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Occurrence and toxicity of microbial foodborne cyclic depsipeptides

Cereulide, beauvericin and enniatins

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LIST of ABBREVIATIONS and SYMBOLS

ACAT	Acyl CoA–cholesterol–acyl transferase
ADI	Acceptable daily intake
ALOP	Appropriate Level of Protection
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ARfD	Acute reference dose
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BEA	Beauvericin
bw	Body weight
CAGs	Cumulative assessment groups
CASA	Computer assisted sperm analysis
CER	Cereulide
ces	Cereulide synthetase
CFU	Colony-forming unit
CNS	Central nervous system
CONTAM	Panel on Contaminants in the Food Chain
CytK	Cytotoxin K
CZE	Capillary zone electrophoresis
DAD	Diode-array detector
D-Hiv	D-2-hydroxyisovaleric acid

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DON	Deoxynivalenol
dSPE	Dispersive solid-phase extraction
EC	European Commission
EC_{50}	50 % effective concentration
ECAR	Extracellular acidification rate
ECDC	European Centre for Disease Prevention and Control
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ENNs	Enniatins
EPA	United States Environmental Protection Agency
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
ETC	Electron transport chain
FAO	Food and Agriculture Organization of the United Nations
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FDA	Food and Drug Administration
FHB	Fusarium head blight
FQPA	Food Quality Protection Act
FUS	Fusaproliferin
HBL	Hemolysin BL

HPLC	High-performance liquid chromatography
IC_{50}	50 % inhibitory concentration
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KCZ	Ketoconazole
LC	Liquid chromatography
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
M(R)L	Maximum (residue) level
MDR	Multidrug resistance
MOE	Margin of exposure
MON	Moniliformin
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHE	Non-hemolytic enterotoxin
NK	Natural killer
NOAEL	No-adverse-effect level
NRPS	Non-ribosomal peptide synthetase
OCR	Oxygen consumption rate
OPP	Office of Pesticide Programs
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
QqQ	Triple quadropole
QSAR	Quantitative structure activity relationship
QuEChERS	"Quick, easy, cheap, effective, rugged, and safe"
R^2	Coefficient of determination
R_{app}	Apparent recovery
REPFED	Refrigerated processed foods of extended durability
RMS_{bias}	Root mean square of the bias
ROS	Reactive oxygen species
RSD	Relative standard deviation
RTH	Ready-to-heat
RTR	Ready-to-reheat
SAM	*S*-adenosylmethionine
SAX	Strong anion exchange
S_{bias}	Accuracy of the bias
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks
SFE	Supercritical fluid extraction
SIDA	Stable isotope dilution analysis
SPE	Solid–phase extraction
SPS	Application of Sanitary and Phytosanitary Measures
SRB	Sulforhodamine B
SRC	Spare respiratory capacity
TCA	Trichloroacetate

TDI	Tolerable daily intake
TOF	Time of flight
U	Combined expanded measurement uncertainty
u	Measurement uncertainty
$U[C_{ref}]$	Uncertainty of the purity of the toxin standard
u_c	Combined standard uncertainty
UV	Ultraviolet
VAL	Valinomycin
WHO	World Health Organization

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"Man's mind, once stretched by a new idea, never regains its original dimensions."

— Oliver Wendell Holmes

And suddenly, 4 years have passed. I started on the day they told me ‘to cool down near the Premier’. By that time, I didn’t realize that my love-hate relationship with the Premier (aka Waters Quattro Premier XE™ Mass Spectrometer) would very soon begin. The last 4 years have been a period of inspiration and curiosity, of surprises and disappointments, of ups and downs, of excitement and frustration. But finally, the moment has come to express my gratitude to all who have supported and encouraged me to sustain this journey.

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Humble but also proud, I present you now my PhD. A piece of work, which was not possible without all your encouragement! And for that: THANK YOU!

Marlies

Summary

Summary

In **Chapter 1,** a general introduction based on literature review is presented in order to provide background for the following experimental parts. An overview is given of the chemical structure, biosynthesis, occurrence in food (and feed), biological and toxic properties and the most common detection techniques of cereulide (CER), beauvericin (BEA) and enniatins (ENNs). The second part of Chapter 1 explains the link between these cyclic depsipeptides originating from a different background (fungal *vs.* bacterial). Due to the possible co-exposure to these toxins, the importance of risk assessment of chemical mixtures is highlighted.

In **Chapter 2** of this study, the development and validation of a multi-mycotoxin LC-MS/MS method for the determination of BEA, ENNs and CER is described in detail. Four matrices were selected based on previously reported occurrence data of BEA, ENNs and CER: maize, wheat, rice and pasta. Although these emerging foodborne toxins are of different microbial origin, the similar structural, toxicological and food safety features provided rationale for their concurrent detection in relevant food matrices. Different approaches for sample preparation were tested with the aim of minimizing the sample treatment with acceptable extraction recoveries (85-105 %). In addition, the LC and MS/MS conditions were optimized in order to achieve a sensitive (LOD ≤ 1.0 $\mu g.kg^{-1}$ and LOQ ≤ 2.9 $\mu g.kg^{-1}$) and rapid (7 min) detection. The obtained sensitivities allow detection of relevant toxicological concentrations. All relative standard deviations for repeatability (intra-day) and intermediate precision (inter-day) were lower than 20 %. Trueness, expressed as the apparent recovery, varied from 80 to 107 %. After the successful development and validation, the multi-mycotoxin LC-MS/MS methods were applied to 59 samples (maize, wheat, pasta and rice) to have a first indication of the occurrence. As expected, no CER was found in the analyzed samples. Moreover, no detectable levels of any of the toxins were observed in the rice samples. In addition, the results indicated that 56 % of the samples were contaminated with at least one mycotoxin and that ENN B was the most prevalent mycotoxin.

