multiple stage fragmentation experiments.

4.2. Experimental

4.2.1. Chemicals and material

MeOH and ACN both of LC-MS grade were obtained from Biosolve (Valkenswaard, The Netherlands), whereas HPLC-grade MeOH and ACN were from VWR International (Zaventem, Belgium). EtOAc, DCM and acetone (dimethyl ketone (DMK)) were purchased from Acros Organics (Geel, Belgium). Sigma-Aldrich (Bornem, Belgium) was the supplier of ammonium formate ($HCOONH_4$). HCOOH from Merck (Darmstadt, Germany) was used. Ultrapure H_2O was produced by a Milli-Q Gradient System (Millipore, Brussels, Belgium). Ultrafree®-MC centrifugal filter units (0.22 µm) from Millipore (Bedford, MA, USA) were used. Sigma-Aldrich was the supplier of agar, uracil, corn steep solids, dextrose, peptone, sucrose, yeast extract, glucose, ammonium tartrate, dipotassium hydrogen phosphate trihydrate ($K_2HPO_4*3H_2O$), magnesium sulfate heptahydrate ($MgSO_4*7H_2O$), iron(II) sulfate heptahydrate ($FeSO_4*7H_2O$). Ammonium sulphate $(NH_4)_2SO_4$, potassium chloride (KCl), sodium nitrate ($NaNO_3$) and sorbitol were from Merck.

Standards of aflatoxins B1, B2, G1 and G2 and cyclopiazonic acid were purchased from Sigma-Aldrich. Ready-made solid portions of commercial standards were dissolved in MeOH to reach the concentration of 1 mg/mL. Stock solutions were stored at -20 °C. Standard of asparasone was kindly supplied by Dr. Victor Sobolev (U.S. Department of Agriculture, Agricultural Research Service, National Peanut Research Laboratory, Dawson, Georgia).

4.2.2. Strains and growth conditions

An *Aspergillus flavus* 70 *niaD−*, *pyrG−* parental strain (referred to as AF70) was used as host for transformation. Three Δ*pks27* (Af70 *niaD−*, Δ*pks27*) mutants were obtained following transformation of the parental strain with the *pks27* knockout vector pks27-pyrG. An AF70 pyrG-1 (*niaD−*, *pyrG+*) strain transformed with the pPG2.8 vector expressing the *A. parasiticus pyrG* gene (Skory *et al*., 1990) was used as a transformation control.

Vector construction and fungal transformation were carried out at the USDA Southern Regional Research Center (New Orleans, LA, USA). A PCR-based method was used to construct the *A. flavus* 70 Δ*pks27* in which a 770 bp region within the *pks27* coding region was replaced by the *A. parasiticus pyrG* selectable marker gene. Briefly, 5' and 3' regions of the *pks27* gene were amplified using oligonucleotide primers. Following PCR amplification of *A. flavus* genomic DNA with ExTaq HS polymerase (Takara, Pittsburg, PA, USA), PCR products of the expected size of 1218 bp for the 5' *pks27* amplification and 1042 bp for the 3' *pks27* amplification were obtained. PCR products were subcloned into TOPO pCR2.1 (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. The 5' and 3' *pks27* PCR products were released from their TOPO vectors using EcoRI-BamHI and SalI-HindIII digestion respectively and subcloned in a stepwise manner into the analogous restriction digested pPG2.8 vector harboring the *A. parasiticus pyrG* selectable marker gene. The resulting knockout vector was designated PKS27-pyrG. Transformation was performed as described in Cary *et al.* (Cary *et al*., 2006) using *A. flavus* 70 (AF70) as the host.

For the investigation of cluster 27 metabolites, conidia were grown for 10 days at 30 °C in the dark onto GMM agar supplemented with 2 % sorbitol (GMMS; per liter: glucose, 10 g; ammonium tartrate, 2.0 g; sorbitol, 2.0 g; Cove's salts, 20 mL; pH 6.5). For LC-Orbitrap-MS method evaluation, the wild-type AF70 was grown on Wickerham medium supplemented

with uracil and ammonium sulphate (WATM-US). The medium contained 2.0 g yeast extract, 3.0 g peptone, 5.0 g corn steep solids, 2.0 g dextrose, 30.0 g sucrose, 2.0 g $NaNO_3$, 1.0 g $K_2HPO_4*3H_2O$, 0.5 g $MgSO_4*7H_2O$, 0.2 g KCl, 0.1 g $FeSO_4*7H_2O$ and 15.0 g agar per liter (pH 5.5) and was supplemented with 1 mg/mL uracil and 10 mM $(NH_4)_2SO_4$.

The strains used in the investigation of asparasone A production by other *Aspergillus* spp. were obtained from the USDA Southern Regional Research Center (New Orleans, LA, USA). These strains were grown on WATM medium as described above.

4.2.3. LC-Orbitrap MS conditions

An Accela™ High Speed LC (UHPLC) (Thermo Fisher Scientific, San José, USA) was used for separation. The column was ZORBAX RRHD Eclipse Plus C18 (1.8 µm, 2.1 x 100 mm) from Agilent Technologies (Diegem, Belgium). The mobile phase consisted of H_2O:MeOH (95:5, v/v) containing 0.1 % HCOOH and 10 mM $HCOONH_4$ [solvent A] and MeOH:H_2O (95:5, v/v) containing 0.1 % HCOOH and 10 mM $HCOONH_4$ [solvent B]. The gradient elution program was applied as follows: 0-0.5 min: 0 % B, 0.5-20 min: 0-99 % B, 20-21 min: 99 % B, 21-24 min: 99-0 % B, 24-28 min: 0 % B. The flow rate was 0.4 mL/min. The column temperature was set at 30 °C and temperature of the autosampler was 10 °C. Five µL of the sample were injected.

Accurate mass measurements of the precursor and fragment ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with ESI and APCI interface. The final MS parameters in the ESI^- were the following: spray voltage 4.5 kV, capillary temperature 250 °C, heater temperature 250 °C, sheath gas flow rate 45 arbitrary units (a.u.), auxiliary gas flow rate 10 a.u. The data were processed using the Xcalibur™ 2.1 and Exactive Tune software (Thermo Fisher Scientific). The instrument was operated in full scan mode with a resolution of 100,000 FWHM. The maximum injection time was 200 ms and the number of microscans per scan was 1. Each full scan was followed by a same-polarity “all ion fragmentation” higher energy collisional dissociation (HCD) scan.

4.2.4. LC-ion trap MS conditions

A Surveyor Plus HPLC System (Thermo Fisher Scientific) was used for separation. The column was an XBridge MS C18 column (3.5 µm, 150 x 2.1 mm) with an XBridge Sentry guard column (3.5 µm, 10 x 2.1 mm i.d.) both supplied by Waters (Milford, MA, USA). The mobile phase was as described in section 4.2.3. The gradient elution program was the following: 0-1 min: 0 % B, 1-24 min: 0-99 % B, 24-25 min: 99 % B, 25-28 min: 99-0 % B, 28-35 min: 0 % B. The flow rate was 0.25 mL/min. The column temperature was set at 40 °C and temperature of the autosampler was 10 °C. Five µL of the sample were injected.

An LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source was used for the investigation of fragmentation behaviour. The set MS parameters were: source voltage 5 kV, capillary temperature 250 °C, heater temperature 175 °C, sheath gas flow rate 40 a.u., auxiliary gas flow rate 10 a.u. The maximum inject time was 200 ms and the number of microscans per scan was 3. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different collision energy levels (CELs) to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

4.2.5. Sample preparation

Sclerotia were collected from the Petri dish by adding 10 mL of sterile deionized H_2O to the agar surface and gently scraping colonies with a cell scraper. The collected sclerotia were washed two times by addition of 40 mL H_2O to remove residual conidia and mycelia. After centrifugation and removal of water, metabolites were extracted with 30 mL MeOH:DCM:EtAOc (10:20:30, v/v/v) containing 1 % (v/v) HCOOH. The samples were shaken during 60 min on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). Four mL of extract was transferred into a glass tube and evaporated till dryness under a stream of nitrogen. The residue was reconstituted with 200 µL MeOH:ACN:H_2O (30:30:40, v/v/v), centrifuged in a Ultrafree®-MC centrifugal device for 5 min at 14,000 g and injected into the MS.

4.2.6. Statistical data treatment

SIEVE 1.3 software (Thermo Fisher Scientific) was used for LC-MS data mining. SIEVE aligns the MS spectra over time for different experimental conditions and determines features in the dataset. Parameters for the SIEVE analysis were set as follows: frame time width 0.05 min, frame *m/z* width 0.020, peak intensity threshold 10,000, maximum number of frames 5000, alignment bypass: False, *m/z* min: 100, *m/z* max: 1,000, retention time start: 0.01 min, retention time stop: 28 min, PCA V1.0. Statistical significance of a frame was calculated within the SIEVE package using a *t*-test.

To identify the extracted frames, initial database searching was performed using the MZLookup function in SIEVE where a home-made database was uploaded. The database was constructed based on literature data and consisted of secondary metabolites produced by *Aspergillus* spp.

4.3. Results and discussion

4.3.1. Development and evaluation of the LC-Orbitrap-MS method

A common practice in the evaluation of targeted LC-MS method parameters (*e.g.*, extraction recovery) is the use of spiked samples (Abia *et al*., 2013; Commission Decision 2002/657/EC, 2002; De Boevre *et al*., 2012; Varga *et al*., 2013). This approach is difficult to apply to untargeted methods, since the number of analytes is unlimited. Moreover, spiking experiments have a disadvantage of not being able to fully mimic analyte interactions with a matrix. In this study, evaluation of some method parameters was performed using cultures of *A. flavus* AF70. The species was inoculated on WATM-US and grown for 10 days at 30 °C. Under these conditions, it produced a number of known metabolites which were identified following the strategy described in Chapter 2. Aflatoxins B1, B2, G1 and G2, and cyclopiazonic acid were confirmed with authentic standards, while aflatrem and ditryptophenaline were identified based on their retention time profile, which was in good agreement with the results of Rank *et al*. (Rank *et al*., 2012), and fragmentation pattern. Fig. 4.1 and Table 4.1 summarize data on the detected metabolites. It is worth to mention that the dimeric character of ditryptophenaline was revealed in the MS spectra; an ion at *m/z*

346.1550 ($C_{21}H_{20}N_3O_2$; -2.0 ppm), corresponding to a monomeric fragment, accompanied the protonated ion at *m/z* 693.3178 ($C_{42}H_{40}N_6O_4$; -0.9 ppm).

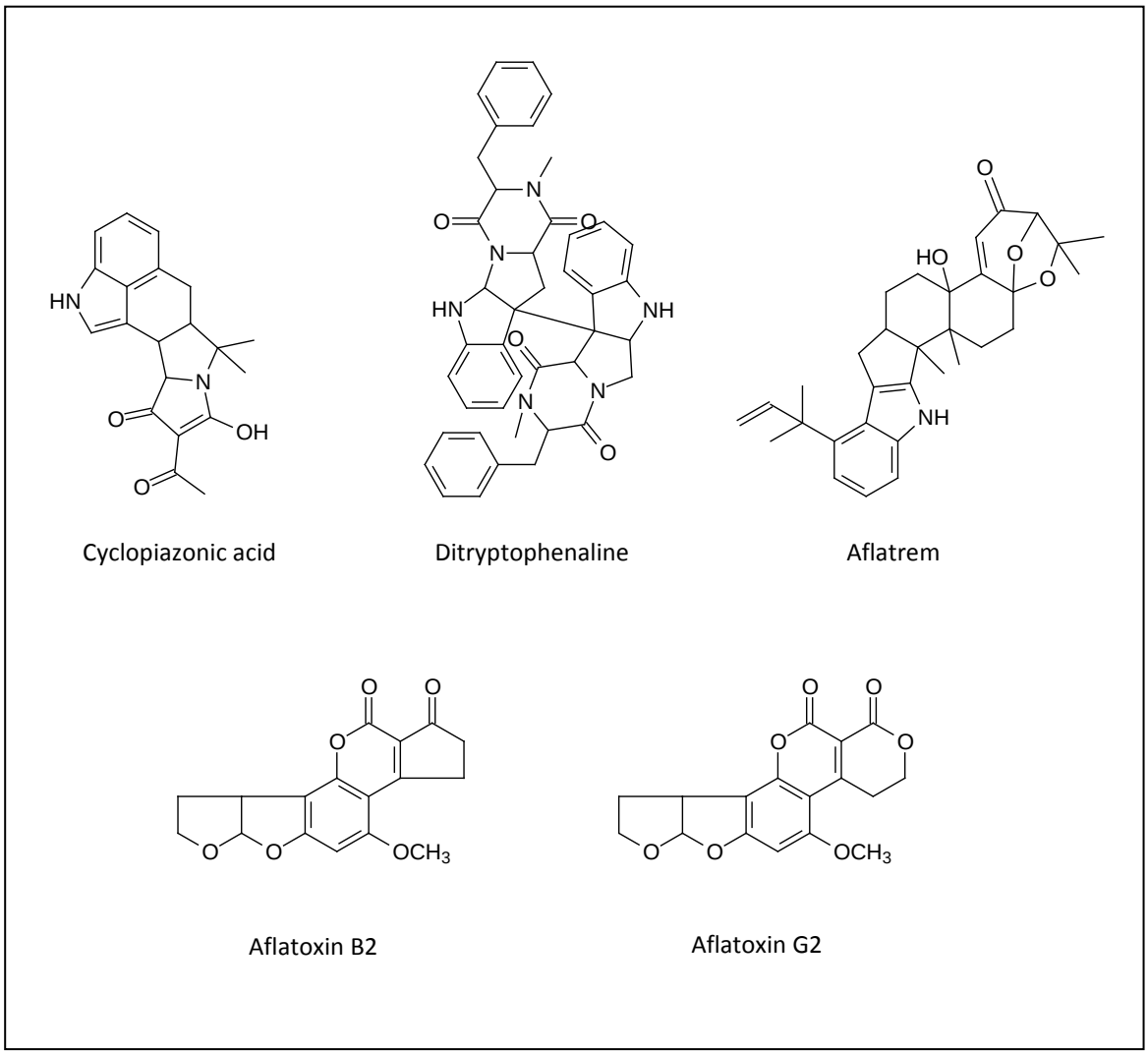

Figure 4.1. *Structures of A. flavus AF70 metabolites identified in the study and used in method evaluation. Structures of aflatoxins B1 and G1 were shown in Chapter 1, Fig. 1.2.*

Table 4.1. *Secondary metabolites identified in cultures of A. flavus AF70 and used for method evaluation.*

Metabolite	Ionization mode	Formula	RT (min)	Calcul. *m/z*	Measur. *m/z*	Mass error (ppm)
Aflatoxin B1	ESI^+	$C_{17}H_{12}O_6$	11.6	313.0707	313.0703	-1.2
Aflatoxin B2	ESI^+	$C_{17}H_{14}O_6$	11.1	315.0863	315.0860	-0.9
Aflatoxin G1	ESI^+	$C_{17}H_{12}O_7$	10.1	329.0656	329.0652	-1.2
Aflatoxin G2	ESI^+	$C_{17}H_{14}O_7$	9.9	331.0812	331.0808	-1.3
Cyclopiazonic acid	ESI^+	$C_{20}H_{20}N_2O_3$	16.3	337.1547	337.1542	-1.4
Aflatrem	ESI^+	$C_{32}H_{39}NO_4$	20.4	502.2952	502.2944	-1.5
Ditryptophenaline	ESI^+	$C_{42}H_{40}N_6O_4$	15.1	693.3184	693.3178	-0.9

4.3.1.1. Optimization of chromatographic conditions

As only volatile mobile phase additives are compatible with MS, the number of mobile phases for LC-MS analysis is limited. Different mobile phase compositions have already been tested for untargeted analysis of secondary fungal metabolites (see Chapter 3) and choice was made towards a H_2O-MeOH-based mobile phase containing HCOOH. However, along with abundant protonated ions, sodium adducts were produced under this condition. In untargeted analysis adduct formation can be considered beneficial for reliable compound identification. Keeping in mind that the aim of untargeted analysis remains detection of as many metabolites as possible in a given sample, efforts were made to increase the signal of (de)protonated ions. Additional literature data revealed that $HCOONH_4$ in combination with HCOOH can be used in mobile phase mixture to enhance signal of (de)protonated ions and reduce formation of sodium adducts (Ediage *et al*., 2012; Hedgepeth and Dahl, 2011; Lacina *et al*., 2012). Compared to CH_3COOH and CH_3COONH_4 as additives in mobile phase, HCOOH and $HCOONH_4$ provided 2-10 times higher MS signal (especially for aflatoxins). At the same time, a drop of HT-2 toxin signal intensity was observed in acidified $HCOONH_4$ (Lacina *et al*., 2012). Since it is not possible to find a universal mobile phase suitable for all compounds, a

mobile phase containing 0.1 % HCOOH and 10 mM $HCOONH_4$ as modifiers was employed in the new LC-MS method.

For increased sensitivity, peak resolution and speed of analysis, UPLC was preferred over HPLC. Structural diversity of secondary fungal metabolites leads to outstanding polarity differences, therefore selection of a suitable stationary phase was of great importance. It was opted for a ZORBAX C18 column which previously demonstrated good separation, improved retention of polar compounds such as glucosinolates and masked metabolites of deoxynivalenol, and excellent reproducibility (De Boevre *et al*., 2012; Ediage *et al*., 2011; Varga *et al*., 2013). In the current study, repeatability (in terms of RSD) of RT on the column for model mycotoxins was calculated for injections on the same day and on different days and generally did not exceed 0.6 %.

4.3.1.2. Evaluation of experimental mass error in Orbitrap-MS analysis

Mass accuracy is one of the crucial parameters of mass spectrometric performance and determines selectivity of database search in a metabolite identification procedure. A typical mass error of 2 ppm was reported for the Orbitrap MS (Hu *et al*., 2005; Muenster *et al*., 2009; Wieghaus *et al*., 2011).

In the study, evaluation of the experimental mass error of the MS was done using repetitive injections of standard solutions of selected mycotoxins as well as extracts of *A. flavus* (Fig. 4.2). The mass accuracy was overall within the typical range for Orbitrap MS, except for a few injections for which the mass error reached a value of -2.5 ppm. This indicates that for positive identification, a mass error slightly higher than 2 ppm is to be expected; but errors above 5 ppm are unlikely to occur. The mass error variation over an LC peak was negligible.

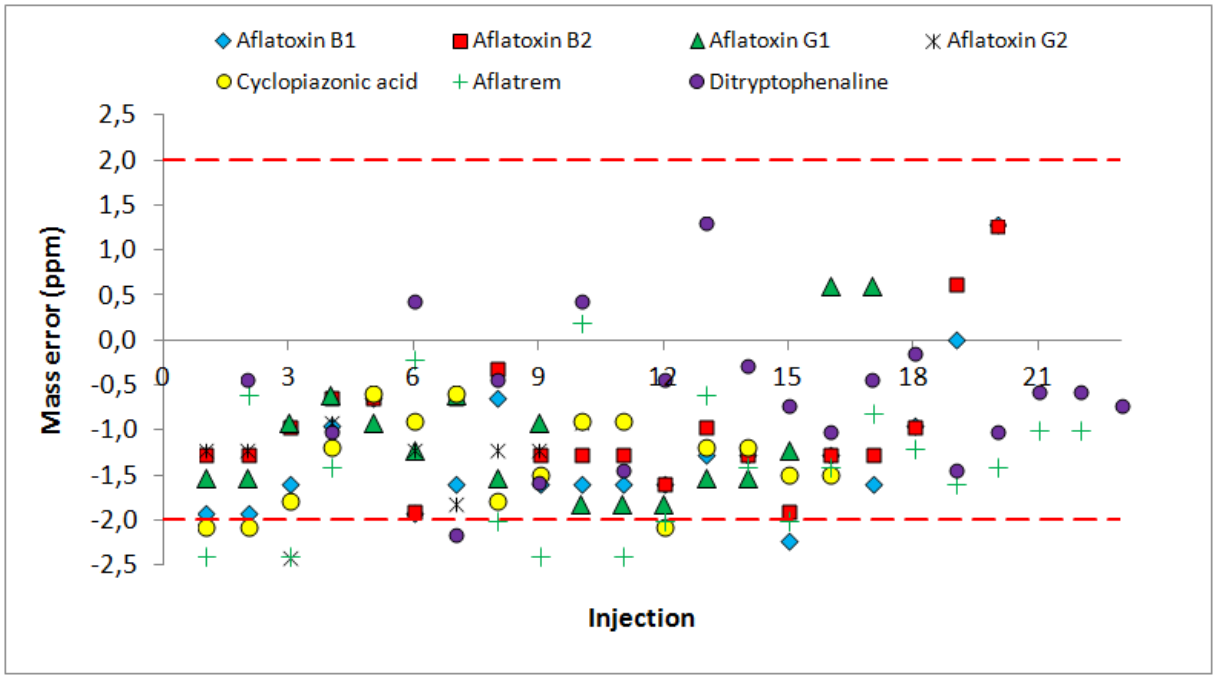

Figure 4.2. *Experimental mass error of Orbitrap-MS. The dashed lines represent a typical mass error of 2 ppm.*

4.3.1.3. Optimization of extraction procedure

Different extraction methods have been applied in the analysis of secondary fungal metabolites. A majority of the methods involved multiple clean-up steps to produce 'clean' extracts (Diana Di Mavungu *et al*., 2009; Ip and Che, 2006; Lattanzio *et al*., 2007; Monbaliu *et al*., 2010), having laboriousness and specificity only towards a selected group of compounds as their main drawbacks. From the other hand, a simple 'dilute-and-shoot' approach ensured extraction and detection of a wide range of analytes, however, yielding 'dirty' extracts (Al-Taher *et al*., 2013; Mol *et al*., 2008; Monbaliu *et al*., 2010; Sulyok *et al*., 2007).

In the current study, a set of commonly used solvents was investigated to develop a fast and generic extraction protocol for the purpose of untargeted secondary fungal metabolite profiling. The tested solvent compositions were the following: *I)* MeOH:DCM:EtOAc (10:20:30, v/v/v) containing 1 % HCOOH (adapted from Smegsgaard, 1997); *II)* MeOH:DCM (50:50, v/v) followed by EtOAc:DMK (50:50, v/v) containing 1 % HCOOH (adapted from Frisvad and Thrane, 1987); *III)* ACN:H_2O (84:16, v/v) (adapted from Berthiller *et al*., 2005; Ren *et al*., 2007); *IV)* ACN:H_2O:HCOOH (79:20:1, v/v/v) (adapted from Mol *et al*., 2008;

Sulyok *et al*., 2007; Varga *et al*., 2013); *V)* DMK; *VI)* DMK followed by DCM and H_2O. To estimate the suitability of the extraction solvent, peak area (n=4) was measured and compared for each condition. Overall good results were obtained for all investigated solvents (Fig. 4.3). Lower areas were noted for aflatoxin G1 and cyclopiazonic acid using solvent VI, while aflatoxin G2 was better extracted using solvent I. This dramatic decrease in the area of cyclopiazonic acid can be possibly attributed to a partitioning step in the procedure VI, where organic phase was used for the analysis (aqueous phase was discarded). Therefore, a partitioning step should be avoided in a generic extraction protocol. Moreover, acidification of extraction solvent can be useful, especially with the prospect of applying the method to different samples (media, food, feed, *etc*.). At neutral conditions, acidic and basic compounds are prone to interact with matrix (its protonated groups). At acidic conditions, acidic compounds are protonated and in their neutral state, and analytes with basic groups are ionized (positively charged), and, therefore, bind less to matrix components and remain dissolved in extraction solvent (Krska *et al*., 2008; Mol *et al*., 2008).

Based on these observations and keeping in mind that the method can be broadened to matrices other than media, solvent I was finally chosen for sample preparation.

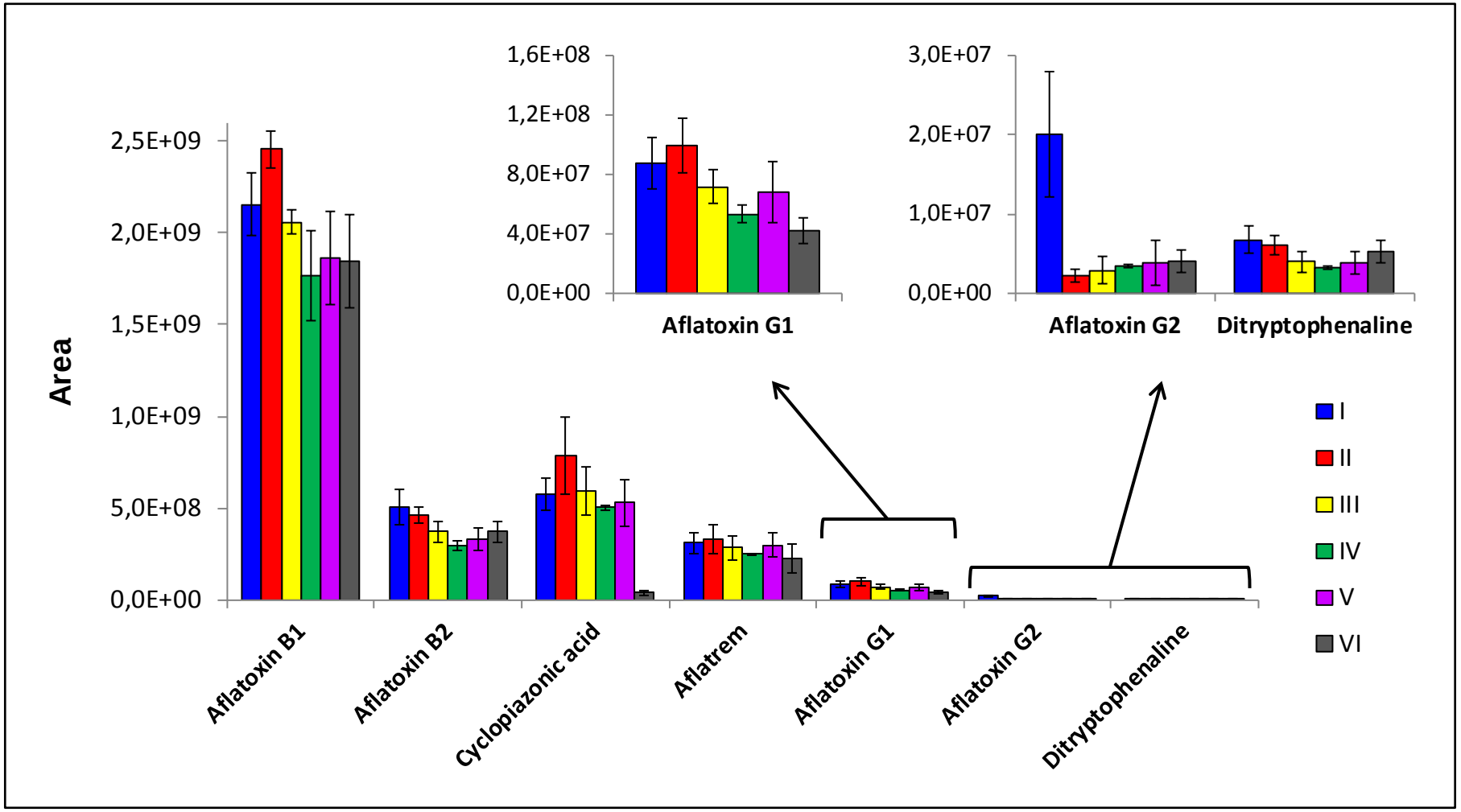

Figure 4.3. *Optimization of extraction solvent.* ***I****: MeOH:DCM:EtAOc (10:20:30, v/v) containing 1 % HCOOH;* ***II****: MeOH:DCM (50:50, v/v) followed by EtOAc:DMK (50:50, v/v) containing 1 % HCOOH;* ***III****: ACN:H_2O (84:16, v/v);* ***IV****: ACN:H_2O:HCOOH (79:20:1, v/v/v);* ***V****: DMK;* ***VI****: DMK followed by DCM and H_2O.*

4.3.2. Metabolic profiling and preliminary metabolite assignment

Recombinational inactivation of the *pks27* resulted in *A. flavus* transformants that no longer produced characteristic dark brown sclerotia but instead produced sclerotia that were greyish-yellow. Metabolite screening of wild-type *A. flavus* AF70 and mutant *A. flavus* AF70 was performed on an LC-Orbitrap-MS in ESI and APCI, both in positive and negative mode. The total ion chromatogram (TIC) for the wild-type and mutant are presented in Fig. 4.4.

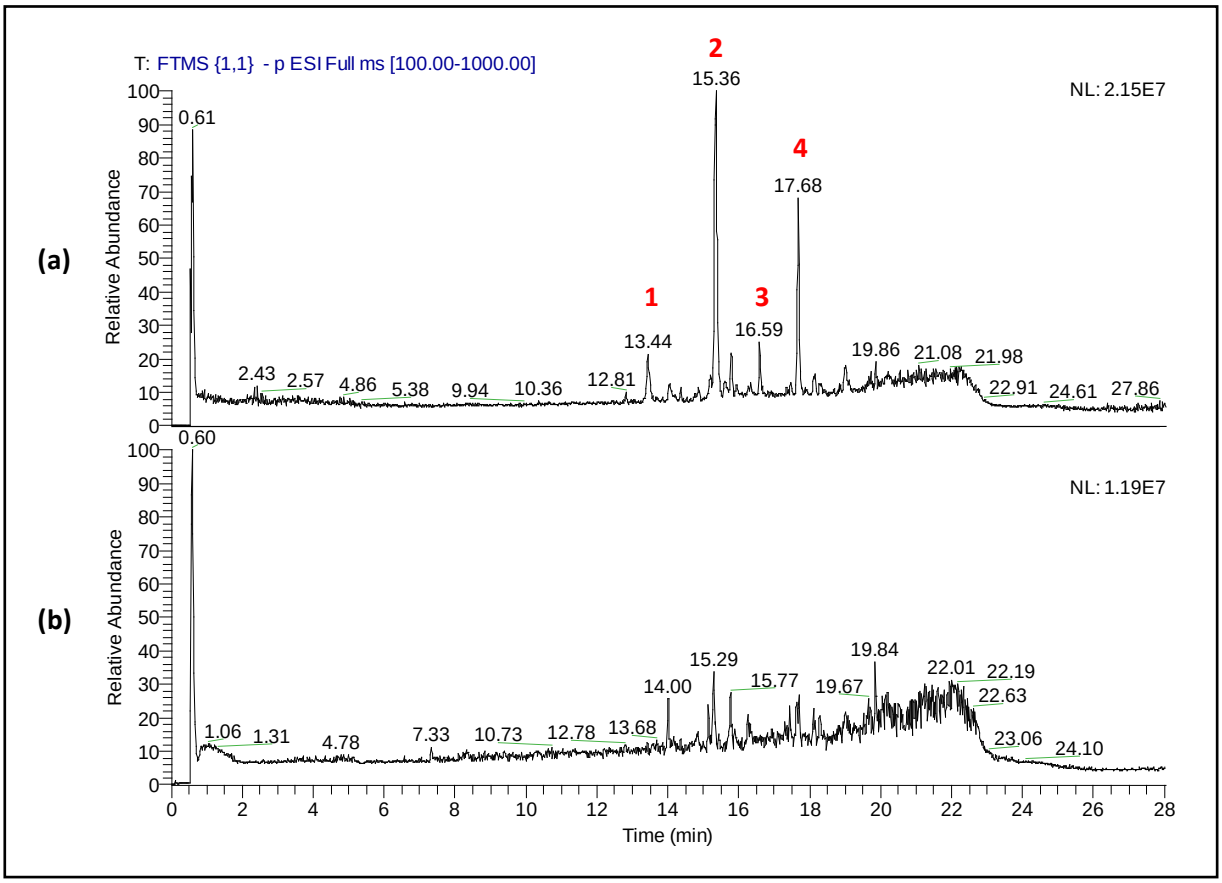

Figure 4.4. *Total ion chromatogram of wild-type A. flavus AF70 (a) and mutant A. flavus AF70 (b) obtained in ESI*$^{-}$*.* ***1*** *corresponds to m/z 373;* ***2****: m/z 357;* ***3****: m/z 315;* ***4****: m/z 339.*

Visual inspection of the TIC of the wild-type revealed presence of four clear peaks, which were seen only in the TIC of the parental strain. As low-abundance compounds might not be apparent, a statistical differential analysis of the LC-MS data was performed using SIEVE software. SIEVE included PCA to highlight differences between the two groups. To compare chromatograms of the groups on the basis of peak intensities, peak alignment was done using wild-type as reference sample. Significant differences in the metabolite profile were observed between the sample groups. On PCA score plot, the wild-type group was clearly separated from the other samples, which included the blank media and replicates of the mutant (Fig. 4.5). On PCA loadings plot (Fig. 4.5), variables relevant for the clustering pattern are shown. A set of four markers, corresponding to 373.0561 (13.4 min) (**1**), *m/z* 357.0612 (15.4 min) (**2**), *m/z* 315.0510 (16.6 min) (**3**), *m/z* 339.0504 (17.7 min) (**4**) could be distinguished for the wild-type. The reconstructed ion chromatograms confirmed absence of these ions in the mutant.

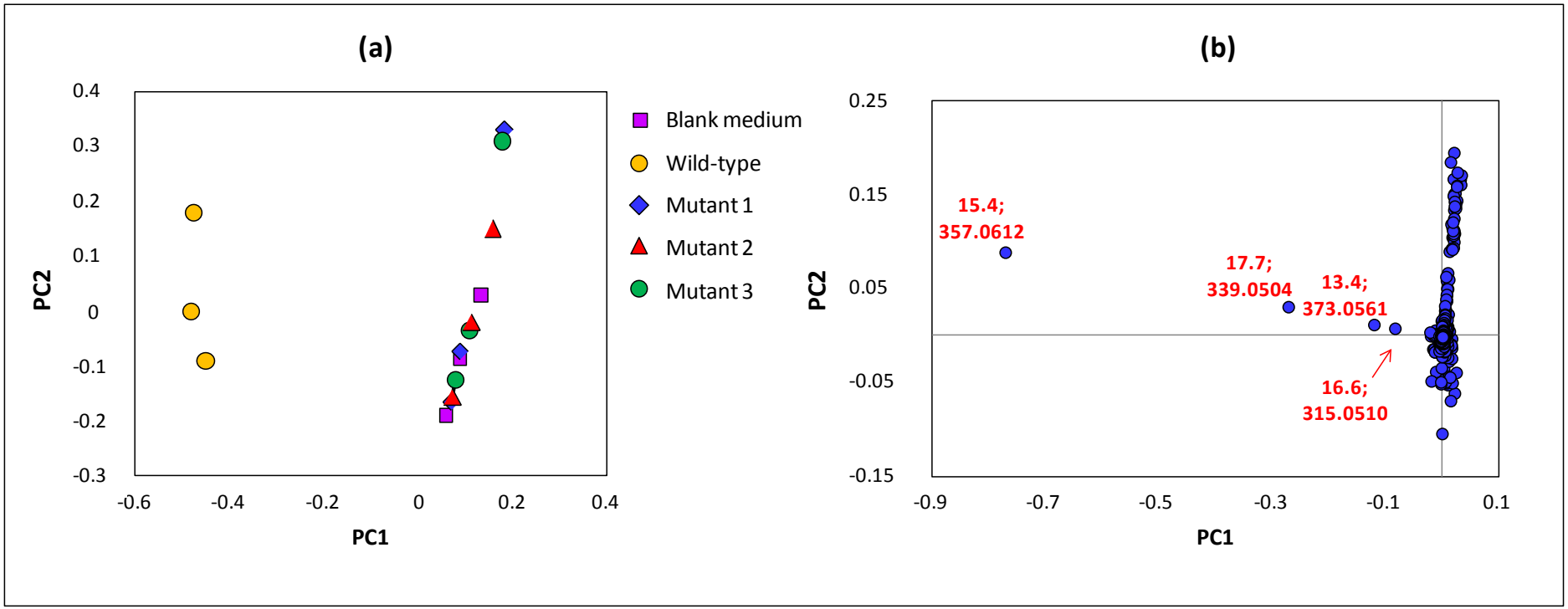

Figure 4.5. *PCA score plot (a) and loadings plot (b).*

4.3.3. Fragmentation pathway and metabolite identification

To identify the detected markers, firstly, accurate mass was used to generate elemental compositions leading to tentative metabolite identification by searching a home-made and public databases. At this stage, a match was found only for *m/z* 357.0612 corresponding to asparasone A (1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)-anthraquinone) with molecular formula $C_{18}H_{14}O_8$, an anthraquinone pigment previously detected in *A. parasiticus* cultures (Sobolev *et al*., 1997). Identification of *m/z* 357.0612 as asparasone A (**2**) was confirmed by comparison with an authentic standard. A complete correspondence of RT, MS and MS^n spectra for the identified metabolite and the standard was observed. MS^2 spectra (Fig. 4.6) were characterized by abundant product ions at *m/z* 339, and *m/z* 299 corresponding to loss of H_2O (-18 Da) and C_3H_6O (-58 Da) from the precursor ion at *m/z* 357, respectively. Subsequent fragmentation of the ion at *m/z* 339 yielded a product ion at *m/z* 297 corresponding to a loss of 42 Da (C_2H_2O), whereas a fragment at *m/z* 271, corresponding to a loss of CO (-28 Da), was formed through fragmentation of the ion at *m/z* 299. MS^n data of the *m/z* 297 and *m/z* 271 (Fig. 4.7) indicated consecutive losses of 28 Da and 44 Da corresponding to CO and CO_2, respectively. The fragmentation mechanism for these losses of CO and CO_2 moieties from the anthraquinone structure was similar to that described by Kang *et al.* (Kang *et al*., 2007) for isoflavone derivatives. This involved a rearrangement of the molecule leading to the loss of the carbonyl and hydroxyl moieties, and closing of ring; however, the order in which CO or CO_2 were eliminated from the structure could not be established. Combining these fragmentation data and accurate mass measurement, a fragmentation pathway of asparasone A in ESI^- was proposed (Fig. 4.8). The above described consecutive losses of CO and CO_2 led to the fragments at *m/z* 141 and 157, and *m/z* 155 from the ions at *m/z* 297 and *m/z* 271, respectively.

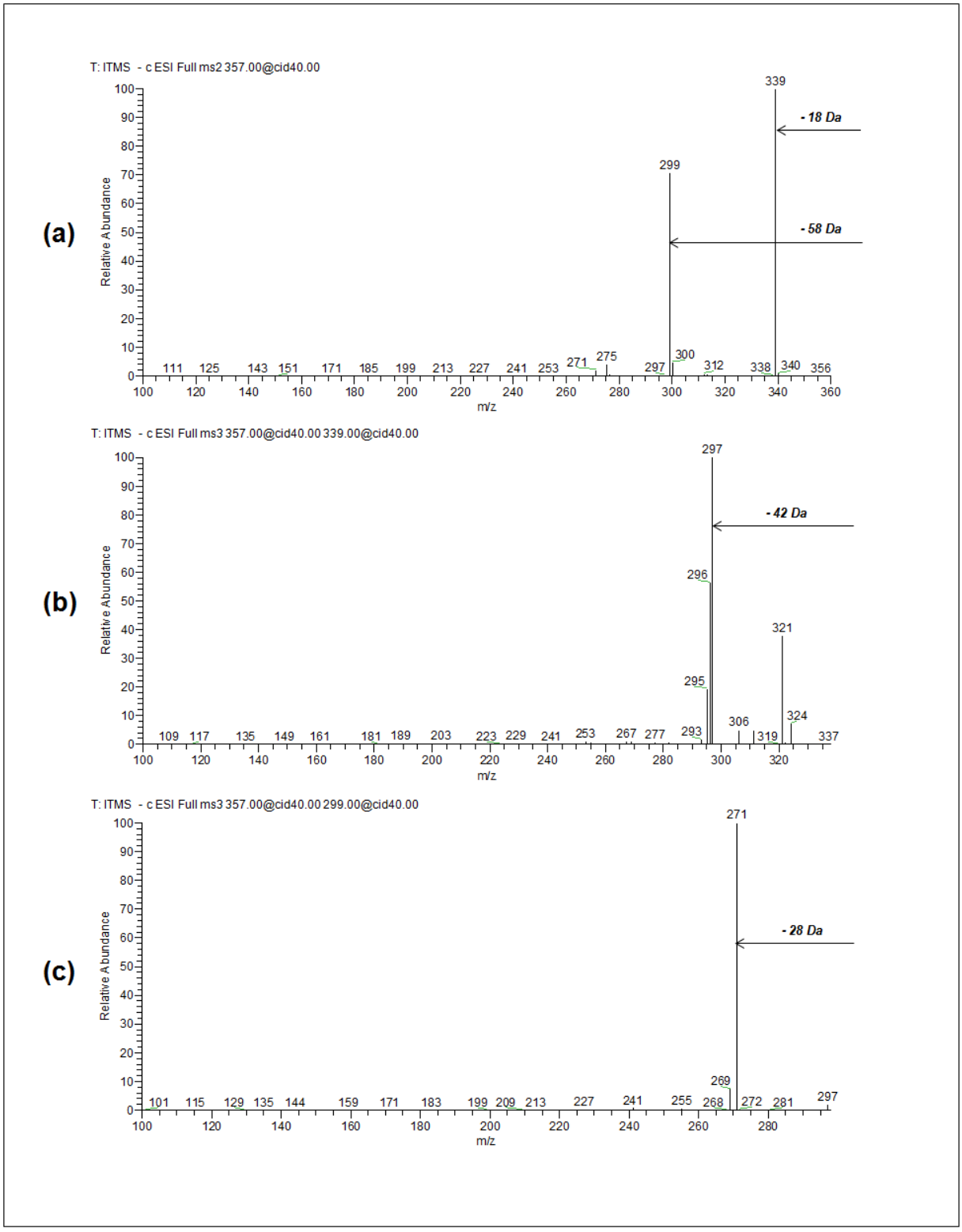

Figure 4.6. *CID spectrum of the precursor ion at m/z 357 (a) and second-generation spectra (b, c) acquired for compound* ***2****. The product ions at m/z 339 (b) and m/z 299 (c) were isolated and collisionally activated at 40 % CEL.*

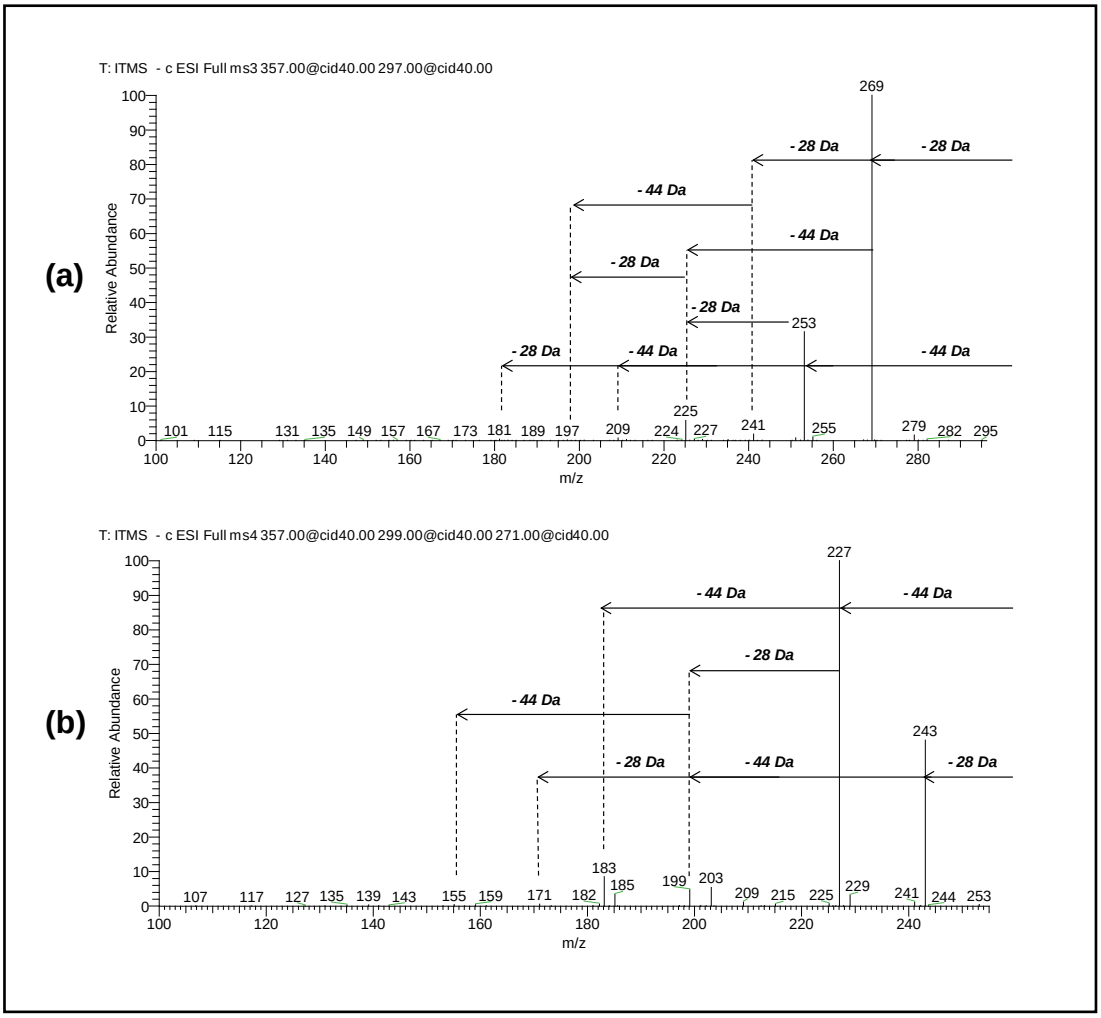

Figure 4.7. *Second- (a) and third- (b) generation spectra acquired for compound* ***2****. The product ions at m/z 297 (a) and m/z 271 (b) were isolated and collisionally activated at 40 % CEL.*

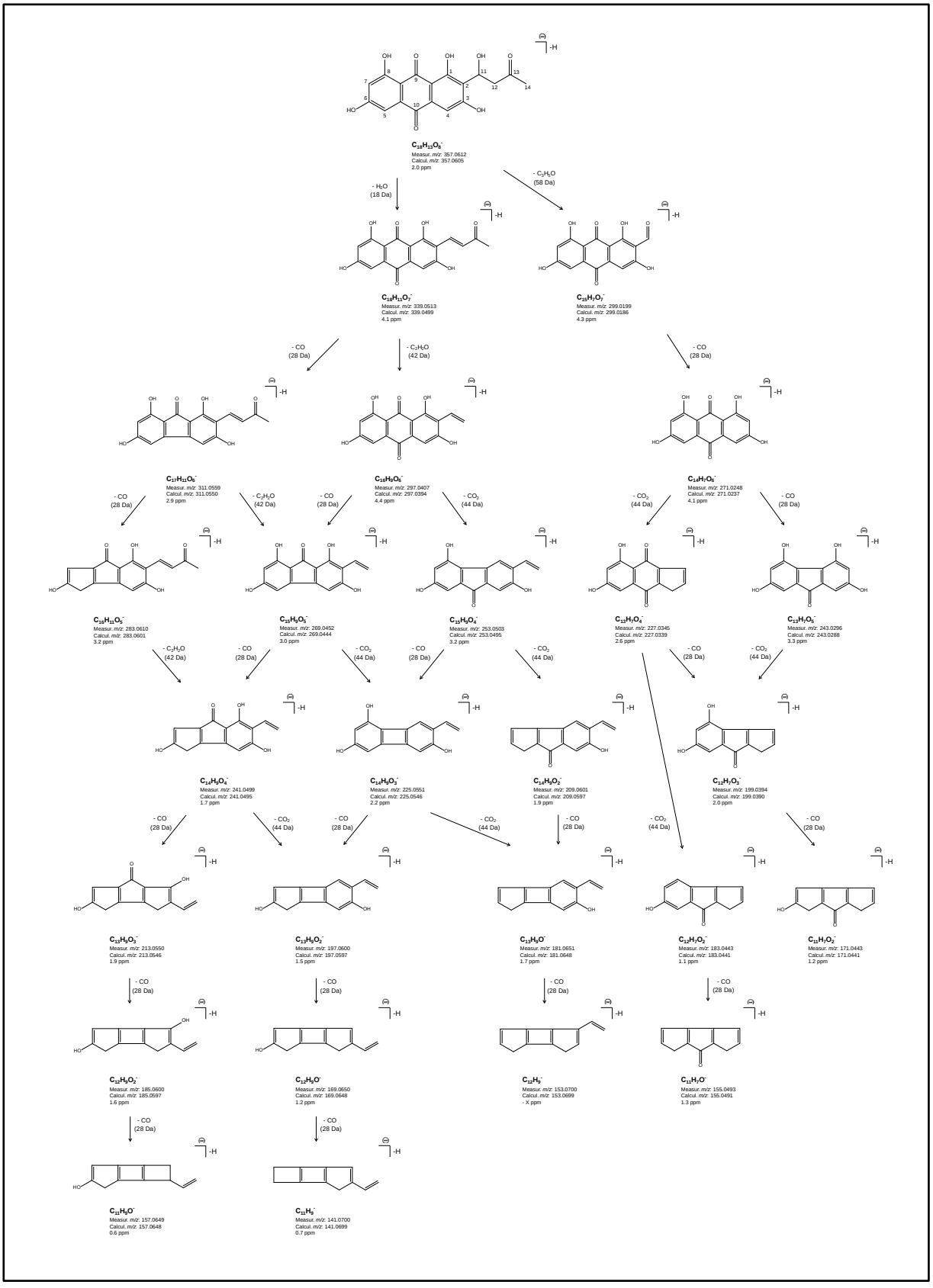

Figure 4.8. *Proposed fragmentation pathway of asparasone A (**2**). The pathway involved consecutive losses of CO and CO_2 moiety, starting from the fragments at m/z 299 ($C_{15}H_7O_7$), m/z 339 ($C_{18}H_{11}O_7$) and m/z 297 ($C_{16}H_9O_6$). The order of these losses could not be established; exemplary structures of fragments are shown.*

It was hypothesized that the four compounds detected in the wild-type *A. flavus* AF70 might be a part of the asparasone A biosynthetic pathway and therefore can be structurally related. Owing to mass differences of the abundant ions observed in the MS spectra, it was assumed that *m/z* 339 (**4**) and *m/z* 315 (**3**) could represent a molecule of asparasone A in which H_2O and C_2H_2O, respectively, were eliminated from the side chain. A difference of 16 Da for *m/z* 373 (**1**) could possibly account for an additional oxygen atom in the structure of asparasone A.

MS^2 spectra of compound **3** (*m/z* 315) showed initially a loss of 18 Da (corresponding to the elimination of a water molecule) yielding *m/z* 297 (Fig. 4.9a), whose fragmentation was identical to that observed for asparasone A. Compound **4** (*m/z* 339) also gave the same fragment at *m/z* 297 (Fig. 4.9b). MS^2 of compound **1** (*m/z* 373) indicated losses of 18 and 58 Da (Fig. 4.10a), as was observed with asparasone A (Fig 4.6a). The resulting fragments (*m/z* 355 and *m/z* 315) had 16 mass units higher than the corresponding fragments in asparasone A, *i.e*. *m/z* 339 and *m/z* 299, respectively. This 16 Da difference pointed to an extra oxygen atom in the molecule, exact position of which in the structure could not be deduced from further MS^n fragmentation of the product ions at *m/z* 355 and *m/z* 315. Nevertheless, these fragmentation data indicated that the substitution with a hydroxyl-group in the side chain was not likely, as the difference of 16 Da between asparasone A (**2**) and compound **1** was still noted after elimination of the side chain from the molecule. Therefore, the hydroxyl-group could be at positions 4, 5 or 7, and more likely at positions 4 or 5. However, to designate the exact position of the hydroxyl-substituent, isolation of the compound and NMR experiments are needed.

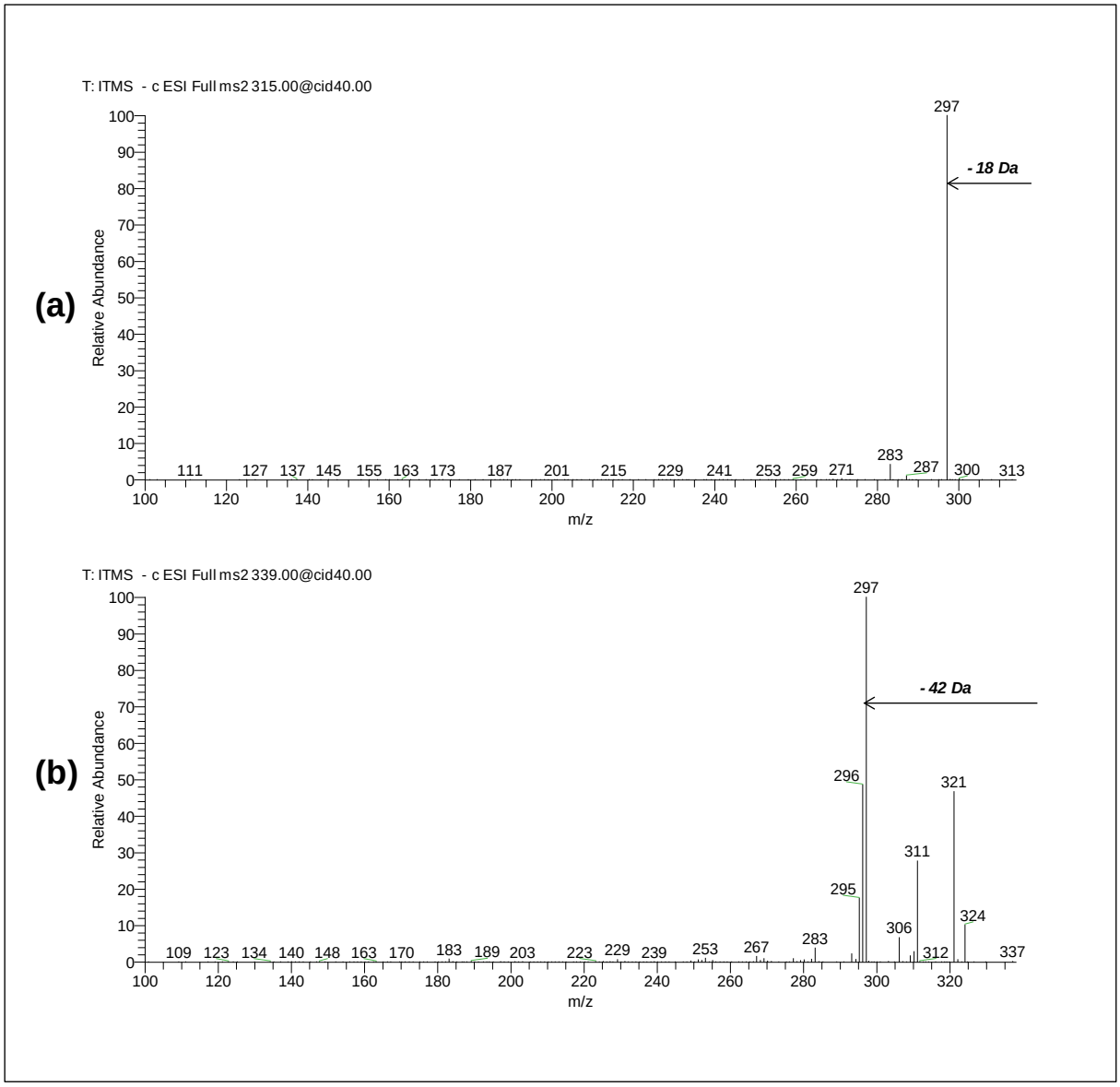

Figure 4.9. *CID spectra of precursor ions at m/z 315 (compound **3**) (a) and m/z 339 (compound **4**) (b).*

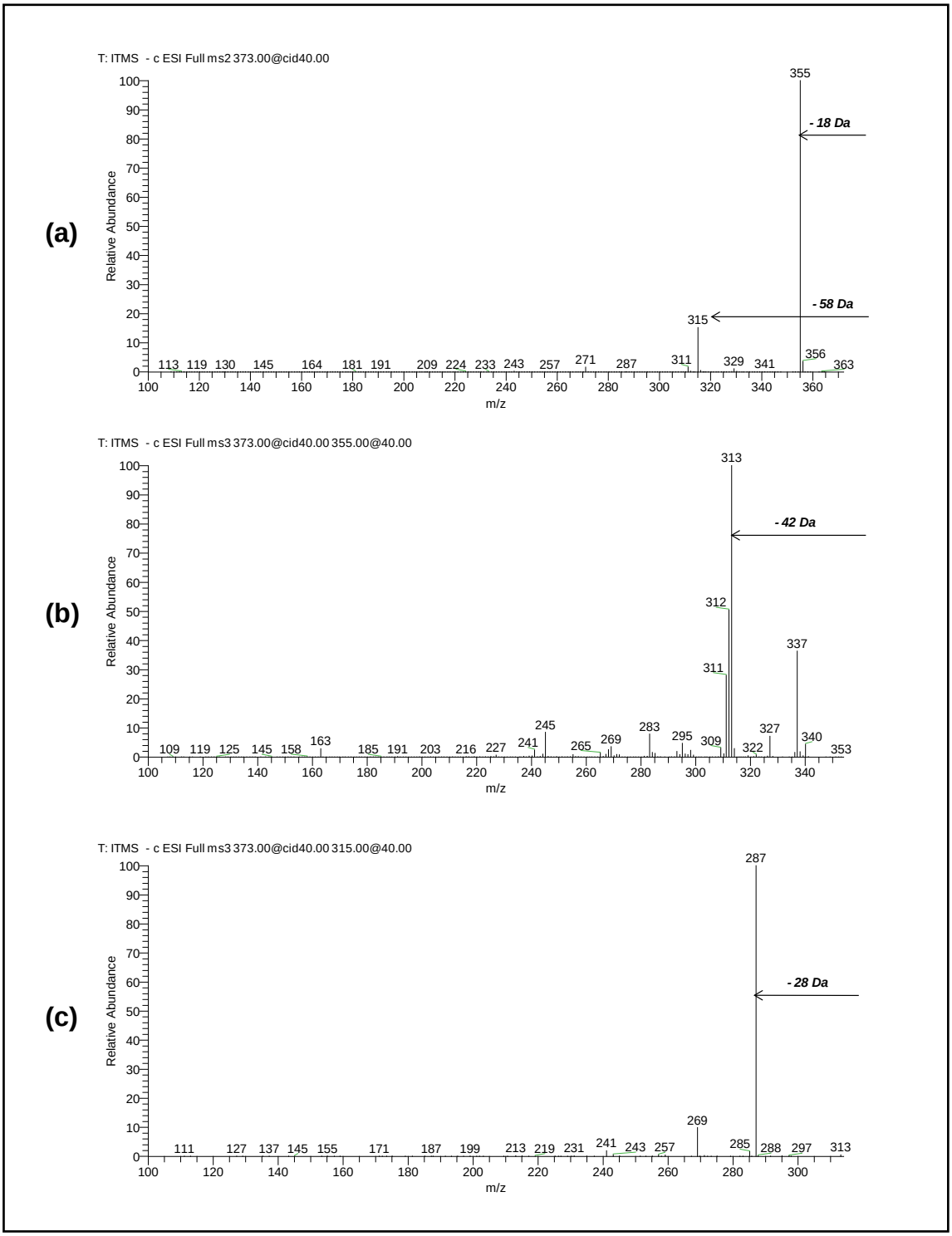

Figure 4.10. *CID spectrum of a precursor ion at m/z 373 (a) and second-generation spectra (b, c) acquired for compound* ***1****. The product ions at m/z 355 (b) and m/z 315 (c) were isolated and collisionally activated at 40 % CEL.*

Based on data described above, compounds **1**, **3** and **4** were assigned respectively as 1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone, 1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone and 1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone (Fig. 4.11). The elution order of these compounds under the applied chromatographic conditions (reversed-phase chromatography) was consistent with the proposed structures. Compounds **4** and **3** eluted later than asparasone A (**2**), being more hydrophobic due to absence of polar hydroxyl- and acetyl-groups, respectively. Compound **3** having a shorter side chain was eluted earlier than compound **4**. Compound **1**, bearing an additional hydroxyl-group is more polar and was eluted before asparasone A (**2**).

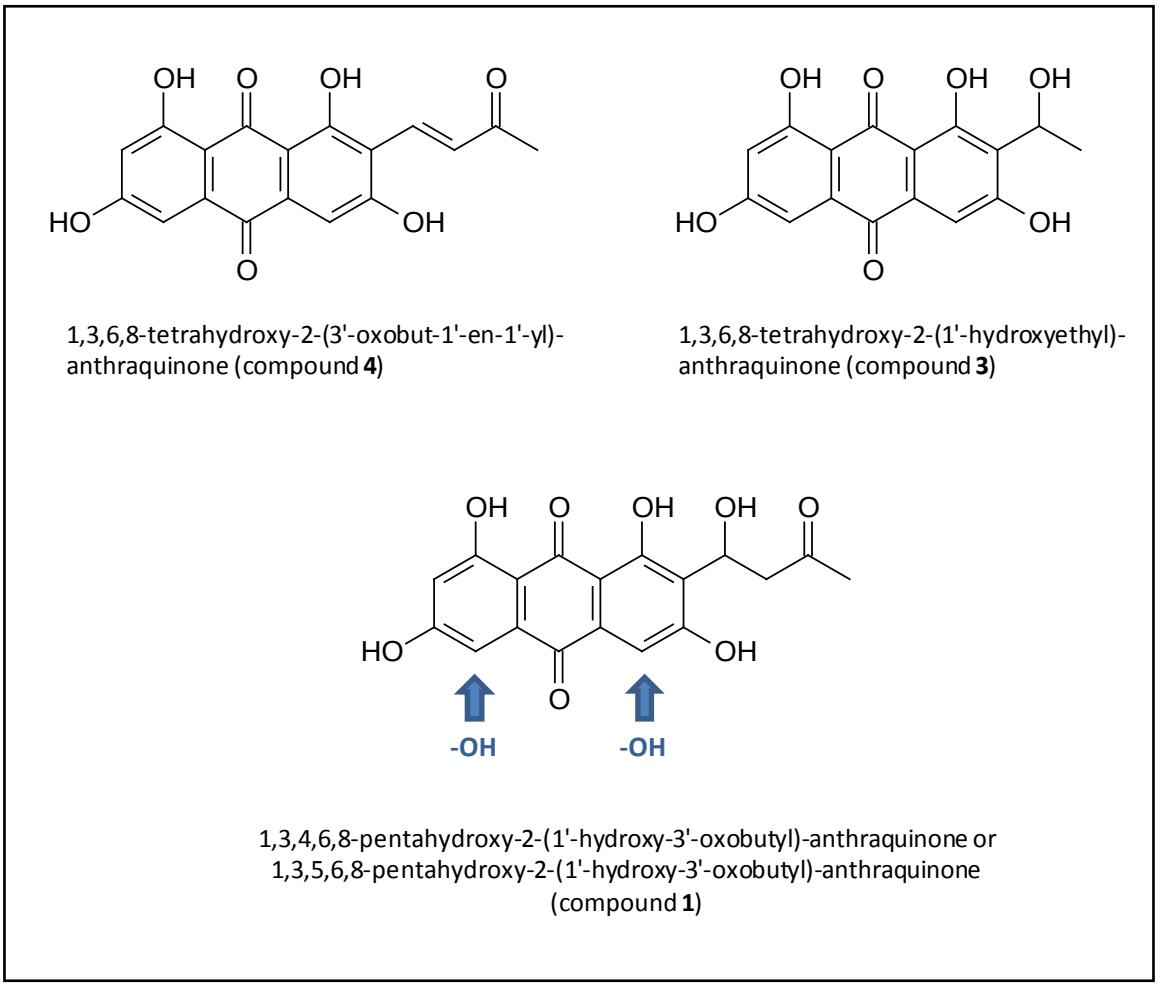

Figure 4.11. *Structures of newly identified metabolites of A. flavus AF70. Probable positions of the additional hydroxyl-group in the structure of compound* ***1*** *are shown with the blue arrows.*

The proposed structures proved to be polyketide products, as was predicted by *in silico* analysis (Georgianna *et al*., 2010). Taking into consideration the *A. flavus* cluster 27 metabolites identified in this study, a biosynthetic pathway was proposed (Fig. 4.12). The biosynthesis begins with acetate as the starter unit and then proceeds with addition of 8 malonyl-CoA units, one more than is incorporated into the aflatoxin *pksA* (Do *et al*., 2007; Korman *et al*., 2010). Following Claisen condensation and thioester cleavage, the anthrone is produced. The resulting anthrone is then oxidized to the anthraquinone and subsequently reduced specifically at the C11 position to produce asparasone A (**2**). This set of reactions requires the presence of an oxidoreductase somewhat similar to that represented by AflD in aflatoxin biosynthesis. Although such an enzyme is not in the SMURF-identified gene cluster, biosynthesis could take advantage of such enzymes, either in the aflatoxin cluster or the enzymes of similar function in the conversion of tetrahydronapthalene to dihydronaphthalene (DHN) in the formation of DHN-derived melanins (Bell and Wheeler, 1986).

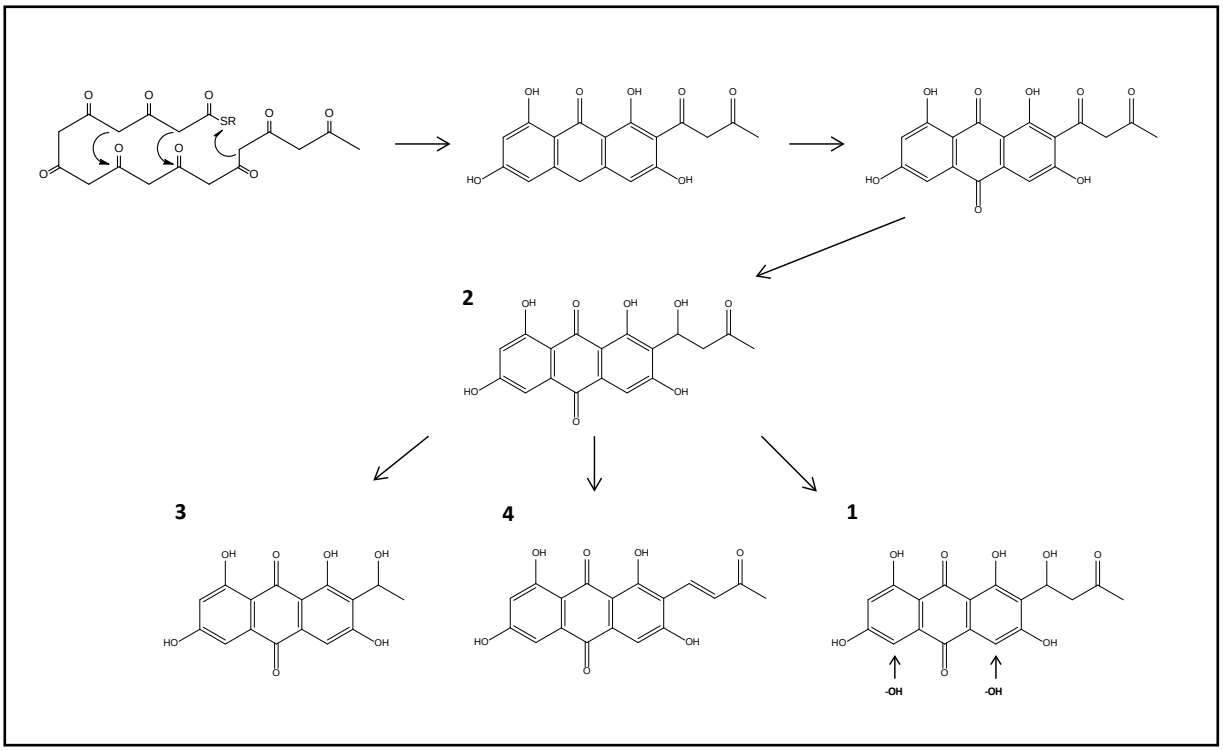

Figure 4.12. *Proposed biosynthetic steps in the formation of cluster 27 pks metabolites: 1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone (**1**), asparasone A (**2**), 1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone (**3**) and 1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone (**4**). Probable positions of the additional hydroxyl-group in the structure of compound **1** are shown with the arrows.*

4.3.4. Production of asparasone A derivatives in selected fungal cultures

Asparasone A was first identified in *A. parasiticus* extracts as a pigment that was structurally related to versicolorin intermediates of the aflatoxin biosynthetic pathway (Sobolev *et al*., 1997). In their study asparasone A was obtained from total extract of *A. parasiticus* culture.

In the investigation of the cluster 27 metabolites described in this work, a AF70 strain was used. Production of asparasone A and its derivatives (compounds **1**, **3** and **4**) was further investigated in extracts of other *Aspergillus* strains (Table 4.3).

Asparasone A was detected in all fungal cultures studied and occurred together with its dehydrated product. In *A. terreus* culture, compounds **3** and **1**, respectively, were not detected simultaneously with asparasone A. Investigation of other known *Aspergillus* metabolites revealed co-occurrence of asparasone A, aflatoxin B1 and cyclopiazonic acid. However, a clear correlation between these metabolites could not be established.

Table 4.3. *Production of asparasone A and its derivatives, and a number of other metabolites by Aspergillus spp.*

Metabolite	Fungi							
	A. nidulans	*A. nomius*	*A. terreus*	*A. parasiticus* BN8	*A. parasiticus* BN9	*A. flavus* AF36	*A. flavus* AF70	*A. flavus* CA14
Asparasone A	++	++	+	++	+++	+++	++	+
1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone	++	++	+	++	++	++	++	+
1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone	++	++	-	++	++	++	++	+
1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone	++	++	-	++	++	++	++	+
Aflatoxin B1	+	+++	++	+++	+++	++	+++	+
Aflatoxin B2	+	+++	+	+++	+++	+	+++	+
Aflatoxin G1	-	+++	++	+++	+++	++	++	-
Aflatoxin G2	-	+++	+	+++	+++	-	++	-
Cyclopiazonic acid	++	++	++	+++	++	+++	+++	+++
Aflatrem	+	-	-	++	++	+++	+++	++
Ditryptophenaline	++	++	++	+++	+	+++	++	+++

-	not detected
+	peak area ≤ 10^6
++	10^6 < peak area ≤ 10^8
+++	peak area > 10^8

4.4. Conclusions

The present study was designed to determine the metabolites produced by the *A. flav*us cluster 27 *pks* gene (*pks27*). Comparative metabolomics, using UHPLC-HR Orbitrap MS, allowed to detect metabolites that were differentially expressed in *A. flav*us wild-type and *Δpks*27 mutant strains. This was supported by a statistical differential analysis of MS data using SIEVE software. Accurate mass data from the Orbitrap MS instrument allowed to determine the elemental composition of the compounds of interest and thereby to perform a preliminary peak assignment, while ion trap multiple stage MS fragmentation trees (up to MS^5) were required for structural information and confirmation of metabolites. In this way, we found that the major metabolite produced by the *A. flav*us cluster 27 is asparasone A, an anthraquinone pigment previously detected in *A. parasiticus* cultures (Sobolev *et al*., 1997). The ESI^- fragmentation pattern of this compound has been elucidated for the first time. Three other compounds, namely the dehydration, deacetyl- and oxy-derivatives of asparasone A, were identified for the first time. Asparasone A and its newly identified derivatives were detected simultaneously with some known *A. flavus* metabolites in other *Aspergillus spp*. The hyphenation of UHPLC with Orbitrap MS and ion trap multi-stage MS proved to be a suitable approach for identification of new or less studied fungal metabolites. The study also showed that asparasone A is a sclerotial pigment. Its function for the fungus still remains to be determined. Further NMR studies are necessary to assign the complete structure of the oxy-derivative of asparasone A identified in this work.

4.5. Acknowledgements

Dr. Jeffrey Cary and Dr. Kenneth Ehrlich (Southern Regional Research Center (New Orleans, LA, USA) are acknowledged for making the *A. flavus* AF70 mutant. Prof. Lynn Vanhaecke (Laboratory of Chemical Analysis, Faculty of Veterinary Medicine, Ghent University, Belgium) is thanked for giving the opportunity to work on Orbitrap and ion trap MS. Natalia Arroyo Manzanares, Stefanie D'heer and Valerie De Brauwer are also acknowledged for their contribution in the realization of this work.

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CHAPTER 5

Ionization strategy for patulin determination by LC-MS/MS: Usefulness in multi-mycotoxin analysis

The content of this chapter was partially derived from:

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*These authors contributed equally to this work

5.1. Introduction

Patulin (Fig. 1.2, Chapter 1) is a toxic fungal metabolite mainly produced by *Penicillium* and *Aspergillus* spp. It naturally occurs in food commodities such as fruits and their processed products (Drusch and Ragab, 2003), wheat bread (Rychlik, 2005), as well as feed (Sabater-Vilar *et al*., 2004). Recently a high content of patulin was found in indoor settled dust (Vishwanath *et al*., 2011).

It has been proven that patulin causes acute and chronic health problems in humans and animals (de Souza Sant'Ana *et al*., 2008; Drusch and Ragab, 2003). Therefore, patulin levels in food are regulated in many countries and an action level in juices is set at 50 µg/kg (Commission Regulation, 2003; FDA, 2001).

Methods for patulin detection include TLC for rapid monitoring of patulin levels (Martins *et al*., 2002; Welke *et al*., 2009), HPLC coupled to UV (Baert *et al*., 2007; Barreira *et al*., 2010; Wu *et al*., 2008) or diode array detection (DAD) (Boonzaaijer *et al*., 2005) and GC-MS (Cunha *et al*., 2009; Roach *et al*., 2000). These LC and GC methods demonstrate satisfactory LODs, but caution has to be taken in the analysis of fruit juices by LC-UV/DAD where interfering peaks are likely to occur (Boonzaaijer *et al*., 2005), while a derivatization is generally required for GC-MS (Kataoka *et al*., 2009).

LC-MS/MS, being considered as the most advanced analytical technique for qualitative and quantitative mycotoxin analysis, is widely used for determination of patulin. There are several methods described allowing reliable detection of this compound in ESI^- (Kataoka *et al*., 2009), APCI (Sewram *et al*., 2000) or APPI (Takino *et al*., 2003) modes. However, methods to simultaneously analyze patulin and other toxins are scarce and require polarity switching of the MS (Sulyok *et al*., 2006). Furthermore, in our group, attempts to achieve a useful ESI-MS signal for patulin using three different generations of triple quadrupole instruments were not successful. Therefore, in line with the approach applied by other authors dealing with multi-analyte detection of mycotoxins, patulin was omitted in previous multi-mycotoxin LC-ESI-MS/MS methods (Diana Di Mavungu *et al*., 2009; Ediage *et al*., 2011; Garon *et al*., 2006; Monbaliu *et al*., 2009).

Besides the food/feed consumption and inhalation routes, mycotoxins can also adversely affect human and animal health through skin penetration (Berthiller *et al*., 2007;

Kemppainen *et al*., 1988-89). However, dermal exposure to mycotoxins currently remains a less studied area of research. Reported studies include mainly *in vitro* experiments of the transdermal penetration of a single mycotoxin using diffusion cells filled with bovine serum albumin (BSA) in phosphate buffered saline (PBS) as receptor fluid (Kemppainen *et al*., 1988-89; Mills and Cross, 2006). Considering the fact that a single mould species can produce several secondary metabolites and that exposure to different moulds is possible, it is important to monitor a wider range of mycotoxins. This necessitates suitable multi-analyte methods.

This study is focused on a closer investigation of the electrospray ionization of patulin with the goal of inclusion of this toxin in a single run multi-analyte LC-ESI^+-MS/MS method. Adduct formation of patulin appeared to be the only possibility to achieve the purpose of the study. The fragmentation behaviour of patulin (methanol adduct) under the applied conditions was thoroughly studied combining the advantageous features of ion trap and Orbitrap MS. The usefulness of the findings was demonstrated by the development of an LC-ESI^+-MS/MS method that allows a rapid analysis of patulin together with other mycotoxins. With the prospect of assessing exposure to mycotoxins through dermal route, the method was validated for fluids from transdermal penetration studies.

5.2. Experimental

5.2.1. Chemicals and material

MeOH and ACN both of LC-MS grade were obtained from Biosolve (Valkenswaard, The Netherlands), whereas HPLC-grade MeOH and ACN were from VWR International (Zaventem, Belgium). EtOAc and DCM were purchased from Acros Organics (Geel, Belgium). Ammonia *p.a.* (25 %) was provided by Vel (Leuven, Belgium). Sigma-Aldrich (Bornem, Belgium) was the supplier of ammonium bicarbonate (NH_4HCO_3), BSA (RIA grade) and PBS. Hydrochloric acid (HCl), CH_3COOH, HCOOH and CH_3COONH_4 from Merck (Darmstadt, Germany) were used. Ultrapure H_2O was produced by a Milli-Q Gradient System (Millipore, Brussels, Belgium). Oasis™ HLB 6 cc (200 mg) SPE extraction cartridges were purchased from Waters (Milford, MA, USA). Ultrafree®-MC centrifugal filter units (0.22 µm) from Millipore (Bedford, MA, USA) were used.

Standards of patulin, ochratoxins A and B, aflatoxins B1, B2, G1 and G2, citrinin, T-2 and HT-2 toxins, zearalenone, zearalanone, neosolaniol, diacetoxyscirpenol, sterigmatocystin, nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, fumonisins B1 and B2, beauvericin, alternariol monomethyl ether were purchased from Sigma-Aldrich. 3-Acetyl-deoxynivalenol was supplied as a 100 µg/mL solution in ACN. Regarding the other compounds, ready-made solid portions of commercial standards were dissolved in MeOH (in ACN:H_2O 50:50, v/v for fumonisins) to reach the concentration of 1 mg/mL. Stock solutions were stored at - 20 °C (at 4 °C for fumonisins). Tuning solutions of the mycotoxins were prepared at a concentration of 10 µg/mL. MS^n experiments and accurate mass measurements were performed on a 50 µg/mL solution of patulin in H_2O:0.2 M NH_4HCO_3 (pH10):MeOH (30:5:65, v/v/v) utilizing the ion trap and Orbitrap MS, respectively.

5.2.2. MS conditions

5.2.2.1. Ionization efficiency of patulin and multi-mycotoxin analysis

A Micromass Quattro LC triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with ESI and APCI sources and Z-spray was used during the ionization efficiency experiments and subsequent multi-mycotoxin LC-MS/MS method development. In the final method ESI was used in the positive mode. The optimized MS parameters were as follows: probe nebuliser gas flow 100 L/h, desolvation gas flow 830 L/h, capillary voltage 3.5 kV, source block temperature 120 °C, desolvation temperature 320 °C, multiplier 700 V. The instrument was controlled by MassLynx^{TM} 3.5 software (Micromass, Manchester, UK).

To evaluate the effects of mobile phase additives on the ionization efficiency, solutions of patulin were introduced into the MS by infusion: the solution of patulin coming from a syringe at a flow rate of 10 µL/min was mixed with the mobile phase (0.15 mL/min) through a T-shaped connector. The mobile phases (MPs) tested during the ionization efficiency experiments are listed in Table 5.1.

Table 5.1. *Mobile phases (MPs) used in the ionization efficiency experiments.*

	Mobile phase composition
MP1	H_2O:MeOH (30:70, v/v) containing 0.1 % HCOOH
MP2	H_2O:ACN (30:70, v/v) containing 0.1 % HCOOH
MP3	H_2O:MeOH (30:70, v/v) containing 1 % CH_3COOH and 5 mM CH_3COONH_4
MP4	H_2O:ACN (30:70, v/v) containing 1 % CH_3COOH and 5 mM CH_3COONH_4
MP5	H_2O:MeOH (30:70, v/v) containing 5 mM CH_3COONH_4 (pH 6.8)
MP6	H_2O:ACN (30:70, v/v) containing 5 mM CH_3COONH_4 (pH 6.8)
MP7	H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (30:5:65, v/v/v)
MP8	H_2O:0.2 M NH_4HCO_3 (pH 10):ACN (30:5:65, v/v/v)

For the multi-analyte LC-MS/MS method, the SRM mode of acquisition was chosen. The optimized MS parameters and the SRM transitions selected for each of the analytes are given in Table 5.2.

5.2.2.2. Fragmentation behaviour

An LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San José, USA) equipped with an ESI source was used for the investigation of patulin fragmentation behaviour. The solution of patulin was infused into the MS as described in section 5.2.2.1. The set MS parameters were: source voltage 2 kV, vaporizer temperature 80 °C, capillary temperature 100 °C, sheath gas flow rate 10 a.u. Nitrogen was used as sheath gas, while helium was used as damping and collision gas in the ion trap. The maximum inject time was 200 ms and the number of microscans per scan was 3. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different collision energy levels to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

Table 5.2. *Optimized MS/MS parameters for the detection of 23 mycotoxins by LC-ESI$^+$-MS/MS.*

Mycotoxin	RT (min)	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Patulin	2.5	169.1 $[M+MeOH-H_2O+H]^+$	30	137.1[a]	10
				81.1	14
Nivalenol	3.2	313.0 $[M+H]^+$	23	205.2[a]	12
				125.2	12
Deoxynivalenol	3.8	297.1 $[M+H]^+$	26	249.3[a]	12
				203.3	15
Neosolaniol	4.7	400.1 $[M+NH_4]^+$	30	215.3[a]	17
				185.3	20
Ochratoxin B	6.6	370.3 $[M+H]^+$	32	205.1[a]	22
				187.3	36
3-Acetyl-deoxynivalenol	6.9	339.1 $[M+H]^+$	24	231.3[a]	11
				203.3	20
15-Acetyl-deoxynivalenol	6.9	339.0 $[M+H]^+$	21	321.3[a]	10
				137.2	10
Fumonisin B1	7.9	722.6 $[M+H]^+$	50	334.7[a]	40
				352.6	34
Aflatoxin G2	8.1	331.0 $[M+H]^+$	49	245.1[a]	30
				189.2	40
Aflatoxin G1	9.1	328.9 $[M+H]^+$	50	243.1[a]	28
				200.2	40
Ochratoxin A	9.2	404.0 $[M+H]^+$	28	239.2[a]	24
				358.5	14
Aflatoxin B2	9.9	315.4 $[M+H]^+$	51	287.3[a]	26
				259.3	31
Aflatoxin B1	11.1	313.4 $[M+H]^+$	47	241.2[a]	34
				285.3	23
Citrinin	11.6	251.3 $[M+H]^+$	28	233.1[a]	16
				205.5	25
Diacetoxyscirpenol	11.9	384.1 $[M+NH_4]^+$	20	307.3[a]	12
				247.3	14
Fumonisin B2	14.1	706.4 $[M+H]^+$	56	336.7[a]	34
				318.6	41
Zearalenone	15.3	319.4 $[M+H]^+$	21	283.5[a]	13
				301.3	10
Zearalanone	15.6	321.4 $[M+H]^+$	24	303.5[a]	16
				189.4	21
HT-2 toxin	16.3	442.4 $[M+NH_4]^+$	17	263.5[a]	16
				215.4	14
Alternariol monomethyl ether	17.4	273.1 $[M+H]^+$	52	128.2[a]	48
				115.1	52
T-2 toxin	17.9	484.3 $[M+NH_4]^+$	19	305.5[a]	15
				215.3	26
Sterigmatocystin	20.1	325.0 $[M+H]^+$	39	281.1[a]	40
				310.1	23
Beauvericin	25.9	783.9 $[M+H]^+$	47	244.3[a]	30
				262.3	27

[a] The most abundant product ion

5.2.2.3. Accurate mass measurement

Accurate mass measurements of the patulin precursor and fragment ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with an ESI interface. The MS parameters were the following: spray voltage 4 kV, capillary temperature 150 °C, heater temperature 100 °C, sheath gas flow rate 30 a.u., auxiliary gas flow rate 5 a.u. The data were processed using the Xcalibur™ 2.1 and Exactive Tune software (Thermo Fisher Scientific). To achieve high mass accuracy, the instrument was calibrated externally each 24 hours using a standard calibration mixture (caffeine, peptide MRFA and Ultramark 1621). The instrument was operated in full scan positive mode with a resolution of 100,000 FWHM. The maximum injection time was 200 ms and the number of microscans per scan was 1.

Each full scan was followed by a same-polarity "all ion fragmentation" HCD scan. The patulin solution was infused into the Orbitrap MS as described in section 5.2.2.1.

5.2.3. LC conditions

For the introduction of the patulin tuning solution into the ion trap and Orbitrap MS, an Accela™ High Speed LC (UHPLC) (Thermo Fisher Scientific) was used.

For the multi-mycotoxin analysis, the chromatographic separation was achieved on a Waters Alliance 2595 XE HPLC (Waters, Milford, MA, USA) using an XBridge™ C18 column (3.5 µm, 150 mm x 2.1 mm i.d.) (Waters). The mobile phase consisted of H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (85:5:10, v/v/v) [solvent A] and H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (5:5:90, v/v/v) [solvent B]. Two different gradient elution programs were applied for the analyses of either a limited (gradient I) or an extended (gradient II) number of analytes. They were as follows: gradient I: 0-2 min: 50-75 % B, 2-7 min: 75-85 % B, 7-9.5 min: 85-90 % B, 9.5-10 min: 90-50 % B, 10-18 min: 50 % B; and gradient II: 0-4 min: 35-50 % B, 4-7.5 min: 50-60 % B, 7.5-9 min: 60-75 % B, 9-14 min: 75-85 % B, 14-16 min: 85-100 % B, 16-19 min: 100 % B, 19-20 min: 100-35 % B, 20-30 min: 35 % B. The flow rate was 0.15 mL/min. The column temperature was set at 30 °C and temperature of the autosampler was 4 °C. Twenty µL of the sample were injected.

5.2.4. Clean-up of transdermal samples

The samples (1 % BSA in PBS) were cleaned-up using Oasis HLB™ solid-phase extraction (SPE) cartridges. Firstly, the SPE cartridges were conditioned with 10 mL of DCM:MeOH (80:20, v/v) containing 50 mM HCOOH, followed by 5 mL MeOH, 20 mL H_2O containing 10 mM HCl and 10 mL H_2O. After the conditioning step, 200 µL of the sample was quantitatively brought onto the SPE cartridge and washed with 10 mL of H_2O. Elution of mycotoxins was achieved by passing 5 mL MeOH. The eluate was collected into glass test tubes and evaporated under a gentle stream of nitrogen at 40 °C and reconstituted with 100 µL of injection solvent H_2O:0.2 M NH_4HCO_3 (pH 8.5):MeOH (69:5:26, v/v/v). The resulting solution was transferred into an Ultrafree®-MC centrifugal device and centrifuged for 5 min at 14,000 g prior to LC-MS/MS analysis.

5.2.5. Optimization of MS parameters by experimental design

The optimization of MS parameters for patulin was supported by experimental design and multivariate analysis, using Modde 5.0 statistical graphic software (Umetrics, Umeå, Sweden). The most influencing factors were determined by a screening experiment and further optimized by a response surface modeling (RSM) experiment.

A Plackett-Burman design was applied for the screening. The lower and the upper levels of the MS parameters in the design were selected on the basis of preliminary experiments. Fourteen variables were included in this study, which resulted in a 16-run design. Applying this design with three replicates of the center point, the total number of runs was 19. The parameter settings are shown in Table 5.3.

After selection of the important factors, further RSM optimization was carried out applying a central composite face-centered (CCF) design. In particular, the central composite design was composed of a two-level full factorial design, axial points and replicated center points, with a number of experiments equal to $2^k + 2k + n$; where k is the number of parameters and n is the number of replicates of the center point. Three parameters (cone voltage, desolvation temperature and ion energy1) were optimized. With this number of parameters and three replications of the center point, the experimental design resulted in a number of experiments equal to 17. The settings of the parameters in the design are given in Table 5.4.

Table 5.3. *Plackett-Burman design for screening experiment.*

N	Cone voltage (V)	Capillary voltage (V)	Desolvation temperature (°C)	Source temperature (°C)	Multiplier (V)	Extractor (V)	LM Resolution1	HM Resolution1	Ion energy1	Entrance (V)	Exit (V)	LM Resolution2	HM Resolution2	Ion energy2
1	55	2.5	150	100	700	2	13	16	3	30	60	13	16	3
2	55	4.5	150	100	600	5	13	13	3	60	30	16	13	3
3	55	4.5	350	100	600	2	16	13	0.5	60	60	13	16	0.5
4	55	4.5	350	150	600	2	13	16	0.5	30	60	16	13	3
5	15	4.5	350	150	700	2	13	13	3	30	30	16	16	0.5
6	55	2.5	350	150	700	5	13	13	0.5	60	30	13	16	3
7	15	4.5	150	150	700	5	16	13	0.5	30	60	13	13	3
8	55	2.5	350	100	700	5	16	16	0.5	30	30	16	13	0.5
9	55	4.5	150	150	600	5	16	16	3	30	30	13	16	0.5
10	15	4.5	350	100	700	2	16	16	3	60	30	13	13	3
11	15	2.5	350	150	600	5	13	16	3	60	60	13	13	0.5
12	55	2.5	150	150	700	2	16	13	3	60	60	16	13	0.5
13	15	4.5	150	100	700	5	13	16	0.5	60	60	16	16	0.5
14	15	2.5	350	100	600	5	16	13	3	30	60	16	16	3
15	15	2.5	150	150	600	2	16	16	0.5	60	30	16	16	3
16	15	2.5	150	100	600	2	13	13	0.5	30	30	13	13	0.5
17	35	3.5	250	125	650	3.5	14.5	14.5	1.75	45	45	14.5	14.5	1.75
18	35	3.5	250	125	650	3.5	14.5	14.5	1.75	45	45	14.5	14.5	1.75
19	35	3.5	250	125	650	3.5	14.5	14.5	1.75	45	45	14.5	14.5	1.75

Table 5.4. *Central composite face-centered design for oprimization experiment.*

Parameter	Experiment																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cone voltage (V)	18	42	18	42	18	42	18	42	10	50	30	30	30	30	30	30	30
Ion energy1	1.2	1.2	3.4	3.4	1.2	1.2	3.4	3.4	2.3	2.3	0.4	4.2	2.3	2.3	2.3	2.3	2.3
Desolvation temperature (°C)	200	200	200	200	320	320	320	320	260	260	260	260	160	360	260	260	260

The other MS parameters were kept constant (see section 5.2.2). Absolute intensity of MS signal was recorded for each combination of the parameter settings.

5.2.6. Evaluation of the multi-analyte LC-ESI$^+$-MS/MS method

For the multi-analyte method development and evaluation, artificially fortified 1 % BSA in PBS samples were analyzed. Sensitivity of the method was characterized by LOD and was determined in spiked samples as a concentration corresponding to a peak of the analyte with a S/N of at least 3. Linearity of the method was evaluated by triplicate analysis of spiked samples at eight different concentrations by fitting a linear model. Apparent recovery was calculated at three concentration levels as a ratio between the actual and the theoretical concentration of analyte in spiked samples. The precision was expressed as relative standard deviation (RSD) of replicate measurements.

5.2.7. Linear regression for the evaluation of analyte stability

A linear regression approach was applied to evaluate analyte stability data using SPSS Statistics 20 statistical software (IBM Corporation, New York, The USA). The hypotheses were stated as follows:

Null hypothesis (H_o): Slope = 0 (no analyte degradation over time)

Alternative hypothesis (H_a): Slope < 0 (analyte is degraded over time)

To test the significance of the regression coefficient (or of the slope), *t*-test (one-tailed) was used. The significance level α was equal to 0.05. The H_o was rejected, if *p*-value ≤ 0.05. If the *p*-value was > 0.05, there was no sufficient evidence to reject the null hypothesis.

5.3. Results and discussion

5.3.1. Optimization of patulin MS signal

A mobile phase consisting of H_2O, MeOH, CH_3COOH and CH_3COONH_4 was previously used for the LC-MS/MS analysis of a wide range of mycotoxins (Diana Di Mavungu *et al*., 2009; Ediage *et al*., 2011). Under these conditions a very poor MS signal was obtained for patulin. Furthermore, the signal was not useful in subsequent CID experiments. In this study, the ionization efficiency of a broader range of solvents and mobile phase additives was thoroughly investigated (Table 5.1).

In preliminary experiments, ESI and APCI interfaces both in positive and negative modes were examined. Comparing the full MS spectra that resulted in the highest precursor ion signal for each combination of ionization mode and solvent, ESI^+ offered the best prospect for further investigation (if MP7 is used) (Fig. 5.1). In some previous studies a better ESI^- or $APCI^-$ signal was reported (Kataoka *et al*., 2009; Sewram *et al*., 2000). However, a limited number of mobile phases was investigated. Furthermore, the formation of precursor ions other than the protonated or the deprotonated molecule was not considered.

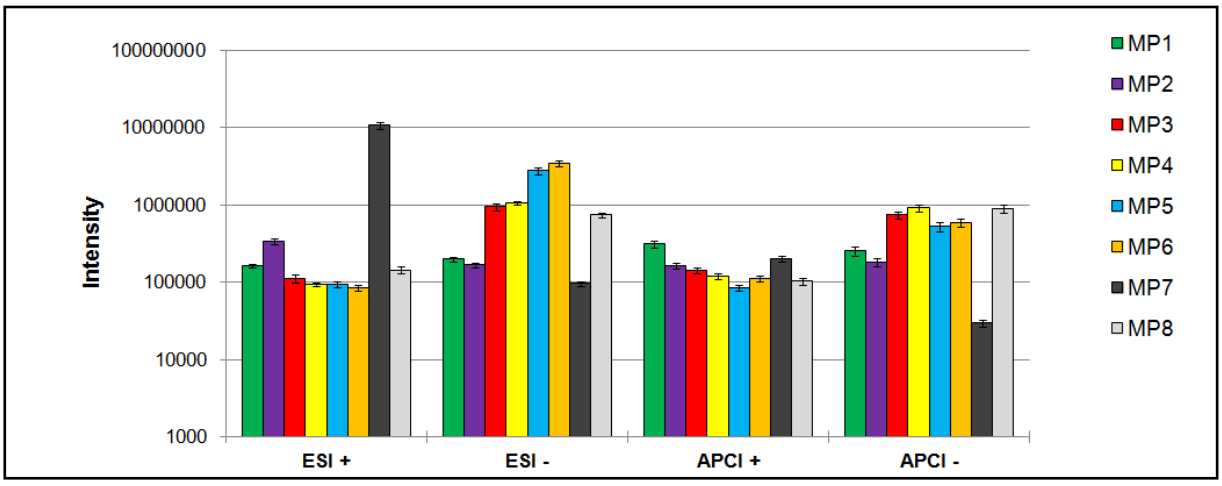

Figure 5.1. *Intensity of patulin MS signal in the tested mobile phases in ESI and APCI modes. The monitored precursor ions in ESI^+ were at m/z 155 (**MP1**-**MP6** and **MP8**) and m/z 169 (**MP7**), in ESI^- - at m/z 153 (**MP1-MP8**), in $APCI^+$ - at m/z 155 (**MP1-MP8**), in $APCI^-$ - at m/z 153 (**MP1-MP8**).*

The mobile phase compositions are as described in Table 5.1.

For visibility reasons a logarithmic scale was applied.

A mobile phase consisting of MeOH, H_2O and NH_4HCO_3 buffer at pH 10 (MP7) resulted in the highest signal (Fig. 5.1). The most abundant ions were at *m/z* 187 and 169 (Fig. 5.2) which were attributed to a protonated methanol-adduct and its dehydrated product, respectively (these ions were observed only with a MeOH-containing mobile phase).

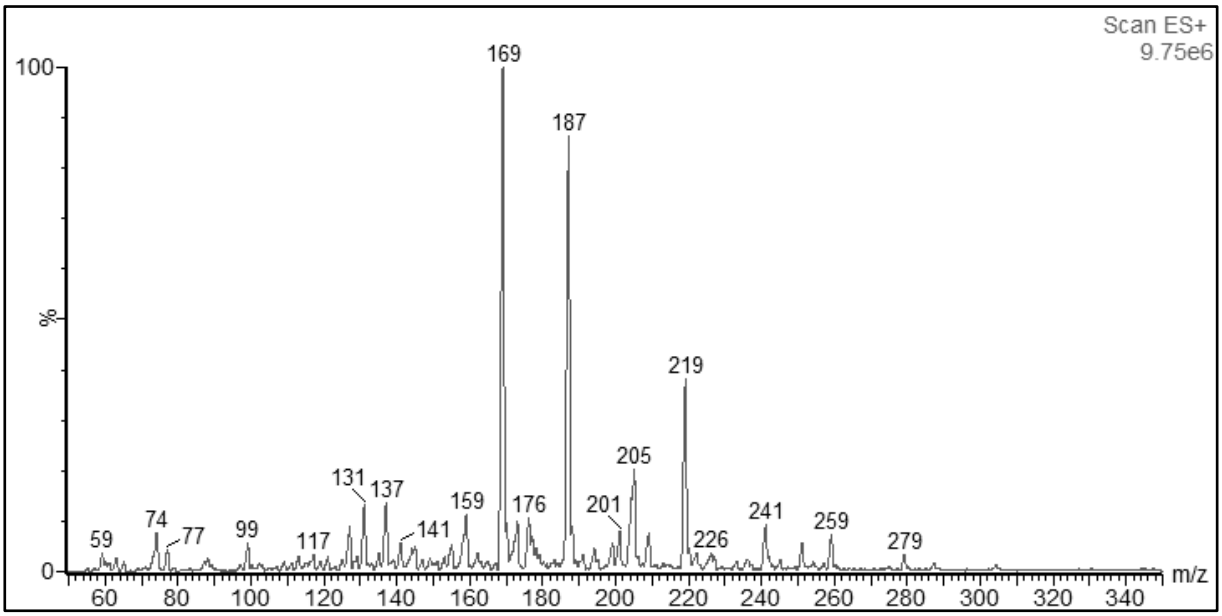

Figure 5.2. *A typical full MS spectrum of patulin in ESI$^+$ in H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (30:5:65, v/v/v).*

The spectrum was acquired utilizing the triple quadrupole MS (see section 5.2.2.1).

The prospect of this mobile phase for ESI$^+$-MS/MS was more apparent when MS^2 experiments were executed using the precursor ions that gave the best full MS signal with each of the mobile phases investigated (Fig. 5.3). This mobile phase was therefore selected for further optimization of the MS signal by experimental design, resulting in the MS parameter settings shown in Table 5.3.

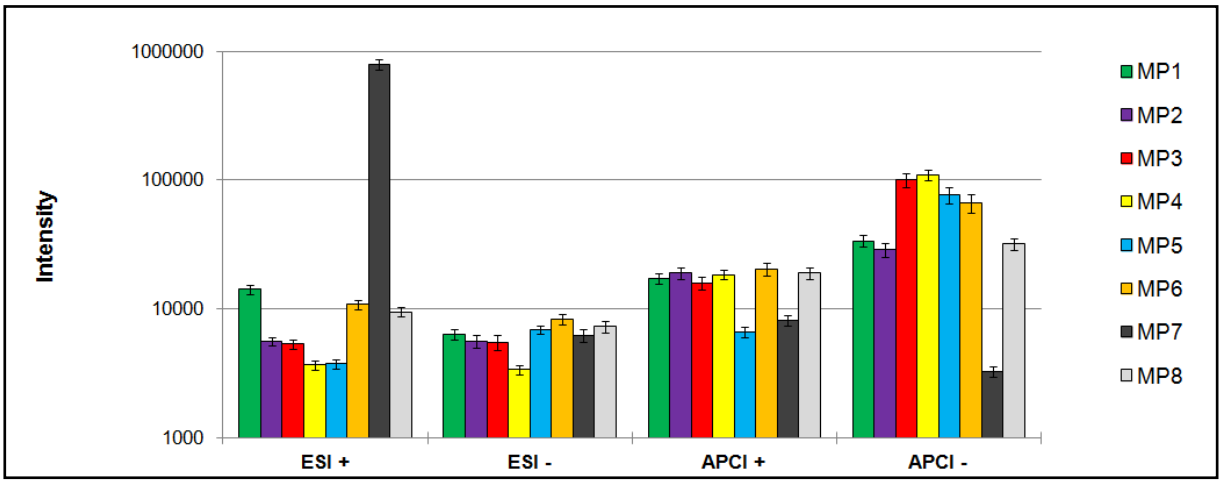

Figure 5.3. *Intensity of patulin MS/MS signal in the tested mobile phases in ESI and APCI modes. The monitored product ions in ESI$^+$ were at m/z 127 (MP1), m/z 109 (MP2-MP4 and MP8), m/z 137 (MP5 and MP7) and m/z 113 (MP6), in ESI$^-$ - at m/z 109 (MP1-MP8), in APCI$^+$ - at m/z 99 (MP1 and MP7), m/z 113 (MP2, MP4, MP6 and MP8), m/z 137 (MP3) and m/z 109 (MP5), in APCI$^-$ - at m/z 109 (MP1-MP8).*

The mobile phase compositions are as described in Table 5.1.

For visibility reasons a logarithmic scale was applied.

5.3.1.1. Optimization of MS parameters by experimental design

5.3.1.1.1. Screening experiment

The MS parameters included in the screening were source temperature, desolvation temperature, cone voltage, capillary voltage, multiplier voltage, extractor voltage, entrance, exit, ion energy1, ion energy2, LM resolution1, LM resolution2, HM resolution1, and HM resolution2. The influence of these parameters on the intensity of the ions at *m/z* 187 and 169 was assessed by means of a Plackett-Burman experimental design. The individual effects of the 14 investigated parameters on the intensity of the signal at *m/z* 169 and 187 are shown in Fig. 5.4.

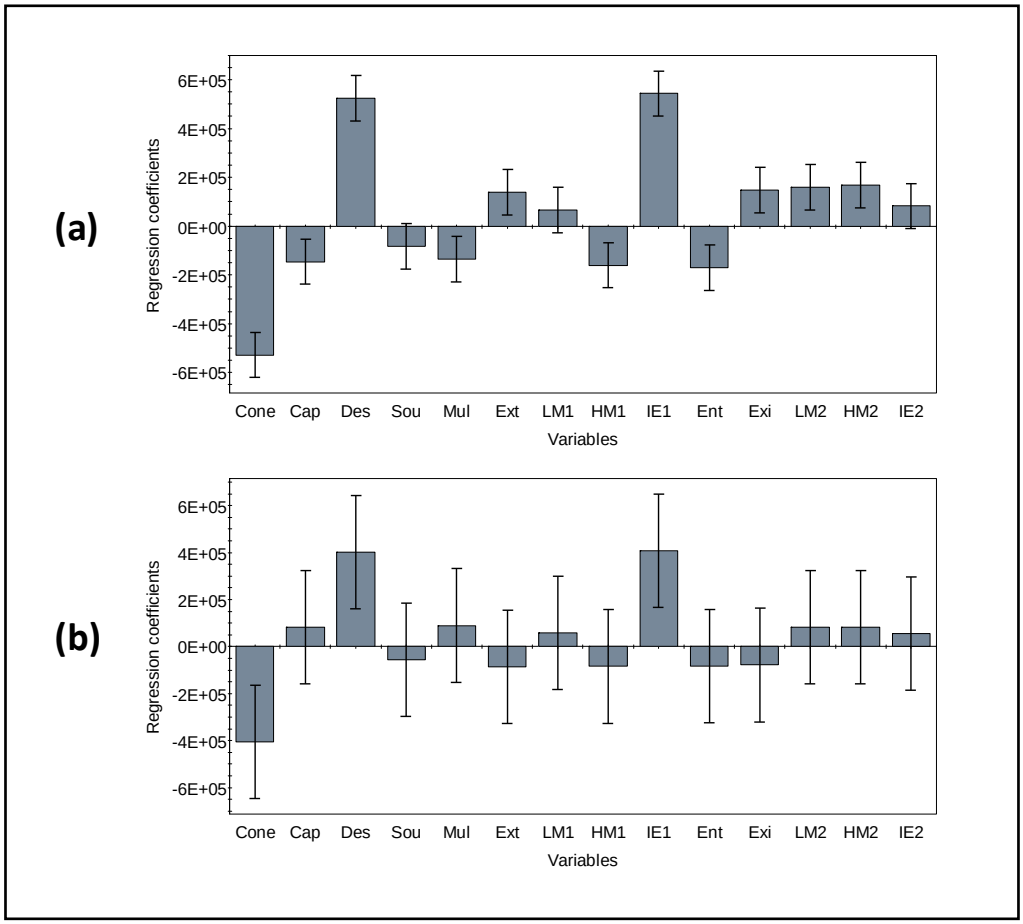

Figure 5.4. *Regression coefficient plots for the ions at m/z 169 (a) and 187 (b).*

Cone: cone voltage; Cap: capillary voltage; Des: desolvation temperature; Sou: source temperature; Mul: multiplier voltage; Ext: extractor voltage; LM1: LM resolution1; HM1: HM resolution1; IE1: ion energy1; Ent: entrance; Exi: exit; LM2: LM resolution2; HM2: HM resolution2; IE2: ion energy2.

The plot consists of bars corresponding to the regression coefficients. The 95 % confidence interval is shown by means of an error bar over the coefficient. A coefficient smaller than the experimental error, indicates that the change in response caused by changing the parameter (from low to high value) is not significant. The magnitude of the effect is proportional to the value of the regression coefficient. A positive regression coefficient reveals a positive effect, while a negative regression coefficient indicates a negative impact of a specific parameter on the MS signal.

The results indicated that the intensity of the MS signal for both ions (*m/z* 169 and *m/z* 187) was mostly influenced by three factors: cone voltage, desolvation temperature and ion energy1. Increasing the cone voltage lead to a reduction of the signal, whereas increasing

the desolvation temperature and ion energy1 had a positive effect on the signal intensity. The effects of the other parameters were less significant.

5.3.1.1.2. Response surface modeling

The parameters that mostly influence the MS signal, namely the cone voltage, desolvation temperature and ion energy1, were further optimized by means of a central composite design (see section 5.2.5). The design allowed to model the response surface by fitting a second-order polynomial model. The statistical analysis of the model gave R^2 values of 0.95 (for ion at *m/z* 169) and 0.90 (ion at *m/z* 187) and Q^2 values of 0.86 (ion at *m/z* 169) and 0.85 (ion at *m/z* 187). The R^2 value corresponds to the fraction of the variation of the response which can be explained by the model, while the Q^2 value corresponds to the fraction which can be predicted by the model. The obtained Q^2 values were close to 1, which indicated the suitability of the model for predicting the optimal conditions.

The response surface plots for the intensity of the ions at *m/z* 169 and 187 as a function of cone voltage, desolvation temperature and ion energy1 are depicted in Fig. 5.5. It was observed that for both ions at *m/z* 169 and 187, increasing the ion energy1 slightly improved the MS signal. The MS signal for both ions reached a maximum when the desolvation temperature was approximately 320 °C. For the cone voltage, a lower value improved the signal at *m/z* 187, while an optimal signal for *m/z* 169 was obtained with a cone voltage around the central value. Based on the observations, a desolvation temperature of 320 °C and ion energy1 of 4 were chosen for both ions; while cone voltages of 30 V and 10 V were selected for the ions at *m/z* 169 and 187, respectively.

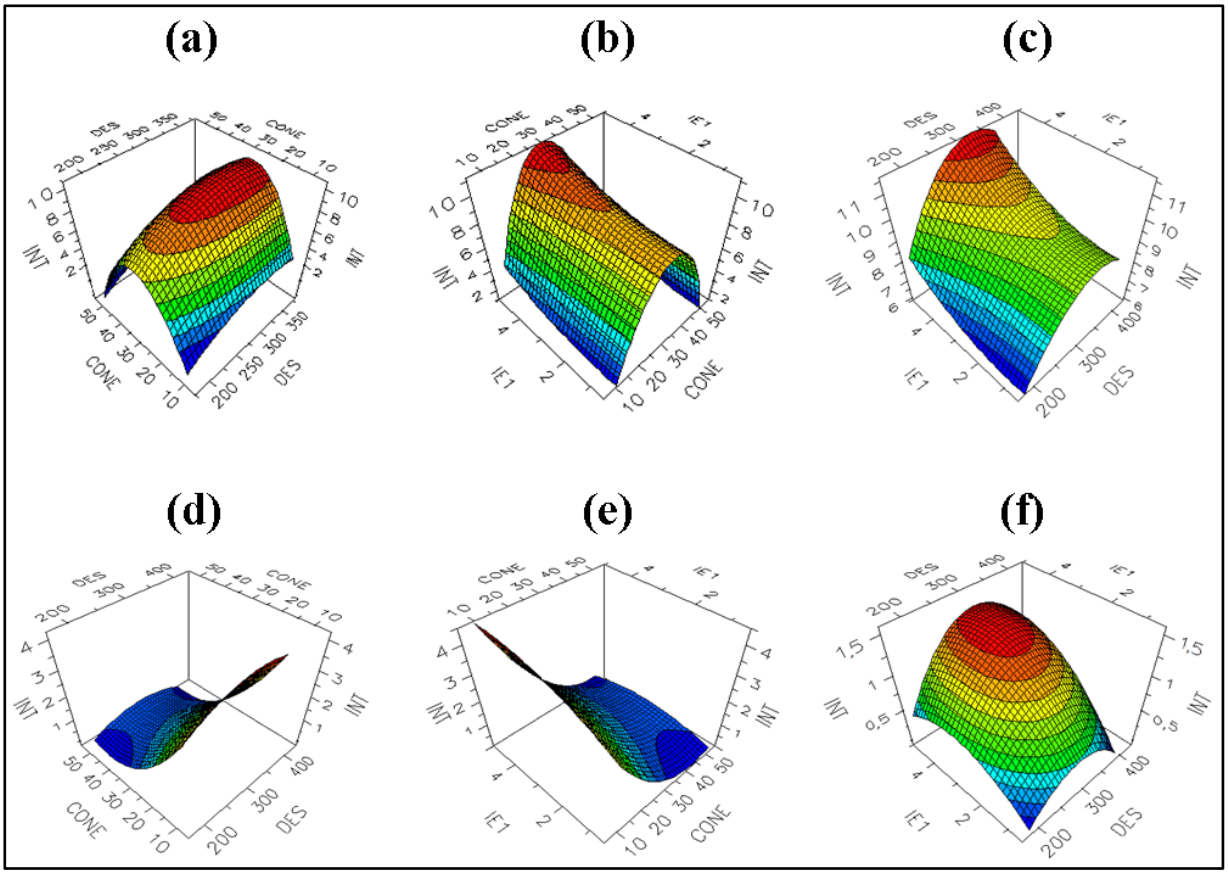

Figure 5.5. *Response surface plots for the intensity of ions at m/z 169 (a, b, c) and 187 (d, e, f) as a function of cone voltage (CONE), desolvation temperature (DES) and ion energy1 (IE1). INT = intensity (x 10^5)*

Using the optimized MS parameters, different collision energies (10-60 eV) were applied in further CID experiments in order to find conditions that lead to the best product ion spectra. In this way the two patulin SRM transitions shown in Table 5.2 were established. The observed signal was stable and the spectra (Fig. 5.6) were overall more useful than those obtained with the protonated or the deprotonated molecule of patulin. The most abundant product ions of the precursor ion at *m/z* 187 were the ions at *m/z* 169, 137 and 109 (Fig. 5.6a). The product ion at *m/z* 137 was also intense in the MS^2 spectrum of *m/z* 169 (Fig. 5.6b).

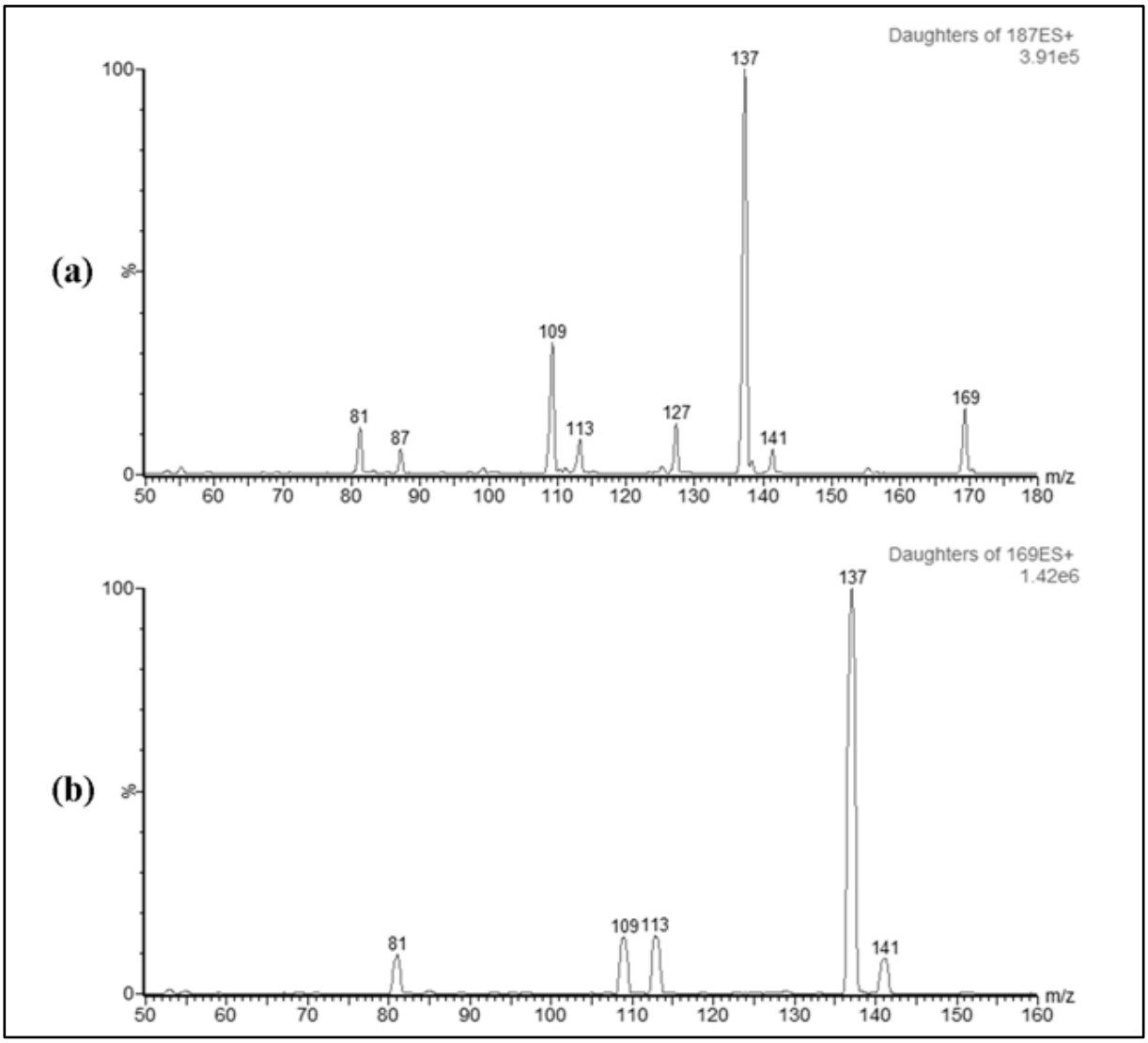

Figure 5.6. *MS^2 spectra of the precursor ions at m/z 187 (cone voltage = 10 V, collision energy = 13 eV) (a) and 169 (cone voltage = 30 V, collision energy = 9 eV) (b).*
The spectra were acquired utilizing the triple quadrupole MS (see section 5.2.2.1.).

5.3.2. Confirmation of the methanol adduct of patulin (*m/z* 187)

A study of the fragmentation behaviour of the ion at *m/z* 187, performed by combining MS^n experiments (ion trap) (Fig. 5.7) and accurate mass measurements (Orbitrap), resulted in the fragmentation pathway shown in Fig. 5.8; thereby confirming the formation of a MeOH-adduct of patulin. As already mentioned, the full MS spectrum of patulin showed among others, an intense ion at *m/z* 187, pointing to a protonated MeOH-adduct of the molecule.

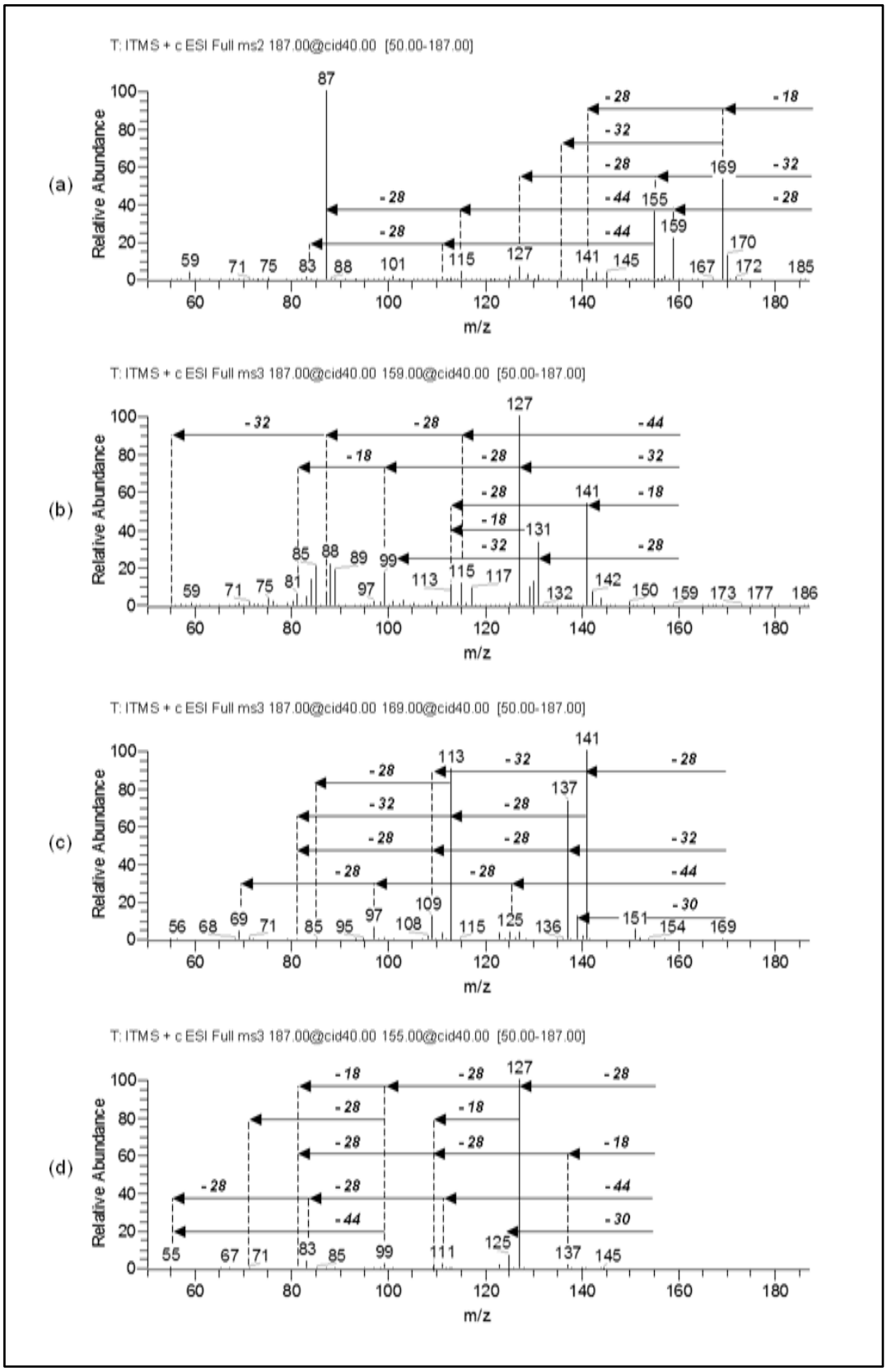

Figure 5.7. *MS^2 spectrum of the precursor ion at m/z 187 (a) and MS^3 spectra of the first generation product ions at m/z 159 (b), 169 (c) and 155 (d).*

The spectra were acquired using the ion trap MS (see section 5.2.2.2.).