Chapter 3 subsequently comprises the application of the previously developed method to Belgian wheat samples harvested in 2015 and 2016 to gather occurrence data on CER, BEA and ENNs. As expected, in none of the samples CER was found. The results revealed that the mycotoxin contamination level in season 2015-2016 was much higher than in season 2014-2015, especially for

ENN B, B1 and ENN A1. Because wheat is commonly infected by various *Fusarium* species leading to Fusarium head blight (FHB), quantitative occurrence data on *Fusarium* species were gathered using qPCR. Subsequently, a correlation study was performed to assess the link between the occurrence of *Fusarium* species and their mycotoxin production. Additionally, the relation of fungicide treatments and wheat genotypes with mycotoxin levels were evaluated. Results indicated significant positive correlations between ENNs and *F. avenaceum* and between BEA and *F. poae*. In search of an explanation for the great inter-season variability in mycotoxin levels, the weather conditions were evaluated. It was observed that more rainfall during the May, June and beginning of July is associated with higher mycotoxin levels. However, this observation was based on only two growing seasons.

In addition, in **Chapter 4** a detection method for emerging *Fusarium* mycotoxins BEA, ENNs and MON was developed for leek samples due to the observation of *F. avenaceum* in Belgian leek samples. A reliable, sensitive and selective method was developed to determine the different *Fusarium* mycotoxins (MON, BEA and ENNs) simultaneously in leek using LC-MS/MS. Sample preparation was based on a standard solvent extraction step using ACN without any further clean-up procedure. The average recoveries ranged from 81 to 91 %. The LOD and LOQ varied respectively between 0.3-1.4 $\mu g.kg^{-1}$ and 0.8-4.2 $\mu g.kg^{-1}$. In addition, the developed method was successfully applied to 76 leek samples that were artificially infected with *F. avenaceum* to investigate potential of this species to produce the selected toxins. Remarkably, *F. avenaceum* mycotoxins were found on non-symptomatic leeks samples.

Chapter 5 describes the *in vitro* evaluation of the (sub)chronic toxicity of low doses of CER using Seahorse XF analyzer. While acute effects have been studied in the aftermath of food poisoning, repeated exposure to low doses of CER might cause unnoticed damages to intestines and liver. The toxicity, which relies on the mitochondrial damage, was assessed on Caco-2 and HepG2 cells after prolonged exposure (ten days) to low doses of CER. Extracellular flux analyses were used to study the impact of low doses of CER on the bioenergetic functions of undifferentiated Caco-2 and HepG2 cells. Both Caco-2 and HepG2 cells experienced measurable mitochondrial damage after prolonged exposure of ten days to 0.25 nM of CER. Observed mitochondrial dysfunction was largely reflected in reduction of maximal cell respiration. At 0.50 nM CER, mitochondrial respiration was almost completely shut down, especially in HepG2 cells. These results corresponded with severe reduction in amount of cells and altered morphology observed by microscopic examination of the cells. This

indicated that Seahorse XF Cell Mito Stress Test is a suitable model for the sensitive identification of mitochondrial toxicity.

As a conclusion, the broader international context, relevance and future perspectives related to this research are discussed in **Chapter 6**.

Samenvatting

Samenvatting

In Hoofdstuk 1 wordt een algemene inleiding gegeven gebaseerd op een literatuurstudie om de nodige achtergrondinformatie te verschaffen voor het daaropvolgend experimenteel gedeelte van dit onderzoek. In dit overzicht ligt de nadruk op de chemische structuur, de biosynthese, het voorkomen in voeding en voeder, de biologische en toxische eigenschappen alsook de meest voorkomende detectietechnieken van cereulide, beauvericine en enniatines. Het tweede deel van Hoofdstuk 1 legt het verband tussen deze cyclische depsipeptides ondanks hun verschillende oorsprong (schimmel *vs.* bacterieel). Aangezien deze toxines mogelijks samen kunnen voorkomen wordt het belang van risicoanalyse van chemische mengsels beschreven.