The MS^2 spectrum of the precursor ion (*m/z* 187) showed an intense ion at *m/z* 169 corresponding to a loss of H_2O (-18 Da) (Fig. 5.7a). An ion corresponding to the protonated molecule of patulin (*m/z* 155) could be seen, resulting from the loss of MeOH (-32 Da). The ion at *m/z* 159 could be attributed to a loss of carbon oxide (CO) (-28 Da); further losses of carbon dioxide (CO_2) (-44 Da) and CO (-28 Da) accounted for the fragment at *m/z* 87. Likewise, consecutive losses of CO_2 and CO from the ion at *m/z* 155 generated the ions at *m/z* 111 and 83, respectively. The ion at *m/z* 137 pointed to the consequential losses of H_2O and MeOH from the precursor ion (*m/z* 187), while the ions at *m/z* 127 and 141 pointed to a loss of CO (-28 Da) from the ions at *m/z* 155 and 169, respectively.

MS^3 data indicated that the fragment at *m/z* 127 and 141 could also be generated from the ion at *m/z* 159 through a loss of MeOH and H_2O, respectively (Fig. 5.7b). The MS^3 data further confirmed that the fragment at *m/z* 137 was produced, on one hand, through a loss of MeOH (-32 Da) from the ions at *m/z* 169 and, on the other hand, through a loss of H_2O (-18 Da) from the ion at *m/z* 155 (Fig. 5.7c and 5.7d). These data also confirmed that the fragments at *m/z* 141 and 127 were generated through a loss of MeOH from the ions at *m/z* 169 and 155, respectively. Furthermore, the MS^3 data revealed that the ion at *m/z* 139 was generated by a loss of formaldehyde (-30 Da) from the ion at *m/z* 169. A corresponding loss (-30 Da) from the ion at *m/z* 155 accounted for the fragment at *m/z* 125.

MS^4 investigation of the second generation product ions (*m/z* 125, 127, 131, 137 and 141) indicated that the fragment at *m/z* 109 was generated by the ions at *m/z* 141, 137 and 127 through a loss of MeOH (-32 Da), CO (-28 Da) and H_2O (-18 Da), respectively. It was also shown that the ion at *m/z* 113 was generated through a loss of CO (-28 Da) from the ion at *m/z* 141, while a loss of ethylene (C_2H_4) (-28 Da) from the ion at *m/z* 127 accounted for the ion at *m/z* 99. This ion could also be generated through a loss of MeOH (-32 Da) from the fragment at *m/z* 131, which in turn produced the ion at *m/z* 113 through a loss of H_2O. The fragment at *m/z* 69 was shown to be generated from the ion at *m/z* 125 through consecutive losses of two CO molecules.

Further MS^5 experiments revealed that the fragment at *m/z* 81 could be obtained from the ion at *m/z* 113 (through a loss of 32 Da), the ion at *m/z* 109 (through a loss of 28 Da) or the ion at *m/z* 99 (through loss of 18 Da). Further loss of C_2H_4 (-28 Da) from the fragment at *m/z* 81 produced an ion at *m/z* 53. It was shown that this ion could also be generated by the

fragments at *m/z* 85 (loss of MeOH) and *m/z* 71 (loss of H_2O), which in turn were generated by the ion at *m/z* 113 (loss of C_2H_4) and *m/z* 99 (loss of CO), respectively. The MS^n experiments also indicated that a loss of 44 Da (corresponding to CO_2) from the fragment at *m/z* 99 generated the ion at *m/z* 55. It was shown that this ion at *m/z* 55 could also be generated, on one hand, from the fragment at *m/z* 159 through the consecutive losses of CO_2 (-44 Da), CO (-28 Da) and MeOH (-32 Da), and, on the other hand, from the fragment at *m/z* 155 through successive losses of CO_2, CO and C_2H_4.

To confirm the assignments proposed above, exact mass measurements were performed using Orbitrap MS. Generally this instrument is not capable to isolate the precursor ion for fragmentation. However, applying the "all ion fragmentation" HCD scan beside the full scan, accurate masses could be recorded for the precursor and product ions. The experimental masses were compared to the calculated (generated elemental compositions) and the mass error was not higher than 2.1 ppm, enabling unequivocal structure elucidation. Using both the MS^n (ion trap) and exact mass measurement (Orbitrap) data in combination with the automated fragmentation generated by simulation in Mass Frontier™ 6.0 software (ThermoFinnigan), a fragmentation pattern was proposed (Fig. 5.8) thereby confirming the identity of the precursor ion at *m/z* 187.

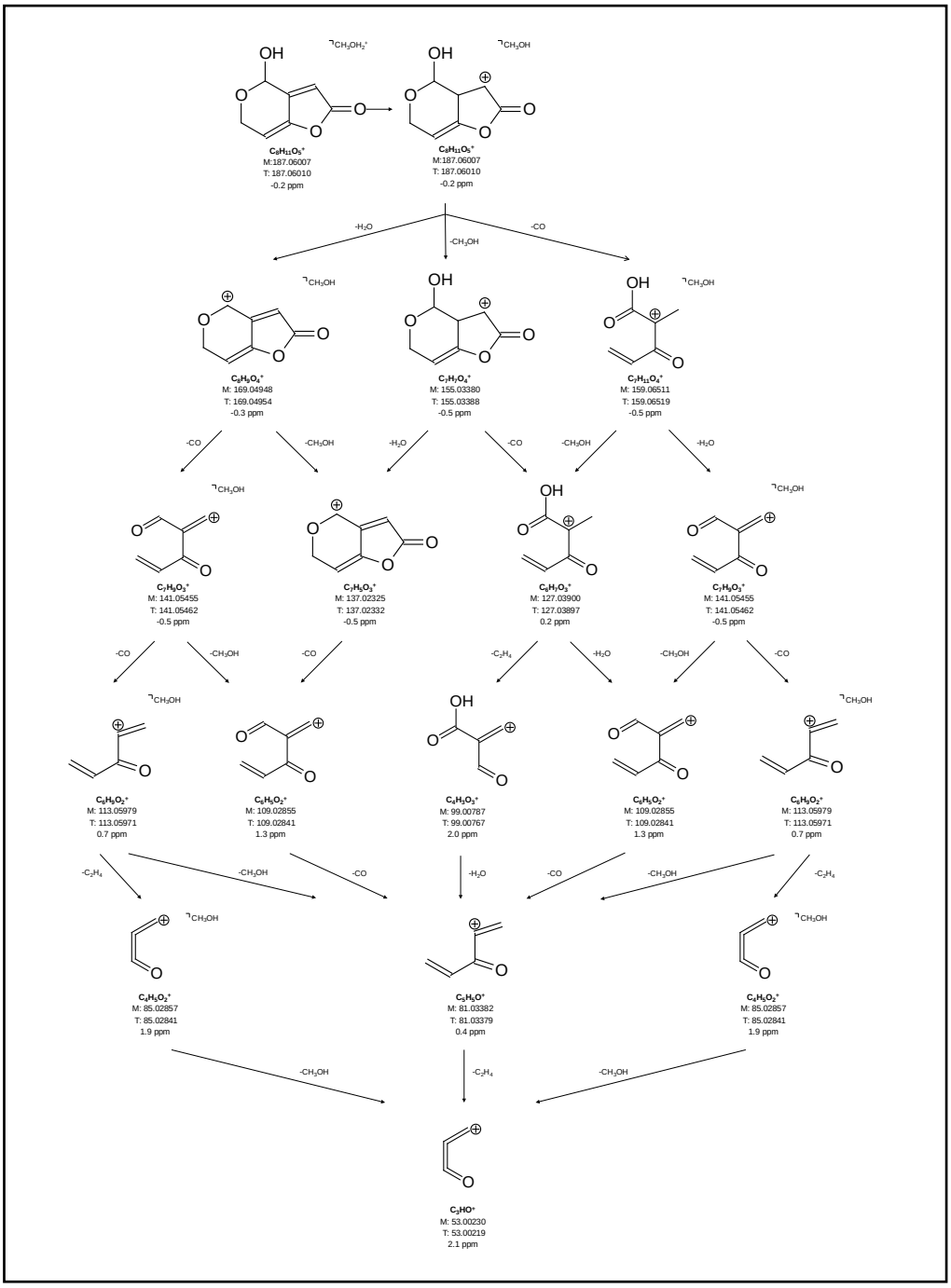

Figure 5.8. *Proposed fragmentation pathway of patulin starting from its methanol adduct (m/z 187).*

The fragment at m/z 159 can alternatively undergo successive losses of CO_2 (-44 Da), CO (-28 Da) and MeOH (-32 Da) to yield an ion with m/z 55. This ion can also be generated on one hand from the fragment at m/z 99 through a loss of CO_2 (-44 Da); and on the other hand from the fragment at m/z 155 through successive losses of CO_2 (-44 Da), CO (-28 Da) and C_2H_4 (-28 Da).
The ion at m/z 53 can also be generated from the fragment at m/z 99 through a primary loss of CO (-28 Da), followed by a loss of H_2O (-18 Da).
The assignment of fragments was supported by the Mass Frontier™ software.

5.3.3. Applicability in multi-mycotoxin LC-MS/MS analysis

The usefulness of the selected ionization approach was demonstrated by the development of an LC-MS/MS multi-analyte method for the simultaneous determination of patulin and other mycotoxins. XBridge C18 stationary phase was chosen, as it has previously shown excellent pH stability (Diana Di Mavungu *et al*., 2012). The SRM ESI^+ chromatograms obtained by analyzing a mixture of 23 mycotoxins are shown in Fig. 5.9. It has to be mentioned that for the purpose of multi-analyte method the ion at *m/z* 169 was chosen for patulin as it was more abundant than the ion at *m/z* 187. Although a baseline separation was not achieved for all adjacent peaks, this was not considered as a problem due to different SRM transitions.

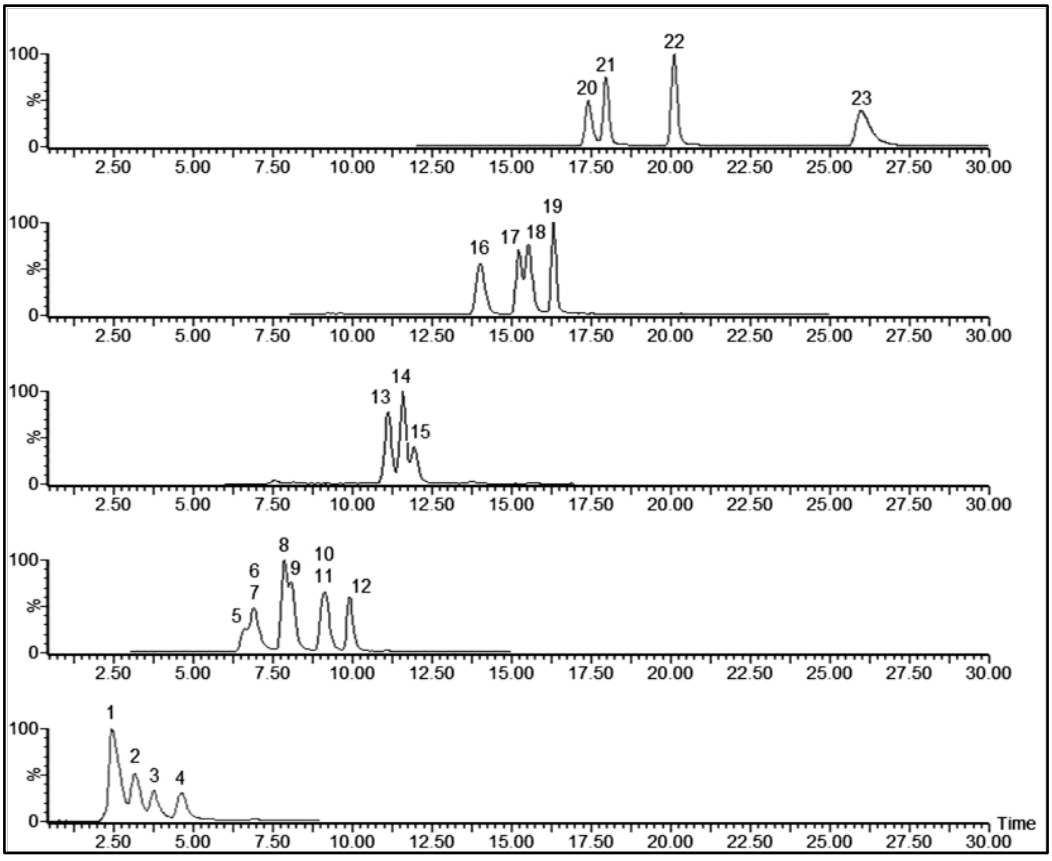

Figure 5.9. *Typical SRM ESI^+ chromatograms of a mixture of 23 mycotoxins, including patulin (1), nivalenol (2), deoxynivalenol (3), neosolaniol (4), ochratoxin B (5), 3-acetyl-deoxynivalenol (6), 15-acetyl-deoxynivalenol (7), fumonisin B1 (8), aflatoxin G2 (9), aflatoxin G1 (10), ochratoxin A (11), aflatoxin B2 (12), aflatoxin B1 (13), citrinin (14), diacetoxyscirpenol (15), fumonisin B2 (16), zearalenone (17), zearalanone (18), HT-2 toxin (19), alternariol monomethyl ether (20), T-2 toxin (21), sterigmatocystin (22), beauvericin (23). The LC-MS conditions are as described in sections 5.2.2.1 and 5.2.3.*

5.3.4. Mycotoxin stability and injection solvent selection

The use of a high pH buffer in the mobile phase raised the question of analyte stability during the LC-MS/MS analysis. Therefore, the effect of the injection solvent on analyte stability and MS signal was investigated. The study was perfomed for a selection of mycotoxins. Five injection solvents were included in the study, namely **(I)** H_2O:MeOH (80:20, v/v), **(II)** H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (69:5:26, v/v/v), **(III)** H_2O:0.2 M NH_4HCO_3 (pH 8.5):MeOH (69:5:26, v/v/v), **(IV)** H_2O:MeOH:CH_3COOH (57.2:41.8:1, v/v/v) containing 5 mM CH_3COONH_4 and **(V)** MeOH:ACN:H_2O (20:40:40, v/v/v).

Mycotoxin standard solutions at relevant concentrations were prepared in the above-listed injection solvents. The samples were analyzed by LC-MS/MS and the MS signal was monitored over time (about 60 hours). The peak areas of mycotoxins were plotted as a function of time and linear regression was applied. The results (Table 5.5) indicated that *p*-value for the slope was greater than 0.05 in the majority of cases.

Patulin has been reported to decompose at high pH (Dombrink-Kurtzman and Blackburn, 2005). Investigation of its stability during analysis, indicated a signal loss only when patulin was dissolved in the mobile phase (pH 10) **(II)** and kept for a long time (appr. 60 hours) before analysis. The patulin signal proved to be stable over the same period of time in an autosampler maintained at 4 °C, when the buffer of the sample solvent was adjusted to pH 8.5 **(III)**. As for the other mycotoxins, their signals in basic injection solvent at pH 8.5 **(III)** were also stable. Finally, injection solvent H_2O:0.2 M NH_4HCO_3 (pH 8.5):MeOH (69:5:26, *v/v/v*) **(III)** was chosen for the LC-MS analysis.

5.3.5. Clean-up procedure and evaluation of the method

In transdermal experiments using diffusion cells, only a limited amount (about 200 µL) of sample is commonly available, which does not allow sample concentration. A direct injection of the samples into the LC-MS instrument is also not possible due to presence of BSA and PBS. It became clear that a sample clean-up was necessary.

Table 5.5. *Linear regression of the data obtained during stability study for mycotoxins dissolved in different injection solvents.*

Mycotoxin	Injection solvent [a]														
	(I)			(II)			(III)			(IV)			(V)		
	r^2	t	*p*-value	r^2	t	*p*-value	r^2	t	*p*-value	r^2	t	*p*-value	r^2	t	*p*-value
Patulin	0.037	-1.201	0.119	0.806	-12.242	0.000	0.037	-1.205	0.118	0.007	0.501	0.310	0.056	1.460	0.077
Fumonisin B1	0.006	0.484	0.316	0.129	-2.371	0.012	0.068	-1.662	0.053	0.055	1.463	0.076	0.001	0.196	0.423
Ochratoxin A	0.272	-3.717	0.001	0.209	-3.125	0.002	0.020	-0.862	0.197	0.010	0.616	0.271	0.005	0.437	0.333
Aflatoxin B1	0.017	0.789	0.218	0.813	-12.672	0.000	0.003	0.338	0.369	0.118	-2.227	0.016	0.061	1.548	0.065
Citrinin	0.00001	-0.020	0.492	0.048	1.368	0.090	0.059	1.538	0.066	0.064	1.592	0.060	0.044	1.307	0.100
Zearalenone	0.019	0.862	0.197	0.050	1.393	0.086	0.0007	0.060	0.477	0.066	1.616	0.058	0.061	1.549	0.065
T2-toxin	0.001	-0.199	0.422	0.859	-15.232	0.000	0.055	-1.482	0.073	0.020	0.866	0.196	0.060	1.541	0.066
Fumonisin B2	0.015	-0.761	0.226	0.101	-2.035	0.025	0.070	-1.671	0.052	0.023	-0.943	0.176	0.031	-1.093	0.141
Ochratoxin B	0.220	-3.272	0.001	0.307	-4.103	0.000	0.068	-1.667	0.052	0.002	0.299	0.383	0.004	-0.370	0.357
Aflatoxin B2	0.069	1.650	0.054	0.783	-11.567	0.000	0.004	-0.390	0.350	0.044	1.664	0.053	0.069	1.652	0.054
Zearalanone	0.062	1.563	0.063	0.001	0.234	0.409	0.006	0.490	0.314	0.042	1.274	0.106	0.067	1.626	0.056
HT2-toxin	0.030	-1.075	0.145	0.069	1.682	0.051	0.0004	-0.116	0.454	0.050	1.388	0.087	0.00004	-0.036	0.486

[a] **(I)** H_2O:MeOH (80:20, v/v); **(II)** H_2O:0.2 M NH_4HCO_3 (pH 10):MeOH (69:5:26, v/v/v); **(III)** H_2O:0.2 M NH_4HCO_3 (pH 8.5):MeOH (69:5:26, v/v/v); **(IV)** H_2O:MeOH:CH_3COOH (57.2:41.8:1, v/v/v) containing 5 mM CH_3COONH_4; **(V)** MeOH:ACN:H_2O (20:40:40, v/v/v)

For the study, seven relevant mycotoxins were selected based on their toxicity and co-occurrence data. In preliminary experiments, both liquid-liquid extraction and SPE using Oasis HLB^{TM} cartridges were evaluated. SPE proved to be more suitable and was therefore chosen for further development.

After the conditioning step, 200 µL of the sample was quantitatively brought onto the SPE cartridge, which was then washed by passing through 10 mL of H_2O. This ensured elimination of BSA and PBS from the sample, while the targeted analytes were retained on the column. For elution of the mycotoxins, two commonly used solvents, namely MeOH and ACN, were compared. Along with the investigation of elution solvent, elution volume was also included. The results indicated that MeOH could be a suitable elution solvent for the majority of analytes, while ACN was superior for the elution of aflatoxins. Since sufficient recovery of the aflatoxins (> 80 %) was obtained with MeOH, this solvent was selected in the final method. Regarding the elution volume, a value of 5 mL could be suggested. Applying the optimized conditions, good extraction recoveries were obtained using the SPE procedure. These recoveries were in the range from 67 % (citrinin) to 95 % (T-2 toxin). Lower recovery values were reported for fumonisins (below 70 % (Sulyok *et al*., 2006; Van Pamel, 2011); and in some cases even below 40 % (Monbaliu *et al*., 2009)). Fumonisins are better extracted using high water content solvent mixtures and are therefore generally poorly recovered when multi-mycotoxin analysis is performed, using more generic extraction solvents. In this study, no extraction was performed prior to SPE, resulting in better recoveries (fumonisin B1: 82 %, fumonisin B2: 93 %). With regard to patulin, 80 % was recovered using the proposed method, while this toxin could not be detected in other studies (Garon *et al*., 2006).

Evaluation of the method (Table 5.6) demonstrated its fit for the detection of the seven targeted mycotoxins in a model transdermal penetration study. LODs were in the range from 1 to 10 ng/mL sample.

The linearity of the method was supported by the high values of coefficients of determination R^2 and lack-of-fit *p*-values above 0.05. The values of the standard error (S_{yx}) were all below 5 % of the estimate, even at low concentrations of the analytes. This feature of method performance is of a high value since some researchers experienced a non-linear signal of patulin and therefore had to omit this mycotoxin from the analysis (Sulyok *et al*., 2006).

Table 5.6. *Evaluation of the LC-ESI$^+$-MS/MS method in support of a transdermal mycotoxin penetration study.*

Mycotoxin	Concentration level (ng/mL)	Apparent recovery (%)	Precision (RSD; n=6)	Linearity							
				LOD (ng/mL)	Concentration range (ng/mL)	Regression equation	R^2	S_{yx}	*p*-value	n_c	n_i
Patulin	Low (10)	114	22	5	10 - 250	y=0.0042x+0.0893	0.920	0.00043	0.945		
	Medium (50)	111	11								
	High (250)	104	7								
Citrinin	Low (2)	117	10	1	2 - 50	y=0.0929x+0.0162	0.994	0.00140	0.440		
	Medium (10)	93	9								
	High (50)	101	11								
Fumonisin B1	Low (20)	98	20	10	20 - 500	y=0.0031x+0.0380	0.937	0.00016	0.574		
	Medium (100)	89	16								
	High (500)	98	11								
Aflatoxin B1	Low (2)	110	21	1	2 - 50	y=0.0384x-0.0018	0.960	0.00155	0.500	8	3
	Medium (10)	104	13								
	High (50)	104	10								
Ochratoxin A	Low (10)	109	10	5	10 - 250	y=0.0088x-0.0162	0.980	0.00021	0.998		
	Medium (50)	103	14								
	High (250)	101	9								
Zearalenone	Low (20)	107	18	10	20 - 500	y=0.0025x+0.0151	0.992	0.00004	0.987		
	Medium (100)	108	7								
	High (500)	97	11								
T2-toxin	Low (5)	110	11	2.5	5 - 125	y=0.0131x+0.0018	0.988	0.00025	0.497		
	Medium (25)	96	13								
	High (125)	106	11								

n_c - number of concentration points; n_i – number of repetitions per concentration point; RSD – relative standard deviation, R^2 – coefficient of determination, S_{yx} - standard error of the estimate

The trueness, referred to as apparent recovery (Thorburn Burns *et al*., 2002), was evaluated by recovery experiments from spiked samples since material with certified concentrations of mycotoxins was not available for intended study. Apparent recoveries for all the analytes were from 89 to 117 % and precision at low concentration levels did not exceed 22 %. These data were considered as appropriate and in good agreement with the Commission Decision 2002/657/EC performance criteria for quantitative methods of analysis (Commission Decision, 2002).

The use of a triple quadrupole (two SRM transitions monitored for each analyte) provided high selectivity and specificity. The specificity of the method was further confirmed through the analysis of blank samples that were passed through the skin obtained from different donors. For each of the SRM transitions, no peak with a S/N of at least 3 was detected at the expected retention time.

Further support of the suitability of the method for its intended use resides in the application of the method in 'real-world' samples from an *in vitro* skin penetration study using diffusion cells conducted in the Laboratory of Drug Quality and Registration (prof. Bart De Spiegeleer), Faculty of Pharmaceutical Sciences, Ghent University. The method allowed to measure simultaneously and in a relatively short time seven selected model mycotoxins (including patulin) not only in the receptor compartment of the diffusion cell, but also in the donor chamber and in the skin, thereby enabling to assess the recovery of the mycotoxins in the mass balance (Boonen *et al*., 2012).

5.4. Conclusions

This study is the first report demonstrating an intense and stable MS signal of patulin in ESI^+ mode by formation of a protonated MeOH-adduct using a MeOH-containing mobile phase under alkaline conditions. The use of high resolution Orbitrap MS in combination with MS^n experiments unambiguously confirmed the authenticity of this adduct and its fragmentation pattern. Under the applied conditions other mycotoxins could also be ionized and gave satisfactory MS signals, thereby allowing inclusion of patulin in multi-mycotoxin LC-MS/MS methods. The usefulness of these findings was clearly demonstrated by the development of

a sensitive, linear and accurate LC-ESI$^+$-MS/MS method in support of transdermal penetration experiments for selected mycotoxins with prior SPE on Oasis^{TM} HLB cartridges.

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CHAPTER 6

Mass spectrometric approaches for targeted and untargeted analysis of ergot alkaloids

The content of this chapter was partially derived from:

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*These authors contributed equally to this work

Malysheva SV*, Diana Di Mavungu J*, Schoeters E, Larionova DA, Goryacheva IYu, Saeger S (2013). Rapid and sensitive LC-MS/MS determination of ergot alkaloids in buffered solutions: Application to *in vitro* testing of clay-based mycotoxin binder. *World Mycotoxin Journal,* 6, 105-115

*These authors contributed equally to this work

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*These authors contributed equally to this work

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*These authors contributed equally to this work

6.1. Background

Ergot alkaloids are mycotoxins produced by fungi of the *Claviceps* genus, mainly by *Claviceps purpurea* (Komarova and Tolkachev, 2001). Infections are mostly prevalent in cereals and wild grasses (Naude *et al*., 2005). Among the cereal species, rye and triticale that have open florets are known to be especially susceptible, but wheat, barley, oats and other cereal grains are also potential fungal hosts (Krska and Crews, 2008). After the infection is established, the fungus replaces the developing grain or seed with an alkaloid-containing hard black tuber-like wintering structure called sclerotium, also known as ergot or ergot body. The alkaloid pattern and individual alkaloid contents in sclerotia vary largely, due to differences in the maturity of the sclerotia and other factors such as the fungal strain, the host plant, the geographical region and the prevailing weather conditions (Lombaert, 2001; Storm *et al*., 2008). Similarly, data from the literature indicate that the total alkaloid content in sclerotia can vary between 0.01 and 1 % (w/w) (Burk *et al*., 2005; Lombaert, 2001; Scott, 2009).

The sclerotia are harvested together with the cereals or grass and can thus lead to contamination of cereal-based food and feed products with ergot alkaloids, the ingestion of which can cause ergotism in humans and animals. Investigations in Germany have indicated an increase in the occurrence of *Claviceps purpurea* infections in the last few years (Krska and Crews, 2008). This increase seems to be associated with the more extensive use of hybrid varieties of rye and perennial rye cultivars (Krska and Crews, 2008). Although nowadays effective cleaning procedures at mills allow to remove up to 82 % of ergots from grain (Krska and Crews, 2008), ergot alkaloids have been detected in surveys of Swiss, Canadian, Swedish, Danish, British and German cereals and cereal products at total levels up to 18,114 µg/kg in German rye flours (Baumann *et al*., 1985; Dusemund *et al*., 2006; Krska and Crews, 2008; Scott, 2009). In the Middle Ages, consumption of grains that were highly contaminated with ergot was found to be the cause of an intoxication described as Holy Fire or St. Anthony's Fire, with respect to the intense pain resulting from vasoconstriction and subsequent gangrene with loss of fingers, hands, feet and even entire limbs. Lower doses can induce symptoms including abdominal pain, vomiting, burning sensation of the skin, insomnia, and hallucinations. Cases of human poisoning from ergot have been reported in more recent times in France (Fuller, 1968), India (Krishnamachari and Bhat, 1976), and

Ethiopia (Urga *et al*., 2002). Nowadays ergotism has practically been eliminated as a human disease, but it remains an important veterinary problem, particularly in cattle, horses, sheep, pigs and chicken.

More than 40 different ergot alkaloids are known. Their common structural feature is the ergoline ring with a nitrogen atom at position 6 (Fig. 6.1). In many toxins, this nitrogen is methylated, and the ring is substituted at C8 and possesses a double bond between C8 and C9 or C9 and C10. The main ergot alkaloids produced by *Claviceps* species are ergometrine, ergotamine, ergosine, ergocristine, ergokryptine, and ergocornine (Fig. 6.1). Ergot alkaloids that have a double bond between C9 and C10 (known as ergolenes) undergo epimerization, with respect to the centre of symmetry at C8, resulting in rotating (C8-(S) configuration) isomers. The left rotating (C8-(R) configuration) and the right rotating (C8-(S) configuration) epimers are named ergopeptines and ergopeptinines, respectively. These epimers differ in biological and physicochemical properties. C8-(R)-isomers (-ines) are biologically active, whereas the C8-(S)-isomers (-inines) are inactive (Krska and Crews, 2008). Both forms are found together in naturally contaminated samples. The conversion of the -ine to the -inine isomers occurs rapidly in aqueous solutions (Komarova and Tolkachev, 2001), and can reverse in some aqueous and organic solvents (Crews *et al*., 2009). It is therefore important to consider both epimers when the ergot alkaloid contamination level of a cereal or cereal product has to be determined.

This chapter deals with different aspects of the ergot alkaloid issue and includes *i)* analytical method development and validation for determination of selected ergot alkaloids and its further application to a European survey of cereals and cereal products, *ii)* investigation of binder efficacy towards ergot alkaloids in feed, as well as *iii)* untargeted screening of other ergot alkaloid derivatives.

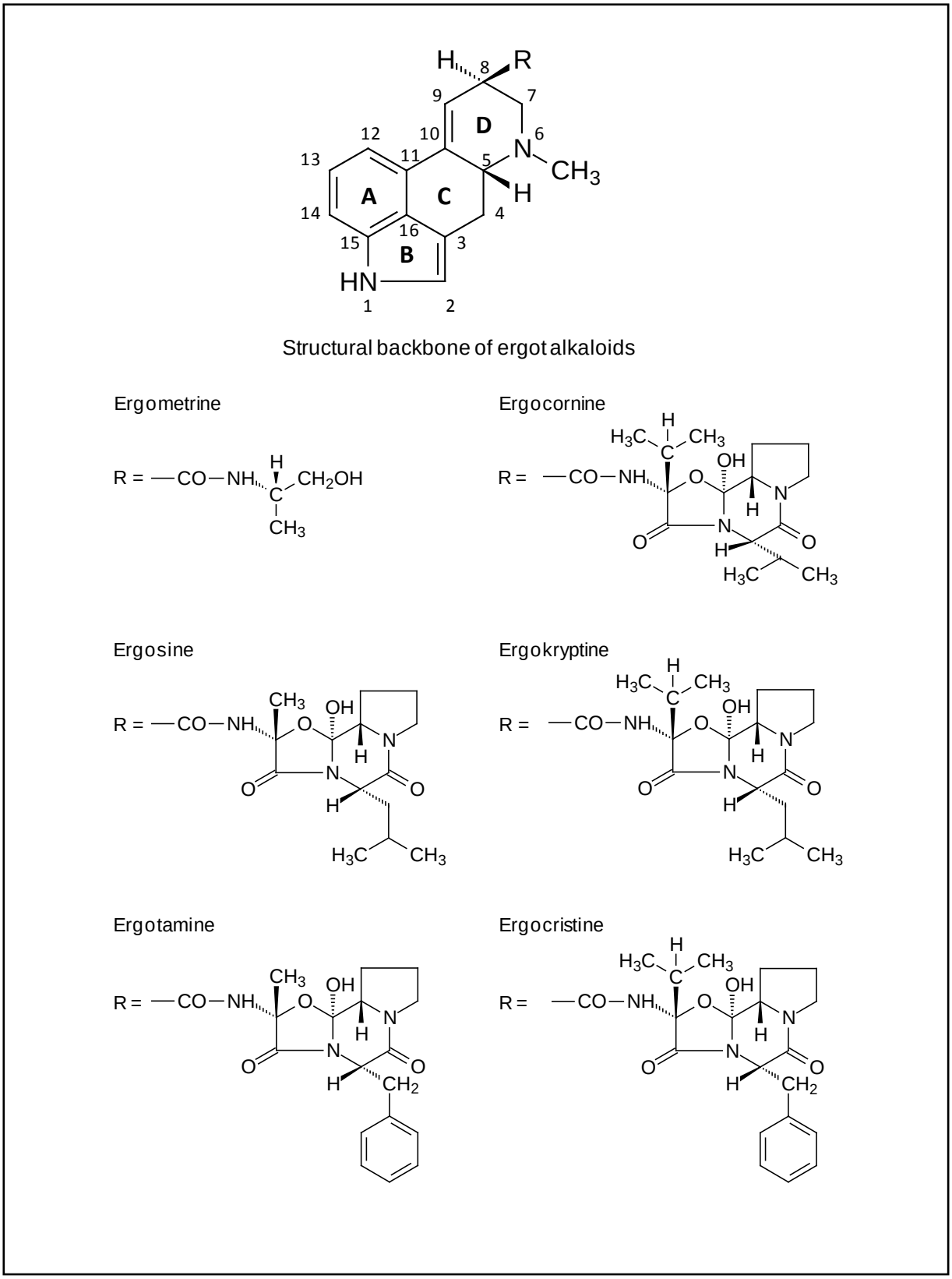

Figure 6.1. *Structures of the most common ergot alkaloids.*

6.2. LC-MS/MS determination of six major ergot alkaloids and their corresponding epimers in cereals and cereal products

6.2.1. Introduction

Different methods have been reported for the analysis of ergot alkaloids, including TLC (Salvat and Godoy, 2001), capillary electrophoresis (Franch and Blaschke, 1998), enzyme linked immunosorbent assay (Hill *et al*., 2001), GC with electron capture detection (Barrow and Quigley, 1975), LC with UV (Veress, 1993), fluorescence (Komarova and Tolkachev, 2001; Storm *et al*., 2008) or MS (Burk *et al*., 2006; Kokkonen and Jestoi, 2010; Krska *et al*., 2008; Mohamed *et al*., 2006) detection. In recent years, LC-MS has become the method of choice for mycotoxin determination (Shephard *et al*., 2011; Spanjer, 2010), and has provided an unequivocal identification of ergot alkaloids in various matrices (Friedrich *et al*., 2004; Krska *et al*., 2008; Lehner *et al*., 2005; Mohamed *et al*., 2006). However, validated LC-MS methods for the simultaneous quantitative determination of the major ergot alkaloids in food and feed are scarce. Such methods are required for the monitoring of these toxins, as well as for further research and study of the ergot problem. A number of the methods mentioned above have been used for biomedical application (Friedrich *et al*., 2004) and/or for identification purposes (Lehner *et al*., 2005). Some include no or only few -inine isomers (Burk *et al.*, 2006; Kokkonen and Jestoi, 2010; Mohamed *et al*., 2006), possibly because of the lack of available standards. Krska *et al.* (Krska *et al*., 2008) reported an LC-MS/MS multi-analyte method including both the -ine as well as the -inine isomers of the six most common ergot alkaloids, which was validated for ten different cereal matrices. ACN was used under alkaline conditions as extraction solvent and as organic modifier in mobile phase. MeOH, the other commonly used organic modifier, was not assessed. On the other hand, although ergot alkaloids have been reported in silages and other feed matrices (Blaney *et al*., 2010; Nagy and Kuldau, 2003), no validated LC-MS method has included these matrices until now.

A maximum level of 1,000 mg/kg of rye ergot (*Claviceps purpurea*) has been established for feed containing unground cereals in the European Union (Directive 2002/32/EC, 2002). Many countries have set limits for ergot in grains, but only a few has regulation for the individual and total ergot alkaloid content; for instance, the current limit for the total ergot alkaloid content in animal feed in Uruguay is 450 µg/kg (EFSA, 2005). However, there are still no limits for ergot alkaloids in food.

The European Food Safety Authority (EFSA) published an opinion on ergot alkaloids as undesirable substance in animal feed in 2005 (EFSA, 2005). In 2012 the Panel on Contaminants in the Food Chain recommended that data on the variability of the ergot alkaloid patterns in food and feed commodities should be collected in Europe with a special attention to processed foods (EFSA, 2012). The available data do not allow yet to identify a marker that could be monitored in all food or feed materials as indicator for ergot alkaloid contamination. It is necessary to generate more data on the presence of ergot alkaloids, not only in unground cereals but also in cereal products and compound feed and food and to obtain reliable data on the ergot alkaloid pattern.

The present study was designed to develop and validate a new LC-MS/MS method that allows simultaneous determination of the six ergot alkaloids defined by the EFSA as among the most common and physiologically the most active (*i.e.* ergometrine, ergotamine, ergosine, ergocristine, ergokryptine and ergocornine), together with their corresponding -inine isomers (ergometrinine, ergotaminine, ergosinine, ergocristinine, ergokryptinine and ergocorninine) in different food and feed matrices including cereals and cereal-based products, as well as grass and corn silages. Furthermore, the method was applied to obtain representative data on the occurrence of ergot alkaloids in different European countries in rye, wheat, triticale and multigrain samples intended for human consumption and animal feeding.

6.2.2. Experimental

6.2.2.1. Standards

Fine film dried ergot alkaloid standards ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine, ergometrinine, ergosinine, ergotaminine, ergocorninine, ergokryptinine and ergocristinine were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). The film dried standards were, as indicated by the manufacturer, reconstituted in 5 mL of solvent (ACN), to give concentrations of 100.0 µg/mL (uncertainty: ± 5.0 µg/mL) and of 25.0 µg/mL (uncertainty: ± 1.5 µg/mL), respectively for the main ergot alkaloids and for the -inine isomers. Definite ergot alkaloids in solution readily undergo epimerization. Therefore, from the freshly prepared standard solutions, deep frozen

standard residues were prepared as follows: defined volumes of individual or mixed standard solutions were pipetted into dark brown or aluminium covered glass tubes, evaporated to dryness at 40 °C under a stream of nitrogen, and deep frozen at -20 °C. Lauber *et al.* (Lauber *et al*., 2005) reported that the ergot alkaloids stored under these conditions are stable for at least one year. The deep frozen standards were reconstituted in the required amount of solvent immediately before use. Methylergometrine (as methylergometrine maleate, purity: 98 %) and dihydroergotamine (as dihydroergotamine tartrate salt, purity: 99 %), used as internal standards, were purchased from Sigma Aldrich (Bornem, Belgium). From the crystalline standards, individual stock solutions were prepared respectively in MeOH:ACN (10:90, v/v) (methylergometrine) or in ACN (dihydroergotamine) at a concentration of 1 mg/mL. These fresh solutions were used to prepare deep frozen standard residues as described above. The residues were reconstituted in the required amount of solvent immediately before use.

6.2.2.2. Chemicals and material

MeOH and ACN used during sample preparation were HPLC-grade and were both obtained from VWR International (Zaventem, Belgium). *n*-Hexane was also obtained from VWR International. For LC-MS analysis, LC-MS grade MeOH and ACN from Biosolve (Valkenswaard, the Netherlands) were used. EtOAc and DCM were purchased from Acros Organics (Geel, Belgium). A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized H_2O. H_3PO_4, HCOOH, ammonium carbonate ($(NH_4)_2CO_3$) and $(NH_4)_2SO_4$ were purchased from Merck (Darmstadt, Germany). NH_4HCO_3 was supplied by Sigma Aldrich (Bornem, Belgium). Concentrated ammonia (NH_3) was purchased from Vel (Leuven, Belgium). The Bakerbond aminopropyl (NH_2) cartridges were from Achrom (Zulte, Belgium). Oasis® HLB extraction cartridges were supplied by Waters (Milford, MA, USA). Bond Elut AccuCAT cartridges were purchased from Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 µm) from Millipore (Bedford, MA, USA) were used.

6.2.2.3. Samples

Method optimization and method validation were performed using sample material (rye) that was tested free from ergot alkaloids. The absence of ergot alkaloids was confirmed as follows: a portion of sample was analyzed as such and another portion was spiked with the target analytes prior to analysis. By comparing with a solution of standards, no peaks corresponding to the target analytes were found in the non-spiked sample, whereas they were found in the spiked sample.

For the investigation of ergot alkaloid occurrence, 1065 cereals and cereal-based products were collected in different European countries over a 3-year period. This included *i)* rye food (flour – 202 samples; crispbread – 6; bread – 16; grain – 2), *ii)* wheat food (flour – 151; biscuit – 4; bread – 32; bran – 137; germs – 2; grain – 6), *iii)* multigrain food (flour – 18; biscuit – 42; crispbread – 11; bread – 81; flakes – 34), *iv)* rye feed (grain – 157), *v)* wheat feed (grain – 137) and *vi)* triticale feed (grain – 27). Conventionally as well as organically grown cereal samples were included. The selection of the countries took into consideration the geographic location. The number of samples for each country was as follows: Belgium – 243 samples, the United Kingdom – 43, Poland – 99, Sweden – 62, Switzerland – 25, the Netherlands – 82, Germany – 92, Finland – 83, Estonia – 48, France – 80, Denmark – 28, Czech Republic – 56, Italy – 12; 112 samples were of unknown origin.

Rye, wheat and triticale feed grains were sampled at the level of feed manufacturers in collaboration with the European Feed Manufacturer's Federation (FEFAC), while collection of wheat and rye flour samples was supported by the European Flour Milling Association. Cereal-based products were randomly collected from retail markets in Belgium.

As a general scheme (Commission Regulation 401/2006/EC, 2006) for cereal (grain and flour) sampling, incremental samples were taken from different places in each sample lot. Subsequently, an aggregate sample was made by combining all the incremental samples taken from the lot. From the aggregate sample, a representative laboratory sample was taken for analysis.

6.2.2.4. Sample preparation

6.2.2.4.1. Cereals and cereal products

Five gram of sample were extracted with 40 mL EtOAc:MeOH:0.2 M NH_4HCO_3 pH 8.5 (62.5:25:12.5, v/v/v) [Step 1] during 30 minutes on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). The sample extract was centrifuged and a phase separation was induced by adding 5 mL of a 0.2 M NH_4HCO_3 buffer pH 10 and 5 mL of a saturated solution of $(NH_4)_2SO_4$ to 15 mL of the extract [Step 2]. 5 mL of the EtOAc-phase was evaporated until dryness, and the residue was reconstituted in 200 µL of MeOH:ACN:H_2O (20:40:40, v/v/v). Subsequently, 200 µL of *n*-hexane were added and the resulting mixture was vortexed and centrifuged in an Ultrafree®-MC centrifugal device for 10 min at 14,000 g. The *n*-hexane was discarded and the aqueous phase was analyzed by LC-MS/MS.

6.2.2.4.2. Corn and grass silages

The procedure was as described in 6.1.2.4.1 but the residue from the EtOAc-phase (5 mL) was redissolved in 1000 µL DCM and 500 µL of 0.2 M NH_4HCO_3 buffer pH 10, and 500 µL of saturated solution of $(NH_4)_2SO_4$ were subsequently added. The resulting mixture was vortexed and centrifuged for 10 min at 14,000 g. 500 µL of the DCM-phase were evaporated to dryness and the residue was redissolved in 200 µL of MeOH:ACN:H_2O (20:40:40, v/v/v) and treated as above prior to LC-MS/MS analysis.

6.2.2.5. Evaluation of extraction and clean-up recovery

The evaluation of the extraction and clean-up recovery was carried out by spiking analyte-free samples before and after the extraction and clean-up step, and subsequent LC-MS/MS analysis. Calculations were performed by comparing peak areas for the same compound in samples spiked ante and post extraction and clean-up.

6.2.2.6. Optimization of method parameters using experimental design

The optimization of MS parameters was supported by experimental design, using Modde 9.1 statistical graphic software (Umetrics, Umea, Sweden). The most influencing factors were determined by a screening experiment and further optimized by an RSM experiment.

A Plackett-Burman design was applied for the screening experiment. The lower and the upper levels of the MS parameters in the design were selected on the basis of preliminary experiments. 15 variables were included in this study, which resulted in a 20-run design. Applying this design with three replicates of the center point, the total number of runs was 23 (Table 6.1).

After selection of the important factors, further RSM optimization was carried out applying a CCF design. Four parameters (desolvation temperature, cone, multiplier and extractor voltages) were optimized. With this number of parameters and 3 replications of the center point, the experimental design resulted in a number of experiments equal to 27 (Table 6.2).

A CCF design was also applied during the optimization of the extraction and clean-up procedure with four variables: pH of the ammonium bicarbonate solution in the extraction solvent, extraction solvent volume, extraction time and solvent evaporation temperature (k = 4) and three replications of the center point (n = 3) giving a total of 27 runs (Table 6.3).

***Table 6.1**. Plackett-Burman design applied for the screening experiment.*

Exp No	Cone voltage (V)	Capillary voltage (V)	Multiplier (V)	RF Lens	Extractor (V)	Exit (V)	Entrance (V)	Ion energy1	Ion energy2	Source temperature (°C)	Desolvation temperature (°C)	LM Resolution1	LM Resolution2	HM Resolution1	HM Resolution2
1	20	2.5	600	0.5	5	30	50	1	5	150	400	12	13	13	13
2	20	3.5	750	0.4	5	30	50	5	1	150	150	13	12	13	13
3	40	3.5	750	0.5	1	30	50	1	1	100	400	12	13	12	13
4	40	2.5	600	0.5	5	70	50	1	1	100	150	13	12	13	12
5	20	3.5	600	0.4	5	70	70	1	1	150	150	12	13	12	13
6	40	3.5	750	0.4	1	30	70	1	5	150	150	12	12	13	12
7	40	2.5	750	0.5	1	70	50	5	5	150	150	12	12	12	13
8	20	2.5	750	0.5	5	70	70	5	1	150	400	12	12	12	12
9	20	3.5	750	0.5	5	30	70	1	5	100	400	13	12	12	12
10	40	3.5	600	0.5	5	30	50	5	5	150	150	13	13	12	12
11	40	3.5	750	0.4	5	70	50	5	1	100	400	12	13	13	12
12	40	3.5	600	0.5	1	70	70	1	1	150	400	13	12	13	13
13	40	2.5	750	0.4	5	70	70	1	5	100	150	13	13	12	13
14	20	3.5	600	0.5	1	70	70	5	5	100	150	12	13	13	12
15	40	2.5	600	0.4	5	30	70	5	5	100	400	12	12	13	13
16	20	3.5	600	0.4	1	70	50	5	5	100	400	13	12	12	13
17	40	2.5	600	0.4	1	30	70	5	1	150	400	13	13	12	12
18	20	2.5	750	0.4	1	70	50	1	5	150	400	13	13	13	12
19	20	2.5	750	0.5	1	30	70	5	1	100	150	13	13	13	13
20	20	2.5	600	0.4	1	30	50	1	1	100	150	12	12	12	12
21	30	3	675	0.45	3	50	60	3	3	125	275	12.5	12.5	12.5	12.5
22	30	3	675	0.45	3	50	60	3	3	125	275	12.5	12.5	12.5	12.5
23	30	3	675	0.45	3	50	60	3	3	125	275	12.5	12.5	12.5	12.5

***Table 6.2**. CCF design applied for the optimization experiment.*

Exp. No	Multiplier (V)	Extractor (V)	Cone voltage (V)	Desolvation temperature (°C)
1	600	1	20	150
2	750	1	20	150
3	600	5	20	150
4	750	5	20	150
5	600	1	55	150
6	750	1	55	150
7	600	5	55	150
8	750	5	55	150
9	600	1	20	400
10	750	1	20	400
11	600	5	20	400
12	750	5	20	400
13	600	1	55	400
14	750	1	55	400
15	600	5	55	400
16	750	5	55	400
17	600	3	37.5	275
18	750	3	37.5	275
19	675	1	37.5	275
20	675	5	37.5	275
21	675	3	20	275
22	675	3	55	275
23	675	3	37.5	150
24	675	3	37.5	400
25	675	3	37.5	275
26	675	3	37.5	275
27	675	3	37.5	275

***Table 6.3**. CCF design applied in the optimization of the extraction and clean-up procedure.*

Exp. No	Solvent evaporation temperature (°C)	Extraction volume (mL)	Shaking time (min)	pH of NH_4HCO_3 solution
1	40	20	10	7
2	60	20	10	7
3	40	40	10	7
4	60	40	10	7
5	40	20	50	7
6	60	20	50	7
7	40	40	50	7
8	60	40	50	7
9	40	20	10	10
10	60	20	10	10
11	40	40	10	10
12	60	40	10	10
13	40	20	50	10
14	60	20	50	10
15	40	40	50	10
16	60	40	50	10
17	40	30	30	8.5
18	60	30	30	8.5
19	50	20	30	8.5
20	50	40	30	8.5
21	50	30	10	8.5
22	50	30	50	8.5
23	50	30	30	7
24	50	30	30	10
25	50	30	30	8.5
26	50	30	30	8.5
27	50	30	30	8.5

6.2.2.7. LC-MS/MS analysis

The LC-MS/MS analyses were performed on an Alliance HPLC 2695 (Waters, Milford, MA, USA) platform coupled to a Micromass Quattro triple quadrupole mass spectrometer (Waters) equipped with a Z-spray ESI interface. Chromatographic separation was achieved using an XBridge MS C18 column (3.5 µm, 150 x 2.1 mm) with an XBridge Sentry guard column (3.5 µm, 10 x 2.1 mm i.d.) both supplied by Waters. The column temperature was set at 30 °C. A mobile phase consisting of eluents A [H_2O:0.2 M NH_4HCO_3 pH 10:MeOH (85:5:10,

v/v/v)] and B [H_2O:0.2 M NH_4HCO_3 pH 10:MeOH (5:5:90, v/v/v)] was used at a flow rate of 0.15 mL/min. A gradient elution was applied as follows: 0-3 min: 30-15 % A; 3-7 min: 15 % A; 7-10 min: 15-0 % A; 10-13 min: 0 % A; 13-14 min: 0-30 % A; 14-23 min: 30 % A. The injection volume was 20 µL. The mass spectrometer was operated in the ESI^+ mode. During the tuning process, the acquisition of the first-order mass spectra as well as the CID experiments were performed on 10 µg/mL solutions of individual ergot alkaloid standards brought into the MS using a syringe pump. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures: 150 °C and 300 °C, respectively; capillary voltage: 3.5 kV; argon collision gas: 1.2 x 10^{-3} mbar; probe nebuliser and desolvation gas flows: 100 and 830 L/h, respectively. After selection of the precursor ions for each analyte, product ions were obtained with a combination of cone voltages and collision energies, parameters that were previously optimized. For increased sensitivity and selectivity, data acquistion was performed working in SRM mode. Two SRM transitions were selected for each of the analytes. For positive identification of ergot alkaloids in the samples, the criteria described in Chapter 2 (section 2.2) were used:

MassLynx and QuanLynx software (Micromass, Manchester, UK) were used for data acquisition and processing.

6.2.2.8. Method validation

The developed method was validated according to the Commission Decision 2002/657/EC (Commission Decision 2002/657/EC, 2002). Method linearity, LOD and limit of quantification (LOQ), decision limit (CC_α), detection capability (CC_β), system and method repeatability, within-laboratory reproducibility and method trueness in terms of recovery were evaluated by spiking experiments using sample material that was tested free from ergot alkaloids. Eventually, samples with very low levels of one or more of the analytes were used. The specificity of the method was assessed through the analysis of unspiked blank samples. The method was initially validated for cereals (rye, wheat, triticale, barley and oats), and was further extended to processed foods (bread, crispbread and biscuits) and feeds (composite feed, corn and grass silages).

The LODs and LOQs were determined as the minimum concentration of analyte in the spiked blank samples inducing SRM traces with a S/N of 3 and 10, respectively. To assess the linearity of the method, the blank samples were spiked with the different ergot alkaloids over a concentration range of 1 – 150 µg/kg. In total, 8 different concentrations were included and the analytical procedure was performed in triplicate at each concentration. A linear regression was applied.

The trueness, referred to as apparent recovery (Thorburn Burns *et al*., 2002), was evaluated by recovery experiments. Since cereals with certified concentrations of ergot alkaloids were not available at the time this study was done, artificially fortified ergot alkaloid-free samples were analyzed and the percent analyte recovery assessed. In detail, samples that were spiked with the different ergot alkaloids over a concentration range of 1 – 150 µg/kg, were analyzed. The observed signal was plotted against the actual concentration. The measured concentration was determined using the obtained calibration curves, and the recovery was calculated as follows (Equation 6.1):

$$\%\ \boldsymbol{Recovery} = \mathbf{100}\ \times \boldsymbol{Measured\ concentration}\ \div \boldsymbol{Actual}\ (\boldsymbol{added})\ \boldsymbol{concentration}$$

(Eq. 6.1)

The precision of the method was studied by repeated analysis of spiked samples. The experiments were carried out at different concentrations of the analyte in the sample on the same day (repeatability) and on 3 consecutive days by different operators (within-laboratory reproducibility). The precision was calculated as RSD of replicate measurements.

The linearity and precision data were also used to calculate CC_α and CC_β. CC_α was determined as the concentration corresponding to 'the y-intercept of the calibration curve plus 2.33 times the standard deviation of the within-laboratory reproducibility' (α= 0.01). CC_β was calculated as the concentration corresponding to 'CC_α plus 1.64 times the standard deviation of the within-laboratory reproducibility' (α= 0.05).

An estimate of the relative standard uncertainty (u_c, %) associated with the results was gained for each alkaloid by combining the within-laboratory reproducibility (S_{RW}) with the

uncertainty associated with the purity of standards ($U(C_{ref})$) as well as the uncertainty associated with the mean recovery (S_{bias}) as follows (Equation 6.2):

$$\boldsymbol{u_c} = \sqrt{\boldsymbol{S_{RW}^2} + \boldsymbol{U(C_{ref})^2} + \boldsymbol{S_{bias}^2}} \qquad \text{(Eq. 6.2)}$$

An estimate of the expanded uncertainty (U, %) corresponding to a confidence interval of approximately 95 % was obtained by multiplying the combined uncertainty by a coverage factor of 2, i.e (Equation 6.3).

$$\boldsymbol{U = 2 \times u_c} \qquad \text{(Eq. 6.3)}$$

6.2.2.9. Method robustness

The assessment of method robustness was based on evaluation of extraction recoveries of ergot alkaloids varying the following method parameters: pH of the extraction buffers, buffer concentration, extraction volume, shaking time and drying temperature. These factors were selected as potential sources of variability when performing the analysis. The intervals of the factors were set at values which slightly exceed the variations which can be expected when a method is transferred from one laboratory to another. The influence of these factors on the response was examined by means of 2-level fractional factorial design using Modde 9.1 statistical software (Umetrics, Umeå, Sweden). Having three replicates of the centre-point and 8 factors, the total number of runs was 19. The experimental design set-up is presented in Table 6.4.

Table 6.4. *Experimental design set-up for evaluation of robustness.*

Exp. No	pH of extraction buffer [Step 1]	Buffer concentration [Step 1] (mol/L)	Extraction volume [Step 1] (mL)	Shaking time (min)	pH of extraction buffer [Step 2]	Buffer concentration [Step 2] (mol/L)	Extraction volume [Step 2] (mL)	Temperature of drying (°C)
1	8.3	0.18	38	28	9.8	0.18	19	37
2	8.7	0.18	38	32	9.8	0.22	21	37
3	8.3	0.18	42	32	10.2	0.18	21	37
4	8.7	0.18	42	28	10.2	0.22	19	37
5	8.3	0.22	38	32	10.2	0.22	19	37
6	8.7	0.22	38	28	10.2	0.18	21	37
7	8.3	0.22	42	28	9.8	0.22	21	37
8	8.7	0.22	42	32	9.8	0.18	19	37
9	8.3	0.18	38	28	10.2	0.22	21	43
10	8.7	0.18	38	32	10.2	0.18	19	43
11	8.3	0.18	42	32	9.8	0.22	19	43
12	8.7	0.18	42	28	9.8	0.18	21	43
13	8.3	0.22	38	32	9.8	0.18	21	43
14	8.7	0.22	38	28	9.8	0.22	19	43
15	8.3	0.22	42	28	10.2	0.18	19	43
16	8.7	0.22	42	32	10.2	0.22	21	43
17	8.5	0.20	40	30	10.0	0.20	20	40
18	8.5	0.20	40	30	10.0	0.20	20	40
19	8.5	0.20	40	30	10.0	0.20	20	40

6.2.3. Results and discussion

6.2.3.1. Optimization of the analytical method

6.2.3.1.1. MS/MS detection

6.2.3.1.1.1. Preliminary experiments

Determination of the optimal SRM transitions and MS settings (mode, cone voltage, collision energy) for the different analytes was performed as described in sections 6.2.2.6 and 6.2.2.7. Krska *et al.* (Krska *et al*., 2008) reported the suitability of a NH_4HCO_3-containing mobile phase for the LC-ESI^+-MS/MS analysis of ergot alkaloids. The authors used ACN as organic modifier, but MeOH, the other commonly used organic modifier, was not assessed. In the current study, different mobile phases were investigated, including MeOH-containing and ACN-containing mobile phases, as well as acidic and basic mobile phases. A mobile phase consisting of H_2O:0.1 M $(NH_4)_2CO_3$ pH 8.5:MeOH (30:5:65, v/v/v) was selected as it showed enhanced sensitivity. Both ESI and APCI interfaces were investigated, working in the positive and in the negative mode of ionization. ESI in the positive mode gave higher sensitivity and was therefore selected for further optimization. Full MS scan for the epimers revealed a signal at *m/z* corresponding to $[M\text{-}H_2O+H]^+$ higher than that of the protonated molecules. Therefore, further optimization of the MS signal was carried out on the ion at *m/z* corresponding to $[M\text{-}H_2O+H]^+$ for the epimers except ergometrinine.

6.2.3.1.1.2. Optimization of MS parameters applying experimental design

The MS parameters included in this study were source temperature, desolvation temperature, cone voltage, capillary voltage, multiplier voltage, extractor voltage, RF lens, entrance, exit, ion energy1, ion energy2, LM resolution1, LM resolution2, HM resolution1 and HM resolution2. The influence of these parameters on the intensity of the ions at *m/z* corresponding to $[M+H]^+$ (for the main compounds and ergometrinine) or $[M\text{-}H_2O+H]^+$ (for the epimers other than ergometrinine) was assessed by means of a Plackett-Burman experimental design. The results (Fig. 6.2) indicated that the intensity of the MS signal for both ions monitored is mostly influenced by four factors: cone voltage, desolvation temperature, extractor voltage and multiplier voltage. Increasing the cone voltage led to a

reduction or increase of the MS signal depending on the analyte. The extractor voltage showed a positive effect on the MS signal of the epimers, whereas the desolvation temperature and the multiplier voltage demonstrated a positive effect on the signal of all analytes. The effects of the other parameters were less significant.

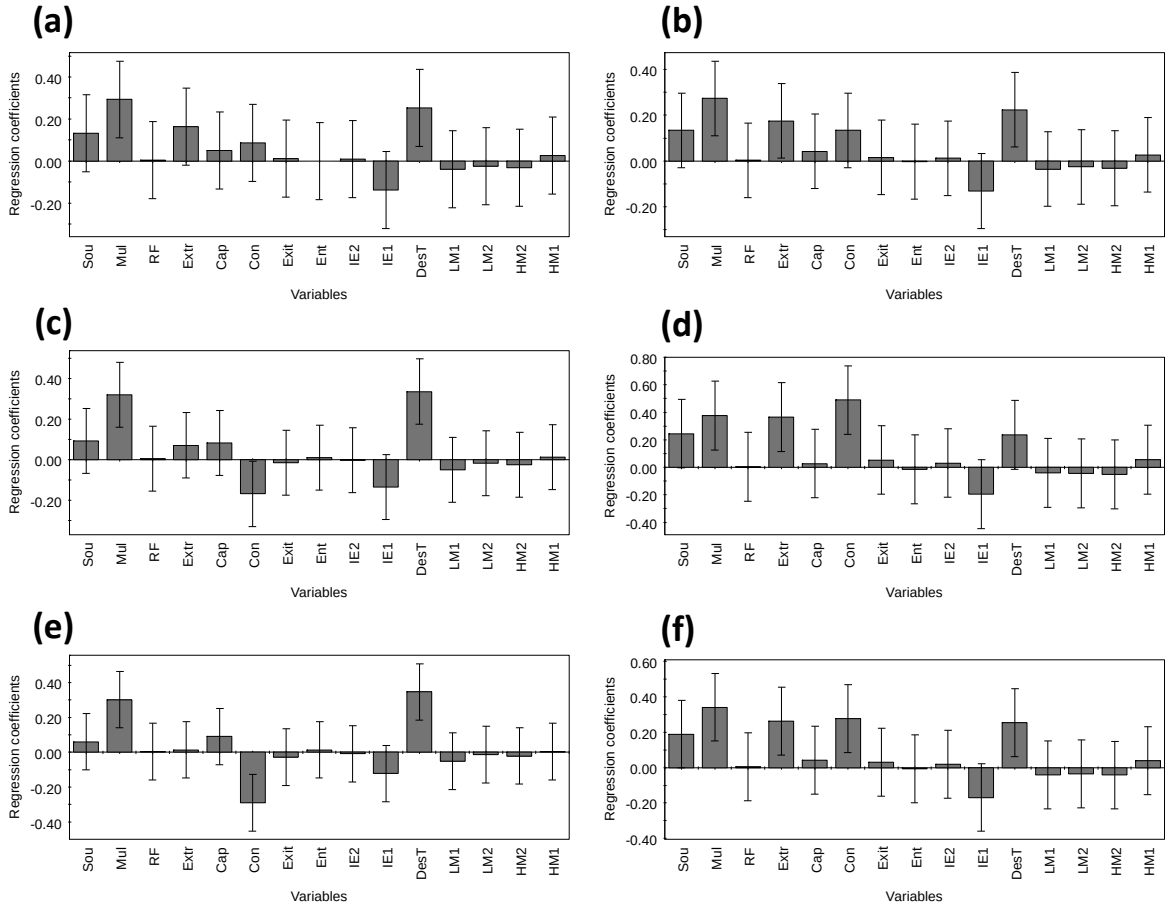

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Figure 6.2. *Regression coefficient plots for different MS parameters for ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l), methylergometrine (m), dihydroergotamine (n).*

A logarithmic transformation of the responses was done.

Sou = source temperature; Mul = multiplier voltage; RF = RF lens; Extr = extractor voltage; Cap = capillary voltage; Con = cone voltage; Exit = exit; Ent = entrance; IE2 = ion energy2; IE1 = ion energy1; DesT = desolvation temperature; LM1 = LM resolution1; LM2 = LM resolution2; HM2 = HM resolution2; HM1 = HM resolution1.

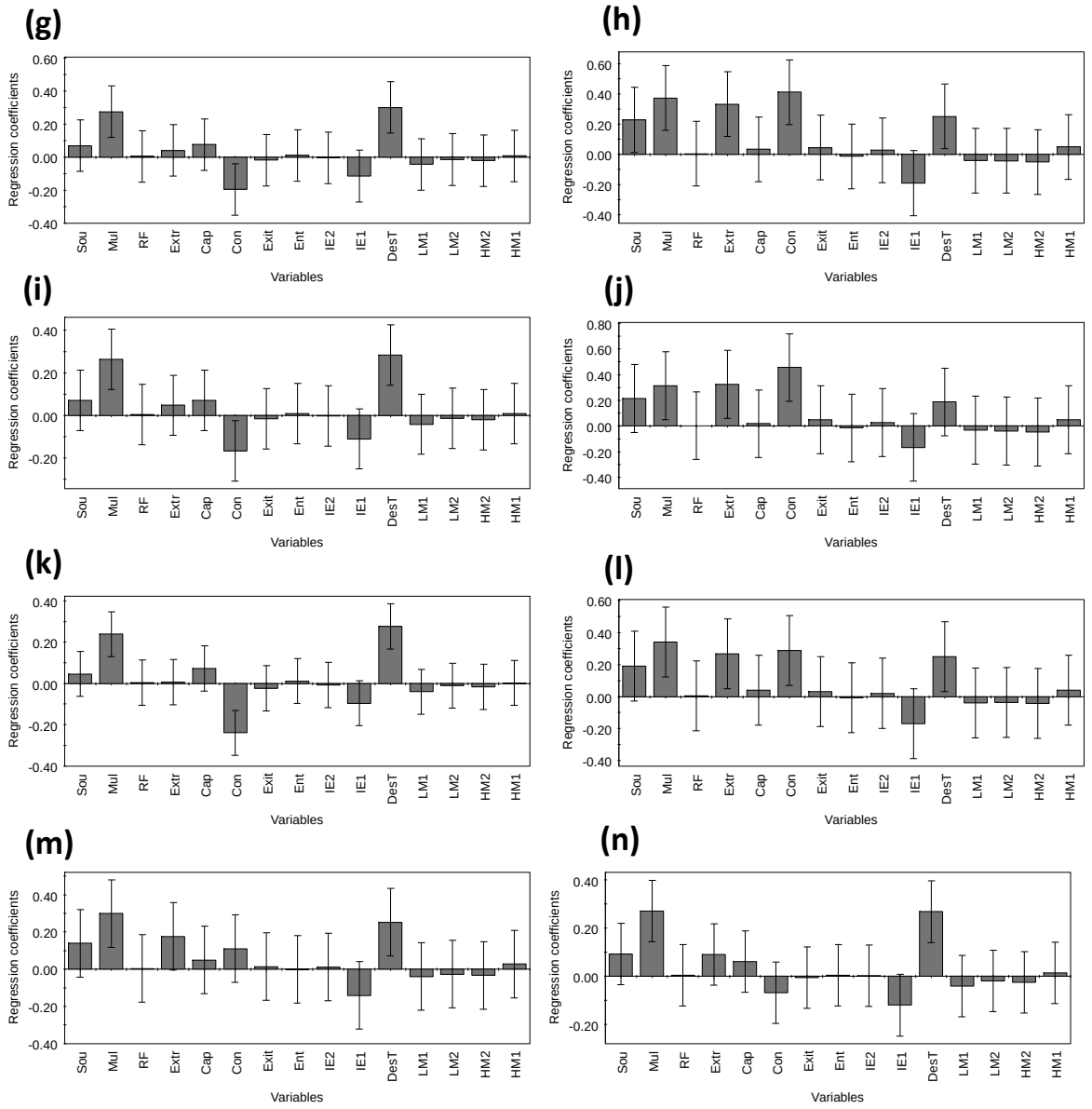

Figure 6.2 (continued)

The parameters that have the most influence on the MS signal, namely the cone voltage, desolvation temperature, extractor voltage and multiplier voltage, were further optimized by means of a central composite design. The design allowed to model the response surface by fitting a second-order polynomial model. The statistical analysis of the model gave R^2 values of 0.97 to 0.99 and Q^2 values of 0.79 to 0.91. The obtained Q^2 values are close to 1, which indicates the suitability of the model for predicting the optimal conditions.

The response surface plots (Fig. 6.3) for the MS signal intensity of the different analytes indicated that cone voltage values around 20 V were optimum for the main ergot alkaloids (-ine compounds) except for ergometrine (cone voltage of 30 V), while values in the range 30 – 40 V were necessary for the –inine compounds as well as for the semi-synthetic compounds methylergometrine and dihydroergometrine used as internal standards.

The CID experiments were carried out using the optimized conditions (Table 6.5) and applying different collision energies. The product ion spectra revealed for most of analytes, an intense signal for the ions at *m/z* 223 and 208 (Table 6.5). These results confirmed those reported by Lehner *et al.* (Lehner *et al*., 2005) and Krska *et al*. (Krska *et al*., 2008). Lehner *et al.* (Lehner *et al*., 2005) attributed these fragments at *m/z* 223 and 208 to the four-ring system of the lysergic moiety and its demethylated counterpart, respectively.

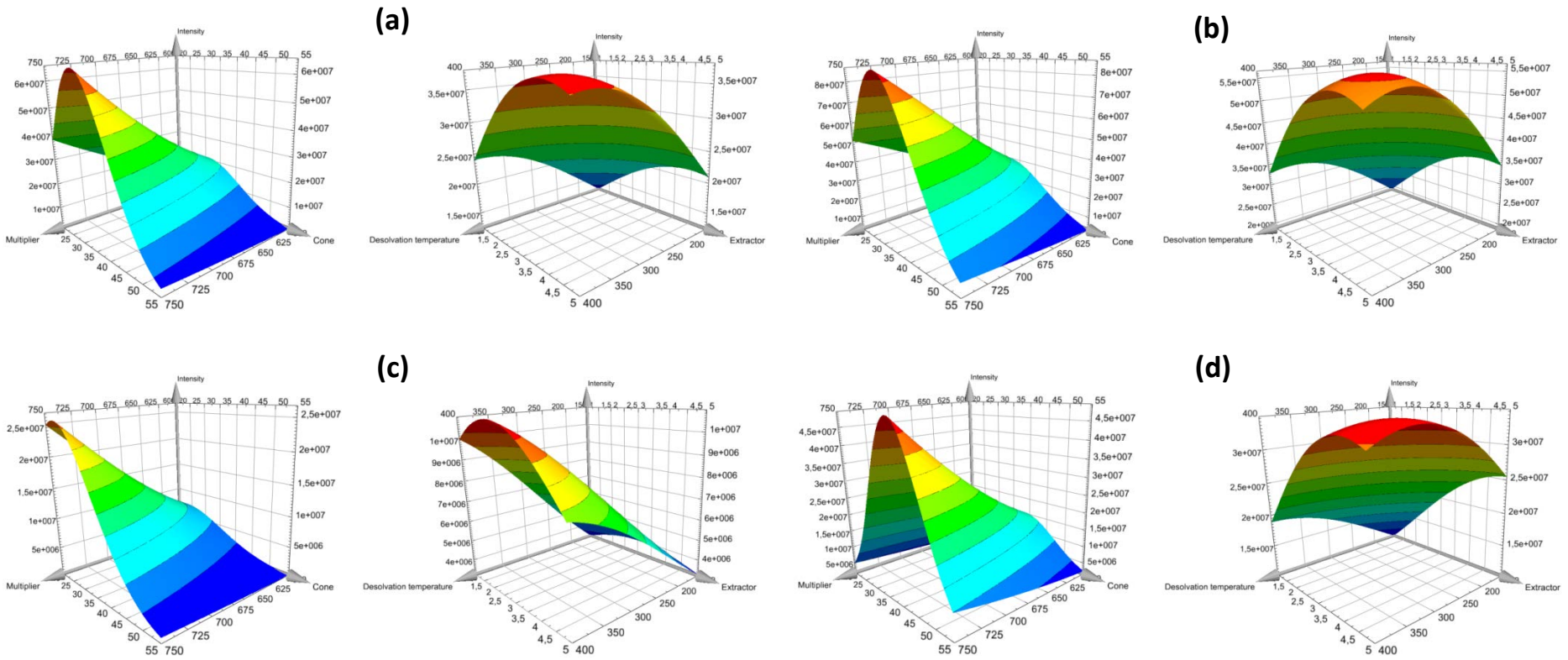

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Figure 6.3. *Response surface plots for MS signal intensity of ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l), methylergometrine (m), dihydroergotamine (n) as a function of multiplier voltage and cone voltage, and desolvation temperature and extractor voltage.*
A logarithmic transformation of the responses was done.

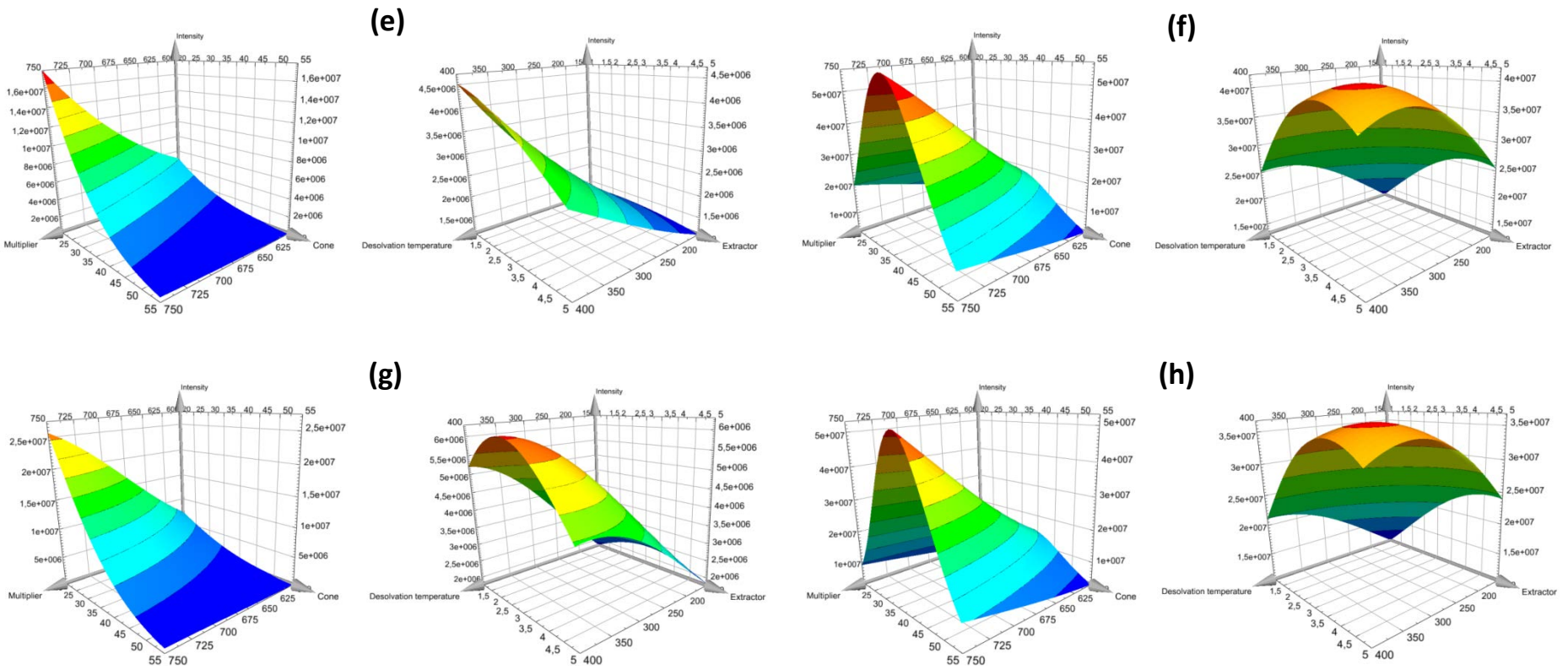

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Figure 6.3 (continued)

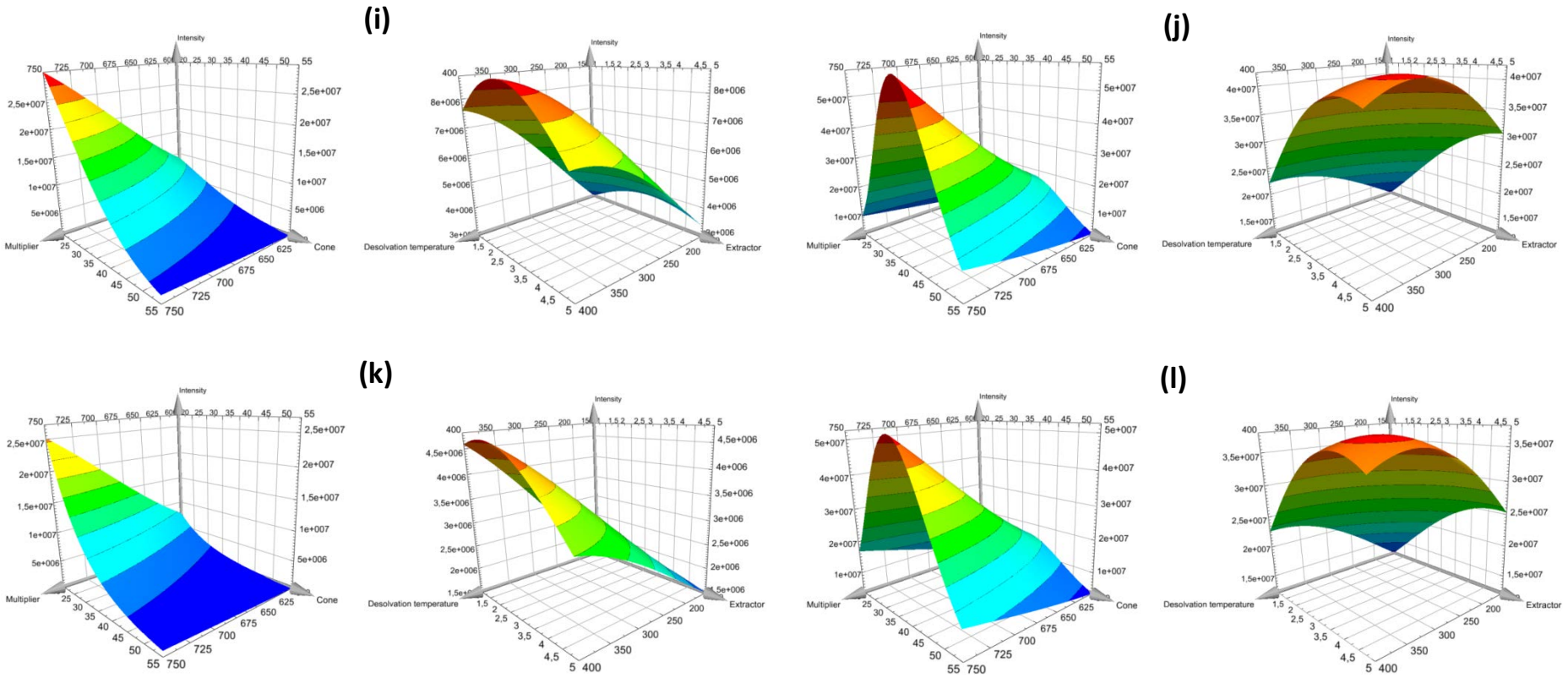

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Figure 6.3 (continued)

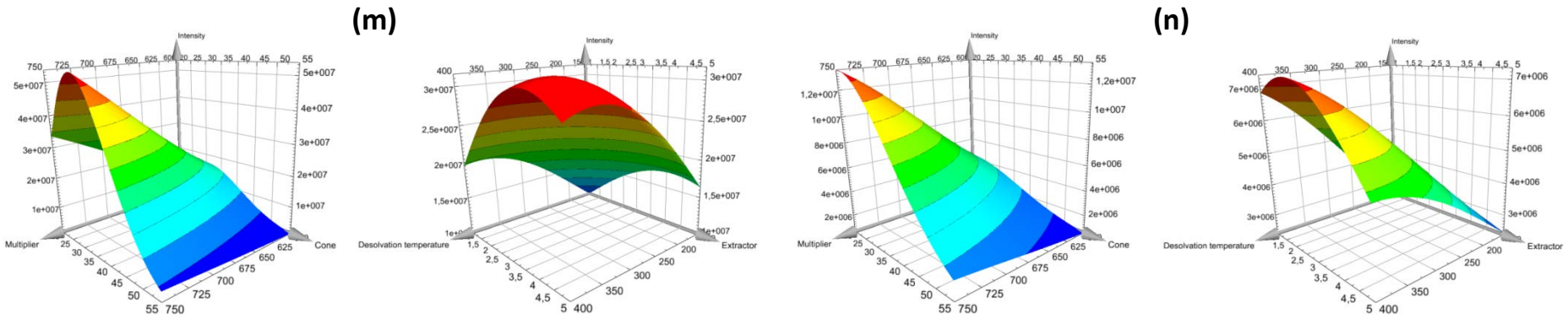

Figure 6.3 (continued)

Table 6.5. *Optimized MS/MS parameters for the analysis of ergot alkaloids in the SRM ESI$^+$ mode.*

Ergot alkaloid	RT (min)	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Ergometrine	3.2	326.3 $[M+H]^+$	30	223.3[a] 208.2	23 30
Methylergometrine	3.5	340.3 $[M+H]^+$	28	223.3[a] 208.2	25 30
Ergometrinine	4.3	326.2 $[M+H]^+$	33	208.0[a] 223.0	25 25
Ergosine	7.6	548.3 $[M+H]^+$	20	208.1[a] 223.3	40 33
Ergotamine	8.5	582.5 $[M+H]^+$	22	208.2[a] 268.4	25 45
Dihydroergotamine	9.1	584.5 $[M+H]^+$	20	270.2[a] 225.3	30 35
Ergocornine	10.4	562.5 $[M+H]^+$	20	268.3[a] 223.3	40 25
Ergokryptine	10.5	576.3 $[M+H]^+$	21	268.4[a] 208.1	45 25
Ergocristine	10.9	610.4 $[M+H]^+$	23	208.1[a] 268.4	25 45
Ergosinine	11.4	530.4 $[M-H_2O+H]^+$	38	223.2[a] 263.2	28 23
Ergotaminine	11.8	564.4 $[M-H_2O+H]^+$	35	223.2[a] 208.2	32 50
Ergocorninine	12.5	544.4 $[M-H_2O+H]^+$	39	277.5[a] 223.3	26 37
Ergokryptinine	14.0	558.5 $[M-H_2O+H]^+$	38	223.3[a] 305.3	35 27
Ergocristinine	14.8	592.4 $[M-H_2O+H]^+$	35	223.3[a] 305.4	35 29

[a] The most abundant product ion.

6.2.3.1.2. LC-MS/MS analysis

The ionogenic nature of ergot alkaloid makes the polarity of both the stationary phase and the mobile phase an important factor to be considered during the development of the chromatographic method. Acidic (Mohamed *et al*., 2006; Smith *et al*., 2009) as well as basic (Krska *et al*., 2008; Müller *et al*., 2009) mobile phases have been used for reverse-phase LC of ergot alkaloids. Although better separation of these analytes is achieved in basic mobile phases, acidic mobile phases are often preferred because conventional silica-based

stationary phases are degraded at high pH. XTerra C18 and XBridge C18 stationary phases have been reported to be stable in the pH range 1 – 12 (Jenkins *et al*., 2005) and were previously used for the analysis of other basic compounds (Diana Di Mavungu, 2005). In this study, the suitability of basic mobile phases in combination with the XTerra or the XBridge stationary phase was investigated. A mobile phase consisting of H_2O, 0.1 M $(NH_4)_2CO_3$ pH 8.5 and MeOH was used as it gave enhanced MS signal. The XBridge column gave an overall better chromatographic separation and proved to be stable over a long period of use. Therefore, this column was selected for further investigation. A gradient elution was needed to elute all analytes within reasonable time. A good separation of the analytes was achieved; however, a shift of retention time was observed during analysis. This was overcome by replacing $(NH_4)_2CO_3$ with NH_4HCO_3 and by further adjusting the buffer pH at 10. The *pKa* values of the ergot alkaloids vary from 4.8 to 6.2 (Krska and Crews, 2008). Therefore, under the pH conditions of the mobile phase applied, nearly 100 % of the molecules are neutral, leading to a more efficient interaction of the analyte with the stationary phase. As a consequence, well-resolved peaks and good peak shape were achieved for almost all components within 15 min, the total run time being 23 min (Fig. 6.4). Ergokryptine and ergocristine were not baseline separated, but this was of minor importance as they have different SRM transitions. Moreover the overall good separation allowed the SRM detection of the ergot alkaloids in five different time window functions, thereby leading to an enhanced sensitivity. An investigation into the analyte stability under the selected analytical conditions revealed that epimerization occurred when samples dissolved in the mobile phase were allowed to stand in the autosampler for several hours before the LC-MS/MS analysis. Ergosine showed the highest tendency to epimerize while ergometrine proved to be the most stable compound among the ergot alkaloids investigated. It has been reported that the epimerization of ergot alkaloids is minimal when standard solutions are prepared in aprotic solvents such as chloroform or ACN (Hafner *et al*., 2008). However, the use of a sample solvent that is stronger than the mobile phase leads to peak distortion. A careful investigation of different solvent mixtures indicated that a sample solvent consisting of MeOH:ACN:H_2O (20:40:40, v/v/v) delivers a good peak shape while keeping the epimerization negligible. Finally, it was shown that by using this sample solvent, maintaining the autosampler temperature at 4 °C and limiting the sample sequence to a maximum of 24 hours, no epimerization occurred during the LC-MS/MS analysis.

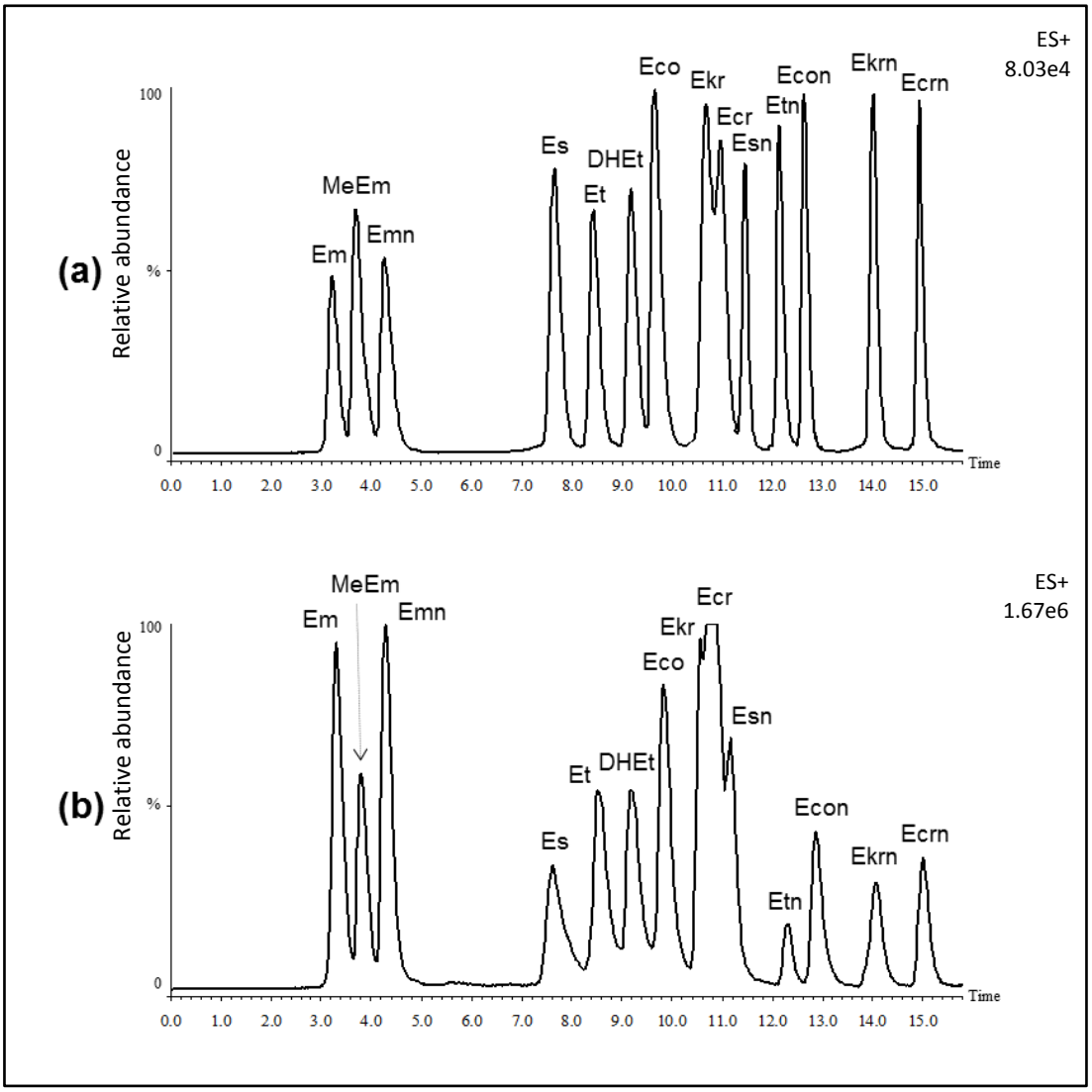

***Figure 6.4**. LC-ESI$^+$-MS/MS total ion chromatogram of a blank rye sample spiked with the ergot alkaloids at 25 µg/kg (a) and a naturally contaminated sample (b).*

Em: ergometrine; MeEm: methylegometrine; Emn: ergometrinine; Es: ergosine; Et: ergotamine; DHEt: dihydroergotamine; Eco: ergocornine; Ekr: ergokryptine; Ecr: ergocristine; Esn: ergosinine; Etn: ergotaminine; Econ: ergocorninine; Ekrn: ergokryptinine; Ecrn: ergocristinine.

6.2.3.1.3. Extraction and clean-up

Ergot alkaloids (*pKa* 4.8-6.2) are positively charged at N6 in acidic solutions and neutral at higher pH. They can therefore be extracted either with non-polar organic solvents under alkaline conditions or with polar solvents under acidic conditions. In this work, different alkaline extraction solvents, namely an ACN:$(NH_4)_2CO_3$ buffer mixture (Krska *et al.*, 2008) and a EtOAc:MeOH:NH_4HCO_3 buffer mixture (Diana Di Mavungu *et al.*, 2009; Müller *et al.*, 2009), as well as an acidic solvent *i.e.* a MeOH:0.25 % H_3PO_4 mixture were evaluated. Blank

rye samples spiked with ergot alkaloids at a concentration of 250 µg/kg were used. To adequately compare the different extraction solvents, no clean-up was performed prior to LC-MS/MS analysis; the extracts were simply diluted (1:10) and filter-centrifuged in an Ultrafree®-MC centrifugal device. The highest SRM responses of the ergot alkaloids were obtained after extraction with ACN:3 mM $(NH_4)_2CO_3$ (84:16, v/v) or EtOAc:MeOH:0.2 M NH_4HCO_3 pH 8.5 (62.5:25:12.5, v/v/v) (Fig. 6.5).

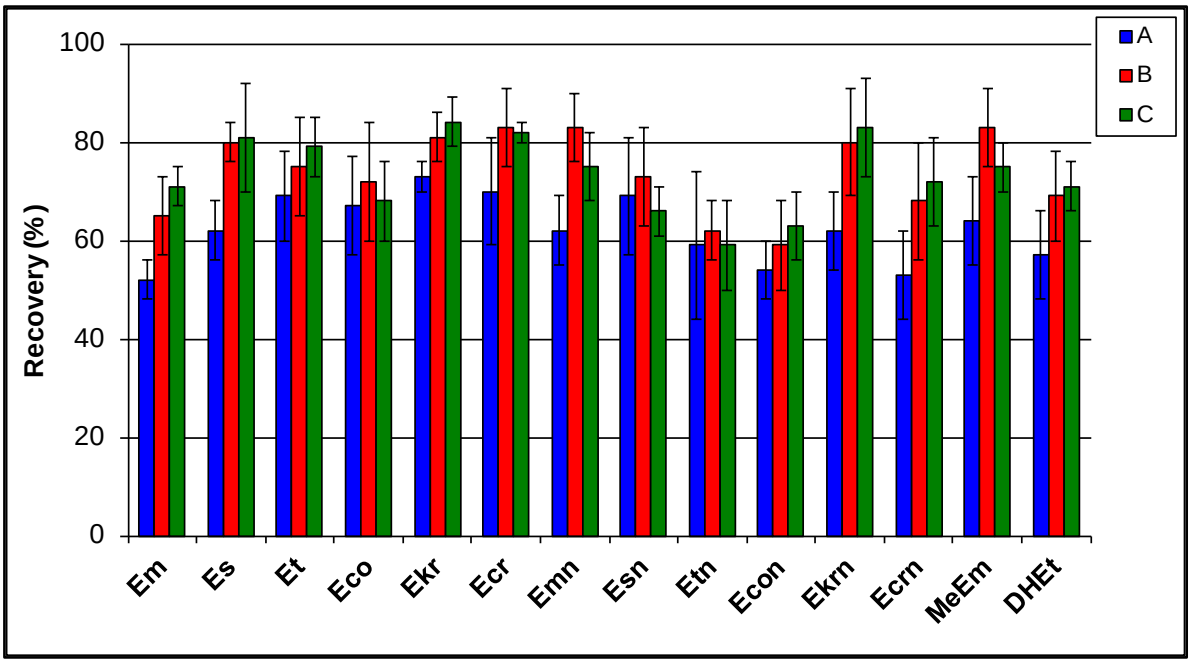

Figure 6.5. *Extraction recoveries for ergometrine (Em), ergometrinine (Emn), ergosine (Es), ergosinine (Esn), ergotamine (Et), ergotaminine (Etn), ergocornine (Eco), ergocorninine (Econ), ergokryptine (Ekr), ergokryptinine (Ekrn), ergocristine (Ecr), ergocristinine (Ecrn), methylergometrine (MeEm), dihydroergotamine (DHEt) applying different extraction solvents: (A) MeOH:0.25 % H_3PO_4 (40:60, v/v), (B) ACN:3 mM $(NH_4)_2CO_3$ (84:16, v/v) and (C) EtOAc:MeOH:0.2 M NH_4HCO_3 (62.5:25:12.5, v/v/v).*

The suitability of SPE exploiting ion exchange mechanism (Bond Elut AccuCAT and Bakerbond aminopropyl cartridges) or hydrophilic-lipophilic interactions (Oasis® HLB column) was investigated, using the ACN:$(NH_4)_2CO_3$ buffer extract. Poor recoveries were obtained with these SPE cartridges. Mohamed *et al.* (Mohamed *et al*., 2006) also reported unsatisfactory results for two different strong cation exchange SPE cartridges. Furthermore, the multiple and tedious steps of conditioning and washing led to the epimerization of the ergot alkaloids (up to 20 % for ergosine) during sample preparation. In further experiments,

the efficiency of a liquid-liquid partition clean-up after extraction with the solvent mixture EtOAc:MeOH:0.2 M NH_4HCO_3 pH 8.5 (62.5:25:12.5, v/v/v) was examined. The components of this extraction solvent as well as their ratio were carefully chosen to allow a quick sample clean-up by simply altering the solvent ratio. Indeed, after extraction of the ergot alkaloids, a phase separation was induced by adding a NH_4HCO_3 buffer to the extract. In this way, polar matrix components were removed with the aqueous phase, leading to partially cleaned extract. The addition of a saturated solution of $(NH_4)_2SO_4$ allowed to improve the transfer of ergot alkaloids in the EtOAc-fraction by reducing the availability of water in the aqueous phase. As a result, the recovery of the relatively more polar ergot alkaloids (ergometrine, ergometrinine, methylergometrine) was improved. Using an EtOAc-containing extraction solvent, several apolar interfering substances (*e.g.*, pigments, essential oils, fatty acids) were coextracted. Therefore, a defatting step with *n*-hexane was considered. Since EtOAc is miscible with hexane, the extract was first evaporated to dryness and redissolved in the solvent mixture MeOH:ACN:H_2O (20:40:40, v/v/v) before adding *n*-hexane.

The parameters that have an effect on the extraction efficiency and/or on the epimerization of the ergot alkaloids, namely the pH of the NH_4HCO_3 solution in the extraction solvent, the extraction solvent volume, the extraction time and the solvent evaporation temperature were optimized by means of experimental design. The R^2 values obtained by fitting a second order polynomial model were above 0.88 and the Q^2 values above 0.6 except for ergosine (Q^2 = 0.49), ergotamine (Q^2 = 0.57), ergokryptine (Q^2 = 0.53) and ergokryptinine (Q^2 = 0.57). The results (Appendix I) indicated that, in the range investigated (pH 7 – pH 10), an increase of pH the NH_4HCO_3 solution in the extraction solvent had a positive effect on the extraction efficiency of all analytes. However, it was also found that increasing the pH also increased the epimerization rate (Appendix II). Therefore, a pH of 8.5 was selected as a compromise. The results also showed that 40 mL of extraction solvent and an extraction time of 30 min were sufficient. Longer extraction time resulted in higher epimerization rate. Regarding the drying temperature (investigated in the range from 40 to 60 °C), less epimerization occurred when a temperature of 40 °C was applied; this temperature was therefore selected in the optimized procedure.

The use of liquid-liquid partition and a careful optimization of the extraction and clean-up parameters allowed to shorten the overall sample preparation procedure, and thereby to

minimize the epimerization process. Other measures to minimize epimerization during the sample preparation step included the use of a brown glass tube or aluminium foil, and the use of a temperature controlled centrifuge.

Applying the optimized conditions, the extraction and clean-up recoveries were assessed for diverse sample matrices. For the different combinations of matrices and analytes, the recoveries were above 50 %, except for ergometrine in biscuits (about 45 %) (Fig. 6.6). In general, little variability in recovery was observed between ergot alkaloids. Relatively low recoveries were observed for biscuits, while the recoveries were much higher for oats and triticale. The low recoveries for biscuits were not considered to be an obstacle for a reliable determination, as the other performance data (discussed in section 6.2.3.2) such as trueness, precision, linearity and detection limits were good.

Application of the developed extraction and clean-up procedure to different cereal-based products was straightforward. However, simply applying the procedure to corn and grass silages resulted in insufficiently cleaned extracts, which could not be directly brought into the LC-MS. Therefore, for these matrices, the residue from the EtOAc-phase underwent further liquid-liquid partitioning using small volume of DCM (1 mL) and of NH_4HCO_3 buffer (0.5 mL) prior to the defatting step with *n*-hexane, resulting in a cleaner extract.

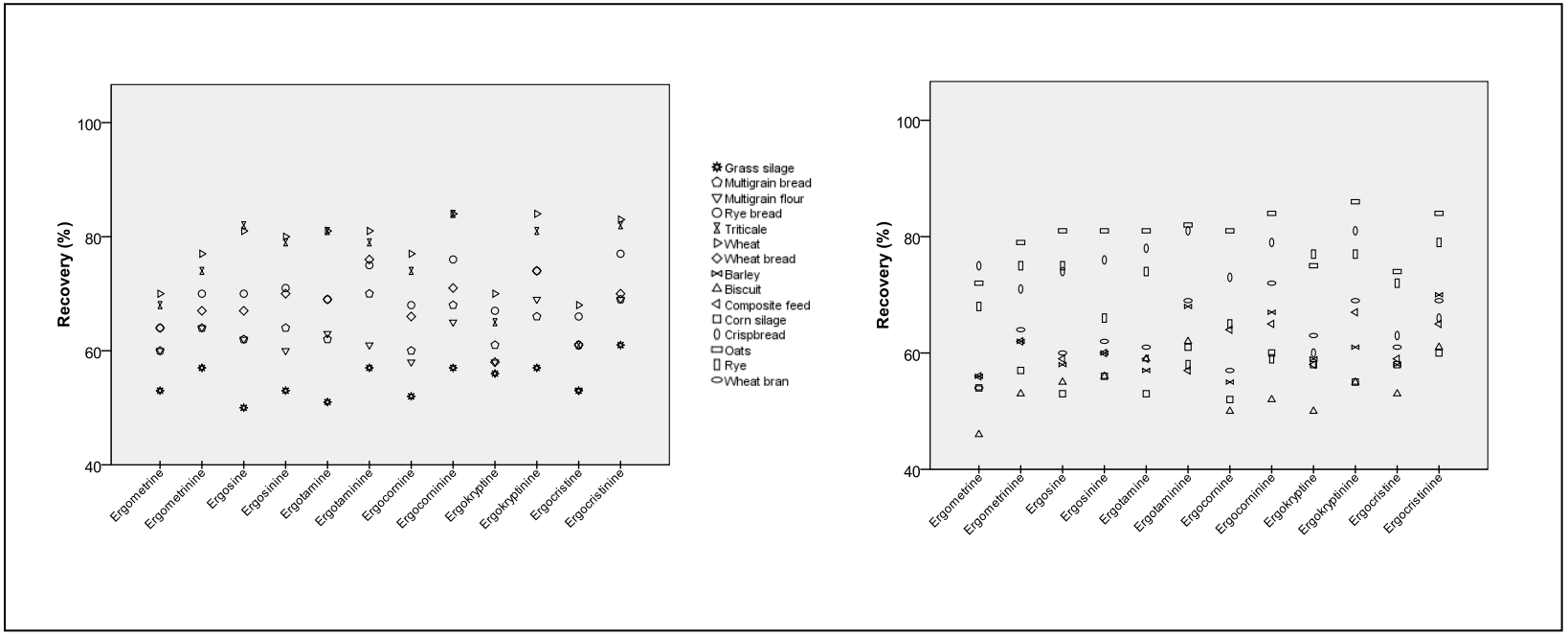

Figure 6.6. *Recovery for individual ergot alkaloids and their corresponding epimers in different matrices.*

6.2.3.2. Method validation

The method validation data for rye matrix are summarized in Table 6.6 as an example. Low LODs and LOQs were obtained for the different ergot alkaloids and matrices investigated. The reported LODs were in the range 0.04 – 0.29 µg/kg, whereas the LOQs were in the range 0.15 – 0.96 µg/kg. Since estimating the sensitivity of the method through LOD and LOQ lacks sufficient statistical significance, the concept of CC_α and CC_β was introduced in Commission Decision 2002/657/EC (Commission Decision 2002/657/EC, 2002). CC_α is defined as the lowest concentration level of the analyte that can be detected in a sample with a chance of 1 % of false positive decision. CC_β is defined as the smallest content of the analyte that can be detected in a sample with a chance of 5 % of false negative decision. The CC_α and CC_β values ranged from 0.57 to 1.13 µg/kg and 1.18 to 2.53 µg/kg, respectively. Although no regulatory limits are set for the content of ergot alkaloids in food and feed, results presented in Table 6.6 indicated that the proposed method is suitable for the detection of ergot alkaloids, even at very low µg/kg levels. Comparing these data to those reported in the literature (Krska *et al*., 2008; Mohamed *et al*., 2006), the method proposed here reaches overall lower LODs and LOQs.

Calibration curves for all the analytes in different matrices were linear over a concentration range of 1 – 150 µg/kg. The calibration data obtained in rye are shown as an example (Table 6.6). The coefficient of determination (R^2) for the eight-point calibration curves ranged from 0.975 (for ergotamine) to 0.996 (for ergometrine). The *p*-values for the lack of fit test (α=0.05) confirmed, for all analytes, the good fit of data with a linear model. Further evidence of linearity was provided by the residual plots, which showed no systematic trend (residuals randomly distributed around zero). The values for the standard error (S_{yx}) were all below 5 % of the estimate, even at low concentrations of analytes.

The trueness results shown in Table 6.6 were obtained for rye spiked at 2.5, 50 and 150 µg/kg and were in good agreement with the Commission Decision 2002/657/EC performance criteria for quantitative methods of analysis: all trueness values were within 80-110 %.

Table 6.6. *Method validation data for ergot alkaloid determination in rye.*

Ergot alkaloid	LOD/LOQ (µg/kg)	CC_α/CC_β (µg/kg)	Linearity			
			Equation	R^2	S_{yx}	Lack of fit test *p* -value
Ergometrine	0.29/0.96	0.65/1.19	y=0.0270x+0.0599	0.996	0.0003	0.07
Ergosine	0.04/0.15	0.71/1.18	y=0.0225x-0.0045	0.992	0.0004	0.70
Ergotamine	0.18/0.60	0.76/1.46	y=0.0189x+0.0009	0.975	0.0006	1.00
Ergocornine	0.06/0.20	0.84/1.43	y=0.0260x-0.0130	0.989	0.0005	1.00
Ergokryptine	0.06/0.19	0.66/1.21	y=0.0295x-0.0570	0.990	0.0006	0.10
Ergocristine	0.10/0.33	0.57/1.13	y=0.0070x-0.0069	0.977	0.0002	0.98
Ergometrinine	0.15/0.50	0.61/1.11	y=0.0756x+0.0010	0.993	0.0011	0.56
Ergosinine	0.06/0.20	0.74/1.38	y=0.0585x+0.0019	0.981	0.0009	0.43
Ergotaminine	0.22/0.75	0.94/2.39	y=0.0159x-0.0077	0.993	0.0003	0.42
Ergocorninine	0.09/0.30	1.06/2.17	y=0.0271x-0.0360	0.984	0.0007	0.46
Ergokryptinine	0.06/0.19	1.18/2.53	y=0.0181x-0.0141	0.988	0.0004	0.86
Ergocristinine	0.05/0.16	0.96/1.57	y=0.0130x-0.0284	0.986	0.0003	0.43

	Repeatability, % RSD			Within-laboratory reproducibility, % RSD		
	2.5 µg/kg	50 µg/kg	150 µg/kg	2.5 µg/kg	50 µg/kg	150 µg/kg
Ergometrine	12	8	10	12	13	14
Ergosine	19	9	5	19	12	10
Ergotamine	21	10	7	19	15	10
Ergocornine	18	13	5	18	14	13
Ergokryptine	26	7	5	24	10	9
Ergocristine	14	13	7	18	15	12
Ergometrinine	13	9	6	18	13	10
Ergosinine	20	11	8	22	15	14
Ergotaminine	16	12	5	19	15	17
Ergocorninine	12	7	2	19	10	14
Ergokryptinine	18	6	2	20	10	12
Ergocristinine	13	5	4	18	11	11

	Trueness (RSD), %			Expanded measurement uncertainty, %		
	2.5 µg/kg	50 µg/kg	150 µg/kg	2.5 µg/kg	50 µg/kg	150 µg/kg
Ergometrine	110 (13)	100 (13)	99 (14)	25	28	30
Ergosine	106 (19)	93 (12)	90 (10)	40	26	21
Ergotamine	106 (19)	101 (15)	91 (10)	39	32	21
Ergocornine	108 (18)	91 (14)	88 (13)	38	30	27
Ergokryptine	91 (24)	88 (10)	92 (9)	51	21	18
Ergocristine	96 (18)	93 (15)	96 (12)	38	32	26
Ergometrinine	103 (20)	96 (15)	93 (11)	41	30	25
Ergosinine	102 (17)	97 (12)	91 (9)	42	35	29
Ergotaminine	110 (23)	91 (15)	88 (17)	40	32	27
Ergocorninine	119 (26)	94 (10)	88 (14)	41	22	30
Ergokryptinine	110 (21)	95 (10)	88 (12)	42	22	26
Ergocristinine	110 (18)	91 (11)	89 (11)	38	24	24

The precision data were calculated as RSD of replicate measurements. The RSD value under repeatability conditions (RSD_r) should be between the half or two thirds of the value calculated by Horwitz equation (Equation 6.4). For mass fractions of 150 µg/kg, this means that the RSD_r should be lower than 14 %. Using this equation, mass fractions of 50 µg/kg and 2,5 µg/kg will results in RSD_r limits of 17 % and 26 %, respectively. With regard to the RSD under within-laboratory reproducibility conditions (RSD_R), the observed values should

not be greater than those calculated using the Horwitz equation. For mass fractions of 150 µg/kg, this means that the RSD_R should be lower than 21 %. However, using this equation, mass fractions of 50 µg/kg and 2,5 µg/kg, will results in RSD_R limits of 25 % and 39 %, respectively. These values are high; therefore, for concentrations below 50 µg/kg, an RSD_R guidance limit of 25 % was chosen.

Data presented in Table 6.6 indicated the good precision of the method.

$$\boldsymbol{CV = 2^{(1-0.5logC)}} \qquad \text{(Eq. 6.4)}$$

Table 6.6 also gives, for each of the ergot alkaloids, the expanded measurement uncertainties calculated at different concentrations, using rye as matrix. The uncertainty was not higher than 51 % at a concentration level of 2.5 µg/kg and not higher than 30 % at concentration levels of 150 µg/kg.

The use of a triple quadrupole provides high selectivity and specificity. Two SRM transitions were monitored for each analyte, which improves specificity. For identification, the ratios of the two SRM transitions were compared with those of the standards. The specificity of the method was further confirmed through the analysis of many blank samples (more than 20 samples for some matrices). For each of the SRM transitions, no peak with a S/N of at least 3 was detected.

Data presented above highlight the performance characteristics of the proposed method for the analysis of ergot alkaloids. The method can be applied to a wide range of food and feed matrices including rye, wheat, triticale, barley, oats, bread, crispbread, biscuits, composite feed, corn and grass silages.

6.2.3.3. Robustness study

Robustness of the method, that plays an important role in ensuring correct quantification, was investigated. The influence of small changes of the sample preparation parameters on the recovery of ergot alkaloids was evaluated. Parameters such as pH and concentration of extraction buffer, shaking time, drying temperature and extraction volumes were included in

the investigation (Appendix III). A fractional factorial design was used. The central values were those specified in the method and the extreme values (low and high) were computed from the nominal ones taking into account possible uncertainty (Table 6.4). It was observed that in the range examined, changes in the above-mentioned parameters did not significantly affect the recoveries of ergot alkaloids ($\alpha = 0.05$); the recovery values for all compounds were within the 95 % confidence interval. Therefore, the applied LC-MS/MS method for determination of ergot alkaloids was considered to be robust and is expected to be easily transferred from one operator/laboratory to another.

6.2.4. Application of the developed LC-MS/MS method to a European survey of cereals and cereal products

The developed method was applied to a survey on the occurrence of ergot alkaloids in cereals and cereal products in European countries. The results revealed that 59 % of samples (a total of 1065 samples were analyzed) were, to some extent, contaminated with ergot alkaloids. Overall, the highest frequencies of contamination were noted for the food samples as compared to the feed, while the highest levels of total ergot alkaloid content were found in the latter (Table 6.7). This observation could be possibly explained by the fact that during food processing (the majority of the analyzed food samples were flours) the ground raw material is homogenously mixed leading to equal distribution of ergot alkaloids (if present) within the sample. It should also be considered that raw materials used for the preparation of food samples could include cereal from different fields characterized by varied ergot contamination; thereby increasing the rate of positive samples (when homogeneously mixed). The number of positive samples was the highest in rye food (84 %), followed by wheat food (67 %), rye feed (52 %), multigrain food (48 %) and triticale feed (44 %), while only 27 % of wheat feed contained ergot alkaloids. Among the food samples, ergot alkaloids were more frequently found in rye in comparison to wheat. The same trend was observed for the feed. The concentration of ergot alkaloids in the investigated samples ranged from < LOQ to 12,340 µg/kg with an average content of 89 µg/kg. It should be noted that samples with an alkaloid content < 1 µg/kg were considered negative, regardless of the actual LOQ (see section 6.2).

Table 6.7. *Descriptive statistics for the total ergot alkaloid content in the different matrix groups.*

	Matrix group					
	Rye food	**Wheat food**	**Multigrain food**	**Rye feed**	**Wheat feed**	**Triticale feed**
Total number of samples	226	332	186	157	137	27
Mean (µg/kg)	87	62	7	311	19	62
Median (µg/kg)	28	7	< LOQ	1	< LOQ	< LOQ
Maximum (µg/kg)	1121	591	123	12340	701	1103
Minimum (µg/kg)	< LOQ[a]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
75th percentile (µg/kg)	87	54	8	54	1	5
90th percentile (µg/kg)	233	246	23	615	12	154
95th percentile (µg/kg)	393	375	36	1456	52	787

[a] LOQ = 1 µg/kg

Rye feed was the only sample type where the ergot alkaloid content exceeded 2,000 µg/kg (4.5 % of samples); and this matrix actually showed the largest variation in the data, from < LOQ to 12,340 µg/kg (Table 6.7). Although the mean total ergot alkaloid concentration in this group was 311 µg/kg, the median was 1 µg/kg.

Fig. 6.7 presents the distribution of the total ergot alkaloid content in different matrix groups per country of production/sampling. The highest levels and frequencies of contamination were noted for rye feed collected in Switzerland (total ergot alkaloid content ranged from 833 to 12,340 µg/kg in 6 out of the 7 samples, although the total alkaloid content in the other sample was 61 µg/kg). The other countries demonstrated a different pattern: the total ergot alkaloid content in the majority of rye feed samples was below 500 µg/kg, with only few samples showing higher levels.

As for the wheat feed, it was observed that in most of these samples ergot alkaloids were not present or present at low levels. All wheat feed samples from Belgium, Finland as well as the only sample from France were free from ergot alkaloids. In more than half of the samples from Germany (64 %), Poland (65 %), Switzerland (75 %) and Czech Republic (85 %) ergot alkaloids were not detected.

More than half of the triticale samples were not contaminated with ergot alkaloids. Most of these non-contaminated samples were from Czech Republic and Germany.

Unlike rye feed samples, ergot alkaloids were found in the majority of rye food samples. Hence, a great part of French (93 %), Estonian (97 %) and Polish (100 %) samples tested positive for the ergot alkaloids.

For the wheat food, samples from Belgium and France were the most contaminated with maximum content of 590 and 544 µg/kg, respectively. The maximum content for other countries were below 100 µg/kg.

For most of multigrain food the country of production was not known. Belgium, Germany and France had a similar distribution, however, the highest maximum alkaloid concentration was noted for the Belgian multigrain samples and amounted to 123 µg/kg.

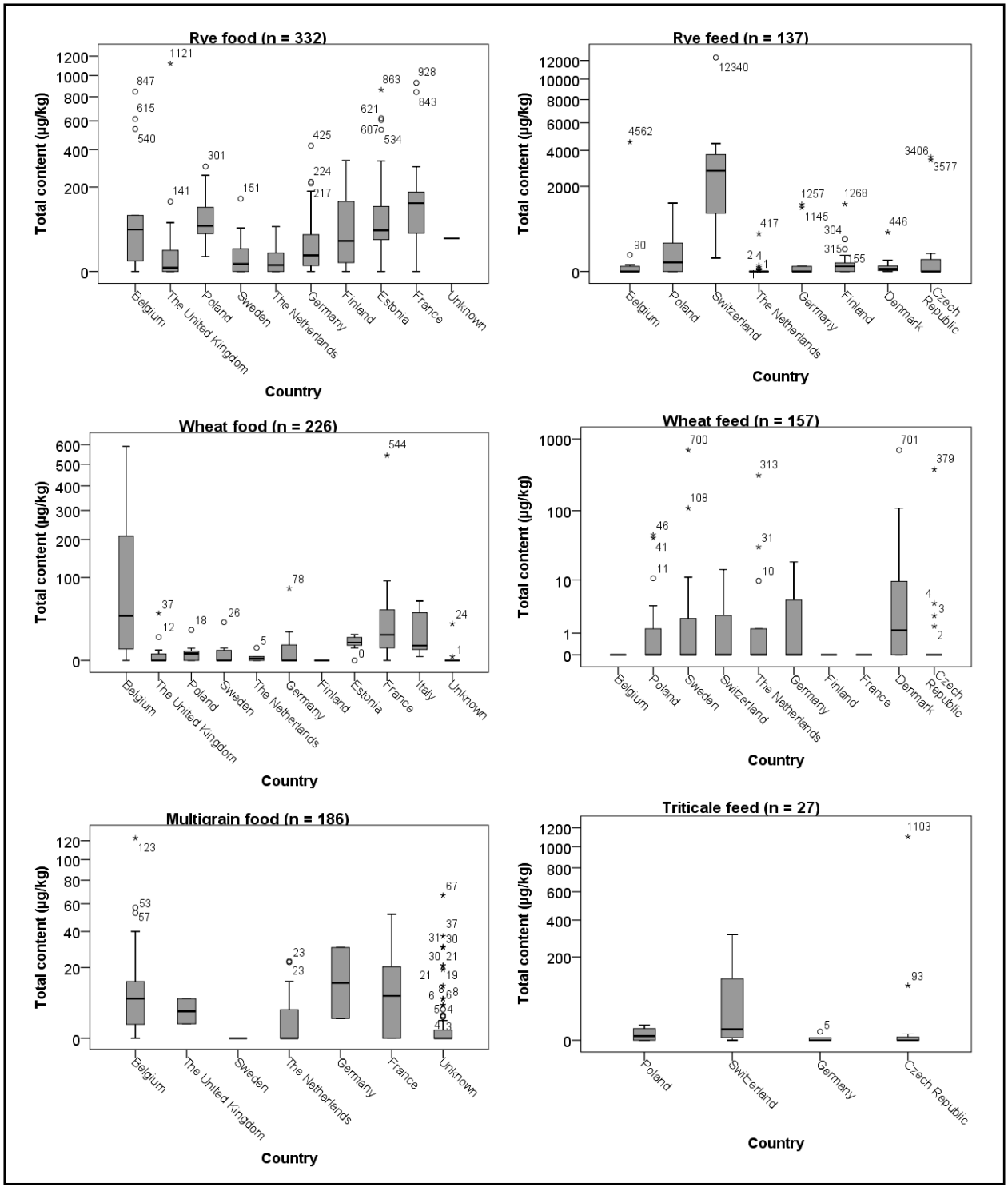

Figure 6.7. *Distribution of the total ergot alkaloid content in different matrix groups per country. n: number of samples. It has to be noted that the 'Country' refers to the country of sampling for the feed and some food (flour from mills) samples, and to the country of production for the food samples collected in retail markets in Belgium.*

Using the full set of data, it was found that all six ergot alkaloids (the main compounds were combined with epimers) co-occurred in 35 % of positive samples. Taking into account the matrix groups, the food and the feed samples showed clear differences in the ergot alkaloid pattern (Fig. 6.8). Presence of all six ergot alkaloids was most common for food samples, especially for rye food (44 %) and wheat food (42 %). At the same time, feed samples were characterized by the predominant presence of a single 'pair' (*i.e.*, a pair 'main compound-epimer'). This was observed in 54 % of positive wheat feed samples and 33 % of triticale feed. Rye feed had almost equal percentage of co-occurrence of a single 'pair' (20 %) and of all six toxins (23 %).