In **Hoofdstuk 2** van dit onderzoek wordt de ontwikkeling en validatie van een LC-MS/MS methode voor de gelijktijdige bepaling van BEA, ENNs and CER in detail beschreven. Hiervoor werden vier soorten stalen geselecteerd nl. mais, tarwe, rijst en pasta. Op basis van de gelijkaardige chemische structuur en eigenschappen kan eenzelfde aanpak worden gehanteerd voor de ontwikkeling van een detectie methode voor deze 'opkomende' toxines. Verschillende procedures werden getest met als doel het minimaliseren van de staalvoorbereiding met aanvaardbare extractie efficiëntie (85-105 %). Bovendien werden de LC en MS/MS condities geoptimaliseerd om een gevoelige (detectielimiet ≤ 1.0 $\mu g.kg^{-1}$ en kwantificatielimiet ≤ 2.9 $\mu g.kg^{-1}$) en snelle (7 min) detectie te bekomen. Deze gevoelige methode laat toe om lage concentraties te detecteren die van belang kunnen zijn bij toxicologisch onderzoek. Alle relatieve standaardafwijkingen voor herhaalbaarheid (op dezelfde dag) en reproduceerbaarheid (tussen verschillende dagen) waren lager dan 20 %. Juistheid, uitgedrukt als de '*apparent recovery*' varieerde van 80 tot 107 %. Na de succesvolle ontwikkeling en validatie van de multi-toxine LC-MS/MS methode werd ze toegepast op 59 stalen (mais, tarwe, pasta en rijst) om een eerste indicatie te krijgen van het voorkomen van deze toxines. Zoals verwacht werd er geen CER gevonden in de geanalyseerde stalen. Bovendien werd in geen enkele van de rijststalen toxines teruggevonden. De resultaten gaven ook aan dat 56 % van de stalen gecontamineerd waren met minstens één mycotoxine en dat ENN B de meest voorkomende mycotoxine was.

De geoptimaliseerde methode werd vervolgens toegepast in **Hoofdstuk 3** op Belgische tarwestalen die geoogst werden in 2015 en 2016 om gegevens te verzamelen over het voorkomen van CER, BEA en ENNs. Opnieuw werd er geen CER gevonden in de tarwe stalen. De resultaten toonden aan dat de

contaminatie in seizoen 2015-2016 veel hoger was dan in het seizoen 2014-2015, vooral voor ENN B, B1 en ENN A1. Omdat tarwe vaak geïnfecteerd wordt door *Fusarium* soorten, werden ook kwantitatieve gegevens verzameld over het voorkomen van verschillende *Fusarium* soorten met behulp van qPCR. Vervolgens werd in een correlatie studie op zoek gegaan naar het verband tussen *Fusarium* soorten en hun mycotoxine productie. Daarbovenop werd de invloed van fungicides en tarwe genotypes op de mycotoxines geëvalueerd. De resultaten toonden een positieve correlatie aan tussen ENNs en *F. avenaceum* en tussen BEA en *F. poae*. Omwille van de grote verschillen in mycotoxine concentraties tussen de verschillende seizoenen werd bijkomend op zoek gegaan naar de impact van weersomstandigeheden. Hierbij werd vastgesteld dat veel neerslag in mei, juni en het begin van juli resulteerde in hogere mycotoxine concentraties. Deze resultaten moeten echter met voorzichtigheid worden geïnterpreteerd aangezien ze slechts over twee seizoenen gaan.

Daarnaast werd er in **Hoofdstuk 4** een detectiemethode ontwikkeld voor de opkomende *Fusarium* mycotoxines BEA, ENNs and MON, nadat *F. avenaceum* werd teruggevonden op geïnfecteerde preistalen. Een betrouwbare, gevoelige en selectieve methode werd ontwikkeld om verschillende *Fusarium* mycotoxins (MON, BEA en ENNs) in prei te bepalen met behulp van LC-MS/MS. Voor de staalvoorbereiding werd ACN als extractie solvent gebruikt. De gemiddelde extractie efficiëntie varieerde van 81 tot 91 %. De detectielimiet en kwantificatielimiet varieerden respectievelijk tussen 0.3-1.4 $\mu g.kg^{-1}$ en 0.8-4.2 $\mu g.kg^{-1}$. De ontwikkelde methode werd met succes toegepast op 76 preistalen die kunstmatig werden geïnfecteerd met *F. avenaceum* om te onderzoeken in welke mate deze schimmel de mycotoxines MON, BEA en ENNs produceert. Opvallend was dat deze mycotoxines werden gevonden in stalen die geen ziektesymptomen vertoonden.

Hoofdstuk 5 beschrijft de *in vitro* evaluatie van de (sub)chronische toxiciteit van lage dosissen van CER met behulp van de Seahorse XF analyzer. De acute effecten van CER werden reeds beschreven in het kader van voedselvergiftigingen. Over de herhaaldelijke blootstelling aan lage dosissen van CER die mogelijks ongemerkt schade kunnen toebrengen aan het gastro-intestinaal stelsel en de lever, is echter

weinig beschreven. De toxiciteit, die berust op de mitochondriale schade, werd geëvalueerd met behulp van Caco-2 en HepG2 cellen na herhaalde blootstelling (tien dagen) aan lage dosissen CER. Seahorse XF Cell Mito Stress Test werd gebruikt om de invloed van lage dosissen CER op de bio-energetische functies van niet-gedifferentieerde Caco-2 en HepG2 cellen te bestuderen. Zowel voor Caco-2 als HepG2 cellen werd mitochondriale schade waargenomen na langdurige blootstelling aan 0.25 nM CER gedurende tien dagen. De geobserveerde mitochondriale schade weerspiegelde zich het meest in een reductie van de maximale celademhaling. Bij 0.50 nM CER was de mitochondriale ademhaling bijna volledig verdwenen, vooral bij HepG2 cellen. Via microscopisch onderzoek bleek dat het aantal cellen duidelijk gedaald was en werd een gewijzigde celmorfologie geobserveerd. Deze studie geeft aan dat Seahorse XF Cell Mito Stress Test een bruikbare method is om op een gevoelge manier mitochondriale toxiciteit te identificeren.

Tenslotte wordt dit onderzoek in **Hoofdstuk 6** in een internationale context geplaatst en worden de relevantie en de toekomstperspectieven besproken.

Chapter 1

Introduction and objectives

Introduction and objectives

1.1 MICROBIAL TOXINS

Microorganisms such as bacteria and fungi can infect and/or contaminate all types of agricultural products throughout the different pre- and post-harvest operations including transport and storage. The production of harmful compounds by these microorganisms unavoidably has implications on food safety and involves acute and chronic health risks. Toxigenesis, or the ability to produce toxins, is one of the underlying mechanisms of both fungal and bacterial foodborne diseases. In times where the production of agricultural crops is barely sustaining the increasing population, naturally occurring toxins pose an important challenge to food safety and directly or indirectly affect food security. Studing the potential impact of these toxins starts with accurate analysis and gathering occurrence data. The scope of this introduction is to provide relevant background for the following chapters in this thesis.

1.1.1 Mycotoxins

History records numerous human and animal diseases in which mycotoxins were retrospectively incriminated. A known example with epidemic proportions in many parts of Europe during the Middle Ages is called St. Anthony's fire or ergotism. This illness was caused by ergot sclerotia from cereals infected by the *Claviceps purpurea* fungus. The consumption of rye flour contaminated with ergot alkaloids as well as the bread made from it, resulted in the death of many thousands of people [1]. However, the scientific interest and research on mycotoxins was not aroused until the 1960s following the severe outbreak of turkey “X” disease in England resulting in the death of more than 100000 turkeys [2]. Later on, this disease was linked to Brazilian groundnut meal contaminated with aflatoxins, the secondary metabolites of *Aspergillus flavus*. In the aftermath of this outbreak and the discovery of the aflatoxins, the term ‘mycotoxins’ was coined. The term mycotoxin is derived from the ancient Greek word ‘mykes’ and Latin word ‘toxicum’, referring to poisonous fungi.

At a global level, mycotoxin contamination frequently occurs in various commodities such as animal feeds, cereal crops, leguminous plants and animal products, resulting in a variety of adverse health effects. Mycotoxins are produced as secondary metabolites under appropriate environmental conditions by filamentous fungi, mainly *Aspergillus*, *Penicillium* and *Fusarium* species. Filamentous

fungi are capable of growing on almost anything, even under harsh conditions, which makes them omnipresent in the environment. The toxic secondary metabolites are commonly found both in the field and during storage in agricultural products, mainly grains (*e.g.* wheat, barley, maize and rice). Moreover, in many regions grains like wheat, maize and rice are the human staple food and represent the main crop grown in that area. This makes the mycotoxin issue of increasing concern for both consumers and producers. So far, the global cereal production in 2017 amounts to 2593 million tonnes (FAO estimation up until 6 July 2017)[3].

Although a large number of compounds can be classified as potential toxic metabolites of fungi, about 400 different mycotoxins have been recognized of which only a few have been thoroughly investigated or legislated. The key mycotoxins which are highly present in contaminated agro-food products are referred to as 'major' or 'traditional' mycotoxins. In recent years, an arsenal of papers has been published on the occurrence of these mycotoxins, in different countries for different food commodities and during different years. After intensive risk assessment based on the collected toxicity, occurrence, and consumption data in Europe, European legislation sets maximum limits (ML) of certain mycotoxins in food and feed products in order to protect the health of consumers and animals. This implies that food business operators are bound to legal obligations to ensure safe food and feed for human and animal consumption. Regulated mycotoxins include aflatoxins, ochratoxin A, fumonisins, zearalenones, patulin and trichothecenes (including deoxynivalenol (DON), T-2 and HT-2 toxin) [4, 5]. In contrast, certain fungal metabolites are nowadays categorized as 'emerging mycotoxins'. Although this term is not clearly defined, mycotoxins are defined as 'emergent' if they have recently become a cause of concern due to an increase in incidence, level, or due to the occurrence in a new area. New insights in toxicology can also be a reason for naming them emerging. It generally regards a group of mycotoxins which are not routinely analyzed and for which no regulations have been set due to lack of background information. Additionally, the evidence of their incidence is rapidly increasing and therefore they are considered emerging risks. This category includes the *Fusarium* metabolites fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs), moniliformin (MON), fusaric acid, culmorin, and butenolide, the *Penicillium* metabolite mycophenolic acid, the *Aspergillus* metabolites sterigmatocystin and emodin, and the *Alternaria* metabolites alternariol, alternariol monomethyl ether and tenuazonic acid [6, 7].

Although some fungal products, such as penicillin, statins and toxic compounds like trichothecenes have been used in the pharmaceutical industries, most mycotoxins have been reported in association with diseases (mycotoxicoses). As an example, ergot alkaloids have both beneficial and harmful characteristics. On the one hand they have been associated with ergotism and on the other hand ergots were used by midwives to hasten labor and induce abortion while the alkaloid ergotamine and its derivatives have been used in controlling migraines [8]. Nonetheless, many problems remain regarding worldwide mycotoxin involvement in livestock, human health and agro-economy. Mycotoxicoses are the toxicity syndromes resulting from the intake of material contaminated with mycotoxins by animals and humans. Through contaminated food and feed, they evoke acute and chronic toxicity leading to a diversity of toxic effects including carcinogenicity, neurotoxicity, hepatotoxicity and immunotoxicity. On top of the health risks, mycotoxins are considered a major financial problem due to the huge economic losses associated with the reduced crop quality, yield loss due to diseases as a consequence of infestation by toxigenic fungi and through the associated human health problems and the decreased productivity of animals [9]. Additionally, managing the mycotoxin problem such as prevention, mitigation, litigation and research expenses, attributes to the total cost of mycotoxins.