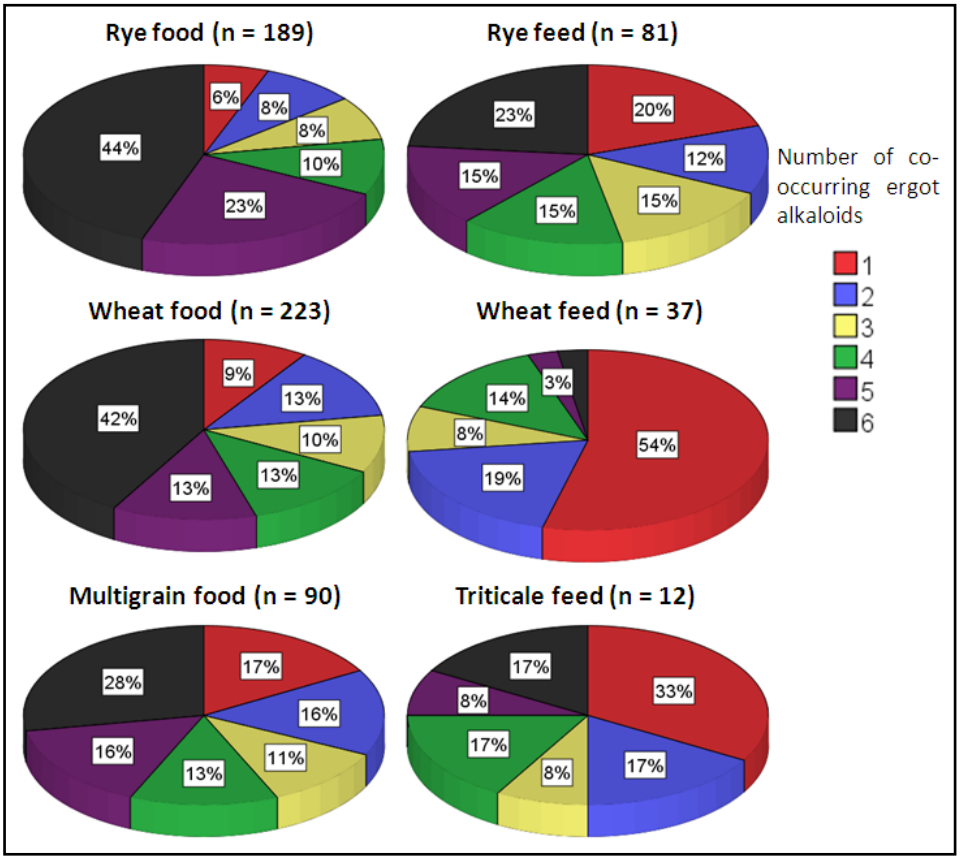

Figure 6.8. *Frequency of co-occurrence of ergot alkaloids in different matrix groups (the main compound was combined with its corresponding epimer; only positive samples are considered). n: number of samples.*

6.2.5. Conclusions

A new rapid and sensitive LC-MS/MS method has been developed and validated for the simultaneous determination of the six most prominent ergot alkaloids, ergometrine, ergosine, ergotamine, ergocornine, ergokryptine and ergocristine, as well as their corresponding epimers in food and feed samples. The suitability of a mixture of EtOAc and MeOH as extraction solvent under alkaline conditions, as well as the efficiency of liquid-liquid partitioning as sample clean-up approach were demonstrated. MeOH also proved to be a suitable alternative to ACN for its use as organic modifier, as the former gave enhanced MS signal. The stability of an XBridge stationary phase allowed the use of an alkaline mobile phase, thereby improving the chromatographic separation of the ergot alkaloids. The application of a simple sample clean-up protocol and a careful selection of the sample solvent allowed minimizing the epimerization of the ergot alkaloids during analysis. A major strength of the proposed method is the low limits of detection and of quantification, and its applicability to a wide range of food and feed matrices. In addition, the method also has the advantages of being rapid, simple, robust and suitable for routinely determination of ergot alkaloids in various matrices. The method represents a suitable tool for research of the ergot problem.

Its application was represented by a European survey on ergot alkaloids in cereals and cereal products intended for human consumption and animal feeding. The results revealed that, though the highest frequencies of contamination were observed for food samples, the feed samples, and in particular the Swiss rye feed, accounted for the highest levels of ergot alkaloids. The co-occurrence of all six ergot alkaloids was noted in 35 % of positive samples.

6.2.6. Acknowledgements

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6.3. A systematic assessment of the variability of matrix effects in LC-MS/MS analysis of ergot alkaloids in cereals

6.3.1. Introduction

A combination of LC with ESI-MS is preferred for the quantitative analysis of ergot alkaloids (Krska and Crews, 2008). However, matrix effects are a major drawback of this technique. Matrix effect (or signal suppression/enhancement (SSE)) is a change in MS signal of an analyte due to co-eluting matrix. The analyte signal can be enhanced or suppressed resulting in inaccurate performance characteristics of the method. Due to possible over- or underestimation of the analyte concentration, matrix effect is a parameter of concern during method development and/or validation.

One of the approaches to deal with the occurrence of matrix effects in quantitative LC-MS analyses is the use of isotopically labelled standards (Kokkonen, 2011). Since these compounds have the same chemical properties and therefore the same retention times as the non-labelled substances, exact correction to the signal suppression or enhancement can be achieved. However, to date, no isotopically labelled standards of ergot alkaloids are commercially available. Hence, in various LC-MS/MS methods, matrix-matched calibration (*i.e.*, preparing the standards in a blank extract of the targeted matrix) is applied to compensate for matrix effects (Krska *et al*., 2008; Mohamed *et al*., 2006). The closeness of match between the matrix to be used for the calibration and the samples to be investigated is of great importance in achieving reliable and accurate results when this approach is applied. Matuszewski *et al*. (Matuszewski *et al*., 2003) demonstrated a high variability of matrix effect among different lots of plasma and highlighted the need to investigate beside the absolute matrix effect, the relative matrix effect which can reveal differences in response among various lots of the same matrix. Cereals which are complex matrices might also result in variability of matrix effects between and within grain types and grain variety.

On the other hand, matrix effects can be minimized by a careful selection of sample preparation, as well as chromatographic and ionization conditions. Krska *et al*. (Krska *et al*.,

2008) proposed primary secondary amine (PSA) as a fast and effective technique for the analysis of ergot alkaloids in different cereals and cereal-based products. A tendency toward underestimation of the analyte concentration was observed for wheat and malted-milk biscuits. SSE values in the range of 80-86 % were obtained for seven ergot alkaloids including ergometrine, ergosine and ergocornine (Krska *et al*., 2008). Importance of a clean-up step in sample preparation of ergot alkaloids was also shown by Mohamed *et al*. (Mohamed *et al*., 2006). The use of C18 SPE cartridges demonstrated lower matrix effects compared to a simple extraction with acetonitrile. Lenain *et al*. (Lenain *et al*., 2012) developed a class-selective molecularly imprinted solid-phase extraction (MISPE) with the prospect of improved efficiency in minimizing matrix effects as compared to conventional SPE.

Kokkonen and Jestoi (Kokkonen and Jestoi, 2010) described the application of MycoSep® 150 Ergot SPE push-through clean-up columns. UPLC was applied in the analysis of ergot alkaloids, which provides high separation efficiency and might play a significant role in occurrence of matrix effects. However, the influence of this separation technique on matrix effects was not investigated.

The ion interface can also affect matrix effect as physico-chemical processes of ion formation vary depending on the ionization technique. ESI, APCI and APPI are widely used in mycotoxin LC-MS analysis (Kokkonen, 2011). For the determination of ergot alkaloids in particular, ESI was mostly applied in daily analysis. A comparison of matrix effects using other modes has not been reported yet, likewise in-depth studies on matrix effects in the analysis of ergot alkaloids.

The aim of this study was therefore a systematic assessment of signal suppression/enhancement in LC-MS/MS analysis of ergot alkaloids in grains, including different grain types and grain varieties using a previously developed LC-MS/MS method (see section 6.2). The effect of the sample preparation procedure, the chromatographic separation and the ionization technique on the matrix effect was also investigated.

6.3.2. Experimental

6.3.2.1. Standards

The standards of ergot alkaloids used in the study were as described in section 6.2.2.1.

6.3.2.2. Chemicals and material

MeOH and ACN of HPLC-grade were both supplied from VWR International (Zaventem, Belgium). n-Hexane, anhydrous disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were also obtained from VWR International. LC-MS grade MeOH and ACN were bought from Biosolve (Valkenswaard, The Netherlands). EtOAc and triethylamine (Et_3N) were purchased from Acros Organics (Geel, Belgium). For purification of demineralized H_2O a Milli-Q purification system (Millipore, Brussels, Belgium) was used. HCl, sodium hydroxide (NaOH), $(NH_4)_2CO_3$ and $(NH_4)_2SO_4$ were bought from Merck (Darmstadt, Germany). Sigma-Aldrich was a supplier of NH_4HCO_3 and citric acid. Concentrated NH_3 (25 %) was purchased from Vel (Leuven, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 µm) from Millipore (Bedford, MA, USA) were used. MycoSep®150 SPE multifunctional columns were provided by Romer Labs® (Tulln, Austria). Discovery® DSC-SCX SPE columns (500 mg, vol. 6 mL) were purchased from Sigma-Aldrich. Bondesil PSA bulk sorbent (40 µm) from Agilent Technologies (Diegem, Belgium) was used for dispersive solid-phase extraction. Paper filters (grade 3 hw, 65 g/m^2) were supplied from Egilabo NV (Kontich, Belgium). Bond Elut empty SPE cartridges (3 mL) and polypropylene frits (diameter 9.5 mm; 20 µm) were provided by Agilent Technologies.

6.3.2.3. Spiking

For evaluation of matrix effects, the ergot alkaloids were spiked in cleaned-up extracts of blank grain samples and in the injection solvent free of matrix at four concentrations (ranging between 5 and 100 µg/kg for ESI mode and between 25 and 500 µg/kg for APCI mode). The samples were prepared and analyzed in triplicate on two different days.

For evaluation of extraction recovery, the ergot alkaloids were fortified in blank grain samples which were then subjected to the corresponding extraction procedure.

For evaluation of epimerization, the ergot alkaloids (main compounds and epimers spiked separately) were spiked at 100 µg/kg in blank grain samples which were then subjected to the corresponding extraction procedure. A mixture of main compounds or epimers prepared in injection solvent was also analyzed to provide a reference.

6.3.2.4. Sample preparation

6.3.2.4.1. Liquid – liquid extraction (LLE)

The LLE procedure exploited in this work was as described in section 6.2.2.4.1. At the last step, the *n*-hexane was discarded and the aqueous phase then used to redissolve the dried residues of ergot alkaloid standards.

6.3.2.4.2. SPE using PSA

The sample preparation procedure using PSA was adapted from (Krska *et al*., 2008). Five gram of fine ground grain sample were extracted with 25 mL of ACN:$(NH_4)_2CO_3$ (200 mg/L) (84:16, v/v) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was filtered through a paper filter (grade 3 hw, 65 g/m^2, Egilabo NV). Consequent dispersive SPE was done by vortex-mixing 4 mL of the extract in a glass tube containing 200 g PSA for 45 sec. Then, the mixture was filtered through a paper filter and 2 mL were dried under a stream of nitrogen at 40 °C. The residue was redissolved in 200 µL of injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v), and 100 µL of it was used to redissolve the dried residues of ergot alkaloid standards.

6.3.2.4.3. SPE using strong cation exchange (SCX) columns

Five gram of fine ground grain sample were extracted with 40 mL of ACN:NH_4HCO_3 (16 g/L) pH 8.5 (85:15, v/v) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was centrifuged for 10 min at 4,000 g; 5 mL of supernatant were brought into a test tube and evaporated till dryness at 40 °C under a stream of nitrogen. The residue was reconstituted with 5 mL of absorption buffer [8 mM citric acid containing 4 mM Na_2HPO_4 pH 4, adjusted with NaOH]. Five mL of *n*-hexane were added to the solution, vortexed and centrifuged for 10 min at 4,000 g. The *n*-hexane phase was discarded and 4 mL of the lower phase was brought onto a preconditioned SCX SPE column. The SPE column was conditioned with 6 mL of 3.2 mM HCl, followed by 3 mL H_2O and 2 x 3 mL absorption buffer. After sample loading, the column was washed with 1 mL H_2O. The ergot alkaloids were eluted with 3 mL MeOH:NH_3 (80:20, v/v). The composition of elution solvent was optimized by means of an

experimental design using Modde 9.1 statistical software (Umetrics, Umea, Sweden) (Appendix IV).

The eluate was collected into a glass tube and evaporated till dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 200 µL of injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v) and centrifuged in an Ultrafree®-MC centrifugal device for 10 min at 14,000 g. This solution was then used to redissolve the dried residues of ergot alkaloid standards.

6.3.2.4.4. SPE using MycoSep® Ergot multifunctional columns

The sample preparation procedure using Mycosep®150 Ergot SPE cleanup columns was according to the instructions given by the manufacturer (Romer Labs®) with only slight adaptations. Five gram of fine ground grain sample were extracted with 25 mL of ACN:$(NH_4)_2CO_3$ (200 mg/L) (84:16, v/v) during 30 minutes on an Agitelec overhead shaker (J. Toulemonde & Cie). The extract was centrifuged for 10 min at 4,000 g and 4 mL of supernatant were brought into the provided glass tube. The extract was cleaned-up by pushing Mycosep® column through the extract till the bottom of the tube. Afterwards, 1 mL of the purified extract was evaporated till dryness at 40 °C under a stream of nitrogen. The dried down residue was redissolved in 100 µL of injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v). This solution was then used to redissolve the dried residues of ergot alkaloid standards.

6.3.2.4.5. SPE using molecularly imprinted polymers (MIPs)

Five gram of ground sample was extracted with 40 mL ACN:NH_4HCO_3 (16 g/L) pH 8.5 (85:15, v/v) for 30 min. Afterwards, the extract was centrifuged for 10 min at 4,000 g and 10 mL of supernatant was evaporated at 40 °C till dryness under a stream of nitrogen. The residue was redissolved with 250 µL ACN:buffer KH_2PO_4-Na_2HPO_4 pH = 7 (80:20, v/v) (pH of a 0.1 M Na_2HPO_4 solution was adjusted till 7 using a 0.1 M KH_2PO_4 solution). A MISPE column was prepared by packing 50 mg MIP between two frits in an empty SPE cartridge. The MIP preparation and extraction procedure were according to Lenain *et al.* (Lenain *et al*., 2012). The packed column was firstly conditioned by passing 2 mL ACN. The sample extract (250 µL)

was then loaded onto the SPE column. Two mL H_2O was used in the following washing step. Elution was finally achieved by passing 3 mL MeOH:Et_3N (95:5, v/v) through the column. The collected fraction was dried under a stream of nitrogen and reconstituted in 500 µL injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v). This solution was then used to redissolve the dried residues of ergot alkaloid standards.

6.3.2.5. HPLC-MS/MS analysis

The HPLC-MS/MS analyses were performed as described in section 6.2.2.7.

For this study, the mass spectrometer was operated in ESI and APCI modes, both in positive and negative polarities. During tuning experiments a 10 µg/mL solution of individual ergot alkaloid standards was infused into the MS using a syringe pump. The MS and MS/MS spectra were recorded. A precursor ion was selected for each analyte, then its product ions were obtained with an optimized combination of cone voltages and collision energies. For increased sensitivity and selectivity, data acquisition was performed in SRM mode. Two SRM transitions were selected for each of the analytes.

MS parameters for the ESI analysis were as follows: ESI source block and desolvation temperatures: 120 °C and 300 °C (400 °C in ESI^-), respectively; capillary voltage: 3.5 kV in ESI^+ and 3.8 kV in ESI^-; argon collision gas: 1.2 x 10^{-3} mbar; probe nebuliser and desolvation gas flows: 100 and 830 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies in ESI^+ are described in section 6.2.3.1.1.2., while the ESI^- parameter settings are presented in Table 6.8.

MS parameters for the APCI analysis were as follows: APCI source block temperature: 150 °C; probe temperature: 550 °C in $APCI^+$ and 500 °C in $APCI^-$; corona voltage: 4 kV in both $APCI^+$ and $APCI^-$; argon collision gas: 1.2 x 10^{-3} mbar; probe nebuliser and desolvation gas flows: 100 and 830 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies are presented in Tables 6.9 and 6.10.

Table 6.8. *Optimized MS/MS parameters for the analysis of ergot alkaloids in the SRM ESI⁻ mode.*

Ergot alkaloid	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Ergometrine	324.3 $[M-H]^-$	25	223.1[a] 180.2	20 30
Methylergometrine	338.2 $[M-H]^-$	25	223.2[a] 180.1	20 35
Ergometrinine	324.2 $[M-H]^-$	26	180.2[a] 223.2	25 15
Ergosine	546.3 $[M-H]^-$	14	296.4[a] 181.1	15 30
Ergotamine	580.5 $[M-H]^-$	10	330.4[a] 215.1	15 30
Dihydroergotamine	582.3 $[M-H]^-$	28	467.2[a] 423.3	20 25
Ergocornine	560.4 $[M-H]^-$	14	195.1[a] 310.3	25 15
Ergokryptine	574.3 $[M-H]^-$	10	324.5[a] 209.1	20 30
Ergocristine	608.3 $[M-H]^-$	10	358.3[a] 243.2	15 30
Ergosinine	546.3 $[M-H]^-$	10	181.0[a] 296.3	25 20
Ergotaminine	580.3 $[M-H]^-$	10	215.2[a] 330.2	30 15
Ergocorninine	560.3 $[M-H]^-$	10	195.3[a] 310.4	25 20
Ergokryptinine	574.3 $[M-H]^-$	10	324.3[a] 209.3	15 30
Ergocristinine	608.2 $[M-H]^-$	10	358.0[a] 243.3	15 30

[a] The most abundant product ion

Table 6.9. *Optimized MS/MS parameters for the analysis of ergot alkaloids in the SRM APCI$^+$ mode.*

Ergot alkaloid	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Ergometrine	326.3 $[M+H]^+$	28	223.1[a] 208.1	25 30
Methylergometrine	340.3 $[M+H]^+$	28	223.1[a] 208.1	25 30
Ergometrinine	326.2 $[M+H]^+$	30	208.4[a] 223.3	25 25
Ergosine	548.3 $[M+H]^+$	10	223.1[a] 208.1	25 45
Ergotamine	582.5 $[M+H]^+$	10	223.1[a] 208.1	35 40
Dihydroergotamine	584.5 $[M+H]^+$	26	322.4[a] 253.2	25 35
Ergocornine	562.5 $[M+H]^+$	10	277.4[a] 223.3	30 40
Ergokryptine	576.3 $[M+H]^+$	18	223.2[a] 305.4	40 30
Ergocristine	610.4 $[M+H]^+$	10	325.5[a] 223.1	30 30
Ergosinine	530.3 $[M-H_2O+H]^+$	37	223.1[a] 263.6	25 25
Ergotaminine	564.4 $[M-H_2O+H]^+$	37	223.2[a] 297.2	25 25
Ergocorninine	544.4 $[M-H_2O+H]^+$	30	348.2[a] 223.4	20 35
Ergokryptinine	558.5 $[M-H_2O+H]^+$	34	291.7[a] 223.2	25 25
Ergocristinine	592.4 $[M-H_2O+H]^+$	34	223.3[a] 305.3	30 30

[a] The most abundant product ion

Table 6.10. *Optimized MS/MS parameters for the analysis of ergot alkaloids in the SRM APCI⁻ mode.*

Ergot alkaloid	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Ergometrine	324.2 $[M-H]^-$	32	223.4[a] 180.2	20 30
Methylergometrine	338.3 $[M-H]^-$	33	223.3[a] 208.2	25 30
Ergometrinine	324.2 $[M-H]^-$	35	223.3[a] 180.2	20 30
Ergosine	546.3 $[M-H]^-$	18	296.3[a] 181.5	20 25
Ergotamine	580.5 $[M-H]^-$	19	215.0[a] 330.0	35 20
Dihydroergotamine	582.5 $[M-H]^-$	34	467.6[a] 423.6	25 25
Ergocornine	560.4 $[M-H]^-$	18	195.4[a] 310.3	30 20
Ergokryptine	574.3 $[M-H]^-$	18	324.2[a] 209.0	15 30
Ergocristine	608.3 $[M-H]^-$	33	358.4[a] 243.4	20 30
Ergosinine	546.3 $[M-H]^-$	18	181.5[a] 296.3	30 20
Ergotaminine	580.5 $[M-H]^-$	23	215.3[a] 330.1	30 20
Ergocorninine	560.5 $[M-H]^-$	18	195.4[a] 310.1	30 20
Ergokryptinine	574.4 $[M-H]^-$	18	209.4[a] 324.1	30 20
Ergocristinine	608.4 $[M-H]^-$	18	358.3[a] 243.0	20 30

[a] The most abundant product ion

6.3.2.6. UPLC-MS/MS analysis

The UPLC-MS/MS analyses were performed on a Waters ACQUITY UPLC system coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters) equipped with a Z-spray ESI. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 column (1.7 µm, 100 x 2.1 mm i.d.) with an ACQUITY BEH C18 VanGuard pre-column (1.7 µm, 5 x 2.1 mm i.d.), both supplied by Waters, or ZORBAX RRHD Eclipse plus C18 column (1.8 µm, 100 mm x 2.1 mm i.d.), supplied by Agilent Technologies. The column temperature was set at 30 °C. A mobile phase consisting of eluents A [H_2O:0.2 M NH_4HCO_3 pH 10:MeOH (85:5:10, v/v/v)] and B [H_2O:0.2 M NH_4HCO_3 pH 10:MeOH (5:5:90, v/v/v)] was used at a flow rate of 0.3 mL/min. A gradient elution was applied as follows: 0-1 min: 50-40 % A; 1-2.5 min: 40-35 % A; 2.5-6.5 min: 35 % A; 6.5-7 min: 35-30 % A; 7-9 min: 30 % A; 9-12 min: 30-10 % A; 12-13 min: 10 % A; 13-14 min: 10-50 % A; 14-17 min: 50 % A. The injection volume was 5 µL.

The mass spectrometer was operated in ESI positive mode. MS parameters for the ESI analysis were as follows: ESI source block and desolvation temperatures: 120 °C and 300 °C, respectively; capillary voltage: 3.5 kV; cone nitrogen and desolvation gas flows: 20 and 500 L/h, respectively. The monitored SRM transitions as well as the optimized cone voltages and collision energies are shown in Table 6.11.

Table 6.11. *Optimized MS/MS parameters for the UPLC-MS/MS analysis of ergot alkaloids in the SRM ESI$^+$ mode.*

Ergot alkaloid	RT UPLC ACQUITY BEH (min)	RT UPLC ZORBAX (min)	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Ergometrine	1.7	1.7	326.2 $[M+H]^+$	26	223.1[a] 208.1	25 26
Methylergometrine	1.9	2.0	340.1 $[M+H]^+$	31	208.1[a] 223.1	26 22
Ergometrinine	2.4	2.5	326.1 $[M+H]^+$	35	208.1[a] 223.0	25 25
Ergosine	4.8	6.0	548.4 $[M+H]^+$	33	208.1[a] 223.3	38 30
Ergotamine	5.6	7.4	582.3 $[M+H]^+$	29	223.3[a] 208.3	31 43
Dihydroergotamine	6.3	8.5	584.1 $[M+H]^+$	40	270.0[a] 297.0	26 27
Ergocornine	6.1	8.0	562.1 $[M+H]^+$	32	268.1[a] 223.0	23 36
Ergokryptine	7.9	11.0	576.4 $[M+H]^+$	29	208.1[a] 268.3	44 25
Ergocristine	8.2	11.8	610.4 $[M+H]^+$	31	208.3[a] 268.3	46 24
Ergosinine	8.4	11.7	530.1 $[M-H_2O+H]^+$	40	223.2[a] 263.0	25 22
Ergotaminine	9.9	12.6	564.3 $[M-H_2O+H]^+$	40	297.2[a] 223.3	24 23
Ergocorninine	10.3	13.0	544.4 $[M-H_2O+H]^+$	38	277.2[a] 223.2	24 24
Ergokryptinine	10.7	13.4	558.3 $[M-H_2O+H]^+$	40	305.0[a] 223.0	25 28
Ergocristinine	10.9	13.6	592.3 $[M-H_2O+H]^+$	37	325.0[a] 305.0	23 24

[a] The most abundant product ion

6.3.2.7. Matrix effects

For evaluation of matrix effects, the ergot alkaloids were spiked in cleaned-up extracts of blank grain samples and in the injection solvent free of matrix at four concentrations, and analyzed by LC-MS/MS. A linear function of the calibrants spiked in cleaned-up extract was compared to that of calibrants spiked in matrix-free solvent. Matrix effects were expressed as SSE and calculated according to Equation 6.5.

$$\boldsymbol{SSE}\ (\%) = \mathbf{100}\ \times \boldsymbol{Slope\ spiked\ extract}\ \div \boldsymbol{Slope\ calibrants\ in\ pure\ solvent}$$

(Eq. 6.5)

An SSE value of 100 % indicates no matrix effect, while a value above 100 % designates signal enhancement and an SSE below 100 % points to signal suppression due to presence of matrix.

Epimerisation of ergot alkaloids, which might have considerable impact on SSE results, was minimized by maintaining the autosampler at 4 °C and immediate analysis of the samples.

For the data treatment a statistical software IBM SPSS Statistics 20 (IBM Corporation, New York, USA) was used.

6.3.3. Results and discussion

6.3.3.1. Grain type and grain variety

As common cereal grains can be infected with *Claviceps* spp. (Krska and Crews, 2008), it was the intention of the study to investigate at first stage matrix effects when analyzing different grain types. Five cereals were chosen for the experiment, namely rye, wheat, triticale, oat and barley. The samples were subjected to LLE and analyzed by HPLC-MS/MS as described in sections 6.3.2.4.1 and 6.3.2.5. The SSE data are visualized in Fig. 6.9. A difference in matrix effects between the grain types was observed with up to 80 % and 90 % decrease of signal for ergometrine in barley and oat, respectively. The late-eluting ergokryptinine and ergocristinine demonstrated nearly no signal suppression/enhancement in rye, wheat and

triticale, whereas in oat and barley the SSE were below or equal to 50 %. An ANOVA test showed that SSE means for the different grain types were significantly different (p-value < 0.001). A Tukey multiple comparison test (Table 6.12) revealed that the mean SSE values of the different combinations of grain types were significantly different (p-values < 0.001) except for the combinations of rye – wheat (p-value = 0.078) and wheat – triticale (p-value = 0.124).

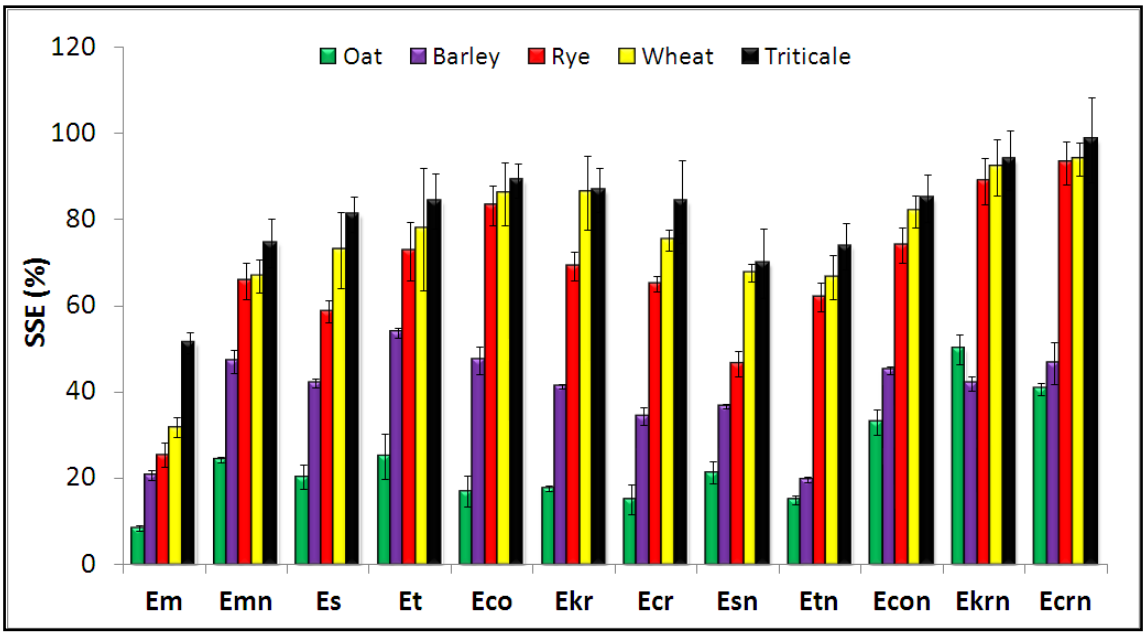


Figure 6.9. *SSE (%) for the different grain types.*

Considering the fact that different grain varieties are cultivated all over the world, at the next stage, an assessment of SSE for different varieties within one grain type was done using rye as an example. The difference in matrix effects between the three varieties of rye investigated (Askari, Conduct and Evolo) was significant (ANOVA p-value = 0.001). Tukey multiple comparison test revealed a significant difference in SSE between Askari and Conduct (p-values = 0.001) (Table 6.12 and Fig. 6.10), while no significant difference could be demonstrated for the combinations Askari – Evolo (p-value = 0.271) and Conduct – Evolo (p-value = 0.063). Overall, it can be stated that the differences in matrix effects between grain varieties were moderate (Fig. 6.10), as compared to grain types. However, the results of this investigation clearly highlight the need for a correct choice of the blank matrix when matrix-matched calibration has to be performed.

Table 6.12. *Tukey multiple comparisons for grain type and grain variety.*

Grain type		Mean difference	*p*-value	95 % confidence interval	
				Lower bound	Upper bound
Rye	Wheat	-6.61	0.078	-13.65	0.43
Rye	Triticale	-12.71*	< 0.001	-19.75	-5.67
Rye	Oat	44.39*	< 0.001	35.77	53.01
Rye	Barley	28.67*	< 0.001	20.04	37.29
Wheat	Triticale	-6.10	0.124	-13.14	0.94
Wheat	Oat	50.99*	< 0.001	42.37	59.61
Wheat	Barley	35.27*	< 0.001	26.65	43.89
Triticale	Oat	57.10*	< 0.001	48.47	65.72
Triticale	Barley	41.37*	< 0.001	32.75	50.00

Grain variety		Mean difference	*p*-value	95 % confidence interval	
				Lower bound	Upper bound
Askari	Evolo	-4.68	0.071	-7.07	-2.29
Askari	Conduct	-11.53*	0.001	-13.92	-9.15
Conduct	Evolo	6.85	0.063	4.46	9.24

*The mean difference is significant at the confidence level α = 0.05

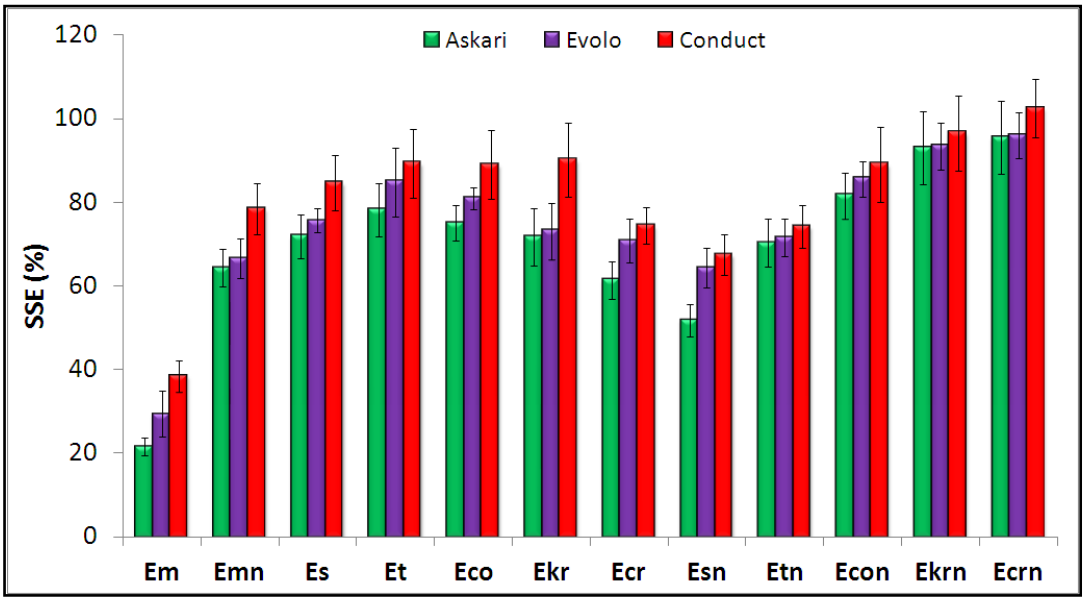


Figure 6.10. *SSE (%) for the different varieties of rye.*

6.3.3.2. Comparison of different ion sources and ionization modes

The commonly used ionization techniques in the LC-MS/MS analysis of mycotoxins are ESI and APCI in either positive or negative mode (Royer *et al*., 2004; Sulyok *et al*., 2007). Due to their different ionization mechanisms, the efficiency of formation of the targeted ions in the presence of the same coeluting compounds can vary depending on the utilized interface. Considering this fact, matrix effects in the LC-MS/MS analysis of ergot alkaloids using different ion sources (as well as different ionization modes) were studied under identical sample preparation and chromatographic conditions. Rye samples were subjected to LLE and analyzed by HPLC-MS/MS as described in sections 6.3.2.4.1. and 6.3.2.5.

It was demonstrated that the type of ion source can have a relevant effect on analyte MS signal (Fig. 6.11, Table 6.13). A suppressive matrix effect was observed in the ESI mode for almost all analytes with ergometrine being the most susceptible. Less signal suppression occurred using the ESI interface in the positive instead of negative ionization mode; the MS signal of the ergot alkaloids dissolved in matrix-free solvent was 10 times higher in ESI^+ than in ESI^-. Using ESI^+, a great signal suppression for ergometrine in rye and wheat was also noticed by Kokkonen and Jestoi (Kokkonen and Jestoi, 2010). The use of APCI instead of ESI resulted in a tremendous signal enhancement for most of the ergot alkaloids (SSE around 200 % for ergometrine) leading to an overestimated ergot alkaloid content in cereal samples.

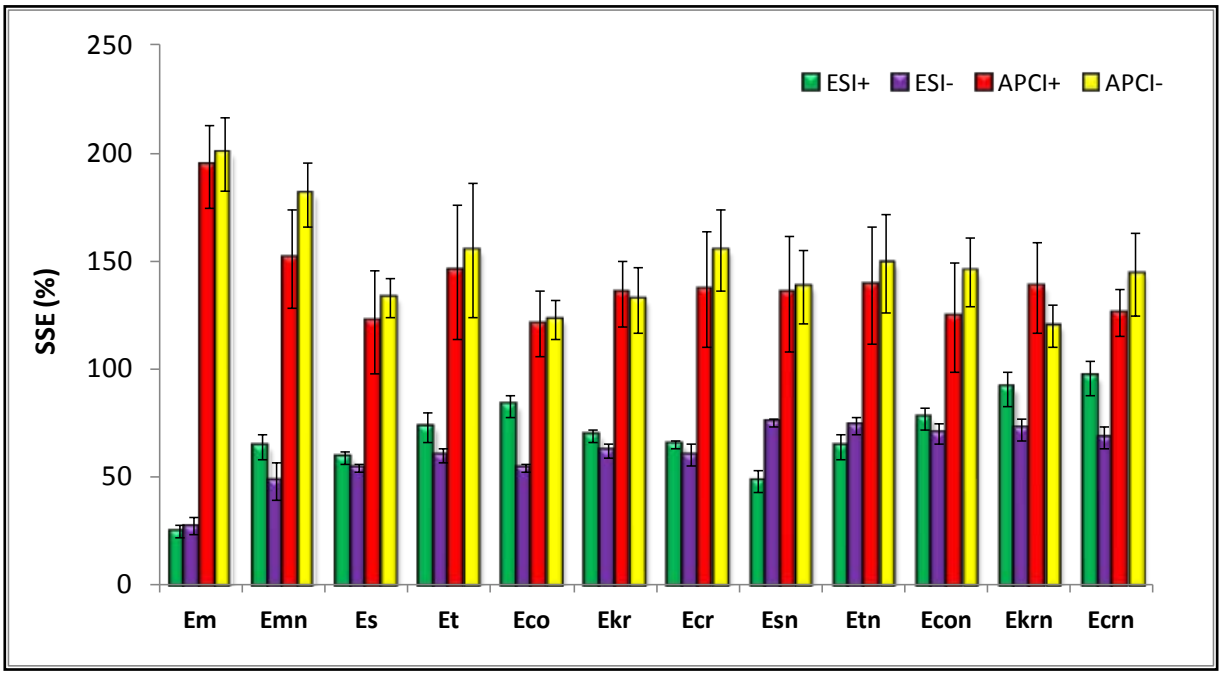


Figure 6.11. *SSE (%) for the different ion sources and ionization modes.*

Table 6.13. *Tukey multiple comparisons for the different ion sources and ionization modes.*

Ionization		Mean difference	*p*-value	95 % confidence interval	
				Lower bound	Upper bound
ESI^+	ESI^-	7.55	0.204	-2.38	17.47
ESI^+	$APCI^+$	-71.04*	< 0.001	-80.89	-61.19
ESI^+	$APCI^-$	-80.50*	< 0.001	-90.35	-70.65
ESI^-	$APCI^+$	-78.59*	< 0.001	-88.51	-68.66
ESI^-	$APCI^-$	-88.05*	< 0.001	-97.97	-78.12
$APCI^+$	$APCI^-$	-9.46	0.065	-19.31	0.39

*The mean difference is significant at the confidence level $\alpha = 0.05$

An ANOVA test demonstrated that the mean SSE values applying different ionization modes significantly differ (*p*-value < 0.001). By further Tukey multiple comparison analysis, it was shown that significant differences were observed between ESI and APCI (*p*-values < 0.001) (Table 6.13). The SSE mean values between polarity modes, namely, ESI^+ and ESI^- (*p*-value = 0.204), and $APCI^+$ and $APCI^-$ (*p*-value = 0.065), were not significantly different (Table 6.13).

When choosing ion source and ionization mode for the LC-MS analysis of ergot alkaloids, it should be considered that these compounds give a much higher signal in ESI^+ than in other modes, as was also observed by other authors (Krska *et al*., 2008). On the other hand, notably high standard deviations of SSE values observed in APCI do not give preference to this ionization mode (Fig. 6.11).

6.3.3.3. Influence of chromatography

To minimize matrix effects, a good chromatographic separation of an analyte from matrix components can play an important role. For this purpose, UPLC (instead of HPLC) can be applied, since the use of a column with smaller particle size leads to sharper peaks and therefore to an improved chromatographic resolution (Kokkonen and Jestoi, 2010). Consequently, a better separation between analytes and matrix components can be expected, leading to reduced matrix effects.

The rye samples fortified with ergot alkaloids underwent the LLE (section 6.3.2.4.1) and were analyzed either by HPLC-MS/MS using a C18 XBridge column or by UPLC-MS/MS using C18 ACQUITY BEH and C18 Zorbax RRHD Eclipse columns (sections 6.3.2.5 and 6.3.2.6).

In the conditions applied, a significant difference between the chromatographic systems was found (ANOVA, *p*-value = 0.002). The Tukey multiple comparison test showed that HPLC and UPLC using ACQUITY BEH (*p*-value = 0.009), or HPLC and UPLC using Zorbax column (*p*-value = 0.012) had significantly different SSE profiles; whereas both UPLC columns demonstrated similar SSE means (*p*-value = 0.994) (Table 6.14). Compared to Zorbax, the ACQUITY BEH column provided higher signal suppression for ergometrinine (about 20 % difference) and ergocorninine (30 %) (Fig. 6.12). In turn, the Zorbax column accounted for lower SSE values for ergokryptine (about 40 % difference) and ergosinine (about 20 %). Regarding the early eluting ergometrine, improvement in matrix effect using UPLC was not achieved; the SSE values were still below 45 %.

In general, UPLC was shown to be a suitable option for minimizing matrix effects and would be preferred in routine ergot alkaloid analysis.

Table 6.14. *Tukey multiple comparisons for the different chromatographic conditions.*

Chromatography		Mean difference	*p*-value	95 % confidence interval	
				Lower bound	Upper bound
HPLC	UPLC BEH	-12.28*	0.009	-21.94	-2.62
	UPLC Zorbax	-11.78*	0.012	-21.44	-2.12
UPLC BEH	UPLC Zorbax	0.50	0.994	-10.66	11.66

*The mean difference is significant at the confidence level α = 0.05

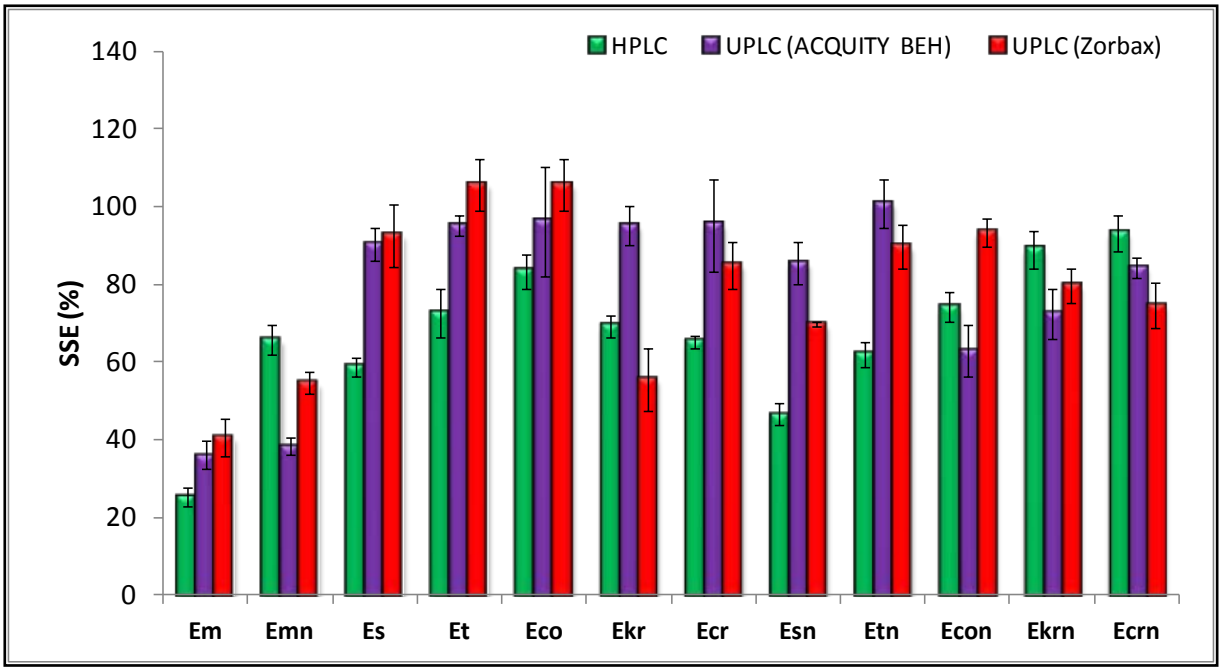


Figure 6.12. *SSE (%) for the different chromatographic conditions.*

6.3.3.4. Comparison of different sample preparation procedures

As matrix effect is caused by interference of matrix components with the analyte peak, it can be reduced by applying a suitable sample preparation. This step in the analysis is the most critical, since grain is considered as a complex matrix. In this study, different sample preparation procedures, namely LLE and SPE using PSA, MIP, MycoSep and SCX columns (section 6.3.2.4) were evaluated with regard to matrix effects (Fig. 6.13).

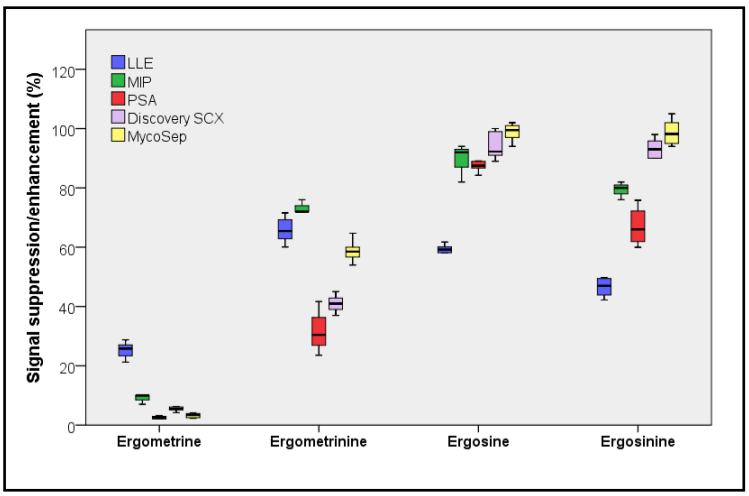


Figure 6.13. *SSE (%) applying different sample preparation procedures (for reasons of clarity only ergot alkaloids with the most pronounced matrix effects are shown).*

A great difference in matrix effects was observed for the different sample preparation procedures tested and the ergot alkaloids studied. Overall, the highest signal suppression was observed for ergometrine and ergometrinine. Applying LLE, the signal suppression observed for these compounds was less pronounced as compared to the other sample preparation procedures. The use of a relatively less polar solvent (EtOAc) could account for these observations.

The use of SCX and multifunctional SPE cartridges resulted in almost no signal suppression or signal enhancement for the later eluting ergot alkaloids. Owing to easy handling and elimination of matrix interferences for the majority of ergot alkaloids, MycoSep® multifunctional columns could be preferred for the clean-up with the only limitation that recovery of ergometrine was below 15 % combined with about 90 % of signal suppression. The use of MIP and PSA as solid sorbents for clean-up was less effective with regards to matrix effects compared to the two above-mentioned SPE columns, but SSE values still exceeded 60 % for the later eluting analytes.

Additionally to matrix effects, the influence of the sample preparation procedure on epimerization rate of the ergot alkaloids was also taken into consideration as it can affect the results of analysis. Ergot alkaloids, having asymmetric carbon at the C8-position, form two epimeric configurations which possess different bioactivity (Pierri *et al*., 1982; Smith and Schappell, 2002). It is known that bidirectional epimerization is observed under both acidic and alkaline conditions (Komarova and Tolkachev, 2001). Fig. 6.14 presents the epimerization rates of the investigated ergot alkaloids applying the different extraction protocols. LLE procedure overall induced lesser epimerization (or no epimerization as in the case of ergometrine and ergometrinine) compared to the other extraction protocols. The composition of the extraction solvent and cautions in the extraction steps could account for this. Although carbonate buffers pH 8.5 and pH 10 (required for optimal extraction) were applied, these were not allowed to interact with the ergot alkaloids for an extended time. In another study, application of LLE for extraction of ergot alkaloids in acidic conditions caused their epimerization with equilibrium shifted towards the epimers (Sulyok *et al*., 2007). Other data showed that almost no epimerization occurred using Oasis® HLB SPE cartridges, however, using Oasis® MCX SPE cartridges, designed for bases, the epimerization (up to 27 % ergosinine) was promoted more strongly due to presence of 5 % NH_3 in the elution solvent

(Krska *et al*., 2008). Current results demonstrated higher degree of epimerization using SCX for clean-up step; the highest rate was noted for ergotaminine (57 %). This can be attributed to elution of ergot alkaloids with a solvent containing a higher concentration of NH_3 (20 %). The same reasoning can be applied for epimerization rates up to 70 % using MIP; here, triethylamine was applied as a component of the elution mixture. It has been previously reported that in alkaline conditions tendency for ergot alkaloid conversion increases (Smith and Schappell, 2002). Krska *et al*. (Krska *et al*., 2008) reported non-significant epimerization employing PSA in the ergot alkaloid sample preparation. Data of the present study showed elevated epimerization rates, up to 40 %. This can be ascribed to the introduction of a drying step in the present experiment set-up. The concentration of extract was required to increase sensitivity; however, temperature has been shown to cause epimerization in other studies (Hafner *et al*., 2008; Smith and Schappell, 2002).

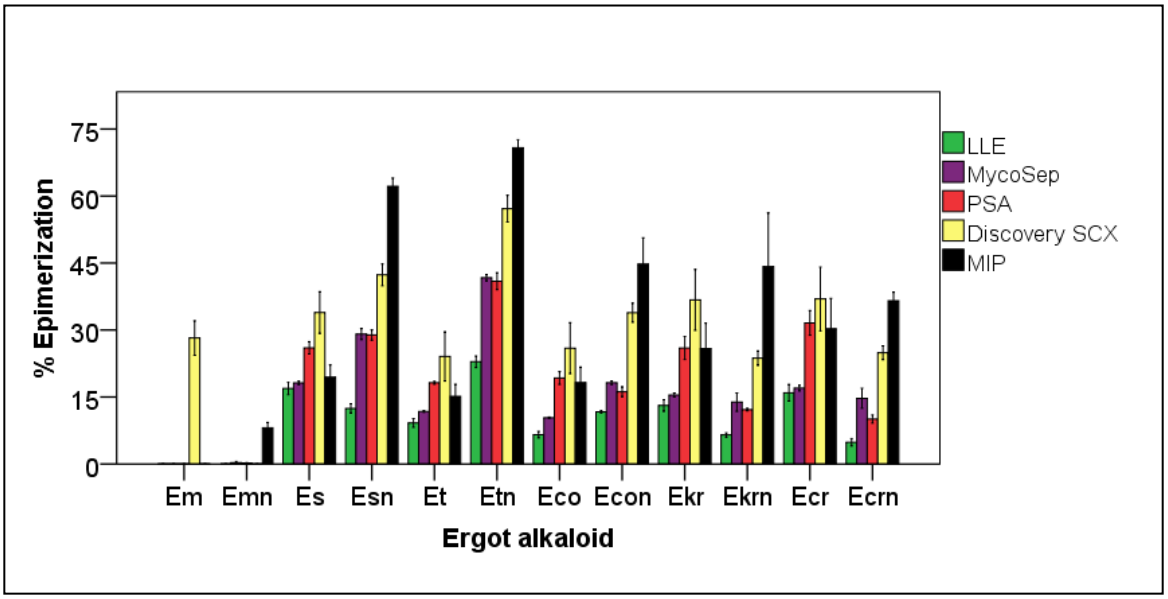


Figure 6.14. *Epimerization rate (%) of ergot alkaloids applying different sample preparation procedures.*

(Difference in MS signal of the main compound and the epimer as well as epimerization in standard mixture was taken into account)

Em: ergometrine; Emn: ergometrinine; Es: ergosine; Esn: ergosinine; Et: ergotamine; Etn: ergotaminine; Eco: ergocornine; Econ: ergocorninine; Ekr: ergokryptine; Ekrn: ergokryptinine; Ecr: ergocristine; Ecrn: ergocristinine.

Generally, the two lysergic acid derivatives, ergometrine and ergometrinine, were more stable with regards to epimerization compared to other ergot alkaloids included in this work. The interconversion of the epimers into their corresponding main ergot alkaloids was observed for all extractions applied and all ergot alkaloids investigated. The exception was ergometrinine, which epimerized to its main compound only using MIP in sample preparation.

6.3.4. Conclusions

Matrix effects can lead to inaccurate quantification. With the goal of ensuring accuracy and robustness in LC-MS/MS analysis of ergot alkaloids, matrix effects and different ways to minimize/eliminate them were systematically investigated. Initially, it was observed that signal suppression or enhancement for ergot alkaloids varied not only between grain types, with the most pronounced effect in oat, but also to a lesser extent within one grain type. These findings should be definitely considered when preparing/searching an appropriate blank grain sample for preparation of a matrix-matched calibration.

The ionization technique was also found to have an influence on matrix effect occurrence. Generally, suppressive effects were characteristic for ESI. The use of APCI instead was not a good option, since it showed a tremendous signal enhancement for most ergot alkaloids leading to overestimation of concentration in a sample. The differences between ionization modes were not significant with regard to matrix effect.

In general, ergometrine was the most susceptible ergot alkaloid to matrix effect which can be attributed to its early and thereby simultaneous elution with matrix interferences in the HPLC system. Application of UPLC in this case did not solve the problem of signal suppression, whereas considering the later eluting compounds, this technique was preferred.

The observed matrix effects were affected by the sample preparation procedure. In this work, an LLE procedure using EtOAc was compared to SPE done on different sorbents. For all the procedures tested, no clear signal enhancement was noted. A more effective clean-up which could be expected from MycoSep® and MIP columns developed especially for ergot

alkaloids did not demonstrate improvements for ergometrine. For the later eluting ergot alkaloids, MycoSep® and SCX cartridges were useful in minimization of signal suppression.

The "dilute-and-shoot" approach, another way of eliminating matrix effect, was not included in the study, since it necessitates the use of a highly sensitive LC-MS instrument to achieve low limits of detection.

6.3.5. Acknowledgements

Astrid Anné is acknowledged for her valuable contribution to the experimental part of this work. Pieterjan Lenain is also acknowledged for providing the MIP for ergot alkaloids.

6.4. Rapid and sensitive LC-MS/MS determination of ergot alkaloids in buffered solutions: Application to *in vitro* testing of clay-based mycotoxin binder

6.4.1. Introduction

Several strategies are known in management of mycotoxins in livestock such as regulation, monitoring, prevention, avoidance, decontamination, detoxification and animal treatments. The ideal strategy consisting of prevention cannot be fully implemented, because toxin production is modulated by environmental factors that are, in fact, uncontrollable. Detoxification strategies are required when contamination cannot be managed (Döll *et al*., 2004). These strategies include physical, chemical and biological methods (Sinha, 1998). Detoxification using binders has great prospects in routinely minimizing mycotoxin exposure. A binder is generally mixed with feed and decontaminates mycotoxins by binding them in the digestive tract and thereby preventing their absorption. The degree of adsorption depends on the chemical nature of the mycotoxin and the surface properties and geometry of adsorbent (Dänicke, 2002).

Mycotoxin binder research has gained its importance and several reviews have been already published (Avantaggiato *et al.*, 2005; EFSA, 2009; Huwig *et al.,* 2001; Kolosova and Stroka, 2011; Ramos and Hernandez, 1997). Efficacy of a mycotoxin binder should be evaluated through *in vitro* and *in vivo* experiments (EFSA, 2010). Addition of modified glucomannans to the ergot alkaloid-contaminated wheat reduced negative effects of mycotoxicosis (reduced

egg production, mortality) in layers (Dvorska, 2005). Clays (Huebner *et al.*, 1999) and hydrated sodium calcium aluminosilicates (Chestnut *et al.,* 1992) demonstrated high affinity for ergotamine *in vitro*, removing more than 90 % of this toxin if the latter binder was used. Another *in vitro* binding study showed that surfactant-modified zeolites could effectively adsorb ergosine, ergotamine, ergocornine, ergokryptine and ergocristine (Tomašević-Čanović *et al.*, 2003).

The aim of the present study was to develop an LC-MS/MS-based strategy to simultaneously monitor six major ergot alkaloids and their corresponding epimers in buffered solutions from *in vitro* studies. The method was validated and applied to an *in vitro* binding efficacy study of a clay-based mycotoxin binder towards ergot alkaloids. Three model ergot alkaloids (ergometrine, ergosine and ergocornine) were selected for the study.

6.4.2. Experimental

6.4.2.1. LC-MS/MS method

6.4.2.1.1. Standards

The standards of ergot alkaloids used in the study were as described in section 6.2.2.1.

6.4.2.1.2. Chemicals and material

LC-MS grade MeOH and ACN were bought from Biosolve (Valkenswaard, the Netherlands). EtOAc was purchased from Acros Organics (Geel, Belgium). H_3PO_4 (85 %), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4*H_2O$) and sodium hydroxide (NaOH) were supplied by Merck (Darmstadt, Germany). Sodium dihydrogen phosphate dodecahydrate ($NaH_2PO_4*12H_2O$) was obtained from Merck Eurolab (Leuven, Belgium). Sigma-Aldrich was a supplier of NH_4HCO_3. Concentrated NH_3 (25 %) was purchased from Vel (Leuven, Belgium). For purification of demineralized H_2O a Milli-Q purification system (Millipore, Brussels, Belgium) was used.

6.4.2.1.3. Buffers

0.1 M phosphate buffer at pH 3.0 and 6.8 was used as the aqueous media in the *in vitro* binding efficacy study. Therefore, these buffers were also applied during the development and optimization of the LC-MS/MS method. They were obtained by mixing the necessary volume of 0.1 M H_3PO_4 and 0.1 M NaH_2PO_4 (for buffer pH 3.0) or 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 (for buffer pH 6.8).

The alkaline buffers examined during extraction optimization were phosphate and bicarbonate buffers both at pH 8.5 and 10; their suitability was tested at two different concentrations (0.1 and 0.5 M). The defined portions of NH_4HCO_3 or Na_2HPO_4 were dissolved in H_2O; the required pH was adjusted with NH_3 conc. or NaOH conc., respectively.

6.4.2.1.4. Sample preparation

Four mL 0.5 M NH_4HCO_3 (pH 10) were added to 4 mL of sample (0.1 M phosphate buffer pH 3.0 or pH 6.8) to adjust sample pH till 10 and the ergot alkaloids were extracted with 20 mL of EtOAc on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France) during 15 min. After centrifugation, 5 mL of supernatant were evaporated till dryness under a stream of nitrogen at 40 °C. Prior to LC-MS/MS analysis, the dried residue was reconstituted in 200 µL injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v).

6.4.2.1.5. LC-MS/MS conditions

The LC-MS/MS conditions applied were as described in section 6.2.2.7.

6.4.2.1.6. Validation

During validation process the following parameters were evaluated: linearity of the method, LOD and LOQ, apparent recovery, repeatability, within-laboratory reproducibility, measurement uncertainty and matrix effects. In addition, evaluation of the method robustness was performed.

The calculation of method parameters were as described in sections 6.2.2.8 and 6.3.2.7.

The assessment of the method robustness was based on evaluation of extraction recoveries of ergot alkaloids varying the following parameters: pH and concentration of extraction buffer, shaking time and drying temperature. These factors were selected as potential sources of variability when performing the analysis. The intervals of the factors were set at values which slightly exceed the variations that can be expected when a method is transferred from one laboratory to another (Table 6.15). To evaluate the effect of the investigated factors on the response, a fractional factorial design was constructed using Modde 9.1 statistical software (Umetrics, Umeå, Sweden). Having included four factors, the total number of runs was equal to 11 including three replicates of the centre-point.