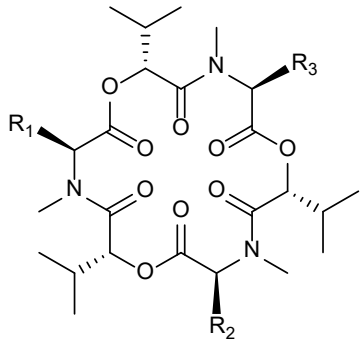
1.1.1.1 EMERGING FUSARIUM MYCOTOXINS

For many years, trichothecenes, zearalenones and fumonisins have been regarded as the so-called 'major' *Fusarium* mycotoxins. Due to intense evaluation, authorities were able to set maximum levels of these mycotoxins in food and feed. Nevertheless, other *Fusarium* derived toxins such as BEA, ENNs and MON have emerged as important contaminants. In this section chemical structures, biosynthesis by *Fusarium* fungi, occurrence in food (mainly cereals) and feed, stability after food processing, biological activity and toxicity and analytical detection methods of BEA and ENNs are described. Since BEA and ENNs types A and B are the most common among the ENNs analogues found in cereals, they are the main focus of this research. In addition, chemical structure, biosynthesis, occurrence in food and feed, biological activity and toxicity and detection methods for MON are presented.

1.1.1.2 BEAUVERICIN AND ENNIATINS

Chemical structure

BEA and ENNs share the same cyclic hexadepsipeptide core structure consisting of alternating N-methyl-L-amino acids and D-2-hydroxyisovaleric acids (D-Hiv) linked by peptide and ester bonds (Figure 1.1). BEA consists of alternating *N*-methylphenylalanine and D-Hiv, while the amino acid residues in enniatins of type A and B are aliphatic N-methyl-valine and/or –isoleucine [10].



Mycotoxin	R1	R2	R3	MW (g.mol^{-1})
BEA	phenylmethyl	phenylmethyl	phenylmethyl	784.0
ENN A	sec-butyl	sec-butyl	sec-butyl	681.9
ENN A1	sec-butyl	sec-butyl	iso-propyl	667.9
ENN B	iso-propyl	iso-propyl	iso-propyl	639.8
ENN B1	iso-propyl	iso-propyl	sec-butyl	653.9

Figure 1.1 Structure of BEA and ENNs A, A1, B and B1 with designated residual chains (R) and molecular weights (MW).

Due to the N-methylation of amino acid moieties, the molecule does not have labile protons nor stabilizing intramolecular hydrogen bonds providing flexibility to the molecule. The presence of the oxygen atoms of the carbonyl groups and tertiary amino nitrogen of the amide bonds provide free electron pairs that can act as weak nucleophiles interacting with cations. Because of this feature and their flexibility, they can form ionophoric lipophilic stable complexes that will alter the normal physiological concentrations of intra- and extracellular cations. Their cation-complexing ability and liphopilic nature explain the effects on membranes and consequently, the toxic actions of these cyclic depsipeptides [11].

Biosynthesis

BEA and ENNs are predominantly produced by various *Fusarium* species, such as *F. avenaceum*, *F. tricinctum*, *F. poae*, *F. culmorum*, *F. subglutinans* and *F. proliferatum*, which commonly grow on cereals [12]. BEA was first isolated from cultures of *Beauveria bassiana* by Hamill *et al.* (1969) and later also from a few other genera such as *Paecilomyces* and *Isaria* [10, 13, 14]. Gäumann *et al.* (1947) first isolated ENN from *Fusarium orthoceras* var. *enniatinum* [15]. Further, they have been

isolated from other fungi as well, such as *Alternaria*, *Halosarpheia* and *Verticillium* [16, 17]. In general, *F. avenaceum* seems to be the main producer of ENNs, while the production of BEA have been related to the presence of *F. poae* in particular but also of *F. subglutinans*, *F. avenaceum* and *F. proliferatum* [12, 18-20].

The biosynthetic pathways of BEA and ENNs are similar as they both are catalyzed by non-ribosomal peptide synthetase (NRPS), often called beauvericin or enniatin synthetases (Esyn). This enzyme group can be considered as hybrid systems of peptide synthetases and integrated N-methyltransferase domains. The biosynthesis starts from the primary precursors D-Hiv and L-phenylalanine for BEA and L-valine or L-isoleucine for ENN A, A1, B and B1 and requires the co-factors adenosine triphosphate (ATP) and *S*-adenosylmethionine (SAM) [21]. The Esyn enzyme consists of groups of active sites called modules EA and EB containing the catalytic binding sites for the substrates D-Hiv and the branched-chain L-amino acid [22]. As depicted in Figure 1.2, these modules contain different domains each responsible for a specific function within the biosynthesis: adenylation (A), thiolation (T), condensation (C), and N-methylation (M). The biosynthesis follows a so-called thiol template mechanism: the substrates (D-Hiv and the L-amino acids) are activated as thioesters via adenylation by the corresponding modules [23-25]. During this stage, N-methylation of the covalently bound L-amino acids occurs prior to the formation of peptide bonds yielding a dipeptidol unit. SAM serves as a methyl group donor. The biosynthesis ends with successive condensation of the three dipeptidol building blocks and a final cyclisation (Figure 1.2). Glinski *et al.* (2002) suggested that this synthesis is an intramolecular process in which all three reaction cycles are catalyzed by a single Esyn molecule [24].

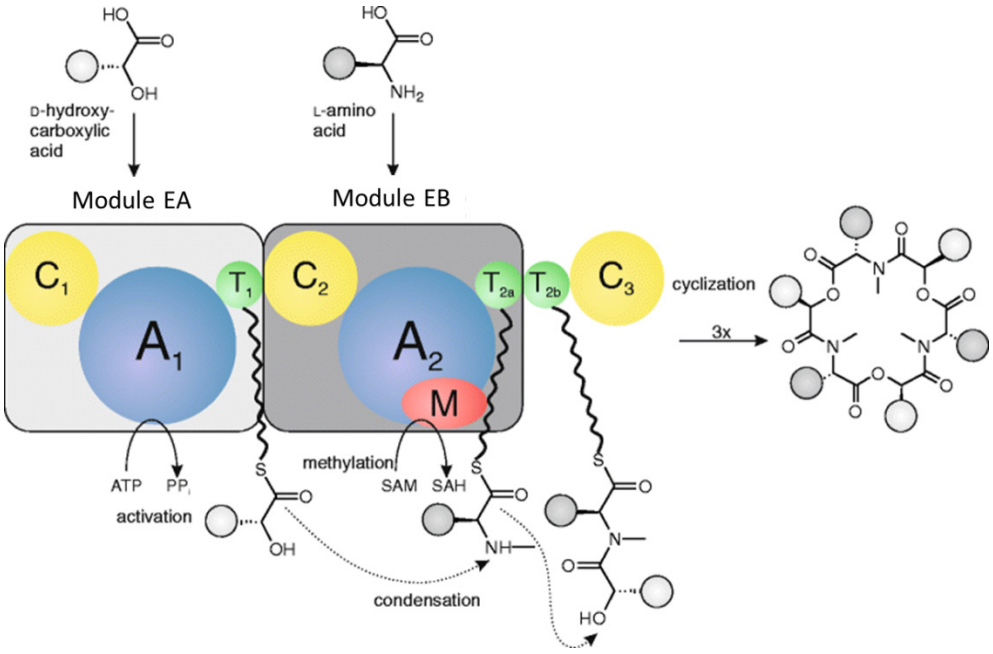


Figure 1.2 Model of ENN biosynthesis (adapted from Richter *et al.*, 2014).
The precursors D-Hiv and L-amino acid become activated via adenlyation at the A-domain (blue) of the corresponding modules (respectively EA and EB). The acyladenylates are then covalently bound to the thiol groups on the T-domain (green). N-methylation of the activated amino acid takes place at the M-domain (red) with SAM. After N-methylation, the amino acid and D-Hiv form a dipeptidol. Peptide bond formation is catalyzed by the condensation domain C_2 (yellow) of module EB. The building blocks are transferred from one module to another by means of T-domains and the dipeptidol unit is ultimately stored at the "waiting position" T_{2b}. The C-domains (yellow) catalyze the condensation of the building blocks, the final cyclization and the release from the enzyme.

Occurrence in food and feed

Many *Fusarium* species have been described as dominant pathogens of maize, wheat and other small grains, causing disease such as Fusarium head blight (FHB), stem and ear rot. The fungal infestation is accompanied by the formation of mycotoxins, which pose health risks to animals and humans [26]. Significant levels of BEA and ENNS have been reported in wheat affected by *F. avenaceum* head blight [12].

During the last decade, more studies have been reported on BEA and/or ENNs occurrence in cereals such as maize, barley, wheat, rice, and oats, as well as in cereal-based food with focus on Northern Europe and Mediterranean countries. In cooler areas of Europe, the ENNs incidence and concentration generally decreases in the following order: ENN B, ENN B1, ENN A1, and ENN A [27]. In Northern Europe, especially ENN B was found with high prevalence in grain and grain-based products. In Norway and Finland, wheat appeared to be highly susceptible to ENN B contamination (< 1000 µg.kg^{-1}) [27, 28]. Similar high concentrations of ENN B were found in Danish maize (up to 2598 µg.kg^{-1}) and in Danish wheat, barley and rye (up to 1600, 2100 and 3900 µg.kg^{-1}, respectively) [29,

30]. In Swedish wheat, ENNs were again the most prevalent toxins occurring at the highest levels compared to DON, ZEA, NIV, T-2 toxin, HT-2 toxins, MON and BEA [18]. In contrast, studies conducted in Mediterranean countries like Morocco, Tunisia or Spain, reported that ENN A1 is the most prominent among the ENNs occurring in high levels (up to 814.42 $mg.kg^{-1}$) [31-34]. Nevertheless, in Italian durum wheat samples and multicereal baby food samples, ENN B again was the most prevalent mycotoxin present (78 % and 70 %, respectively) with concentrations up to 1826 and 1000 $\mu g.kg^{-1}$ [35, 36]. For the vulnerable group of infants mean values of 7.29 to 573.14 ng.kg bw^{-1} per day were found for the estimated daily intake. However, no conclusion could be drawn concerning the risk represented by the consumption of multicereal baby food due to the lack of a provisional maximum tolerable daily intake (PMTDI) for infants [36].