Epimerization of ergot alkaloids was minimized by maintaining the autosampler at 4 °C and immediate analysis of the samples.

Table 6.15. *Experimental design for robustness evaluation.*

Exp. No	pH of extraction buffer	Buffer concentration (mol/L)	Shaking time (min)	Temperature of drying (°C)
1	9.8	0.48	17	43
2	10.2	0.48	13	37
3	9.8	0.48	17	37
4	10.2	0.48	13	43
5	9.8	0.52	13	43
6	10.2	0.52	17	37
7	9.8	0.52	13	37
8	10.2	0.52	17	43
9	10.0	0.50	15	40
10	10.0	0.50	15	40
11	10.0	0.50	15	40

6.4.2.2. *In vitro* testing

6.4.2.2.1. Experimental set-up

The *in vitro* testing was based on an aqueous binding experiment at isothermal conditions *i.e.*, including single-concentration sorption. The experiment was performed at two different pH (3.0 and 6.8) to encompass the pH range encountered in the gastro intestinal tract. An

ergot alkaloid binder comprising of a mixture of activated clays was obtained from Kemin Europa NV (Herentals, Belgium). 0.1 M phosphate buffer at pH 3.0 and 6.8 was used as the aqueous media in the *in vitro* binding efficacy study. These buffers were obtained by mixing the necessary volume of 0.1 M NaH_2PO_4 and H_3PO_4 (for buffer pH 3.0) or 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 (for buffer pH 6.8). A toxin working solution (separate for each ergot alkaloid) was prepared in the buffers by diluting ergometrine (Coring System Diagnostix GmbH), ergosine (Coring System Diagnostix GmbH) and ergocornine (Sigma-Aldrich, Buchs, Switzerland) in buffer pH 3.0 at a concentration of 250 µg/L.

Initially, mycotoxin adsorption was evaluated. Toxin binder (100 ± 5 mg) was placed in a 50 mL Nalgene™ oak ridge PPCO centrifuge tube (Nalge Nunc International, USA) with two glass boiling stones. Twenty mL of the ergot alkaloid working solution (prepared in the phosphate buffer pH 3.0) were added into the tube. The tube was incubated on an orbital shaker (GFL3500, GFL, Burgwedel, Germany) for 60 min at ambient temperature followed by centrifugation (MR1822 centrifuge (Jouan, USA)) for 10 min at 10,000 g. An aliquot of supernatant was collected in a test tube, labeled as 'adsorption'. To investigate mycotoxin desorption, the remaining supernatant was decanted and precipitates were resuspended in 20 mL phosphate buffer pH 6.8 free of ergot alkaloids. The following steps were as described above (evaluation of mycotoxin adsorption). An aliquot of supernatant was transferred into a test tube, labeled as 'desorption'. The binding experiments were executed in triplicate.

6.4.2.2.2. Calculation of binding efficacy

Calculation of the ergot alkaloid concentration was done using a matrix-matched calibration prepared in the phosphate buffer (all samples were analyzed by LC-MS/MS as described in section 6.4.2.1.). The adsorption and desorption percentage, as well as the binding efficacy were computed as follows:

$$Adsorption\ (\%) = \frac{\frac{Response\ 250\ ppb\ toxin\ in\ buffer\ pH\ 3.0 - b}{a} - \frac{Response\ toxin\ in\ supernatant\ 'adsorption' - b}{a}}{\frac{Response\ 250\ ppb\ toxin\ in\ buffer\ pH\ 3.0 - b}{a}} \times 100$$

$$Desorption\ (\%) = \frac{\frac{Response\ toxin\ in\ supernatant\ 'desorption' - b}{a}}{\frac{Response\ 250\ ppb\ toxin\ in\ buffer\ pH\ 6.8 - b}{a}} \times 100$$

$$Efficacy\ (\%) = Adsorption\ (\%) - Desorption\ (\%)$$

where $\boldsymbol{a}$ *is the slope and* $\boldsymbol{b}$ *is the intercept of the equation of the respective linear trend lines at pH* 3.0 *and pH* 6.8.

6.4.3. Results and discussion

6.4.3.1. Optimization of sample preparation

In vitro testing of binding efficacy implies analysis of buffered sample solutions. The major focus in the optimization of the sample preparation procedure was sample concentration as well as the removal of salts (that are not compatible with MS-detection) without significant loss of analytes. As *pKa* values of the investigated ergot alkaloids vary from 4.8 to 6.2 (Lorenz, 1979), these compounds are positively charged at the N6-position in acidic solutions and neutral at higher pH. Considering this, extraction can be performed either with non-polar organic solvents under alkaline conditions or with polar solvents under acidic conditions. In this work, a H_2O-immiscible organic solvent, namely EtOAc, was chosen. To alkalinize the sample, phosphate and bicarbonate buffers were evaluated at different concentrations and pH. Buffer volumes were experimentally determined to achieve the required pH of the sample. As it was the intention to develop a universal extraction for the samples in aqueous media buffered at both pH 3.0 and 6.8, the optimization was oriented towards the more acidic buffer (pH 3.0). Fig. 6.15 shows that use of the phosphate buffer did not provide sufficient extraction of ergot alkaloids from the aqueous medium at pH 3.0.

The extraction was more efficient using bicarbonate buffer, especially at higher pH (pH 10) with recoveries of all ergot alkaloids above 70 % (for 0.5 M bicarbonate buffer). As for the samples in medium at pH 6.8, overall good results were achieved using both buffers (Fig. 6.16). Noticeably high variation in recoveries (up to 29 % RSD) was observed for 0.1 M NH_4HCO_3 pH 8.5. Using 0.5 M NH_4HCO_3 pH 10, recoveries in the range from 93 to 105 % for the main ergot alkaloids and their corresponding epimers were obtained with good precision.

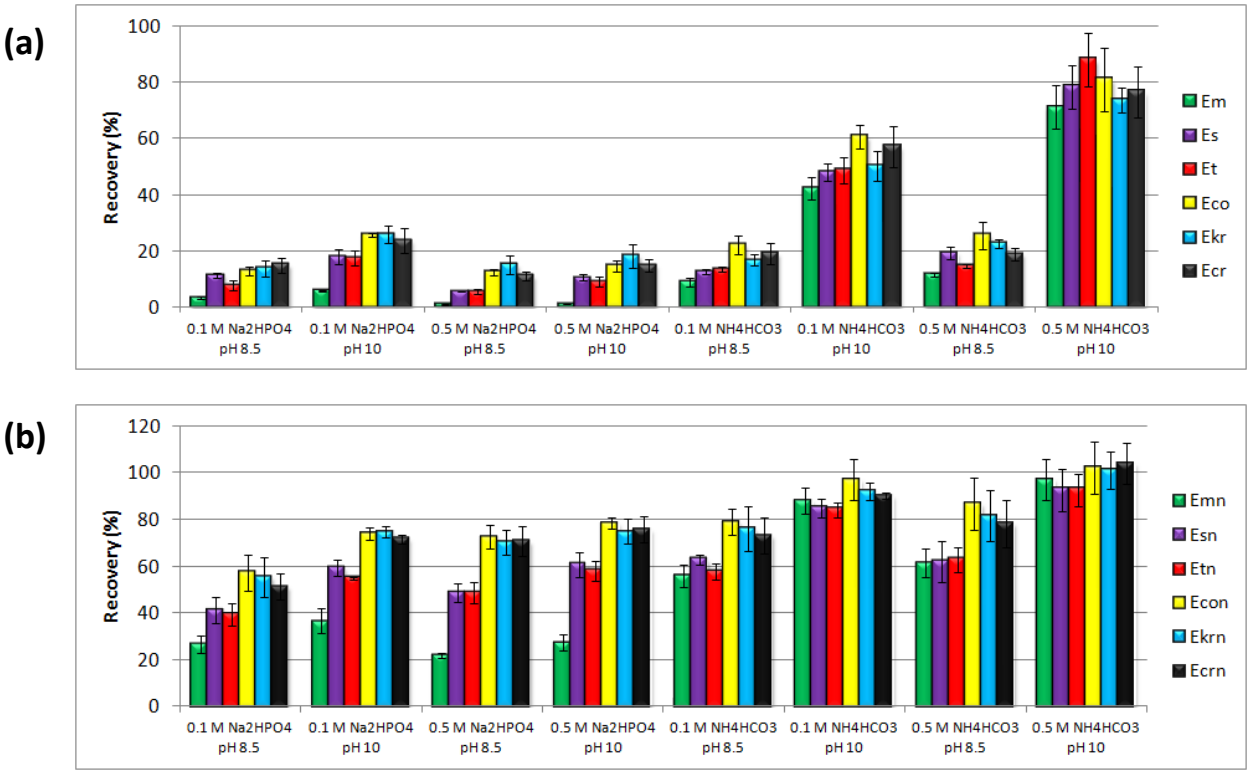


Figure 6.15. *Optimization of extraction buffer for main ergot alkaloids (a) and their corresponding epimers (b) in aqueous medium at pH 3.0.*

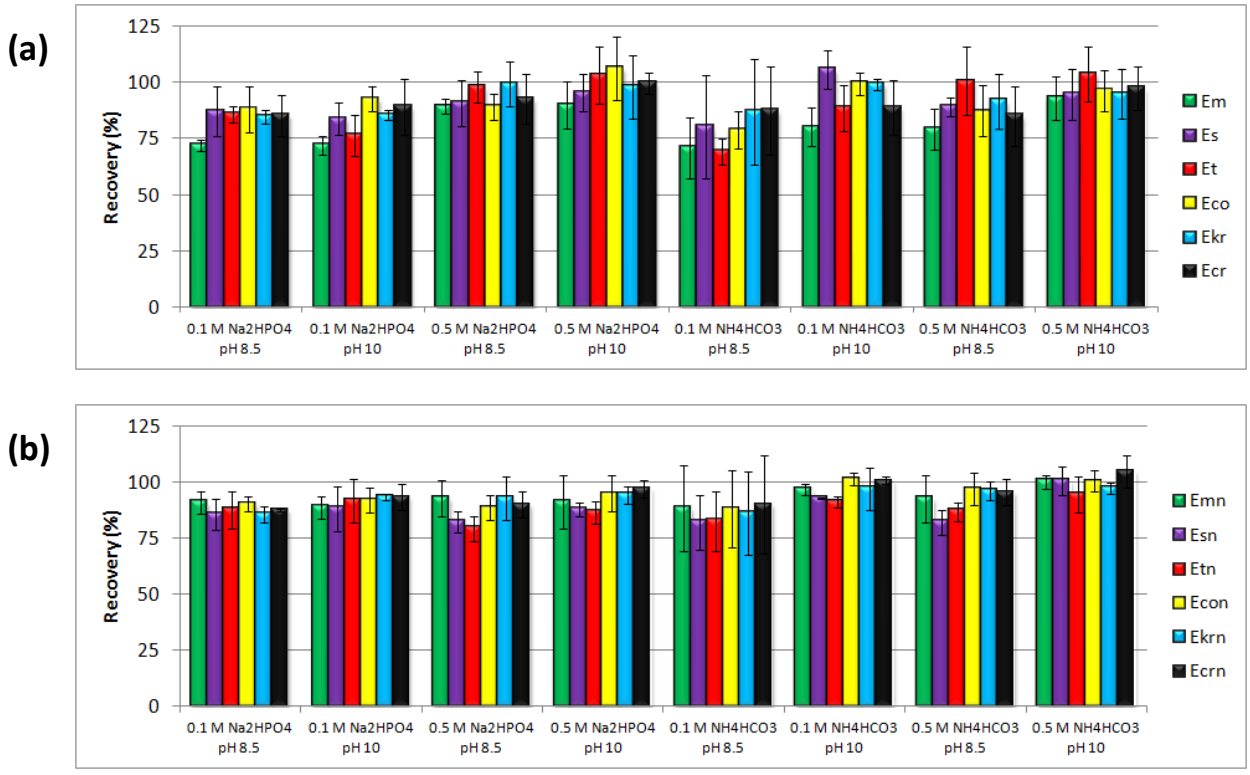


Figure 6.16. *Optimization of extraction buffer for main ergot alkaloids (a) and their corresponding epimers (b) in aqueous medium at pH 6.8.*

Finally, 0.5 M NH_4HCO_3 pH 10 was chosen for pH adjustment prior to liquid-liquid extraction of the ergot alkaloids from both sample types (pH 3.0 and pH 6.8).

6.4.3.2. Method validation

The developed LC-MS/MS method for the binding efficacy study proved to be sensitive; LOD values for both media were overall below 0.5 µg/L, whereas LOQ values ranged from 0.19 to 1.45 µg/L (Tables 6.16 and 6.17).

Table 6.16. *Validation data for the medium at pH 3.0.*

Ergot alkaloid	LOD (µg/L)	LOQ (µg/L)	Linearity			
			Equation	R^2	S_{yx}	Lack of fit test *p*-value
Ergometrine	0.13	0.44	y = 0.0499x - 0.0221	0.999	0.00037	0.78
Ergometrinine	0.06	0.19	y = 0.0851x + 0.0253	0.999	0.00064	0.93
Ergosine	0.33	1.08	y = 0.0048x + 0.0006	0.997	0.00006	1.00
Ergosinine	0.11	0.36	y = 0.0075x + 0.0043	0.995	0.00012	0.98
Ergotamine	0.44	1.45	y = 0.0029x - 0.0005	0.998	0.00003	1.00
Ergotaminine	0.25	0.83	y = 0.0063x + 0.0014	0.998	0.00007	0.99
Ergocornine	0.13	0.44	y = 0.0060x + 0.0016	0.997	0.00008	0.98
Ergocorninine	0.16	0.53	y = 0.0038x - 0.0029	0.969	0.00016	0.71
Ergokryptine	0.23	0.75	y = 0.0039x - 0.0015	0.994	0.00007	0.99
Ergokryptinine	0.15	0.49	y = 0.0027x + 0.0048	0.972	0.00010	0.97
Ergocristine	0.36	1.21	y = 0.0020x - 0.0010	0.988	0.00005	1.00
Ergocristinine	0.14	0.48	y = 0.0033x + 0.0112	0.953	0.00017	0.46

	Repeatability, (% RSD)			Within-laboratory reproducibility, (% RSD)		
	2.5 µg/L	50 µg/L	200 µg/L	2.5 µg/L	50 µg/L	200 µg/L
Ergometrine	10	5	0.3	12	5	0.5
Ergometrinine	7	3	0.2	9	5	0.3
Ergosine	12	4	0.5	13	5	0.6
Ergosinine	16	7	0.4	14	10	0.7
Ergotamine	15	4	1	19	5	1
Ergotaminine	9	6	0.9	10	8	0.9
Ergocornine	15	4	0.9	16	4	0.9
Ergocorninine	20	9	2	20	18	3
Ergokryptine	18	10	0.9	18	10	0.8
Ergokryptinine	5	9	3	12	11	3
Ergocristine	19	11	1	21	11	1
Ergocristinine	19	17	4	19	17	4

	Apparent recovery, (%)			Expanded measurement uncertainty, (%)		
	2.5 µg/L	50 µg/L	200 µg/L	2.5 µg/L	50 µg/L	200 µg/L
Ergometrine	103	104	100	25	11	3
Ergometrinine	96	105	100	20	12	5
Ergosine	107	105	100	28	11	4
Ergosinine	101	104	100	30	22	6
Ergotamine	105	100	100	40	11	3
Ergotaminine	113	105	100	22	18	7
Ergocornine	101	103	100	34	9	4
Ergocorninine	110	94	100	43	38	8
Ergokryptine	99	100	100	38	21	3
Ergokryptinine	123	104	99	26	24	9
Ergocristine	98	99	100	45	24	6
Ergocristinine	112	104	98	41	36	10

Table 6.17. *Validation data for the medium at pH 6.8.*

Ergot alkaloid	LOD (µg/L)	LOQ (µg/L)	Linearity			
			Equation	R^2	S_{yx}	Lack of fit test *p*-value
Ergometrine	0.28	0.93	y = 0.0274x + 0.0115	0.999	0.00018	0.07
Ergometrinine	0.14	0.48	y = 0.0735x + 0.0634	0.999	0.00062	0.07
Ergosine	0.28	0.93	y = 0.0048x - 0.0008	0.996	0.00007	0.99
Ergosinine	0.13	0.44	y = 0.0081x - 0.0013	0.995	0.00014	1.00
Ergotamine	0.30	0.99	y = 0.0040x + 0.0001	0.993	0.00008	1.00
Ergotaminine	0.13	0.44	y = 0.0086x + 0.0014	0.993	0.00016	1.00
Ergocornine	0.26	0.87	y = 0.0059x - 0.0028	0.999	0.00003	0.88
Ergocorninine	0.22	0.74	y = 0.0095x - 0.0024	0.966	0.00042	0.85
Ergokryptine	0.18	0.61	y =0.0047x + 0.0012	0.968	0.00020	1.00
Ergokryptinine	0.08	0.28	y = 0.0085x + 0.0013	0.997	0.00011	0.93
Ergocristine	0.34	1.15	y = 0.0023x + 0.0033	0.991	0.00005	0.89
Ergocristinine	0.08	0.28	y = 0.0104x + 0.0035	0.996	0.00016	0.86

	Repeatability, (% RSD)			Within-laboratory reproducibility, (% RSD)		
	2.5 µg/L	50 µg/L	200 µg/L	2.5 µg/L	50 µg/L	200 µg/L
Ergometrine	6	4	0.4	6	4	0.6
Ergometrinine	10	4	0.4	9	5	0.7
Ergosine	6	8	0.6	8	7	0.7
Ergosinine	8	6	1	9	6	1
Ergotamine	15	7	2	14	6	1
Ergotaminine	8	9	0.9	9	10	0.9
Ergocornine	8	7	0.6	7	7	0.6
Ergocorninine	23	8	0.4	22	10	0.7
Ergokryptine	19	22	3	20	21	4
Ergokryptinine	13	13	0.6	21	14	0.6
Ergocristine	18	10	1	17	13	2
Ergocristinine	7	6	1	7	7	0.9

	Apparent recovery, (%)			Expanded measurement uncertainty, (%)		
	2.5 µg/L	50 µg/L	200 µg/L	2.5 µg/L	50 µg/L	200 µg/L
Ergometrine	98	102	99	13	9	3
Ergometrinine	98	106	99	20	12	5
Ergosine	100	100	100	17	15	4
Ergosinine	108	102	100	20	14	6
Ergotamine	101	101	100	30	13	3
Ergotaminine	106	104	100	20	22	7
Ergocornine	96	98	100	15	15	3
Ergocorninine	100	109	99	47	22	6
Ergokryptine	100	97	101	42	44	9
Ergokryptinine	109	103	99	45	30	6
Ergocristine	91	102	100	36	28	7
Ergocristinine	114	110	99	16	16	6

Linearity of the method over a concentration range of 1-200 µg/L was supported by high coefficients of determination (R^2) which were between 0.953 (ergocristinine) and 0.999 (ergometrine) (Tables 6.16 and 6.17). A good fit of the data with a linear model was confirmed by *p*-values for the lack of fit test above 0.05. Additionally, residual plots demonstrated no systematic trend as the residuals were randomly distributed around zero. The standard errors (S_{yx}) were all below 1.5 % of the estimate as calculated by expressing S_{yx} as a percentage of y at the different concentration levels of the analyte.

Apparent recoveries of the investigated ergot alkaloids in the medium at pH 3.0 and 6.8 were tested at three concentration levels and were in the range 94-123 % and 91-114 %, respectively (Tables 6.16 and 6.17).

Precision of the method expressed as RSD was determined at three concentration levels. Repeatability did not exceed 23 % (ergocorninine) at low concentration level and was overall below 4 % at 200 µg/L (Tables 6.16 and 6.17). Within-laboratory reproducibility ranged from 0.3 % (ergometrinine) to 22 % (ergocorninine). Generally, the corresponding values of repeatability and within-laboratory reproducibility were similar for both media used in the study.

Expanded measurement uncertainties for each of the ergot alkaloids are presented in Tables 6.16 and 6.17. At lowest concentration level, the uncertainty did not exceed 47 %, whereas it was below 10 % at the highest level.

Matrix effects were also investigated for the two sample types – buffered solution pH 3.0 and pH 6.8 (Fig. 6.17). For most of the ergot alkaloids, the influence of matrix components on the MS signal was moderate. However, substantial matrix effects were observed for ergometrine and ergometrinine (SSE below 60 %). Furthermore, slight differences in SSE values were noted between the two sample types, therefore, a matrix-matched calibration was applied to ensure correct quantification.

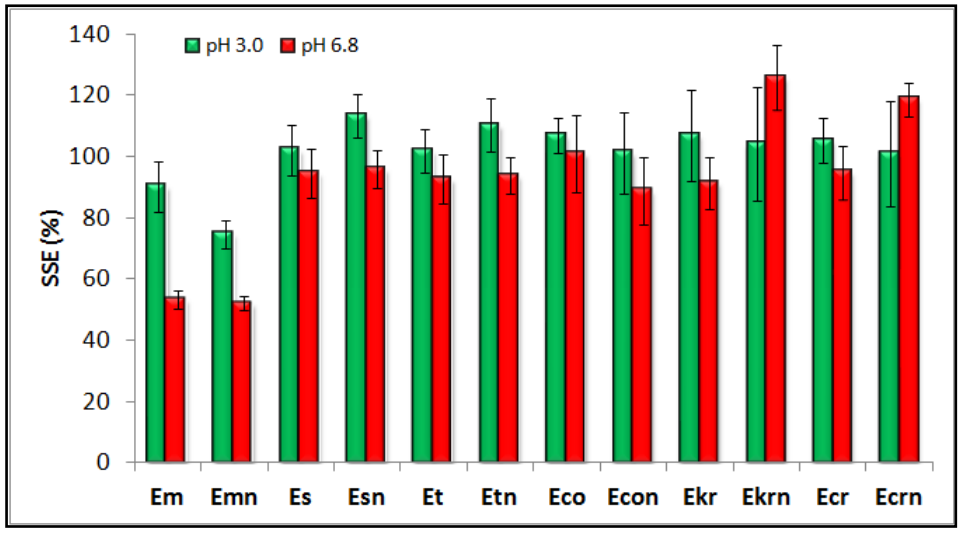


Figure 6.17. *Signal suppression-enhancement (SSE) data. Em: ergometrine; Emn: ergometrinine; Es: ergosine; Esn: ergosinine; Et: ergotamine; Etn: ergotaminine; Eco: ergocornine; Econ: ergocorninine; Ekr: ergokryptine; Ekrn: ergokryptinine ; Ecr: ergocristine; Ecrn: ergocristinine.*

Further validation implied the investigation of method robustness. A fractional factorial design was used to assess the effect of small changes in the sample preparation parameter settings on the accuracy of the results. It was observed that for each of the investigated parameters (extraction buffer pH, extraction buffer concentration, shaking time and drying temperature), the changes in recovery were not significant ($\alpha = 0.05$) when varying the method parameters from their low to high values (Appendix V). Therefore, the developed LC-MS/MS method was considered to be robust and is expected to be easily used in a different laboratory or operated by a different analyst.

6.4.3.3. Application of the method for *in vitro* efficacy testing of a mycotoxin binder

The method was applied to monitor the ergot alkaloid content in solutions from an *in vitro* model aiming at determining the efficacy of a clay-based binder towards ergot alkaloids. As examples, a lysergic acid derivative (ergometrine) as well as two peptide ergot alkaloids (ergosine and ergocornine) were included in the investigation. Data (Table 6.18) indicated a binding efficacy above 93 % for the peptide ergot alkaloids, while a binding efficacy of 24 % was observed for ergometrine. The lower binding efficacy of this lysergic acid derivative can be attributed to its relatively higher solubility in aqueous media. It has to be noted that no or negligible epimerization of the main ergot alkaloids occurred during *in vitro* study and LC-MS/MS analysis.

Table 6.18. *Binding efficacy of a clay-based binder towards ergometrine, ergosine and ergocornine.*

Ergot alkaloid	Adsorption (%)	Desorption (%)	Binding efficacy (%) ± SD
Ergometrine	69.74	66.58	23.60 ± 22.32
	80.16	59.93	
	81.73	34.32	
Ergosine	95.19	2.69	93.28 ± 0.86
	95.67	1.46	
	95.18	2.07	
Ergocornine[a]	97.97	1.01	96.97 ± 0.00
	97.97	1.01	
	97.97	1.01	

[a] The responses were below the response of the lowest calibration standard. Therefore, the responses were set to the same level as for that standard.

6.4.4. Conclusions

Mycotoxin contamination of feed is a great concern in livestock as it can harmfully affect the animal whealth and in some cases lead to death. One of the ways to cope with this is the use of detoxifying agents which prevent digestive absorption of feed-borne mycotoxins. Before applying the adsorbent, it has to be evaluated for its binding efficacy. Such study necessitates suitable methodologies for analyte determination. Here, a validated rapid and sensitive LC-MS/MS method is proposed suitable for monitoring of six main ergot alkaloids with their corresponding epimers in buffered solutions. The method was successfully applied in an *in vitro* efficacy testing of a clay-based binder towards ergot alkaloids.

The applied extraction procedure demonstrated good recoveries (more than 70 %) of the ergot alkaloids and allowed a simple and fast analysis. An alkaline bicarbonate buffer (pH 10) was required for sufficient extraction of the compounds. The validation report demonstrated that the method was sensitive, precise and robust.

Additionally, the developed LC-MS/MS method can easily be used in *in vitro* evaluation studies of a wider range of binders for ergot alkaloids, as well as in other *in vitro* biomedical applications implying ergot alkaloid-containing buffered solutions; for instance, Franz diffusion cell (FDC) experiments (Boonen *et al*., 2012).

Binder efficacy should also be tested in an *in vivo* experiment, as adsorbents *in vitro* might not necessarily demonstrate the same result when applied in a living organism due to influence of physiological variables. Furthermore, the binder safety has to be proven as well.

6.4.5. Acknowledgements

Eva Buyse is acknowledged for her valuable contribution to the experimental part of the work, as well as Aimée Teunckens and Ilse Mast for their practical support for the *in vitro* evaluation of mycotoxin binding potential.

6.5. Holistic approach based on high resolution and multiple stage mass spectrometry to investigate ergot alkaloids in cereals

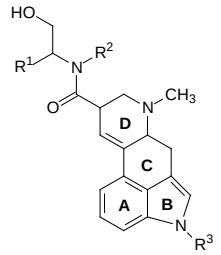
6.5.1. Introduction

The majority of ergot alkaloids are commonly comprised of ergoamides, ergopeptines (or ergopeptides), also called cyclol ergot alkaloids, and the lactam ergot alkaloids, also named ergopeptams (Bennet and Klich, 2003; Komarova and Tolkachev, 2001). The ergoamides are D-lysergic acid amides (Fig. 6.18a), whereas the ergopeptines are D-lysergic acid peptides containing lysergic acid and three amino acids in their structure (Fig. 6.18b). The ergopeptams are tripeptidic non-cyclol ergot alkaloids (Fig. 6.18c). Their structure is similar to that of ergopeptines except that L-proline is exchanged by D-proline, and the tripeptide chain is a non-cyclol lactam (Komarova and Tolkachev, 2001).

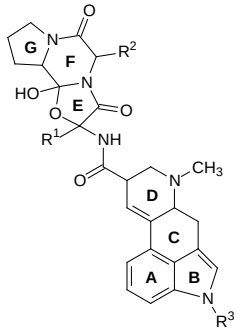
To date, more than 40 ergot alkaloids are known. Several analytical techniques have been used to study the fragmentation of these compounds and attempts have been made to identify novel derivatives. In the last years, a number of new ergot alkaloids have been discovered (Cvak *et al*., 2005; Lehner *et al*., 2005; Lehner AF *et al*., 2011; Uhlig *et al*., 2011). Mohamed *et al.* (Mohamed *et al*., 2006) used triple quadrupole and multiple stage MS to characterize six ergot alkaloids belonging to lysergic acid and peptide-type derivatives and could confirm the presence of ergosine in a rye flour extract at trace levels. Lehner *et al.* (Lehner *et al*., 2004; Lehner AF *et al*., 2011) using HPLC coupled to tandem quadrupole MS and ion trap MS was able to establish the fragmentation patterns of eight ergot alkaloids and subsequently elucidate a new ergot alkaloid-related compound. Uhlig and Petersen (Uhlig and Petersen, 2008) obtained structural information of four ergopeptams using HPLC-ion trap MS.

In the above-mentioned studies, fragment assignment was supported by hydrogen/deuterium exchange (Mohamed *et al*., 2008) and/or comparison of the fragmentation behaviour of known ergot alkaloids, using unit mass resolution data acquired by triple quadrupole and ion trap instruments. Along with fragmentation trees, accurate mass measurement is a highly important feature for correct structure elucidation (Kind and Fiehn, 2010). Orbitrap MS is becoming a more and more popular platform for identification purposes in natural product analysis.

(a)

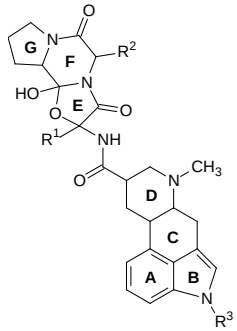


	R^1	R^2	R^3
Ergometrine (M= 325 Da)	$-CH_3$	-H	-H
Methylergometrine (M=339 Da)	$-CH_2CH_3$	-H	-H
Methysergide (M=352 Da)	$-CH_2CH_3$	-H	$-CH_3$



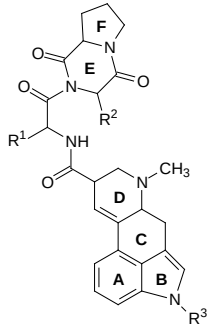
	R^1	R^2	R^3
Ergosine (M=547 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergotamine (M=581 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergocristine (M=609 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Ergokryptine (M=575 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornine (M=561 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H

(b)



	R^1	R^2	R^3
Dihydroergotamine (M=583 Da)	$-CH_3$	$-CH_2Ph$	-H
Dihydroergocristine (M=611 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Dihydroergocryptine (M=577 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H

(c)



	R^1	R^2	R^3
Ergocristam (M=593 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Ergocryptam (M=559 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornam (M=545 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H
Ergostam (M=579 Da)	$-C_2H_5$	$-CH_2Ph$	-H
Ergoptam (M=546 Da)	$-C_2H_5$	$-CH_2CH(CH_3)_2$	-H
Ergonam (M=531 Da)	$-C_2H_5$	$-CH(CH_3)_2$	-H
Ergotamam (M=565 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergosam (M=531 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergovalam (M=517 Da)	$-CH_3$	$-CH(CH_3)_2$	-H

Figure 6.18. *Common structures of ergoamides (a), ergopeptines (b) and ergopeptams (c) and representative ergot alkaloids for each class. M: molecular weight.*

Regarding the secondary fungal metabolites and more specifically ergot alkaloids, this type of MS has been utilized only in targeted analysis (Zachariasova *et al*., 2010) or in a pre-selected screening using a limited database (Lehner SM *et al*., 2011). The full-scan accurate mass capability of Orbitrap MS remains to be exploited in untargeted screening of ergot alkaloid derivatives.

In this work, a method based on high resolution mass spectrometry (HRMS) and ion trap MS technology is proposed for the study of the fragmentation pattern of ergot alkaloids and the identification of less studied or novel ergot alkaloid derivatives. In particular, Orbitrap MS was used, which allows to achieve high mass resolution (up to 100,000 FWHM) and high mass accuracy (mass error < 2 ppm), thereby leading to higher sensitivity, dynamic range and selectivity for the analysis of complex matrices (Hu *et al*., 2005). The fragmentation of twelve ergot alkaloids, namely ergometrine, ergosine, ergotamine, ergocornine, ergokryptine, ergocristine, methylergometrine, methysergide, dihydroergotamine, ergocornam, ergocryptam and ergocristam was studied with the aim of establishing a simple strategy for identification of novel ergot alkaloid derivatives.

6.5.2. Experimental

6.5.2.1. Standards

The standards of ergot alkaloids were as described in section 6.2.2.1. Methysergide (MeErgi) (as methysergide maleate) was purchased from Sigma-Aldrich (Bornem, Belgium).

6.5.2.2. Chemicals and material

MeOH and ACN (both of LC-MS grade) were supplied by Biosolve (Valkenswaard, the Netherlands). NH_4HCO_3 was obtained from Sigma-Aldrich. $(NH_4)_2SO_4$ and NH_3 (25 %) were supplied by Merck (Darmstadt, Germany). ACN and MeOH (both of HPLC grade), and *n*-hexane were purchased from VWR International (Zaventem, Belgium). EtOAc was obtained from Acros Organics (Geel, Belgium).

A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized H_2O. Ultrafree®-MC centrifugal filter units (0.22 µm) were purchased from Millipore (Darmstadt, Germany).

6.5.2.3. Sample preparation

Sample preparation procedure was as described in section 6.2.2.4.1.

6.5.2.4. HPLC-MS/MS analysis

The HPLC-MS/MS analysis was performed as described in section 6.2.2.7.

The gradient elution was as follows: 0-30 min: 55 % B; 30-35 min: 55-65 % B; 35-90 min: 65 % B; 90-100 min: 65-55 % B; 100-120 min: 55 % B.

ESI^+ mode was used throughout the work as it provided higher MS signal for ergot alkaloids (see section 6.2).

6.5.2.5. HPLC-MS^n analysis

LC analyses were performed on a Surveyor Plus HPLC System (Thermo Fisher Scientific, San Jose, CA, USA). The column was XBridge MS C18 column (3.5 µm, 150 x 2.1 mm) with an XBridge Sentry guard column (3.5 µm, 10 x 2.1 mm i.d.) both supplied by Waters (Milford, MA, USA). The mobile phases were as described in section 6.2.2.7. Flow rate was 0.25 mL/min. The gradient elution profile was as follows: 0-10 min: 10-35 % B; 10-30 min: 35 % B; 30-45 min: 35-60 % B; 45-50 min: 60-99 % B; 50-55 min: 99 % B; 55-57 min: 99-10 % B; 57-65 min: 10 % B. The temperature of the column was 40 °C and the injection volume was 5 µL.

Mass spectra were acquired using an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source. The mass spectrometer was operated in the ESI^+ mode. The MS parameters were as follows: spray needle voltage 5 kV, capillary voltage 17 V, capillary temperature 200 °C, nitrogen sheath gas flow 35 a.u., auxiliary gas flow 10 a.u. When

performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different CELs to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

6.5.2.6. UHPLC-HRMS analysis

LC analyses were performed on a Thermo Accela UHPLC system (Thermo Fisher Scientific). The mobile phases were as described in section 6.5.2.5. The column used was ZORBAX RRHD Eclipse Plus C18 (1.8 µm, 2.1 x 100 mm) from Agilent Technologies (Diegem, Belgium). The gradient elution program was set as follows: 0-1 min: 0 % B; 1-5 min: 0-25 % B; 5-15 min: 25-35 % B; 15-25 min: 35-40 % B; 25-40 min: 40-70 % B; 40-47 min: 70-98 % B; 47-50 min: 98 % B; 50-51 min: 98-0 % B; 51-55 min: 0 % B. The mobile phase flow rate was 0.4 mL/min and the injection volume was 5 µL.

Accurate mass measurements of the precursor and product ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with an ESI interface. The mass spectrometer was operated in the ESI^+ mode. The MS parameters were the following: spray voltage 4.5 kV, capillary temperature 250 °C, heater temperature 250 °C, sheath gas flow rate 45 a.u., auxiliary gas flow rate 10 a.u. The data were processed using Xcalibur™ 2.1 (Thermo Fisher Scientific). The instrument was operated in full scan mode with a resolution of 100,000 FWHM. The maximum injection time was 200 ms and the number of microscans per scan was 1. Each full scan was followed by a same-polarity “all ion fragmentation” higher energy collisional dissociation scan.

6.5.3. Results and discussion

6.5.3.1. Fragmentation pattern of ergot alkaloids

6.5.3.1.1. Ergopeptine and ergoamide alkaloids

The ergopeptines and ergoamides are structurally related compounds that share a common skeleton (see Fig. 6.18). This characteristic was exploited to establish a strategy for the screening and identification of unknown ergot alkaloid derivatives. This was achieved through a careful study of the fragmentation pattern of known derivatives, including six ergopeptines (ergosine, ergotamine, ergocornine, ergokryptine, ergocristine and dihydroergotamine) and three ergoamides (ergometrine, methylegometrine and methysergide).

MS^n fragmentation data of the ergopeptine alkaloids are summarized in Table 6.19. It was observed that all the studied ergopeptines initially underwent a loss of a water molecule (-18 Da). Subsequently, in MS^3 and MS^4 experiments, neutral losses of 28 and 18 Da (corresponding to CO and H_2O) were observed. In further MS^5 experiments, losses of 91 Da (ergocristine, ergotamine and dihydroergotamine), 56 Da for ergosine and ergokryptine; 42 Da for ergocornine) occurred (Fig. 6.19). Taking into consideration the structural differences between the ergopeptines studied and the accurate mass data, these losses of 91, 56 and 42 Da were attributed to the radical R^2. The observed losses are consistent with a homolytic cleavage of the implied C-C bond. This would represent a violation of the even-electron fragmentation rule, however such a phenomenon has previously been described (Mohamed *et al*., 2006). While the loss of 91 Da (ergocristine, ergotamine and dihydroergotamine) could be inferred from the homolytic cleavage in a straightforward fashion, the fragmentation mechanism for ergosine, ergokryptine and ergocornine implied the transfer of one hydrogen atom to the leaving fragment, leading to the formation of a double bond in the lost entity (Fig. 6.20).

Table 6.19. *MS^n fragmentation data for ergotamine (Et), ergocornine (Eco), ergocristine (Ecr), ergokryptine (Ekr), ergosine (Es) and dihydroergotamine (DHEt).*

Es	Ekr	Ecr	Eco	Et	DHEt	Fragment assignment[a]
548	576	610	562	582	584	$[M+H]^+$
530	558	592	544	564	566	$[M+H]^+ - H_2O$
502	530	564	516	536	538	$[M+H]^+ - H_2O - CO$
484	512	546	498	518	520	$[M+H]^+ - H_2O - CO - H_2O$
428	456	455	466	427	429	$[M+H] - H_2O - CO - H_2O - R^2$
320	348	348	348	320	322	Cleavage within and loss of most of peptide ring system $[M+H]^+ - C_7H_{11}N_2O_3-R^2$
292	320	320	320	292	294	Loss of CO from 320, 348 or 322 $[M+H]^+ - C_7H_{11}N_2O_3 - R^2 - CO$
277	305	305	305	277		Cleavage within the lysergic D ring system
		268			270	Loss of R^1 from 292, 320 or 294
		251			253	Loss of NH_3 from 268 or 270
		223			225	Loss of CO from 251 or 253
		208			210	Loss of $-CH_3$ from 223 or 225
		208				Cleavage within the lysergic D ring and loss of R^1-C(CO)-NH
		197			197	Cleavage within and loss of D ring from 251 or 253
		194				Cleavage within the lysergic D ring and loss of $N-CH_3$ from 223
		182			182	Cleavage within lysergic D ring and loss of $–CH-N-CH_3$ from 223
		182			182	Loss of $-CH_3$ from 197
		180				Cleavage within lysergic D ring and loss of $N-CH_2-$ from 208 or 210

[a] R^1 and R^2 are as defined in Fig. 6.18

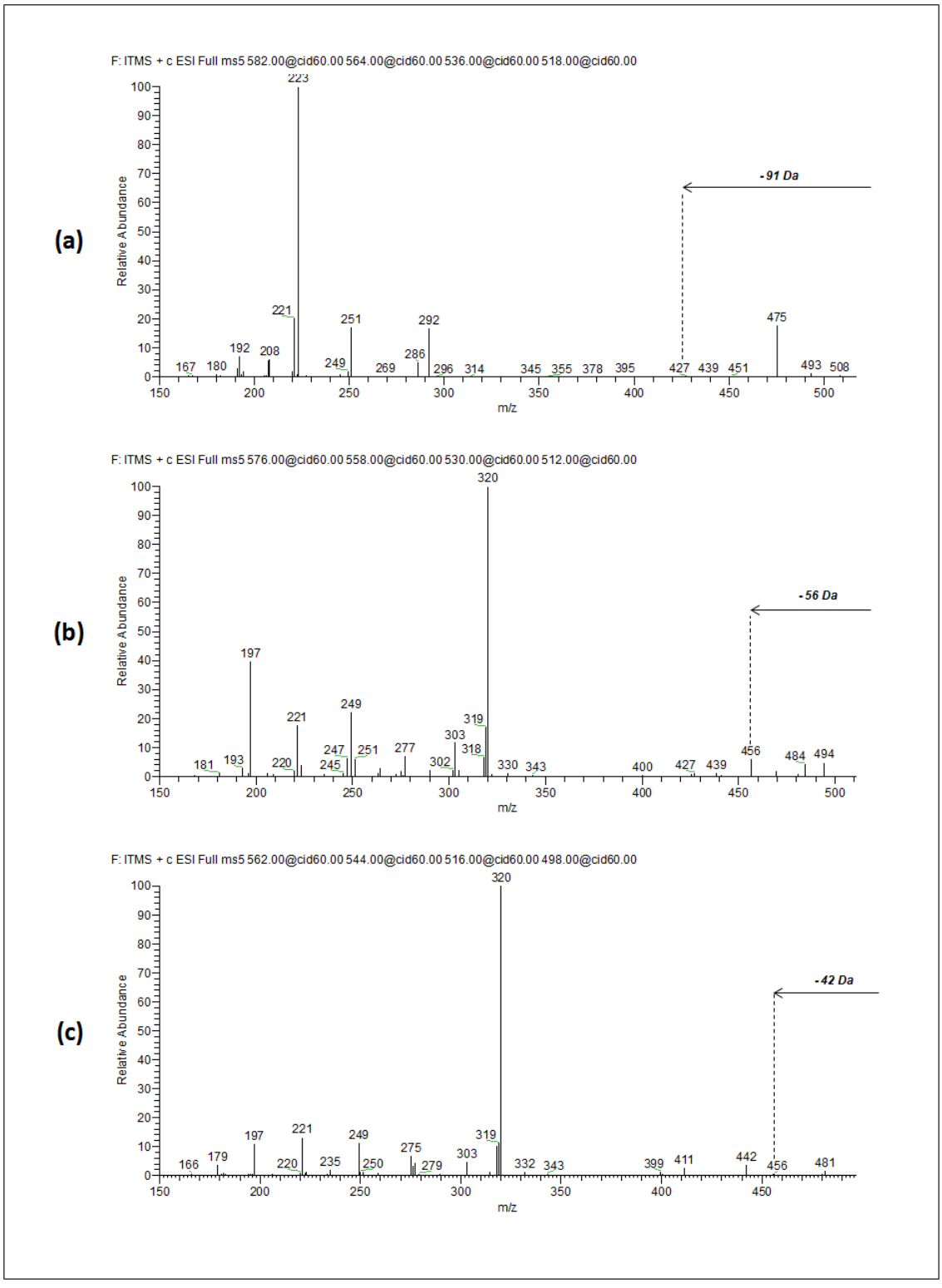


Figure 6.19. *Fourth-generation CID spectra acquired for ergotamine (a), ergokryptine (b) and ergocornine (c) demonstrating the loss of the R^2 substituent from the main skeleton. R^2: $-CH_2Ph$ (for ergotamine), $-CH_2CH(CH_3)_2$ (for ergokryptine) and $-CH(CH_3)_2$ (for ergocornine).*

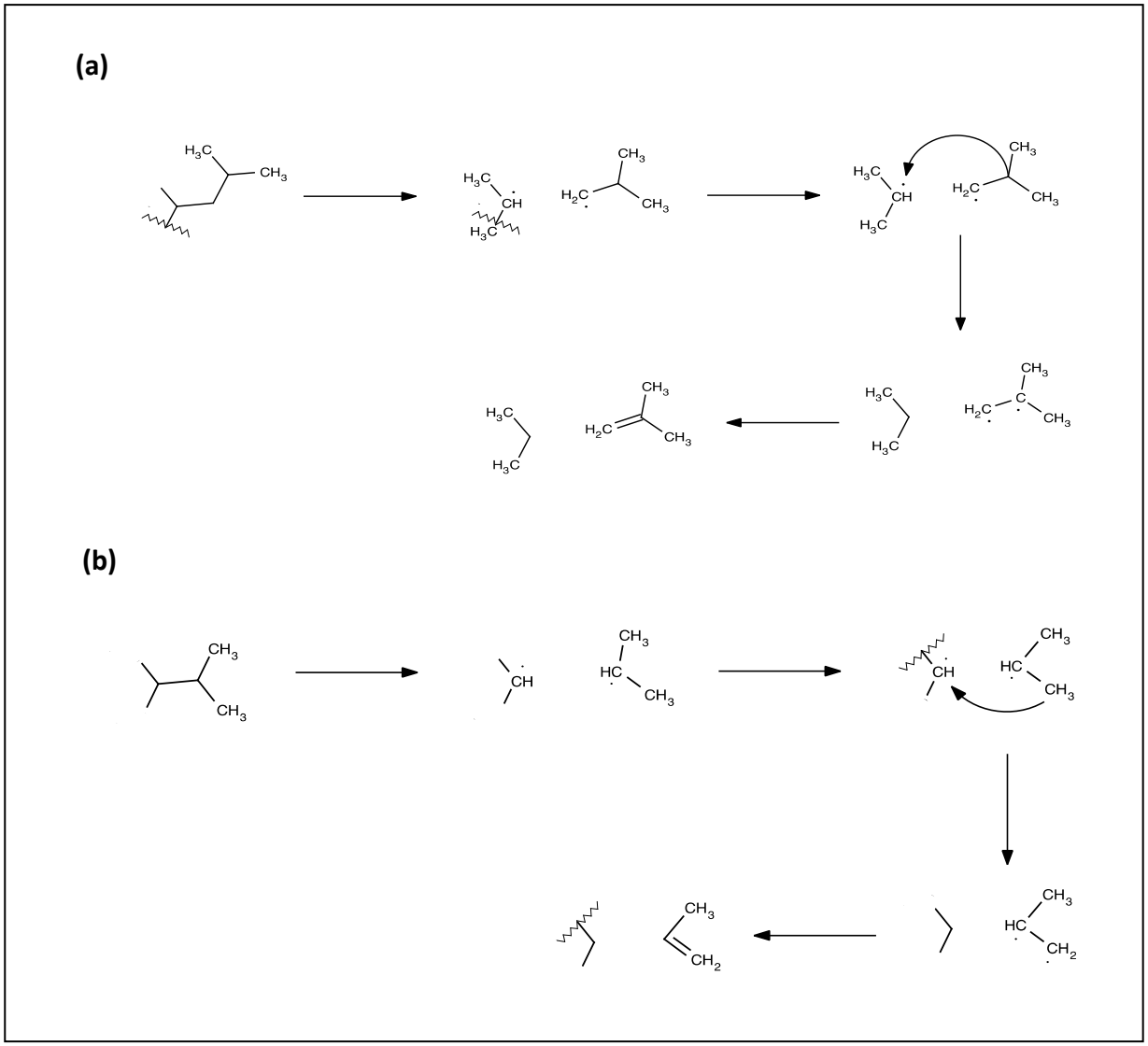


Figure 6.20. *Proposed fragmentation mechanism for the loss of R^2 substituent from the main skeleton of ergosine and ergokryptine (a), and ergocornine (b).*

Besides the loss of R^2 radical, the ergopeptines underwent, subsequent to the losses of H_2O and, CO and H_2O, a loss of most of the peptide ring system (Fig. 6.21). For ergokryptine, ergocristine and ergocornine (where R^1 is an isopropyl radical), an ion with a *m/z* of 348.1698 Da was obtained, corresponding to $C_{21}H_{22}N_3O_2$ (mass error: -2.4 ppm). The ergopeptines having a methyl group at R^1 position (*i.e*. ergotamine, ergosine) produced a fragment with *m/z* 320.1388 Da ($C_{19}H_{18}N_3O_2$; -1.7 ppm). Dihydroergotamine (an ergotamine-derivative having a saturated C9-C10 bond) rather produced an ion at *m/z* 322.1549 assigned as $C_{19}H_{20}N_3O_2$ (-0.5 ppm). The

resulting fragments further underwent a loss of CO (- 28 Da), yielding *m/z* 320.1750 ($C_{20}H_{22}N_3O$, -2.2 ppm) and *m/z* 292.1439 ($C_{18}H_{18}N_3O$, -1.8 ppm) for the derivatives with an isopropyl and methyl R^1 radicals, respectively (Fig. 6.22). A fragment at *m/z* 294.1597 (assigned as $C_{18}H_{20}N_3O$; -0.4 ppm) was obtained for dihydroergotamine. Subsequent loss of the R^1-C-NH moiety gave an ion at *m/z* 251.1175 corresponding to $C_{16}H_{15}N_2O$ (-1.5 ppm) for all ergopeptines, except for dihydroergotamine, for which the corresponding ion at *m/z* 253.1332 ($C_{16}H_{17}N_2O$; -0.3 ppm) was obtained. Fragmentation of the ion at *m/z* 251 yielded, among others, an ion at *m/z* 223.1227 ($C_{15}H_{15}N_2$; -1.4 ppm) through the loss of CO (- 28 Da). The corresponding ion for the dihydro-derivatives (*m/z* 253) yielded a fragment at *m/z* 225.1384 ($C_{15}H_{17}N_2$; -0.2 ppm). The product ion at *m/z* 223 (*m/z* 225 for dihydroergotamine) proved to be the most abundant common fragment of the different ergopeptines. The product ion spectrum (Fig. 6.22) of this common ion indicated that subsequently, a homolytic cleavage of the N–CH_3 bond in the D ring took place, giving rise to the radical cation at *m/z* 208.0993 ($C_{14}H_{12}N_2$; -0.8 ppm) for the ergopeptines and *m/z* 210.1151 ($C_{14}H_{14}N_2$; -0.1 ppm) for the dihydroergopeptines, as also reported by Mohamed *et al.* (Mohamed *et al*., 2006).

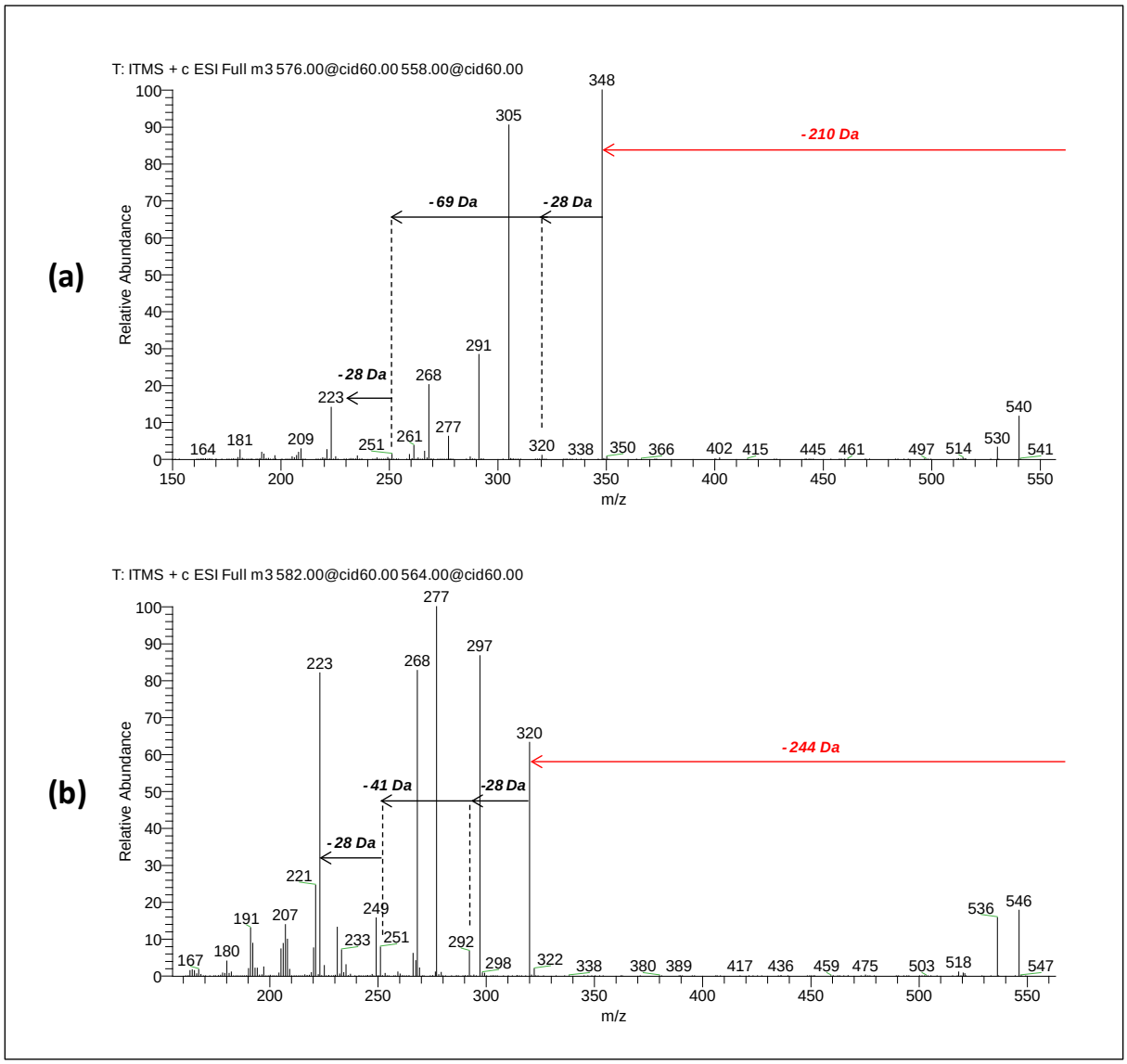


Figure 6.21. *Second-generation CID spectra acquired for ergokryptine (a) and ergotamine (b) demonstrating loss of most of the peptide ring system (shown in red).*

The resulting fragments, i.e. m/z 348 (ergokryptine) and m/z 320 (ergotamine) underwent a loss of CO (-28 Da) followed by the loss of the R^1-C-NH moiety (69 Da for ergokryptine and 41 Da for ergotamine), yielding a common ion at m/z 251.

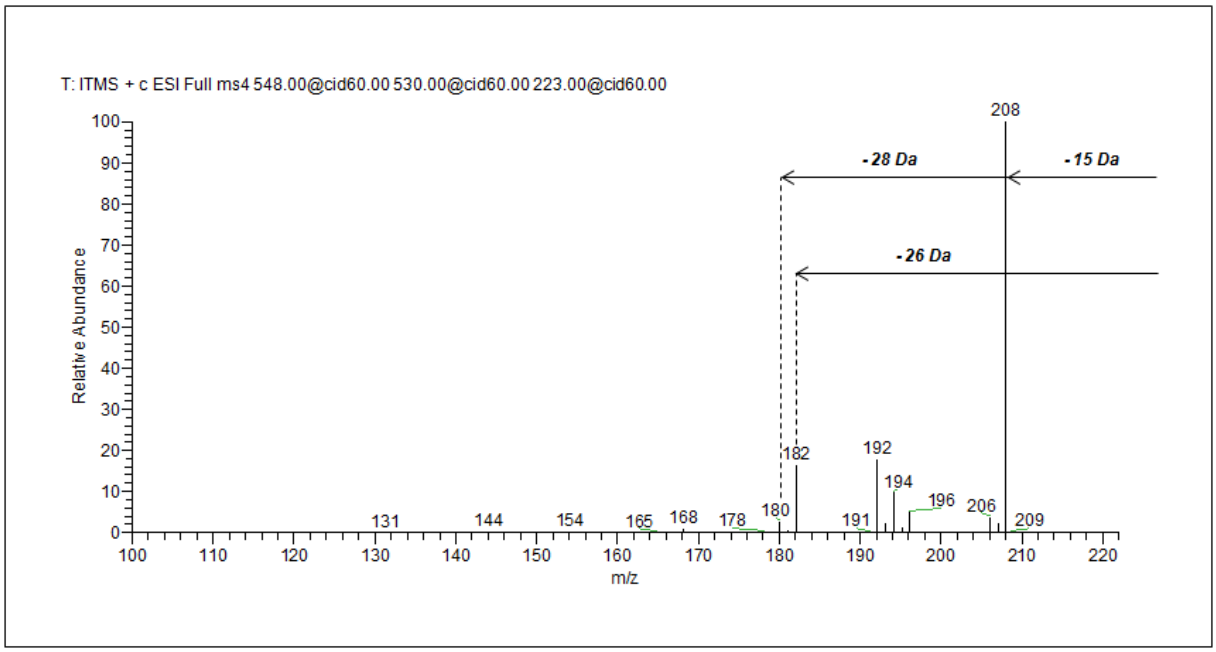
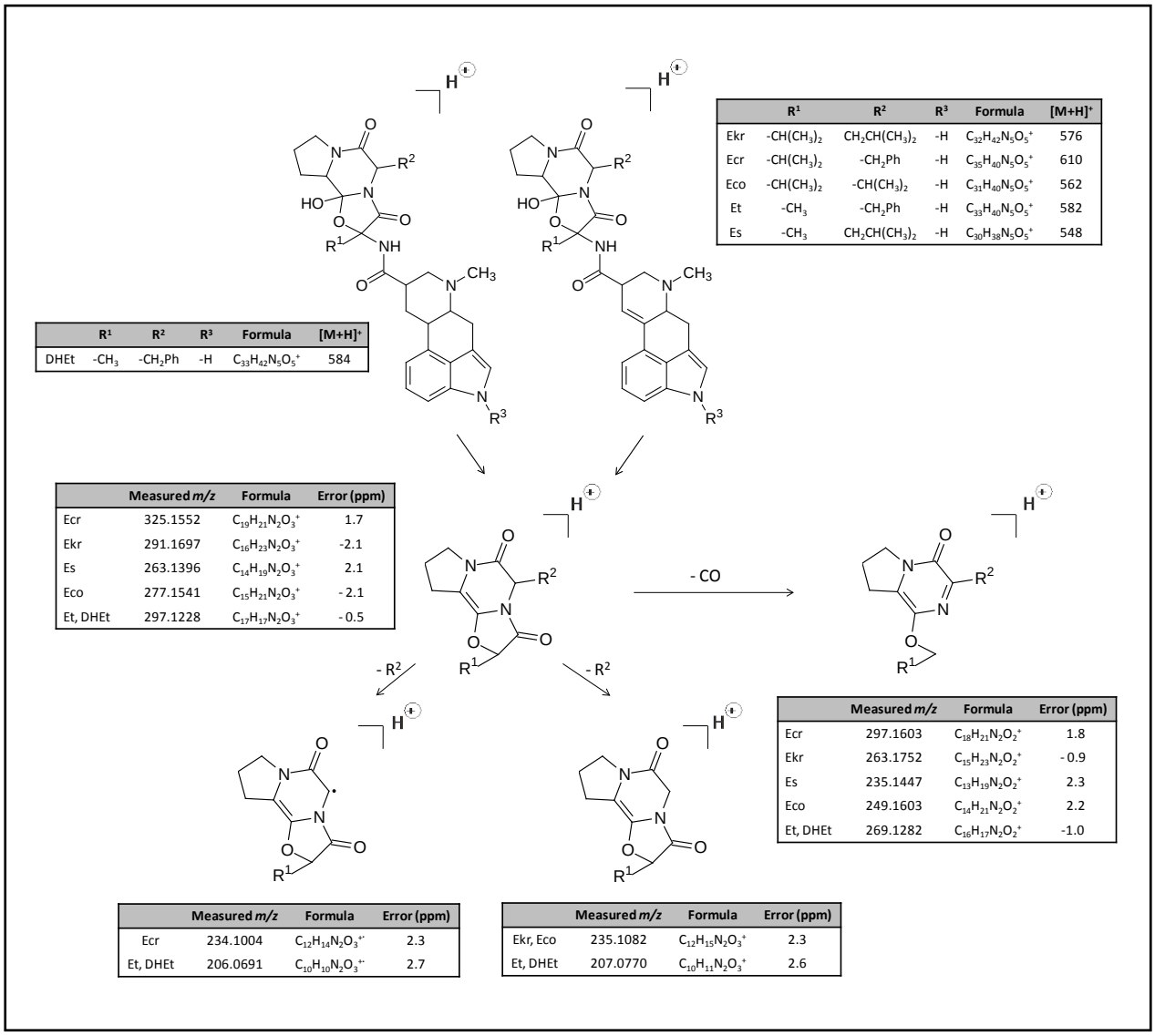


Figure 6.22. *Third-generation CID spectrum acquired for ergosine shown as an example of the fragmentation pattern of the common ion at m/z 223. This product ion at m/z 223 was isolated and collisionally activated at 60 % CEL.*

It was observed that the ions at *m/z* of 348 (ergokryptine, ergocristine) and *m/z* 320 (ergocornine, ergotamine and ergosine), described above, followed an alternative fragmentation pathway. A fragment at *m/z* 305.1280 ($C_{19}H_{17}N_2O_2$; -0.4 ppm) or *m/z* 277.0969 ($C_{17}H_{13}N_2O_2$; -0.2 ppm) was obtained by cleavage within the lysergic D ring system for derivatives with isopropyl R^1 radical (ergokryptine, ergocristine) or methyl R^1 radical (ergocornine, ergotamine, ergosine), respectively. A subsequent loss of CO (- 28 Da) gave *m/z* 277.1332 (ergokryptine, ergocristine) and *m/z* 249.1020 (ergocornine, ergotamine, ergosine) (assigned as $C_{18}H_{17}N_2O$; -0.3 ppm and $C_{16}H_{13}NO$; -0.3 ppm, respectively). A loss of the R^1-C(CO)-NH moiety from these fragments yielded a common ion at *m/z* 208.0756 ($C_{14}H_{10}NO$; -0.1 ppm).