In Central European countries such as Poland, Czech Republic and Germany, these *Fusarium* mycotoxins have also been observed. In the Czech Republic, 270 samples were collected during a three-year study and ENN B appeared to be the most prevalent toxin among the ENNs, present in 80 % of the samples, followed by ENN B1 (73 %) and BEA (54.4 %) [37]. Of particular concern was the fact that in Poland ENN B, B1 and A1 were found in 100% and ENN B1 in 70 % of the tested samples (oats, barley and triticale) [38]. Another example was reported in Germany in barley malt samples from three different years: the samples were contaminated with ENN B (93 %) and ENN B1 (83 %) with an average and maximum ENN B concentration of 3 $mg.kg^{-1}$ and 60 $mg.kg^{-1}$, respectively. ENN A1 and ENN A only reached maximal 1/5 of the ENN B concentrations. The incidence of BEA was low as well as the concentration levels (<48 $\mu g.kg^{-1}$) [39]. Recently, the levels of ENNs and BEA were evaluated in wheat and wheat-based products collected from Romania. The results indicated that ENN B presented the highest incidence (41 % in wheat and 32 % in wheat-based products), with maximum levels of 815 $\mu g.kg^{-1}$ and 170 $\mu g.kg^{-1}$ in wheat and wheat-based products, respectively.

The reported BEA concentrations are highly variable worldwide and the observed concentrations might be connected to climate and the prevalence of BEA-producing fungi [40]. In general, BEA contamination is more prominent with higher incidence and concentrations in Southern Europe and Northern Africa compared to cooler climates [27]. BEA has shown to be a natural contaminant of maize. For example in Moroccan maize, a concentration of 59 $mg.kg^{-1}$ was reported [34]. In cereals and cereal-based products from the Mediterranean region (Spain, Italy, Morocco and Tunisia) BEA was the predominant mycotoxin with a maximum level of 844 $\mu g.kg^{-1}$ found in wheat pasta from

Tunisia. Outside Europe, BEA was detected in 96 % of Brazilian maize samples with a maximum of 160 $\mu g.kg^{-1}$ while the detected ENN concentrations were much lower (maximal level of 5.0 $\mu g.kg^{-1}$ for ENN B) [41]. Recently, a study conducted on rice samples from Iran reported that the natural occurrence of BEA was 40 % but at low levels (up to 0.47 $\mu g.kg^{-1}$). In addition, ENN A1 was the ENN analogue found in the samples [42].

Due to the co-occurrence of several ENNs on the same food product, some studies reported total ENNs as the sum of ENN A, A1, B and B1 which has to be considered regarding the cumulative and aggregate exposure and the potential health risks to both humans and animals [18, 32, 37].

Impact of food processing

The occurrence data confirmed an ubiquitous presence of BEA and ENNs in cereals. However, food processing techniques may alter the final concentrations in products available for consumption. To unravel the fate of BEA and ENNs during food processing, the transfer of the mycotoxins from raw materials into the final products have been studied. The first study on the impact of heat treatment on BEA evaluated the degradation in crispy bread during heat treatment at three different temperatures (160, 180 and 200 °C) and at several incubation times (3, 6, 10, 15 and 20 min). To simulate the BEA degradation in food, crispy breads were prepared using six different flours (corn, integral, wheat, durum wheat, soy and rice flour). The degradation of BEA was proportional to the temperature and the incubation time. At 160 °C, BEA decreases with an average of 43 % after 20 min. Increasing the temperature to 180 °C resulted in an average reduction of 66 % after 20 min. At 200 °C, the average percentage of reduction further increased to 83 %. The percentage of reduction was dependent on the flour matrix that was used in the experiment. A maximal reduction of 88 % was evidenced in the experiment carried out with integral flour, whereas for the soy flour the reduction was 78 % for the same exposure time (20 min) and temperature (200 °C). [43]. The fate of ENNs during beer making (malting and brewing) and bread production (milling and baking) was studied by Vaclavikova *et al.* (2013). In this study naturally contaminated raw material (barley and wheat) were used. The levels of ENNs were reduced during the two steps of beer making, resulting in no detectable levels of mycotoxins in the final beer. Milling of wheat grains during bread making decreased the ENN B and ENN B1 concentrations with 71 % and 79 %, respectively. Baking of bread from the wheat flour contributed to further decrease of ENNs levels (50-60 %) resulting in traces of ENN B and B1 in the final bread samples [44]. Similar results were obtained by Habler *et al.* (2016)

during the malting process of barley inoculated with *Fusarium* species. During the steeping step, ENNs levels decreased to 30 - 60 % of the initial levels [45]. In another study, the stability of ENNs was evaluated during food processing (boiling process of pasta) using a food model simulating the composition of pasta (pasta resembling system). After 5 min of treatment, all ENNs were reduced with at least 81 % regardless the pH of the solution. The percentages of reduction even reached 95 % and higher after 15 min [46]. In some of these studies, degradation products formed during the heat treatment has been identified with LC-MS [43, 46]. However, further investigation is needed for isolation and the study of the toxicological activity of the BEA and ENNs degradation products on several cell lines.