Loss of the lysergic ring-$CONH_2$ moiety from protonated molecule was also observed. The resulting fragment (Fig. 6.23) subsequently lost a CO moiety or the radical R^2. Interestingly, the fragment obtained upon loss of R^2 confirmed the homolytic cleavage fragmentation mechanism

described above for ergokryptine, ergosine and ergocornine, *i.e.*, the transfer of one hydrogen atom to the leaving fragment (Fig. 6.20).



	R¹	R²	R³	Formula	[M+H]⁺
Ekr	-CH(CH$_3$)$_2$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{32}H_{42}N_5O_5^+$	576
Ecr	-CH(CH$_3$)$_2$	-CH$_2$Ph	-H	$C_{35}H_{40}N_5O_5^+$	610
Eco	-CH(CH$_3$)$_2$	-CH(CH$_3$)$_2$	-H	$C_{31}H_{40}N_5O_5^+$	562
Et	-CH$_3$	-CH$_2$Ph	-H	$C_{33}H_{40}N_5O_5^+$	582
Es	-CH$_3$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{30}H_{38}N_5O_5^+$	548

	R¹	R²	R³	Formula	[M+H]⁺
DHEt	-CH$_3$	-CH$_2$Ph	-H	$C_{33}H_{42}N_5O_5^+$	584

	Measured *m/z*	Formula	Error (ppm)
Ecr	325.1552	$C_{19}H_{21}N_2O_3^+$	1.7
Ekr	291.1697	$C_{16}H_{23}N_2O_3^+$	-2.1
Es	263.1396	$C_{14}H_{19}N_2O_3^+$	2.1
Eco	277.1541	$C_{15}H_{21}N_2O_3^+$	- 2.1
Et, DHEt	297.1228	$C_{17}H_{17}N_2O_3^+$	- 0.5

	Measured *m/z*	Formula	Error (ppm)
Ecr	297.1603	$C_{18}H_{21}N_2O_2^+$	1.8
Ekr	263.1752	$C_{15}H_{23}N_2O_2^+$	- 0.9
Es	235.1447	$C_{13}H_{19}N_2O_2^+$	2.3
Eco	249.1603	$C_{14}H_{21}N_2O_2^+$	2.2
Et, DHEt	269.1282	$C_{16}H_{17}N_2O_2^+$	-1.0

	Measured *m/z*	Formula	Error (ppm)
Ecr	234.1004	$C_{12}H_{14}N_2O_3^{+\cdot}$	2.3
Et, DHEt	206.0691	$C_{10}H_{10}N_2O_3^{+\cdot}$	2.7

	Measured *m/z*	Formula	Error (ppm)
Ekr, Eco	235.1082	$C_{12}H_{15}N_2O_3^+$	2.3
Et, DHEt	207.0770	$C_{10}H_{11}N_2O_3^+$	2.6

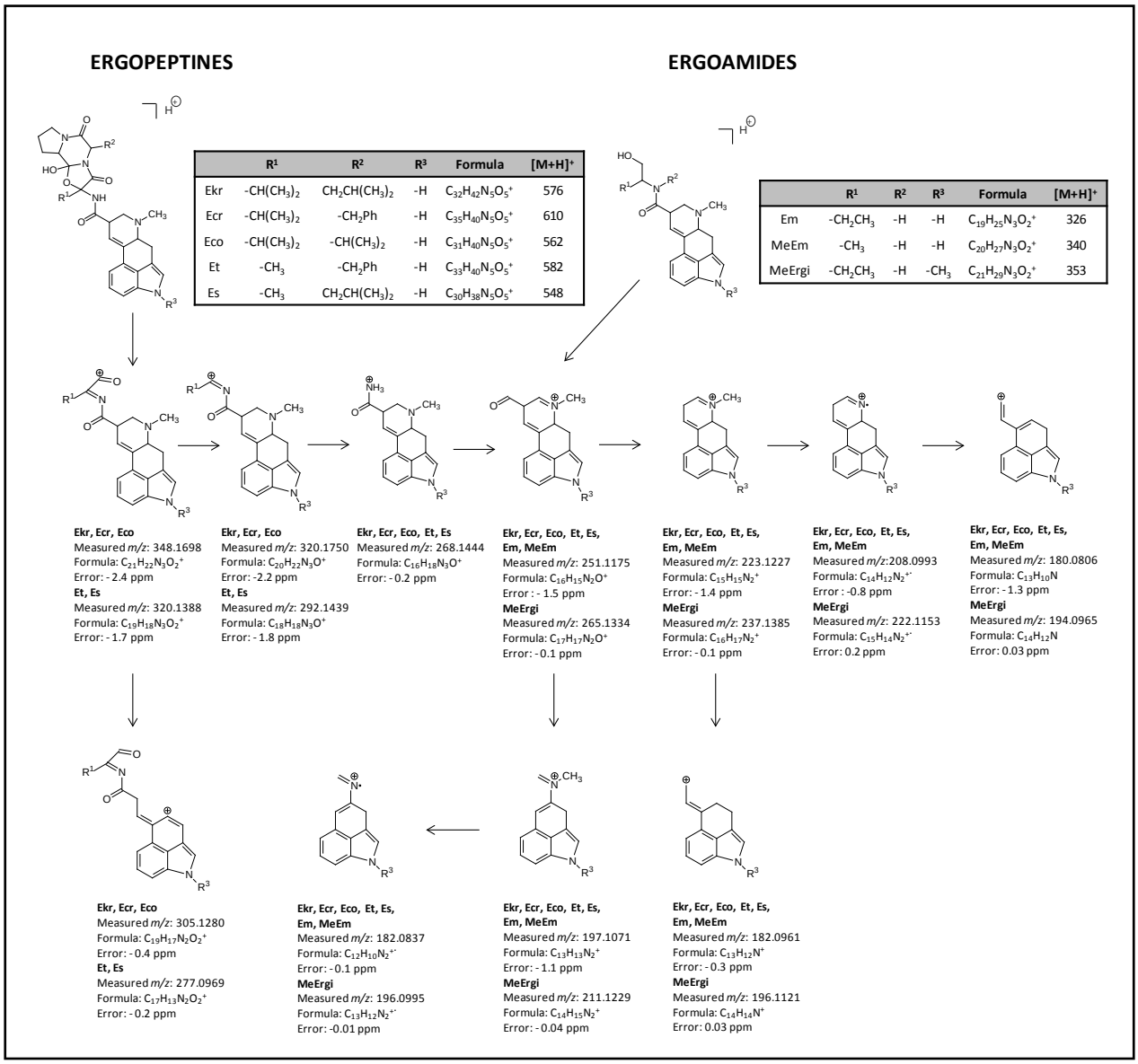
Figure 6.23. *Fragmentation mechanism of ergopeptines showing the loss of the lysergic acid moiety.*

MS^n fragmentation data of the ergoamide alkaloids are summarized in Table 6.20. A fragmentation pattern similar to that of ergopeptines was observed for this group of ergot alkaloids. Initially, all the studied ergoamides lost a water molecule or $–CH_3$ by homolytic cleavage. The fragment at *m/z* 251 (*m/z* 265 for MeErgi) was obtained through a loss of R^1-$CH(CH_2OH)$-NH_2 from a protonated ion $[M+H]^+$, and followed a common pathway with the ergopeptines (Fig. 6.24). The ergoamides also underwent a cleavage within the lysergic D ring as shown in Fig. 6.25.

Table 6.20. *MS^n fragmentation data for ergometrine (Em), methylergometrine (MeEm) and methysergide (MeErgi).*

Em	MeEm	MeErgi	Fragment assignment[a]
326	340	354	$[M+H]^+$
311	325	339	$[M+H]^+$- CH_3
308	322	336	$[M+H]^+$- H_2O
283	297	311	Cleavage within the lysergic D ring and loss of C_2H_5N
265	279	293	Loss of H_2O from 283, 297 or 311
251		265	Loss of R^1-$CH(CH_2OH)$-NH-R^2 from $[M+H]^+$
223		237	Loss of CO from 251 or 265
208		222	Loss of -CH_3 from 223 or 237
197		211	Cleavage within the lysergic D ring from 251 or 265
194		208	Cleavage within the lysergic D ring and loss of N-CH_3 from 223 or 237
182		196	Cleavage within the lysergic D ring and loss of –CH-N-CH_3 from 223 or 237
182		196	Loss of -CH_3 from 197 or 211
180		194	Cleavage within the lysergic D ring and loss of N-CH_2- from 208 or 222

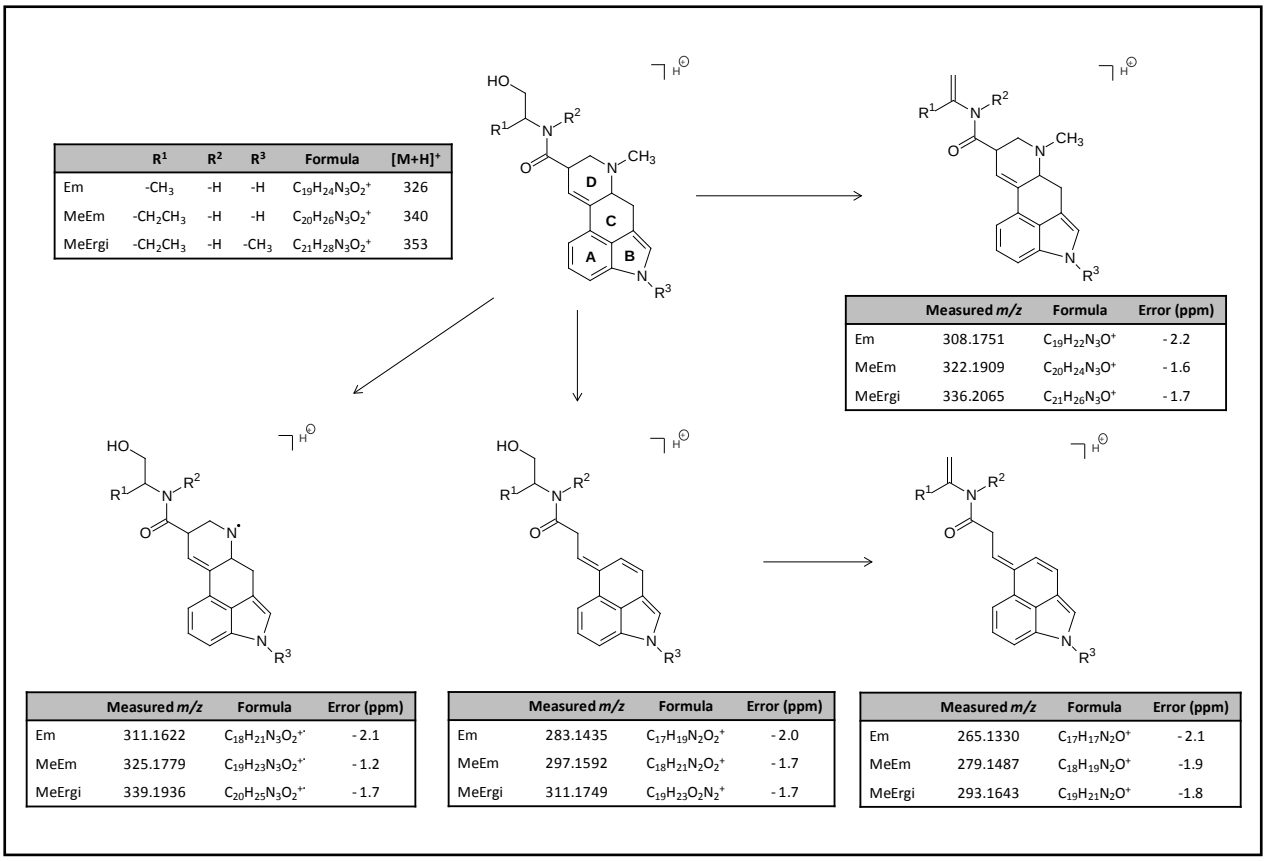
[a] R^1 and R^2 are as defined in Fig. 6.18



	R¹	R²	R³	Formula	[M+H]⁺
Ekr	-CH(CH$_3$)$_2$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{32}H_{42}N_5O_5^+$	576
Ecr	-CH(CH$_3$)$_2$	-CH$_2$Ph	-H	$C_{35}H_{40}N_5O_5^+$	610
Eco	-CH(CH$_3$)$_2$	-CH(CH$_3$)$_2$	-H	$C_{31}H_{40}N_5O_5^+$	562
Et	-CH$_3$	-CH$_2$Ph	-H	$C_{33}H_{40}N_5O_5^+$	582
Es	-CH$_3$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{30}H_{38}N_5O_5^+$	548

	R¹	R²	R³	Formula	[M+H]⁺
Em	-CH$_2$CH$_3$	-H	-H	$C_{19}H_{25}N_3O_2^+$	326
MeEm	-CH$_3$	-H	-H	$C_{20}H_{27}N_3O_2^+$	340
MeErgi	-CH$_2$CH$_3$	-H	-CH$_3$	$C_{21}H_{29}N_3O_2^+$	353

Figure 6.24. *Proposed common fragmentation pathway of ergopeptines and ergoamides.*



	R^1	R^2	R^3	Formula	$[M+H]^+$
Em	$-CH_3$	-H	-H	$C_{19}H_{24}N_3O_2^+$	326
MeEm	$-CH_2CH_3$	-H	-H	$C_{20}H_{26}N_3O_2^+$	340
MeErgi	$-CH_2CH_3$	-H	$-CH_3$	$C_{21}H_{28}N_3O_2^+$	353

	Measured *m/z*	Formula	Error (ppm)
Em	308.1751	$C_{19}H_{22}N_3O^+$	- 2.2
MeEm	322.1909	$C_{20}H_{24}N_3O^+$	- 1.6
MeErgi	336.2065	$C_{21}H_{26}N_3O^+$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	311.1622	$C_{18}H_{21}N_3O_2^{+\cdot}$	- 2.1
MeEm	325.1779	$C_{19}H_{23}N_3O_2^{+\cdot}$	- 1.2
MeErgi	339.1936	$C_{20}H_{25}N_3O_2^{+\cdot}$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	283.1435	$C_{17}H_{19}N_2O_2^+$	- 2.0
MeEm	297.1592	$C_{18}H_{21}N_2O_2^+$	- 1.7
MeErgi	311.1749	$C_{19}H_{23}O_2N_2^+$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	265.1330	$C_{17}H_{17}N_2O^+$	- 2.1
MeEm	279.1487	$C_{18}H_{19}N_2O^+$	-1.9
MeErgi	293.1643	$C_{19}H_{21}N_2O^+$	-1.8

Figure 6.25. *Fragmentation mechanism of ergoamides showing cleavage within the lysergic D ring.*

6.5.3.1.2. Ergopeptam alkaloids

Reference standards for the ergopeptams were not available during the course of this work. The study of their fragmentation pattern was achieved using a sample that was presumed to contain these ergot alkaloids based on the levels and pattern of ergot alkaloids for which commercial standards were available. Using accurate mass measurements and fragmentation data, three known ergopeptams (Komarova *et al*., 2002; Olsovska *et al*., 2008; Uhlig and Petersen, 2008), namely ergocornam ($C_{31}H_{40}N_5O_4$, *m/z* 546.3072; -0.5 ppm), ergocryptam ($C_{32}H_{42}N_5O_4$, *m/z* 560.3234; 0.5 ppm) and ergocristam ($C_{35}H_{40}N_5O_4$, *m/z* 594.3078; 0.5 ppm) were detected in this sample. The fragmentation of these compounds is summarized in Table 6.21. As expected from their structures, the initial loss of 18 Da (corresponding to the

elimination of a water molecule) observed for ergopeptines did not occur for the ergopeptam derivatives. The studied compounds underwent a loss of the dipeptide ring system in MS^2 experiments of the protonated ions, resulting in a common and abundant fragment at *m/z* 350 (Fig. 6.26). This ion at *m/z* 350 was attributed to the fragment depicted in Fig. 6.27, where the radical R^1, in accordance with the structure of the studied compounds, is $–CH(CH_3)_2$. A loss of CO from this fragment yielded an ion at *m/z* 322. A subsequent fragmentation yielded the ion at *m/z* 251, identical to that obtained for ergopeptines (Fig. 6.24), as revealed by the further fragmentation. Based on the above-mentioned data, a fragmentation pathway of the ergopeptams was proposed (Fig. 6.27).

Table 6.21. *MS^n fragmentation data for ergopeptam alkaloids.*

Ergocornam	Ergocryptam	Ergocristam	Fragment assignment[a]
546	560	594	$[M+H]^+$
	350		Loss of the dipeptide ring system
	322		Loss of CO from 350
	307		Cleavage within the lysergic D ring system
	279		Cleavage within the lysergic D ring and loss of CO
	251		Loss of NH-CH-R^1 from 322
	223		Loss of CO from 251
	208		Loss of -CH_3 from 223
	208		Cleavage within the lysergic D ring and loss of R^1-C(CO)-NH
	197		Cleavage within and loss of D ring from 251
	194		Cleavage within the lysergic D ring and loss of N-CH_3 from 223
	182		Cleavage within the lysergic D ring and loss of -CH-N-CH_3 from 223
	182		Loss of -CH_3 from 197
	180		Cleavage within the lysergic D ring and loss of N-CH_2- from 208

[a] R^1 is as defined in Fig. 6.18

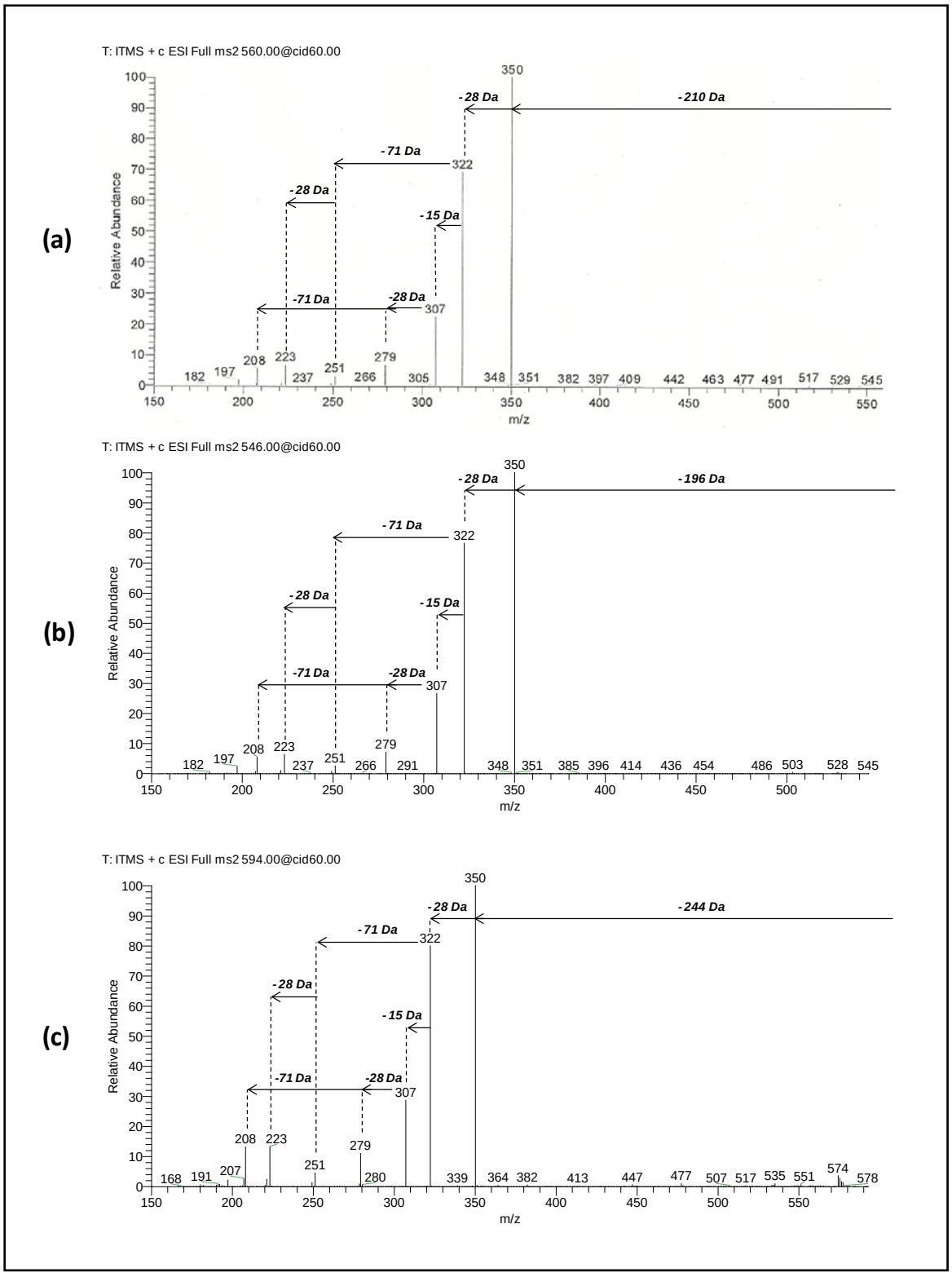
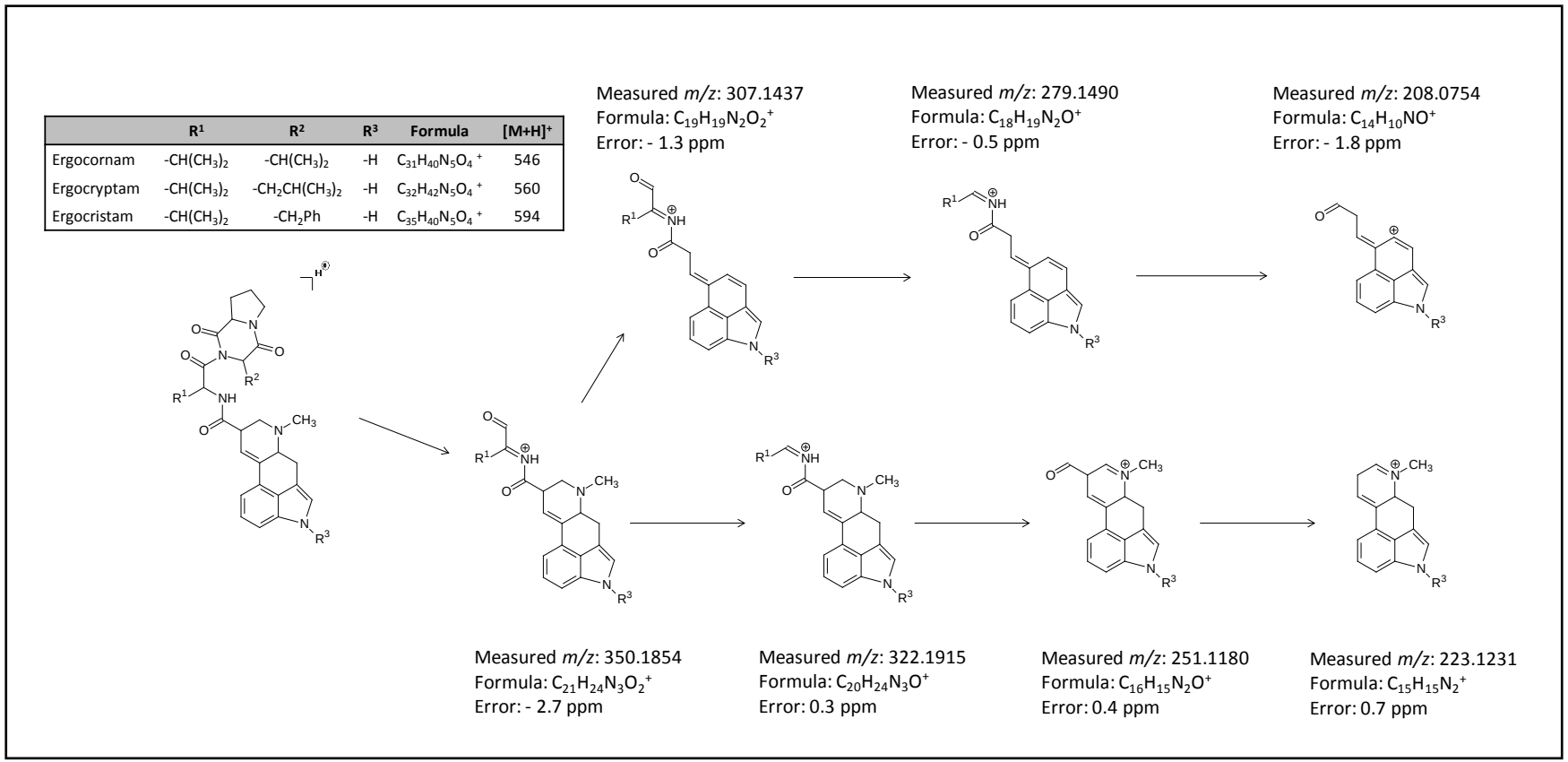


Figure 6.26. *First-generation CID spectra acquired for ergocryptam (a), ergocornam (b) and ergocristam (c).*



	R¹	R²	R³	Formula	[M+H]⁺
Ergocornam	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H	$C_{31}H_{40}N_5O_4^+$	546
Ergocryptam	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H	$C_{32}H_{42}N_5O_4^+$	560
Ergocristam	$-CH(CH_3)_2$	$-CH_2Ph$	-H	$C_{35}H_{40}N_5O_4^+$	594

Figure 6.27. *Proposed fragmentation pathway of ergopeptam ergot alkaloids. The fragment at m/z 223 was identical to that shown in Fig. 6.24, thereby following the same pathway.*

6.5.3.2. Strategy for identification of novel ergopeptams and/or ergopeptines

A strategy for the identification of novel or less studied ergot alkaloids was proposed (Fig. 6.28).

Based on fragmentation pathways described in section 6.5.3.1, the ions at *m/z* 223 and *m/z* 251 were found to be common for all the ergopeptines, ergoamides and ergopeptams, while for the dihydroergopeptines, ions *m/z* 225 and *m/z* 253 were characteristic. Therefore, it was proposed that monitoring of these ions can be used as the first step in screening of ergot alkaloids. Initially, the samples with possible ergot alkaloid derivatives were analysed using HRMS in MS^2 mode to screen for the fragment at *m/z* 223.1230 ($C_{15}H_{15}N_2$) or *m/z* 225.1386 ($C_{15}H_{17}N_2$). Eventually, this screening can be performed by applying a parent scan of the ions at *m/z* 223 and *m/z* 225. The fragmentation of the possible ergot alkaloid derivatives is further studied by LC-MS^n. Compound identification is performed according to the scheme indicated in Fig. 6.27, and is supported by accurate mass data.

If the ions at *m/z* 223 or *m/z* 225 were observed in the spectrum, the next step is to check whether the molecule of interest looses water upon fragmentation of the protonated ion. Formation of dehydrate will indicate that the studied compound refers to ergopeptines or ergoamides. Noteworthy, if the cations at *m/z* 223 or *m/z* 225 were formed in HRMS analysis, but the loss of H_2O from the protonated ion did not occur, the compound can be a possible ergopeptam.

Assignment of R^1 and R^2 radicals can be performed following the steps shown in Fig. 6.28. As described in section 6.5.3.1, losses of 28 Da and 18 Da were characteristic features for ergopeptines during MS^3 and MS^4 fragmentation, respectively. Therefore, from the fragmentation spectrum of the ion corresponding to ($[M+H]^+$-64), the radical R^2 can be deduced (Fig. 6.28, **step 1**). Subsequently, R^1 can be determined (see **step 2** in Fig. 6.28), based on its mass calculated using Equation 6.6.

$$R^1 = [M+H]^+ - 448 - R^2 \qquad \text{(Eq. 6.6)}$$

where 448 corresponds to the ergopeptine structure without radicals

For ergopeptams, the ion with the greatest *m/z* value (Mg), originating from MS^2 of the protonated ion, should be further fragmented. At this stage, a loss of 28 Da should be noted. Considering that the fragment at m/z 251 is obtained from consecutive losses of a 28 Da-moiety, R^1 and another 28 Da-moiety from the greatest fragment, R^1 can be calculated as follows (Equation 6.7):

$$R^1 = Mg - 307 \quad \text{(Eq. 6.7)}$$

where Mg is the greatest fragment

R^2 is in turn calculated according to Equation 6.8.

$$R^2 = [M+H]^+ - Mg - 153 \quad \text{(Eq. 6.8)}$$

where 153 corresponds to the ergopeptam dipeptide ring withour R^2

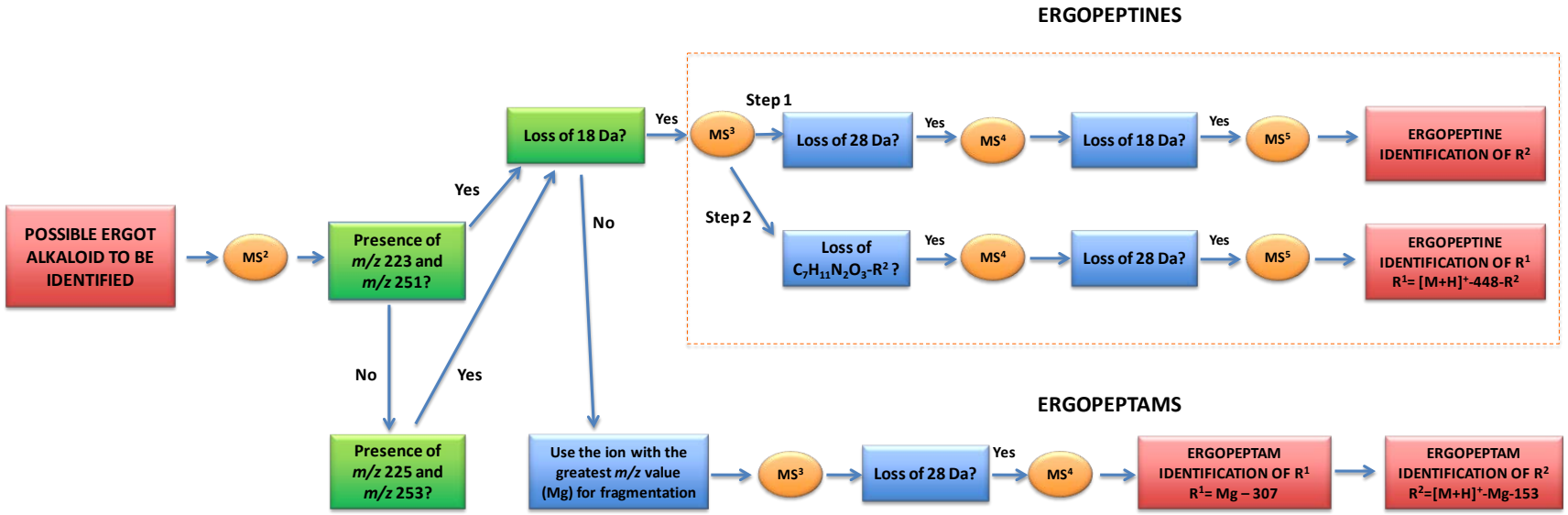


Figure 6.28. *Proposed strategy for identification of novel ergopeptines and ergopeptams.*

6.5.3.3. Screening and identification of ergot alkaloid derivatives in grain samples

The fragmentation study of ergot alkaloids standards showed that the ion at *m/z* 223 was common for all the ergopeptines, ergoamides and ergopeptams. This observation has been previously described (Lehner *et al*., 2004; Mohamed *et al*., 2006). Therefore, detection of MS signal in *m/z* 223-parent scan experiments can point to possible occurrence of ergot alkaloids in a sample. The proposed identification strategy was applied for the screening of ergot alkaloid derivatives in grain samples. Parent scan monitoring of the *m/z* 223 ion revealed the presence of possible ergot alkaloid derivatives in several feed samples. Among 19 analyzed samples, four were free of ergot alkaloids. Ergometrine, ergosine, ergotamine, ergocornine, eegokryptine, ergocristine and their corresponding -inine epimers were identified in the samples by comparison with reference standards. Other presumed ergot alkaloid derivatives could not be identified at this stage. These unknowns were studied by fitting their mass spectral data into the proposed fragmentation pathway (described in section 6.5.3.1). Firstly, the authenticity of the ion at *m/z* 223 was checked through accurate mass measurement. Then, the ion trap fragmentation study was carried out for each unknown ergot alkaloid derivative. The obtained information was coupled with the exact mass data obtained with Orbitrap instrument.

A TIC of a rye feed sample (sample 1) is given as example (Fig. 6.29). Besides the known ergot alkaloids, ten putative ergot alkaloid derivatives were detected in this sample. Among these unknown derivatives, four (**Unk**s **1**, **3**, **4** and **5**) followed the same fragmentation pathway as observed for the ergopeptams, while the others, **Unk**s **2**, **6**, **7**, **8, 9** and **10**, showed similarity with the fragmentation pathway of ergopeptines. Eventually, these compounds were identified as described in Table 6.22. These compounds, namely ergocornam, ergovaline, ergocryptam, ergocristam, ergostine, ergoptine and ergogaline, have been previously reported in grain and grass samples (Cvak *et al*., 1994; Lehner *et al*., 2005; Uhlig and Petersen, 2008). Overview of ergot alkaloid derivatives identified in other grain samples is presented in Table 6.22.

Among the identified ergot alkaloids, some were accompanied by their respective epimers. Epimerization, with respect to the centre of symmetry at C8, is a characteristic feature of ergot alkaloids that have a double bond between C9 and C10, resulting in rotating (C8-(S) configuration) isomers (Komarova and Tolkachev, 2001; Krska and Crews, 2008). Since both

forms are found together in naturally contaminated samples (Komarova and Tolkachev, 2001), this feature was used as additional confirmation of the identification.

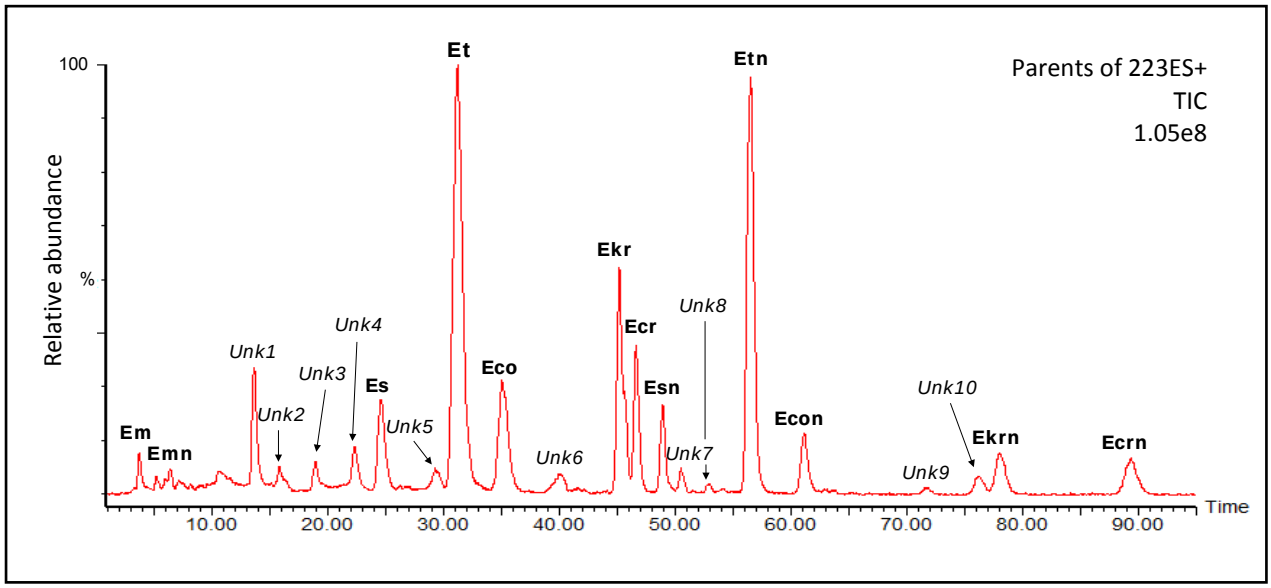


Figure 6.29. *Parent scan total ion chromatogram of a rye feed sample.*

Table 6.22. *Ergot alkaloid derivatives identified in grain samples (only positive samples are shown).*

Metabolite	Elemental composition	Measured *m/z*	Calculated *m/z*	Error (ppm)	Sample[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ergometrine	$C_{19}H_{24}N_3O_2$	326.1858	326.1863	-1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergometrinine	$C_{19}H_{24}N_3O_2$	326.1857	326.1863	-1.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergosine	$C_{30}H_{38}N_5O_5$	548.2863	548.2867	-0.7	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergosinine	$C_{30}H_{38}N_5O_5$	548.2862	548.2867	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergotamine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	-1.5	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Ergotaminine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	-1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergocornine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergocorninine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	-0.9	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergokryptine	$C_{32}H_{42}N_5O_5$	576.3174	576.3180	-1.0	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergokryptinine	$C_{32}H_{42}N_5O_5$	576.3175	576.3180	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocristine	$C_{35}H_{40}N_5O_5$	610.3016	610.3024	-1.3	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocristinine	$C_{35}H_{40}N_5O_5$	610.3017	610.3024	-1.1	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocornam (= Unk 1)	$C_{31}H_{40}N_5O_4$	546.3072	546.3075	-0.5	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+
Ergovaline (= Unk 2)	$C_{29}H_{36}N_5O_5$	534.2708	534.2716	-1.5	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+
Ergocryptam (= Unk 3)	$C_{32}H_{42}N_5O_4$	560.3233	560.3231	0.4	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Ergocryptam isomer (= Unk 4)	$C_{32}H_{42}N_5O_4$	560.3231	560.3231	0.0	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Ergocristam (= Unk 5)	$C_{35}H_{40}N_5O_4$	594.3078	594.3075	0.5	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+
Ergostine (= Unk 6)	$C_{34}H_{38}N_5O_5$	596.2886	596.2873	2.2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ergoptine (= Unk 7)	$C_{31}H_{40}N_5O_5$	562.3036	562.3029	1.2	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+
Ergogaline (= Unk 8)	$C_{33}H_{44}N_5O_5$	590.3331	590.3342	-1.9	+	-	-	+	-	-	+	-	+	+	+	-	-	-	+
Ergostinine (= Unk 9)	$C_{34}H_{38}N_5O_5$	596.2888	596.2873	2.5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ergokryptine isomer (= Unk 10)	$C_{32}H_{42}N_5O_5$	576.3195	576.3186	1.6	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+

[a] '+': detected; '-': not detected

6.5.4. Conclusions

A holistic approach based on high resolution and multiple stage MS was developed for identification of less studied or novel ergot alkaloid derivatives. Initially, the fragmentation of nine known ergot alkaloids was studied to establish a strategy for the identification of novel ergot alkaloids. The ions at *m/z* 223 and *m/z* 251 were found to be common for all the ergopeptines, ergoamides and ergopeptams. Subsequently, the parent scan experiments using these ions were performed to screen grain samples for the presence of possible ergot alkaloid derivatives. Besides the six most common ergot alkaloids and their corresponding epimers (for which standards were available), ten other ergot alkaloid derivatives were identified following the proposed strategy.

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CONCLUSIONS AND PERSPECTIVES

Secondary fungal metabolites are an enormous group of structurally-diverse natural compounds. Due to their beneficial (*e.g.*, used as drugs) and non-favourable (*e.g.*, mycotoxins) impact on the society, these chemicals have been a focus of many studies. Considering the great number of fungi distributed world-wide and that each of them is able to produce several secondary metabolites, a considerable part of the work in the field of fungal metabolites still remains to be done.

The current PhD dissertation represents a valuable contribution into the field of secondary fungal metabolites providing sensitive analytical methods for detection and quantification of targeted toxic metabolites in different matrices, as well as strategies for untargeted screening of less studied or novel compounds. Liquid chromatography - mass spectrometry (LC-MS) was chosen as a main tool for the secondary fungal metabolite investigation owing to its broad applicability, sensitivity and selectivity. Advantageous features of several mass analyzers, namely triple quadrupole, time-of-flight (TOF), Orbitrap and ion trap MS were utilized in the study aiming at collecting as complete as possible information on the analytes present in a given sample. The most important considerations of LC-MS analysis (*e.g.*, epimerization of ergot alkaloids during sample preparation, choice of chromatographic conditions, ionization issues of patulin, matrix effects) were extensively studied and suggestions were made to overcome the problems. Among these issues, it is worth to point out the case of the mycotoxin patulin, which was omitted in some previously developed LC-MS methods due to low MS signal. In this study, a significant improvement in LC-MS/MS analysis of this compound, *i.e.* a higher and more useful MS signal in positive electrospray ionization (ESI) compared to the commonly used negative ESI, was achieved through a careful optimization of the mobile phase composition, thereby making possible inclusion of this toxin into multi-analyte methods.

Given the overall distribution of fungi, the analytical methods developed in the framework of the study are applicable to a variety of matrices. The most important exposure route to secondary fungal metabolites, specifically to mycotoxins, ingestion through consumption of contaminated food or feed, was studied in more detail. In this regard, the LC-MS/MS method developed for toxic metabolites of *Claviceps* spp., ergot alkaloids, in grain and grain products can be emphasized. This fast and sensitive method was applied in a comprehensive survey of cereal foods and feeds disclosing occurrence and pattern of ergot alkaloids in products

(grain, flour, bread, bran and biscuit) consumed within the European Union Member States. Furthermore, the method was optimized for buffered solutions. Subsequently, its applicability was demonstrated in an *in vitro* efficiency study of a clay-based binder towards ergot alkaloids in feed. Promising results were obtained for the model ergot alkaloids using this binder. To uncover ergot alkaloid derivatives, which were not caught by the targeted methods, an approach based on high resolution and multiple stage MS was applied. Eventually, identification of a number of ergot alkaloids, for which commercial standards were not available, was proposed. During the investigation, a fragmentation pathway of the different classes of ergot alkaloids as well as a simple strategy for identification of ergot alkaloid-related compounds were established. This information can be of a great value for other researchers studying ergot alkaloids.

An LC-MS/MS method for a selected group of toxic metabolites (aflatoxin B1, T2-toxin, fumonisin B1, ochratoxin A, patulin, zearalenone and citrinin) was established for the fluids from an *in vitro* study of mycotoxin penetration through the human skin. Solid-phase extraction using polymeric sorbent was exploited for the clean-up, more specifically for elimination of salts, incompatible with the MS equipment, from the samples. The samples from the *in vitro* study were applied directly, without additional pre-treatment, onto the cartridge, which speed up the procedure.

Keeping in mind that the inhalation route is one of the ways of mycotoxin exposure, indoor environment was also under consideration in this study. Initially, a workflow for identification of untargeted compounds (without a need of a reference standard) was evaluated using extracts of fungal cultures, and further applied to “real-world” samples. A screening of the samples collected in mouldy houses where inhabitants experienced non-specific health problems, revealed presence of some secondary fungal metabolites. Thus, the obtained results enriched the existing information on fungal metabolite occurrence indoor, aiding in understanding the not fully-described relationship “fungi - indoor environment - health”.

Besides the direct untargeted screening of metabolites (collect → extract → analyze) which was described above, discovery/detection of novel/less studied metabolites can also be performed with prior gene manipulation experiments. The scope of the work also included investigation of a secondary metabolism gene cluster (namely cluster 27) in *Aspergillus*

flavus using comparative metabolomics. Through inactivation of the cluster 27 *pks* gene and using high resolution MS, a number of metabolites differentially expressed in the wild-type and mutant strains were detected. Eventually, these metabolites were found to be only specific to the wild-type *A. flavus,* and were assigned as asparasone A (an anthraquinone pigment previously isolated from *A. parasiticus* cultures) and three novel metabolites having structures that are likely to derive from asparasone A. Using ion trap MS, the ESI fragmentation pattern of asparasone A has been elucidated for the first time. The proposed approach can be used straightforward, without the need of tedious isolation techniques, to study other gene clusters in *A. flavus* and in other fungi. The acquired data can help to fill the missing blocks in a scheme of secondary fungal metabolism.

Although a considerable work on determination of secondary fungal metabolites has been done in the scope of this PhD thesis, there is still room for future research.

First of all, the function of metabolites newly identified in the PhD study has to be determined. For this purpose, a response to changing environmental stimuli can be suggested for understanding the role of these products in the biology of the fungus. Isolation of the compound of interest and subsequent *in vitro* toxicity studies should also be carried out. This may ultimately lead to discovery of activities potentially interesting to humans with regards to medicine, industry or agriculture.

The predicted genes in the *A. flavus* gene cluster 27 did not include an oxidoreductase that is required in the set of reactions leading to the production of asparasone A from the anthrone precursor. We hypothesized that asparasone A biosynthesis could take advantage of the AflD oxidoreductase in aflatoxin biosynthesis. This can be verified through the investigation of metabolites affected by knocking out the *AflD* gene, using the comparative metabolomics approach described in this thesis.

The cluster 27 *pks* represents only one of the genes that were found to be significantly downregulated upon knock out of the *A. flavus* global regulator VeA. Other biosynthetic gene clusters significantly downregulated in the *downregulated* mutant include clusters 5, 20, 39, 41, 42 and 51. The function of these gene clusters can also be determined using an MS-based comparative metabolomics approach. Genome analysis using the SMURF software predicted many other putative secondary metabolite gene clusters in *A. flavus*. Some of these gene clusters are presumably "silent", *i.e.*, the genes in the cluster are not expressed

under laboratory growth conditions, and therefore the necessary biosynthetic enzymes are not made. By over-expressing key regulatory genes (identified by conserved domain search in GenBank) for many of the putative metabolite-producing gene clusters, "silent" gene clusters can be activated by stimulating the overproduction of enzymes critical for metabolite formation from gene clusters that may be active but only at a very low level of production and only under certain growth conditions. Subsequently, the metabolites of interest can be identified applying MS-based comparative metabolomics approach. Recently, chromatin remodelling has been shown to alter the expression of many secondary metabolites, and to induce the expression of cryptic gene products. Shwab *et al.* (Shwab *et al*., 2007) demonstrated the importance of histone acetylation on the regulation of natural products in *Aspergillus*. Disruption of the *hdaA* gene encoding an *A. nidulans* histone deacetylase led to the increased production of sterigmatocystin and penicillin. Chromatin remodelling can therefore be applied in the context of the MS-based comparative metabolomics to discover new fungal metabolites.

The number of sequenced fungal genomes is growing, and as for *A. flavus*, their analysis has also revealed the existence of a large number of genes putatively involved in secondary metabolite biosynthesis that is much greater than anticipated even in strains that have been extensively studied for the formation of natural products. Hence, the true biosynthetic potential of most secondary metabolite producing fungi remains to be determined, using the approaches mentioned above and advanced MS techniques structure elucidation of metabolites can be more feasible. The use of Fourier transform ion cyclotron resonance MS, possessing the highest mass accuracy among the currently available MS analyzers, can significantly improve the compound identification process. The next generation of TOF instruments, triple TOF, can be introduced into an arsenal of MS instruments used in secondary fungal metabolite identification. Apart from the accurate mass measurements, this MS analyzer provides higher sensitivity compared to commonly used TOF instruments, and at the same time high resolution with fast acquisition speed. It is important to mention that a combination of several analytical techniques might be necessary for the structure elucidation. In particular, NMR can be used, as a complementary tool to MS, to ascertain compound identification. These NMR studies and others, namely bioactivity studies, can be

facilitated by using fungal strains in which key genes are overexpressed to produce large quantities of the metabolites of interest.

Occurrence data for the known toxic fungal metabolites should be continuously collected for monitoring of year-to-year contamination in order to prevent unexpected outbreaks of toxicosis (especially in animals). With the global warming, there may be new opportunities for fungal contamination of crops that were not previously affected. In this regard, untargeted analysis of toxic fungal metabolites should be favoured. In addition, attention should be paid to (re)-emerging issues, such as ergot alkaloids and *Alternaria* toxins. The significance of ergot alkaloid problem was demonstrated in this thesis. Regarding *Alternaria* toxins, it is worth noting that the fungus *Alternaria alternata* is becoming increasingly important due to its ability to produce a diverse range of mycotoxins and its presence in different food and feed commodities. Therefore, a global survey on *Alternaria* toxins would be of great value to establish the pattern of contamination. Risk assessment should be performed for some toxins followed by establishment of regulatory limits in food and feed commodities (example of ergot alkaloids).

The MS based untargeted metabolic profiling approach applied in this thesis is useful for the identification of untargeted compounds from complex biological matrices. Traditionally, this is a tedious and difficult task. A trend is to use a network based analysis approach coupled with a high resolution MS instrument capable of MS/MS data acquisition. The network analysis relies on MS/MS fragmentation, is created through similarity scoring, and can be displayed as a network of nodes and edges. In this way structurally related analogues can be identified. Such analysis implies the acquisition of MS/MS data for all analytes present in the sample. In this regard, MS/MS data independent acquisition (DIA) algorithms will be preferred to data dependant acquisition (DDA). Traditionally, MS/MS DIA lacks specificity. The availability of high speed and resolution quadrupole TOF instruments has allowed the implementation a new DIA algorithm called SWATH (sequential window acquisition of all theoretical fragment ion spectra). SWATH acquisition overall results in improved specificity. SWATH is currently being applied in proteomics studies and is expected to also be an important acquisition approach for the untargeted study of fungal metabolites. In particular, this acquisition mode will play a crucial role in applications where the simultaneous identification of unknown metabolites and their (relative) quantification are desired.

More data are still required to fully understand the association of secondary fungal metabolites with health problems of inhabitants of mouldy environments. Therefore, a closer collaboration of clinical doctors, residents of problematic mouldy environments and analysts should be established; comprehensive surveys of health complaints from inhabitants of mouldy buildings combined with mycotoxin analysis of samples collected in these environments are strongly recommended.

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SUMMARY

Secondary fungal metabolites, a diverse group of organic compounds possessing a range of bioactivity, were the main focus of the current PhD dissertation. The emphasis of the study was specifically put on analytical tools and strategies which can help to acquire a broader knowledge of these natural products.

The definition of secondary fungal metabolites and an overview on their classification were given in **Chapter 1**. Due to enormous diversity in chemical structures, biosynthetic origins and biological effects, classification of secondary fungal metabolites is a challenging task. Although only exemplary classifications were discussed, they formed a complete and clear picture of similarities and differences between the metabolites. Their impact on human and animal health was also mentioned. The different routes of human and animal exposure (ingestion, inhalation, dermal route) to the secondary fungal metabolites were further presented and supported by literature occurrence data.

In **Chapter 2** different analytical techniques applied in the analysis of secondary fungal metabolites were described. Eventually, for the study, it was opted for liquid chromatography coupled to mass spectrometry (LC-MS) owing to its high sensitivity, high-throughput and possibility to confirm the identity of the components present in complex samples. The most advanced mass analyzers as well as major considerations in the MS analysis were discussed. Furthermore, a good insight was given into targeted and untargeted methods developed so far.

Quadrupole time-of-flight MS (QTOF-MS) was utilized in **Chapter 3** to investigate possible occurrence of secondary fungal metabolites in indoor environments. The study was undertaken in relation to the Sick Building Syndrome (SBS), a set of non-specific symptoms related to living or working in a particular building. Fungi and, as a consequence, their secondary metabolites were pointed as one of the causative agents of the SBS. An LC-QTOF-MS method and strategy for identification of untargeted compounds were established and applied to fungal cultures grown on buildings materials and to samples collected in water-damaged mouldy houses where inhabitants experienced health problems (allergy). The method implied full scan data acquisition with accurate mass measurement and statistical analysis (Principal Component Analysis) to extract potential markers from the LC-MS data. The screening revealed presence of a number of secondary metabolites. Some limitations of the method with regards to samples from mouldy houses were described.

The aim of **Chapter 4** was the metabolic profiling of *Aspergillus flavus*, one of the most important species in the *Aspergillus* genus. *A. flavus* is distributed world-wide and has been detected in various foods and feeds. *In silico* analysis computed 55 gene clusters associated with secondary metabolism in *A. flavus*; however, to date, many of these clusters remain uncharacterized with metabolites being assigned to only six clusters. In this study, a comparative metabolomics approach, based on high resolution (Orbitrap) and multiple stage (ion trap) MS, was applied to identify the metabolites associated with *A. flavus* cluster 27 polyketide synthase (*pks*) gene. Several known compounds were yielded by wild-type and mutant *A. flavus*. Interestingly, four metabolites, asparasone A and its newly identified derivatives, were characteristic only to the wild-type, suggesting that *pks* 27 could possibly regulate their production. Moreover, the fragmentation pathway of asparasone A was elucidated for the first time. It was also found that asparasone A was a sclerotial pigment.

The focus of **Chapter 5** was on the improvement of LC-MS analysis of the mycotoxin patulin mostly produced by *Penicillium* spp. Methods to simultaneously analyze patulin and other toxic secondary fungal metabolites, described in the literature, are scarce and require polarity switching of the MS; patulin was omitted in some previous multi-mycotoxin LC-MS/MS methods. In this work, a closer investigation of the electrospray ionization (ESI) of patulin was conducted with the goal of inclusion of this toxin in a single run multi-analyte LC-ESI^+-MS/MS method. Adduct formation of patulin appeared to be the only possibility to achieve the purpose of the study. Combining the advantageous features of ion trap and Orbitrap MS, a fragmentation pathway was proposed confirming the authenticity of the adduct. Further, patulin and 22 other toxic secondary metabolites were successfully included in an LC-ESI^+-MS/MS method. The established method was validated for fluids from transdermal penetration studies with the prospect of assessing exposure to mycotoxins through dermal route.

The issue of ergot alkaloids, toxic secondary metabolites of *Claviceps* spp., in cereals and cereal products was comprehensively studied in **Chapter 6**. Initially, a rapid LC-MS/MS method was developed and validated for the determination of six main ergot alkaloids and their corresponding epimers in cereals and cereal products. The method was applicable to a wide range of cereal matrices as well as grass and corn silages with, as major strength, the low limits of detection and quantification. Careful investigation of method parameters

served in minimizing undesired epimerization of the ergot alkaloids during analysis. A systematic assessment of matrix effects in the LC-MS analysis of these contaminants was carried out providing useful advices in choosing appropriate extraction, chromatographic and detection conditions to reduce MS signal suppression or enhancement, therefore leading to accurate quantitative measurements. The established method was further applied to a European survey of cereals and cereal products intended for human consumption and animal feeding. Representative data on the occurrence of ergot alkaloids were collected and statistically evaluated. The pattern of ergot alkaloids was defined in the different food and feed samples.

A method for monitoring of the 12 ergot alkaloids in buffered solutions was developed. The method was validated and applied to an *in vitro* binding efficacy study of a clay-based binder towards ergot alkaloids with ergometrine, ergosine and ergocornine as model compounds.

Finally, a holistic approach based on high resolution and multiple stage MS was proposed for identification of less studied or novel ergot alkaloid derivatives. Using ion trap MS, fragmentation trees of ergoamides, ergopeptines and ergopeptams were established. Applying the strategy, a number of ergot alkaloids were identified in grain samples.

SAMENVATTING

Secundaire schimmelmetabolieten, een diverse groep van organische componenten, die een verschillende bioactiviteit bezitten, waren de focus van het huidige proefschrift. Meer bepaald, er werd in dit onderzoek gestreefd naar het ontwikkelen van analytische strategieën die kunnen helpen om een beter inzicht over deze natuurlijke producten te verkrijgen.

De definitie van secundaire schimmelmetabolieten en een overzicht van hun classificatie werden in **Hoofdstuk 1** gegeven. Vanwege de enorme diversiteit in chemische structuren, biosynthetische oorsprong en biologische effecten, is de indeling van secundaire schimmelmetabolieten een uitdagende taak. Hoewel slechts een aantal voorbeelden van classificaties werden besproken, geven zij een volledig en duidelijk beeld van gelijkenissen en verschillen tussen de metabolieten. Hun invloed op de menselijke en dierlijke gezondheid werd ook besproken. De verschillende manieren van menselijke en dierlijke blootstelling (oraal, inademen, huidcontact) aan de secundaire schimmelmetabolieten werden verder gepresenteerd en werden door literatuurgegevens omtrent hun voorkomen onderbouwd.

In **Hoofdstuk 2** werden verschillende analytische technieken beschreven, die toegepast zijn voor de analyse van secundaire schimmelmetabolieten. Uiteindelijk werd er in de studie voor vloeistofchromatografie gekoppeld aan massaspectrometrie (LC-MS) gekozen vanwege de hoge gevoeligheid, snelheid van de analyse en de mogelijkheid om de identiteit van de componenten in complexe matrices te bevestigen. De meest geavanceerde massaspectrometers als ook de belangrijke punten in de MS analyse werden besproken. Verder werd een inzicht gegeven van targeted en untargeted methoden die tot nu toe ontwikkeld werden.

Quadrupool time-of-flight MS (QTOF-MS) werd gebruikt in **Hoofdstuk 3** om het mogelijke voorkomen van secundaire schimmelmetabolieten binnenshuis te onderzoeken. De studie werd uitgevoerd in verband met het Sick Building Syndrome (SBS), een reeks van niet-specifieke symptomen die gerelateerd zijn aan het wonen of het werken in een bepaald gebouw. Schimmels en als bijgevolg hun secundaire metabolieten werden beschouwd als één van de oorzaken van het SBS. Een LC-QTOF-MS methode en strategie voor de identificatie van untargeted stoffen werden opgesteld en toegepast op schimmelculturen gegroeid op bouwmaterialen en monsters van vochtige, beschimmelde huizen waar bewoners gezondheidsproblemen (allergie) ervaren. De methode werd gebaseerd op full

scan data acquisitie met accurate massa metingen en statistische analyse (Principal Component Analysis) om potentiële merkers van de LC-MS gegevens te extraheren. De screening heeft de aanwezigheid van een aantal secundaire metabolieten aangetoond. Enkele beperkingen van de methode met betrekking tot monsters van beschimmelde huizen werden beschreven.

Het doel van **Hoofdstuk 4** was metabole profilering van *Aspergillus flavus*, één van de belangrijkste soorten van het *Aspergillus* genus. *A. flavus* is wereldwijd verspreid en wordt gedetecteerd in verschillende voedingsmiddelen en diervoeders. *In silico* analyse heeft 55 gen clusters aangetoond die met een secundair metabolisme in *A. flavus* geassocieerd worden; toch zijn vele van deze clusters nog niet gekarakteriseerd: metabolieten zijn bekend voor slechts zes clusters. In deze studie werd hoge-resolutie (Orbitrap) en multi-stage (ion trap) MS gebaseerde metabolomics toegepast om de metabolieten geassocieerd met het *A. flavus* cluster 27 polyketidesynthase (*pks*) gen te identificeren. Een aantal bekende stoffen werden geproduceerd door wild-type en mutant *A. flavus*. Vier metabolieten, asparasone A en zijn nieuw geïdentificeerde derivaten, waren alleen aanwezig in het wild-type, en dit suggereert dat *pks* 27 een belangrijke rol speelt in hun productie. Bovendien werd het fragmentatiegedrag van asparasone A toegelicht voor de eerste keer. Er werd ook vastgesteld dat asparasone A een sclerotiaal pigment is.

De focus van **Hoofdstuk 5** was de verbetering van de LC-MS analyse van het mycotoxine patuline dat meestal door *Penicillium* spp. wordt geproduceerd. Methoden voor gelijktijdige analyse van patuline en andere toxische secundaire schimmelmetabolieten, die in de literatuur beschreven worden, zijn schaars en vereisen polariteit omschakeling van de MS; patuline werd weggelaten in sommige vorige multi-mycotoxine LC-MS/MS methoden. In dit werk werd een nader onderzoek van de electrospray ionisatie (ESI) van patuline uitgevoerd met als doel dit toxine op te nemen in één run multi-analiet LC-ESI^+-MS/MS methode. Adductvorming van patuline bleek de enige mogelijkheid om het doel van de studie te bereiken. Door het combineren van de gunstige eigenschappen van ion trap en Orbitrap MS werd een fragmentatie route voorgesteld die de identificatie van het adduct heeft bevestigd. Verder werden patuline en 22 andere toxische secundaire metabolieten met succes opgenomen in één LC-ESI^+-MS/MS methode. De ontwikkelde methode werd gevalideerd

voor vloeistoffen van transdermale penetratie studies met het oog op de inschatting van de blootstelling aan mycotoxinen via de dermale route.

Het probleem van ergotalkaloïden, toxische secundaire metabolieten van *Claviceps* spp., in granen en graanproducten werd in detail bestudeerd in **Hoofdstuk 6**. In eerste instantie werd een snelle LC-MS/MS methode ontwikkeld en gevalideerd voor de bepaling van de zes belangrijkste ergotalkaloïden en hun overeenkomstige epimeren in granen en graanproducten. De methode is toepasbaar op een breed range van graan matrices als ook gras- en maïskuilen met de lage detectie- en kwantificatielimieten als groot voordeel. Nauwkeurig onderzoek van de methodeparameters, die ongewenste epimerisatie van de ergotalkaloïden tijdens de analyse kunnen minimaliseren, werd voorgesteld. Een systematische evaluatie van matrix effecten in de LC-MS analyse van deze contaminanten werd uitgevoerd die bruikbare tips opleverde in de keuze van geschikte extractie, chromatografie en detectie condities om MS signaalonderdrukking of -versterking te verminderen en accuraatheid te bevorderen. Als gevolg worden nauwkeurige kwantitatieve resultaten bekomen. De ontwikkelde methode werd verder toegepast op een Europese survey over het voorkomen van ergotalkaloïden in granen en graanproducten bestemd voor menselijke consumptie en diervoeding. Representatieve gegevens over het voorkomen van deze ergotalkaloïden werden verzameld en statistisch geëvalueerd. Het patroon van ergotalkaloïden werd bepaald in de monsters van verschillende voedingsmiddelen en diervoeders.

Een methode voor de bepaling van de 12 ergotalkaloïden in bufferoplossingen werd ontwikkeld. De methode werd gevalideerd en toegepast op een *in vitro* binding effectiviteitsstudie van een klei-gebaseerde binder voor ergotalkaloïden, met ergometrine, ergosine en ergocornine als modelstoffen.

Tot slot werd een holistische benadering op basis van hoge resolutie en multi-stage MS voorgesteld voor de identificatie van minder bestudeerde of nieuwe ergotalkaloid derivaten. Met behulp van ion trap MS werd het fragmentatiegedrag van ergoamides, ergopeptines en ergopeptams vastgesteld. Door het toepassen van deze strategie werden een aantal van ergotalkaloïden in graan monsters geïdentificeerd.

CURRICULUM VITAE

Svetlana V. Malysheva works as a doctoral researcher at the Laboratory of Food Analysis (Faculty of Pharmaceutical Sciences, Ghent University) since November 2008.

PERSONAL DATA:

Citizenship: Russian

Birth place: Saratov, Russia

Birth date: 16 December 1985

WORK ADDRESS:

Laboratory of Food Analysis, Department of Bio-Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

CONTACT:

Tel.: + 32 9 264 81 16; Fax: + 32 9 264 81 99

email: Svetlana.Malysheva@UGent.be; svetlana.vl.malysheva@gmail.com

HIGHER EDUCATION:

Ecologist (in the field of chemistry), Chemistry Faculty, Saratov State University, Saratov, Russia, duration of study: 2005-2008

Chemist with the major in chemistry (Master diploma with honors), Chemistry Faculty, Saratov State University, Saratov, Russia, duration of study: 2003-2008

GRANTS:

Finalizing PhD grant from Ghent University for a period of 12 months awarded by the Research Council on 25 August 2011; grant code: 01DI2611, starting date: 1 January 2012

SUPERVISION OF PRACTICUMS AND MASTER THESES:

Practicum

"Bepaling van ergot alkaloïden in granen met behulp van een LC-MS/MS method" ("Determination of ergot alkaloids in grains by means of LC-MS/MS"), Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University, November 2012

"Totaal D-iso-citroenzuurgehalte" ("Total D-iso-citric acid content"), Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, Ghent University, November 2010

Master theses

"Ontwikkeling en validatie van een UPLC-MS/MS methode ter ondersteuning van in vitro testen op bindingefficiëtie van kleibinders op ergot alkaloïden" ("Development and validation of an UPLC-MS/MS method in support of *in vitro* testing of binding efficiency of binders for ergot alkaloids"),

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   *These authors contributed equally to this work

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   *These authors contributed equally to this work

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   *These authors contributed equally to this work

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    *These authors contributed equally to this work
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PARTICIPATION IN SCIENTIFIC CONFERENCES:

Oral presentations:

35th Mycotoxin Workshop (Ghent, Belgium), 22-24 May 2013: "Metabolic profiling of *Aspergillus flavus*: identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multiple stage mass spectrometry", Malysheva SV, Arroyo-Manzanares N, Cary JW, Ehrlich KC, Vanden Bussche J, Vanhaecke L, Bhatnagar D, Diana Di Mavungu J, De Saeger S

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HTC-12: Twelfth International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analyzers (Bruges, Belgium), 1-3 February 2012:

"A class targeted metabolomic approach based on liquid chromatography-mass spectrometry for the screening and identification of ergot alkaloids in cereals and cereal products", Diana Di Mavungu J, Malysheva SV, Vanhaecke L, De Saeger S

"LC-MS/MS analysis of ergot alkaloids in cereals: Robustness testing and evaluation of between and within commodities matrix effects", Malysheva SV, Diana Di Mavungu J, Robbens J, Dubruel P, De Saeger S

5th International Symposium on Recent Advances in Food Analysis (RAFA) (Prague, Check Republic), 1-4 November 2011: "A systematic assessment of the variability of matrix effects in LC-MS/MS analysis of ergot alkaloids in cereals", Diana Di Mavungu J, Malysheva SV, Larionova D, Robbens J, Dubruel P, Van Peteghem C, De Saeger S

4th International Symposium Mycotoxins: Challenges and Perspectives (Ghent, Belgium), 24 May 2011: "New insights into electrospray ionization of patulin", Malysheva SV, Diana Di Mavungu J, Boonen J, De Spiegeleer B, Goryacheva IYu, Van Peteghem C, De Saeger S

BSMS meeting (Grand Hornu historical industrial site, Hornu-Mons, Belgium), 29 April 2011: "Mass spectrometric investigation of the fragmentation pattern of patulin in different mobile phases", Malysheva SV, Diana Di Mavungu J, Boonen J, De Spiegeleer B, Van Peteghem C, De Saeger S

The World Mycotoxin Forum – 6th conference (Noordwijkerhout, the Netherlands), 8-10 November 2010: "Simultaneous determination of six major ergot alkaloids and their corresponding epimers by LC-MS/MS: Method development, validation and application to various food and feed matrices", Diana Di Mavungu J, Sanders M, Larionova D, Malysheva SV, Robbens J, Dubruel P, Van Peteghem C, De Saeger S

Indoor air quality in different living settings. Results of investigation and consequences in terms of decision making (Committee of the Regions, Brussels, Belgium), 18 October 2010: "Mycotoxins – indoor air pollutants?!", Malysheva SV, Polizzi V, Adams A, Delmulle B, Van Peteghem C, De Kimpe N, De Saeger S

32nd Mycotoxin Workshop (Technical University of Denmark, Copenhagen, Denmark), 14-16 June 2010: "Untarget screening of fungal metabolites produced by *Penicillium* by means of LC-Q-TOF-MS", Malysheva SV, Polizzi V, Van Peteghem C, Adams A, De Kimpe N, Moretti A, Goryacheva IYu, Van Bocxlaer J, De Saeger S

6th International Symposium on Hormone and Veterinary Drug Residue Analysis (Ghent University, Ghent, Belgium), 1-4 June 2010: "Possibility of untarget screening by LC-Q-TOF-MS", Malysheva SV, Polizzi V, Van Peteghem C, Adams A, De Kimpe N, Deforce D, Van Bocxlaer J, De Saeger S

BSMS meeting (Woluwé Campus, Catholic University of Louvain, Brussels, Belgium), 16 April 2010: "Untarget screening by LC-Q-TOF-MS applied to fungal metabolites", Malysheva SV, Polizzi V, Van Peteghem C, Adams A, De Kimpe N, Deforce D, Van Bocxlaer J, De Saeger S

3rd Symposium on Mycotoxins: Threats and Risk Management (Het Pand, Ghent University, Ghent, Belgium), 28 April 2009: "Mycotoxins in mouldy interiors from water-damaged buildings", Malysheva SV, Polizzi V, Van Peteghem C, De Kimpe N, Van Bocxlaer J, De Saeger S

PARTICIPATION IN SEMINARS, WORKSHOPS AND COURSES:

RIC Seminar Life Sciences (Kennedyhotel, Kortrijk, Belgium), 24 January 2013

Symposium "Towards smaller particles in HPLC: Fused-core and UPLC" (Ghent University, Ghent, Belgium), 16 November 2012

Applying for a postdoctoral job, 6h (UCT, Ghent University, Ghent, Belgium), 4, 11, 18 September 2012

Advanced Academic English: Writing Skills, 20h (UCT, Ghent University, Ghent, Belgium), 2 March-1 June 2012

Project Management, 6.5h (Institute for Continuing Education in Science, Ghent University, Belgium), 8 and 10 May 2012

Workshop "High Resolution Mass Spectrometry in Quantitative Analysis and Screening of Organic Contaminants in Food and Environment" (RIKILT, the Netherlands), 16 February 2012

Personal Efficiency, 20h (Doctoral Schools of Life Sciences and Medicine, Ghent University, Ghent, Belgium), 8, 15 and 22 February 2012

Advanced Academic English: Conference Skills, 20h (UCT, Ghent University, Ghent, Belgium), 21 February-23 May 2011

Seminar on HPLC by Sigma-Aldrich (Ghent University, Ghent, Belgium), 26 October 2010

Workshop "Pipetting and Calibration" (Louvain, Belgium), 21 October 2010

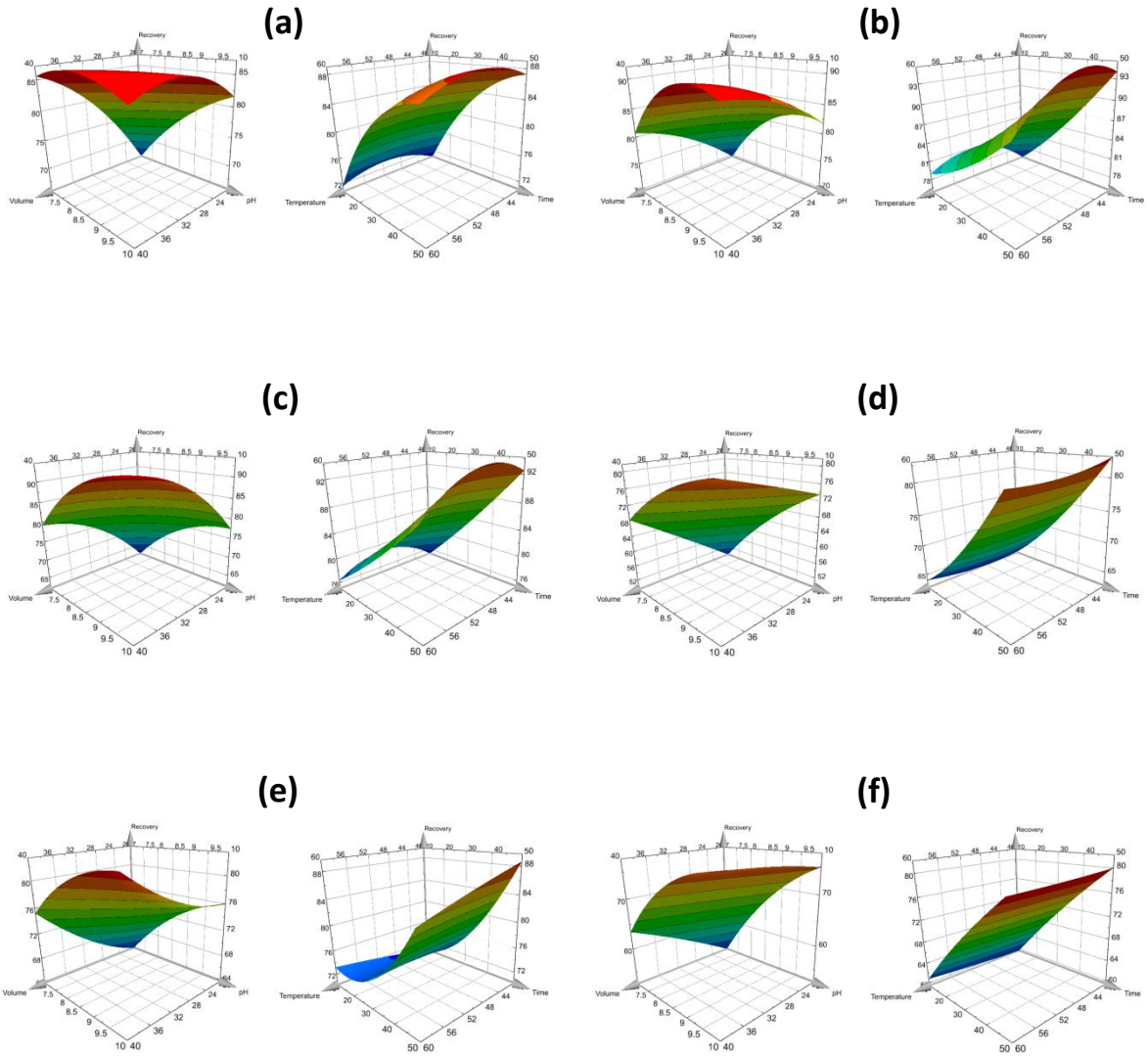
Seminar "How confident are you with your results" (Scientific Institute of Public Health, Brussels, Belgium), 4 December 2009

Module 3: Introductory Statistics. Basics of statistical inference. Theory 14h, practice 14h. (Institute for Continuing Education in Science, Ghent University, Belgium), 24, 26 November, 1, 3, 8, 10, 15 December 2009

"Waters Metabolic Profiling Seminar" (VIB Department of Plant Systems, Ghent University, Ghent, Belgium), 27 October 2009

APPENDICES

Appendix I: *Response surface plots for recovery of ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l), methylergometrine (m), dihydroergotamine (n) as a function of extraction solvent volume and pH of the NH_4HCO_3 solution in the extraction solvent, and solvent evaporation temperature and extraction time.*



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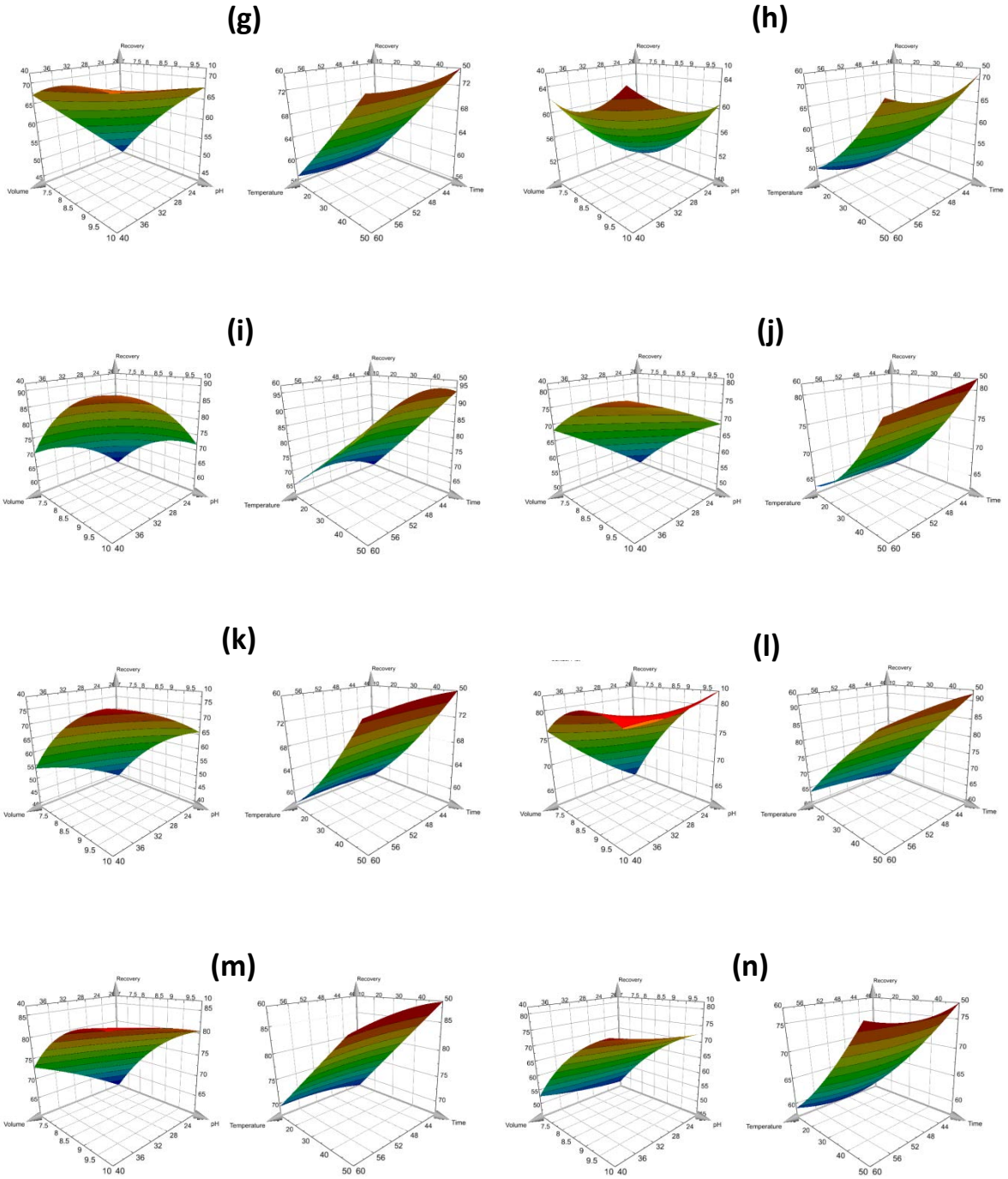
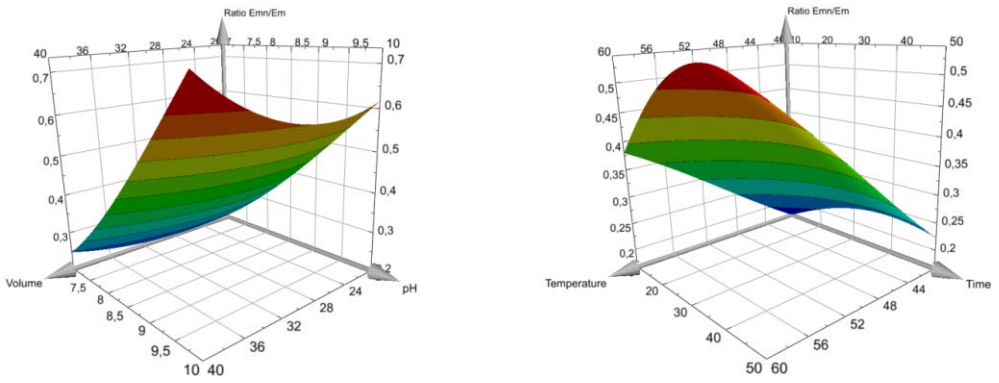
Appendix I (continued)

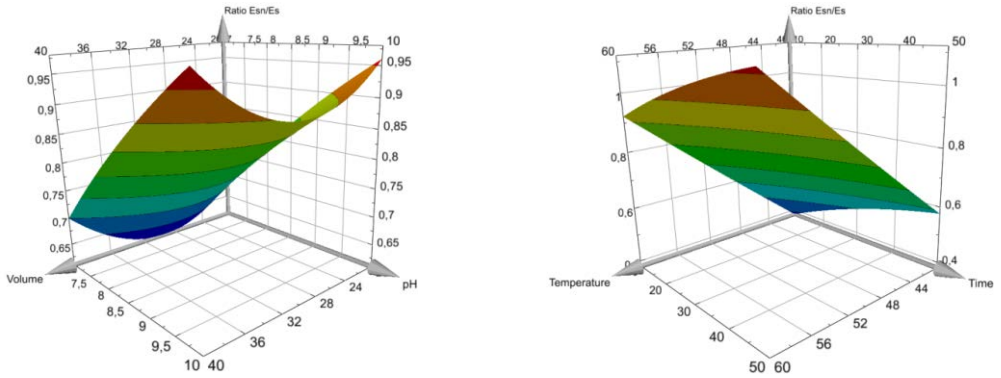


Appendix II: *Response surface plots for the ratio between ergometrinine (Emn) and ergometrine (Em) (a), ergosinine (Esn) and ergosine (Es) (b), ergotaminine (Etn) and ergotamine (Et) (c), ergocorninine (Econ) and ergocornine (Eco) (d), ergokryptinine (Ekrn) and ergokryptine (Ekr) (e), ergocristinine (Ecrn) and ergocristine (Ecr) (f) as a function of extraction solvent volume and pH of the NH_4HCO_3 solution in the extraction solvent, and solvent evaporation temperature and extraction time.*

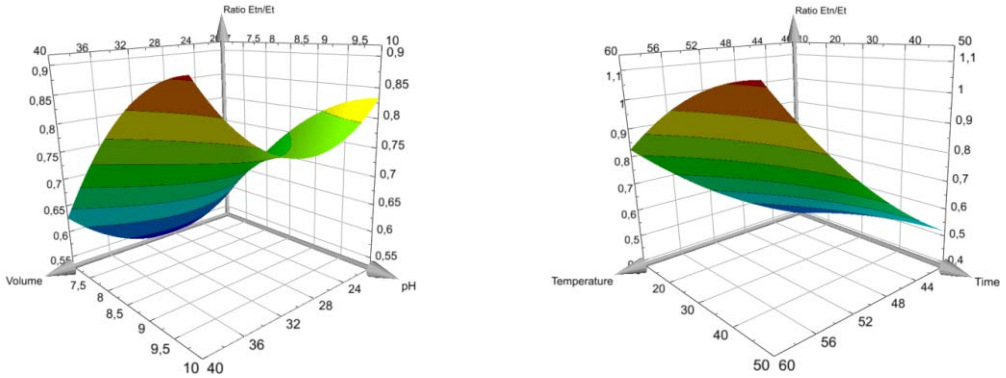
(a)



(b)



(c)



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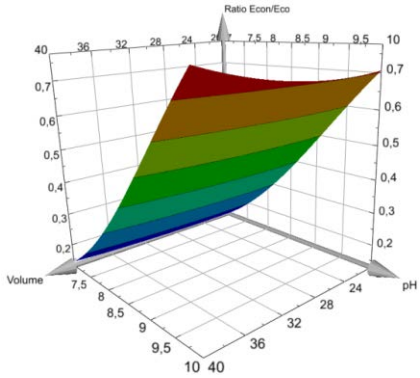
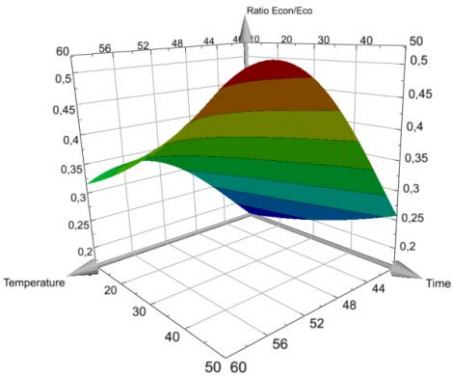
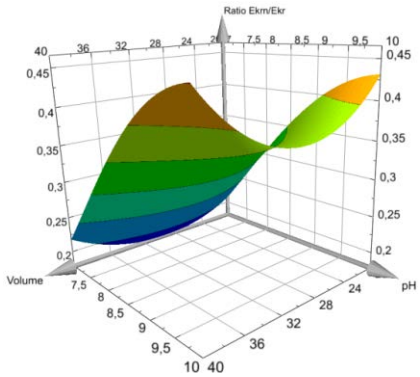
Appendix II (continued)

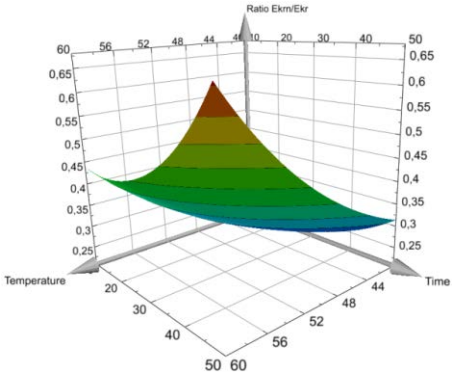
(d)





(e)





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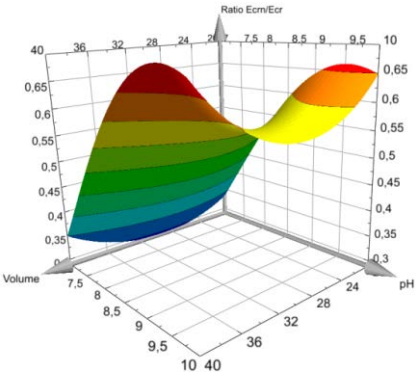
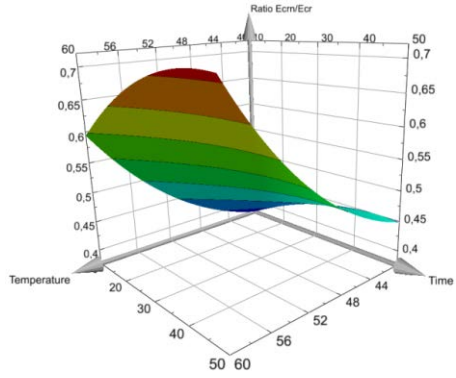
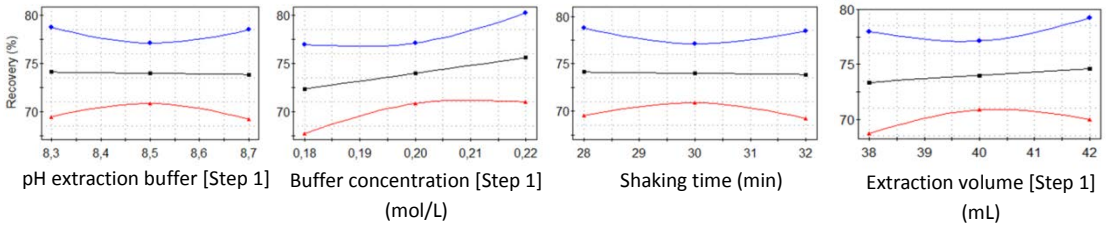
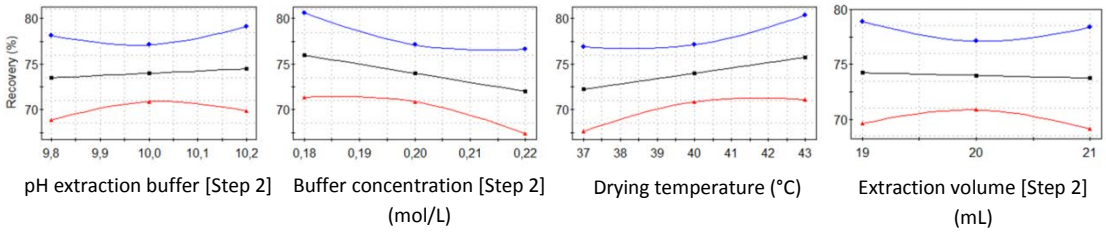




Appendix III: *Robustness evaluation of extraction buffer pH and concentration, shaking time, drying temperature and extraction volume for ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l), methylergometrine (m), dihydroergotamine (n). The 95 % confidence interval is shown.*

(a)





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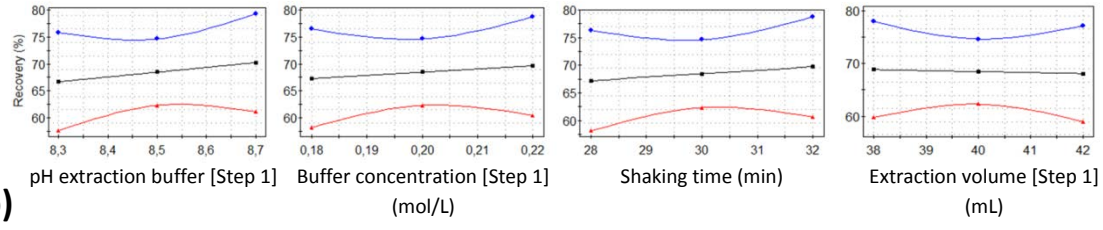

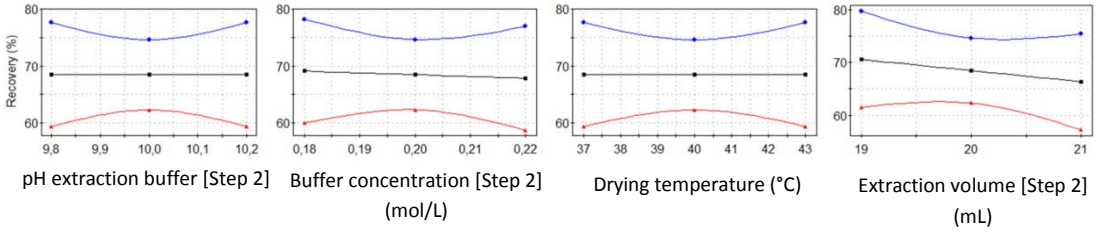

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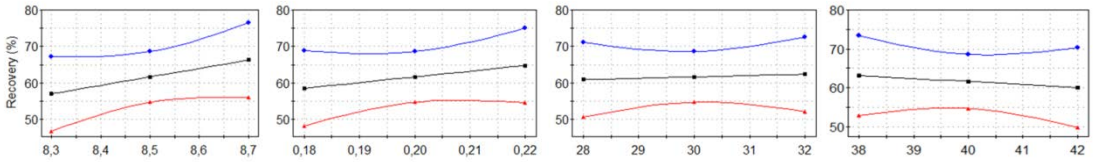
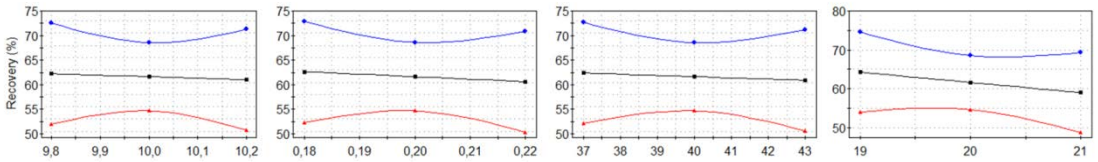
Appendix III (continued)

(c)





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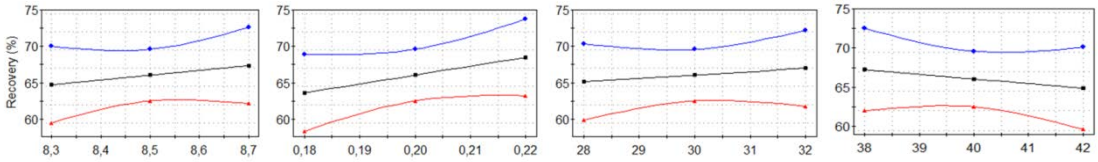
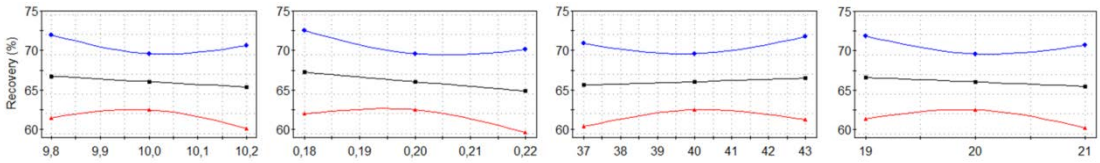
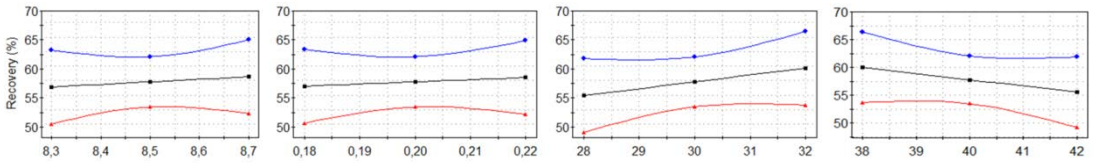
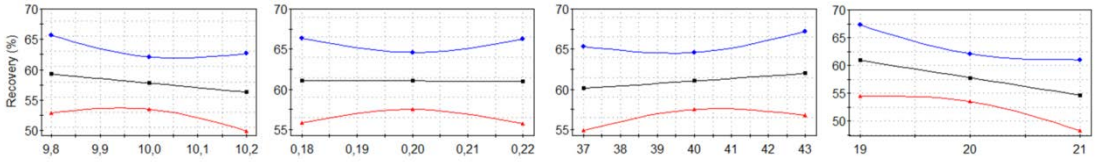




(e)





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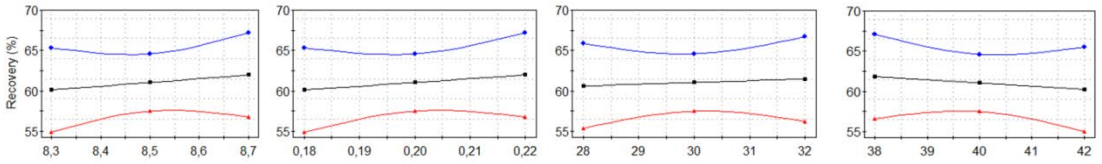
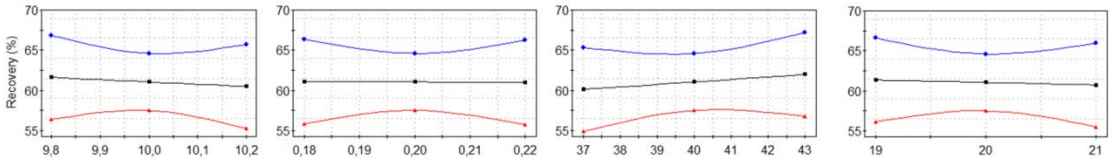
Appendix III (continued)

(f)





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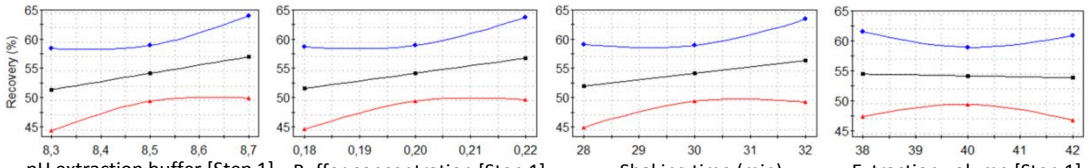

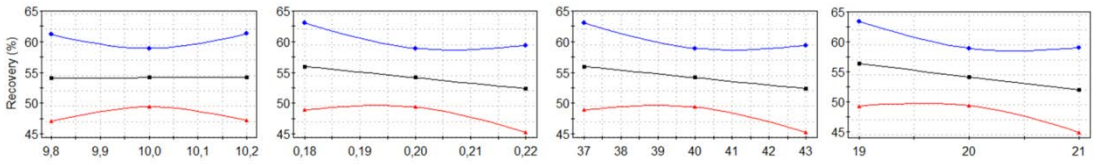

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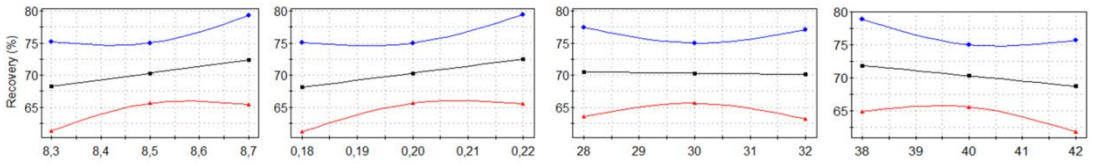

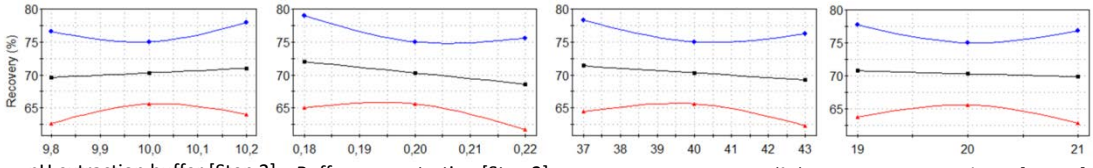

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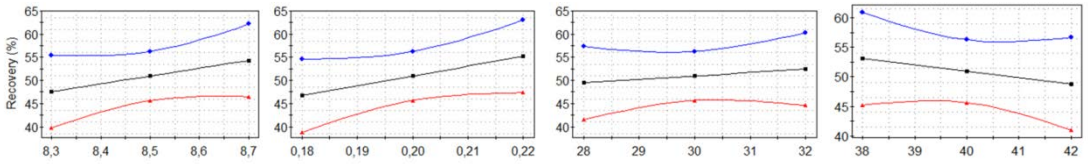
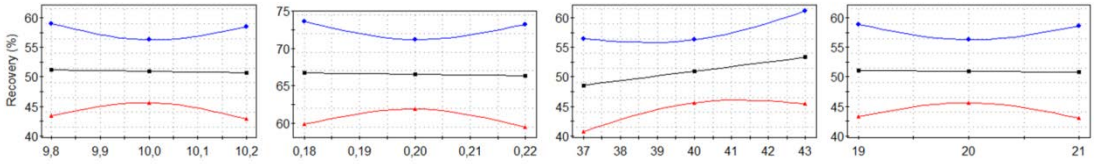
(i)





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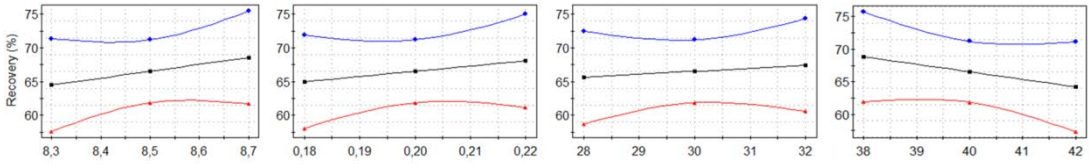

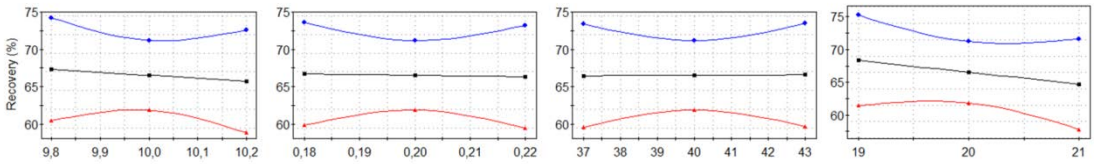

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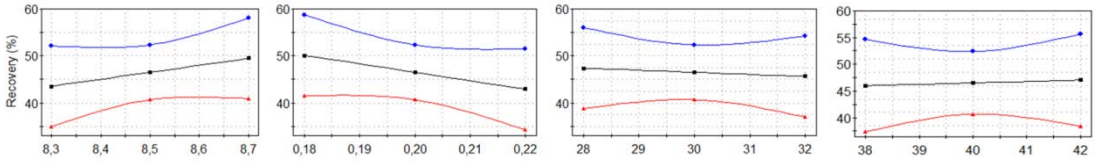

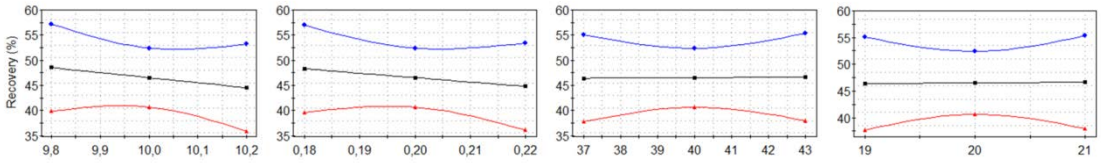

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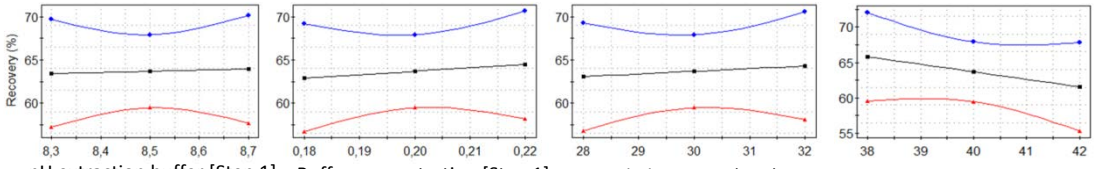
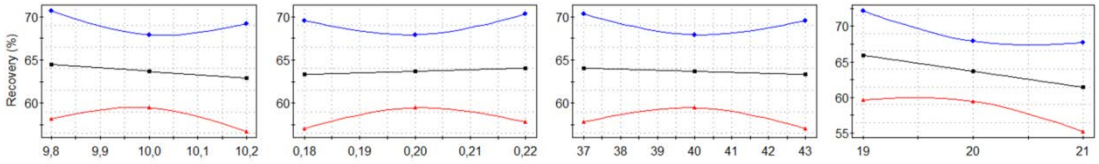
Appendix III (continued)

(l)





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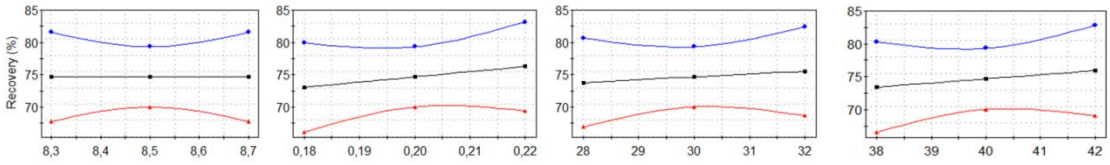

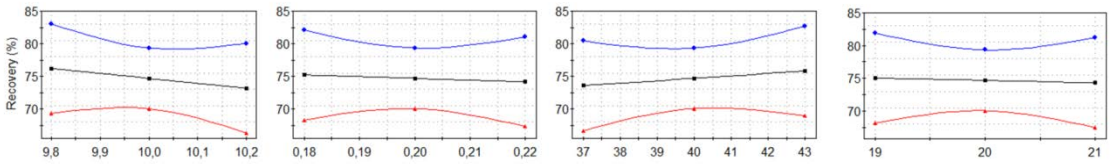

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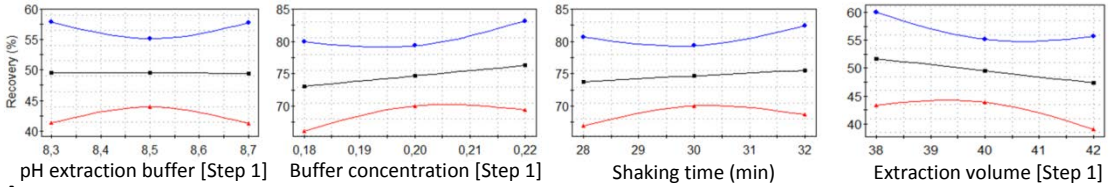
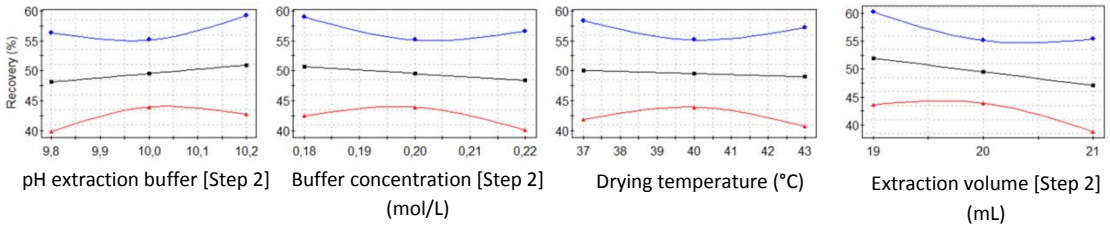
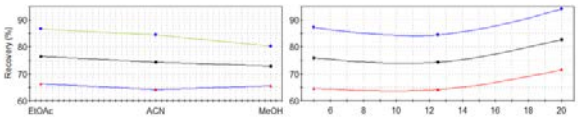


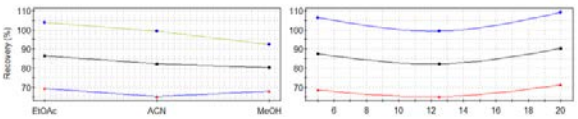


Appendix IV: *Optimization of elution solvent composition applying a D-optimal design for ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l). The 95 % confidence interval is shown.*

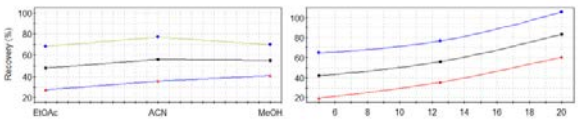
(a)



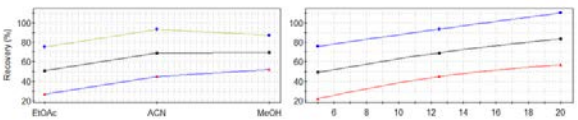
(b)



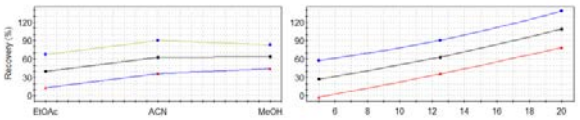
(c)



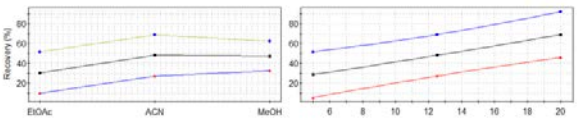
(d)



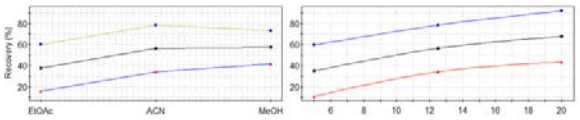
(e)



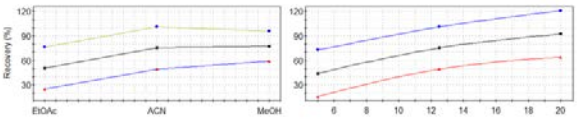
(f)



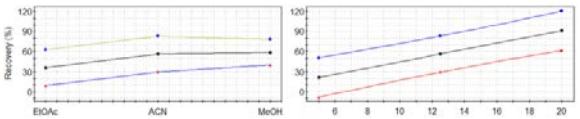
(g)



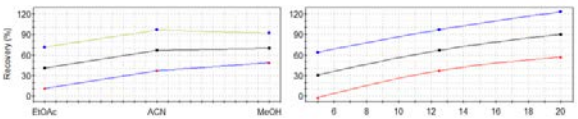
(h)



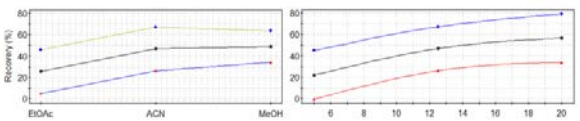
(i)



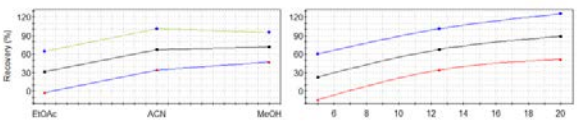
(j)



(k)

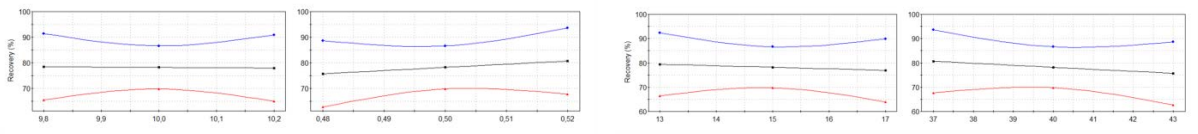


(l)

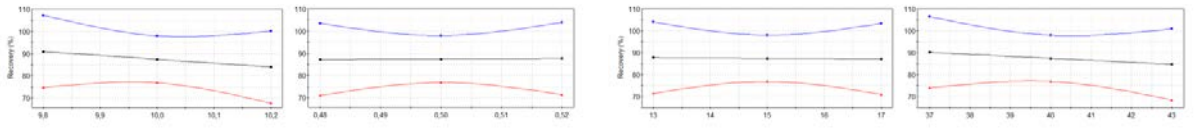


Appendix V: *Robustness evaluation indicating the change of recovery for ergometrine (a), ergometrinine (b), ergosine (c), ergosinine (d), ergotamine (e), ergotaminine (f), ergocornine (g), ergocorninine (h), ergokryptine (i), ergokryptinine (j), ergocristine (k), ergocristinine (l), methylergometrine (m), dihydroergotamine (n) as a function of pH and concentration of the buffer, shaking time and temperature of drying. The 95 % confidence interval is shown.*

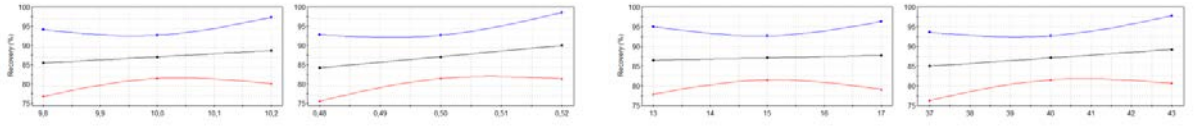
(a)



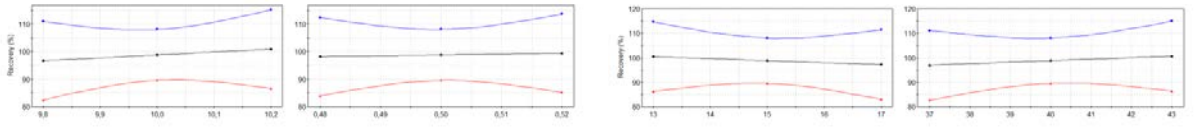
(b)



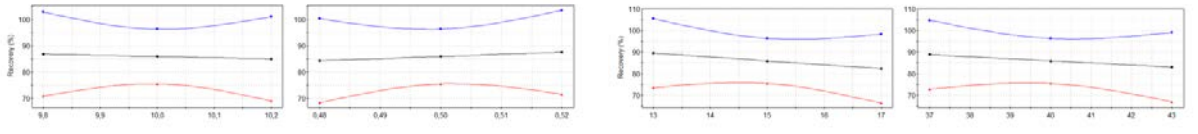
(c)



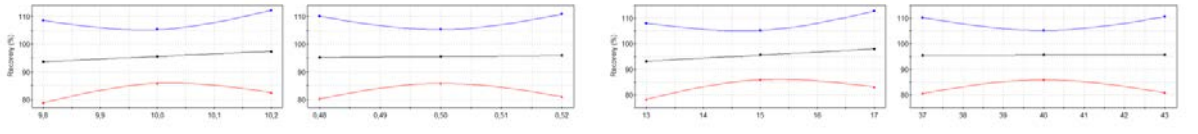
(d)



(e)



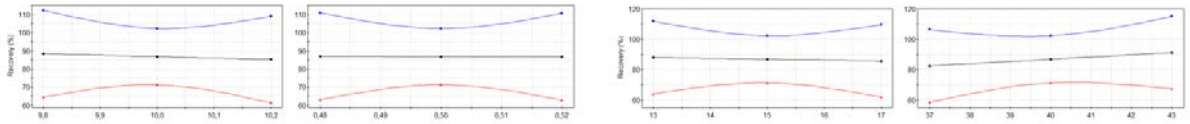
(f)



Continue

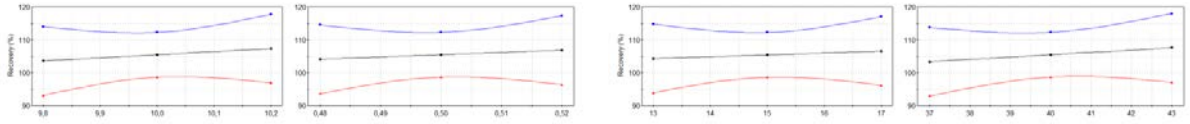
Appendix V (continued)

(g)



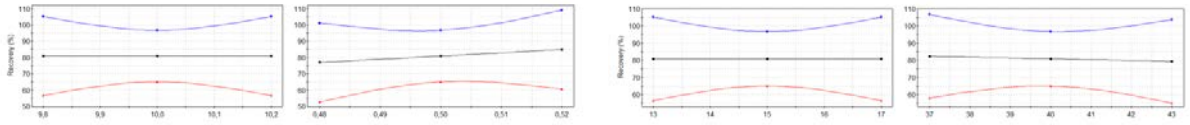
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(h)



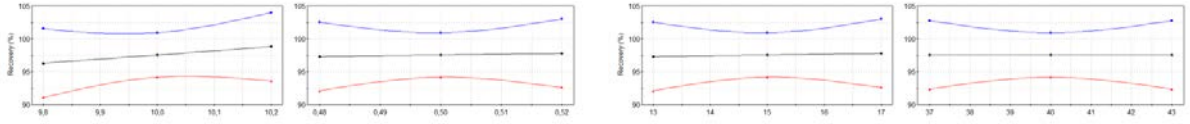
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(i)



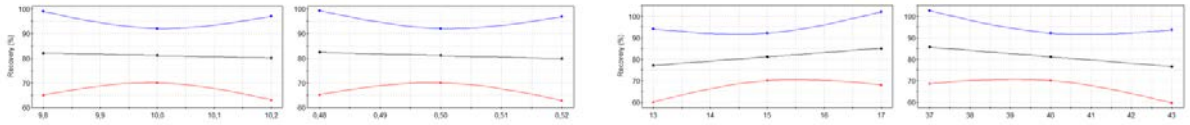
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(j)



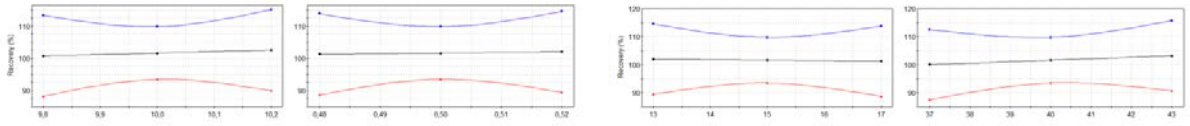
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(k)



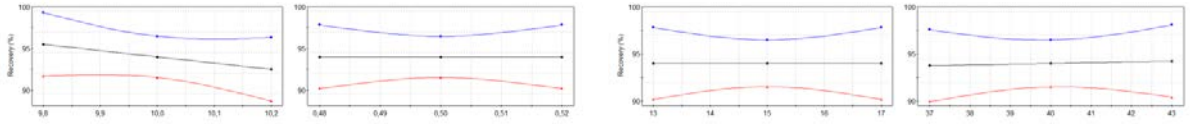
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(l)



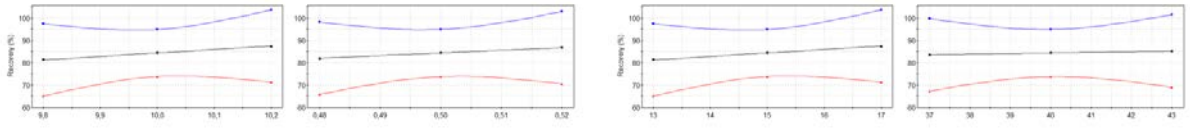
pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(m)



pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)

(n)



pH of extraction buffer | Concentration of buffer (mol/L) | Shaking time (min) | Temperature of drying (°C)