So far, the studies focusing on the fate of BEA and ENNs during food processing are scare. Nonetheless, all findings indicated that the degradation of BEA and ENNs was accelerated with increasing exposure times and temperatures, in particular. Since processing steps such as milling, steeping, cooking and baking, have proven to reduce the mycotoxin content, they can be adapted as mitigation strategy. However, despite the significant reductions of the mycotoxin concentrations, generally, the mycotoxins were not eliminated completely. Since the studied food processes techniques (such as cooking, milling and baking) represent realistic procedures applied in the food industry or at home, it is important to assess the final concentration of the mycotoxins in processed and prepared food products and not only in the raw grains. Remarkably, when solutions models were compared to food matrices, the data indicated that degradation was lower in food matrices, which might suggest protective effects of the food toward the mycotoxins. In addition, the type of matrix 'protects' the mycotoxin to a greater or lesser extent, which needs to be considered in an experimental setting [43].

Biological activity and toxicity

Studies have confirmed that ENNs and BEA can exert many different biological activities such as antibacterial, insecticidal and phytotoxic properties [47, 48]. One of the first effects described was the antimicrobial activity against *Mycobacterium spp., Staphylococcus spp.*, *Bacillus spp.*, *Pseudomonas aeruginosa* and *Escherichia coli* [48]. Furthermore, Meca *et al.* (2012) evidenced the inhibition of the growth of several mycotoxigenic moulds [49]. The insecticidal effect of BEA was investigated on microgram level on several insects [50]. The toxic effects induced by BEA and ENNs were observed in the insect cell line lepidopteran *S. frugiperda* (SF-9) [51]. Phytotoxic effects of BEA

have been demonstrated in tomato protoplast resulting in its death and decreased ascorbate level [52].

The primary mode of action of these cyclic depsipeptides is based on their ionophoric properties by forming carrier sandwich complexes or pore forming channels that transport cations (K^+, Na^+, Ca^{2+}) through lipid membranes across biological membranes. The ENN molecules are capable of forming either 1:1, 2:1 or 3:2 (sandwich) enniatin:cation complexes [53]. This results in uncoupling oxidative phosphorylation and mitochondrial dysfunction [54, 55]. Subsequently, induction of apoptosis causes mediated cell death. In addition, some studies indicated that ENNs are able to increase intracellular Ca^{2+}, which as well may lead to induction of cell death [54]. Besides the ionophoric properties, they inhibit various enzymes such as acyl CoA–cholesterol–acyl transferase (ACAT) and cyclic nucleotide phosphodiesterase [56]. ACAT inhibitory activity has been demonstrated in an enzyme assay using rat liver microsomes. Among the ENNs, BEA showed the most potent inhibitory activity (IC_{50} value of 3.0 µM compared to 22-110 µM for the other ENNs). Furthermore, Hiraga *et al.* (2005) found that ENN B, B1, and D showed potential to inhibit drug efflux by Pdr5p in *Saccharomyces cerevisiae*. Pdr5p is one of the major sources of drug resistance because overexpression of this efflux pump contributes to multidrug resistance (MDR). By inhibiting this efflux pump, ENNs are able to reverse the MDR phenomenon caused by Pdr5p [57]. Additionally, beside the inhibition of cyclic nucleotide phosphodiesterase, ENN B was found to bind calmodulin. Interaction with calmodulin might affect the signal transduction mechanism required for calmodulin-dependent enzymes, such as cyclic nucleotidase, protein kinases and phospholipase A2 [58]. Ivanova *et al.* (2012) demonstrated that ENN B exposure of Caco-2-cells resulted in progressing lysosome swelling followed by disruption, which is characteristic for the lysosomal death pathway. During this necrosis-like process induced by ENN B, lysosomal destabilization, mitochondrial membrane permeabilization and increased reactive oxygen species (ROS) production were observed. However, it was suggested that cathepsins and oxidative stress were not the main factors for the cytotoxicity but rather an early event in the cell death cascade [59]. The fact that cellular ROS production was not a critical determinant for the ENN-mediated cytotoxicity was supported by a previous study predicting that ROS were not directly involved in the cytotoxic effects [60]. Ficheux *et al.* (2013) studied *in vitro* the effect on development of human dendritic cells and macrophages. The data indicated that exposure to ENN B and BEA disturbs the human monocytes differentiation process into macrophages. They also interact with the human dendritic cells maturation process which could imply a decrease of the immune response in

case of infection [61]. Pharmacological behavior and *in vivo* toxicity have been tested in mice and in poultry (turkey and broilers) [62-64]. A study by Taevernier *et al.* (2016) showed that BEA and ENNs were able to cross the blood-brain barrier in mice with a dose corresponding to 0.2 $mg.kg^{-1}$. Once that these mycotoxins reach the systemic circulation, they can exert central nervous system (CNS) effects [63].

The potent cytotoxic activity has been demonstrated in several mammalian cell line models including colorectal (Caco-2, HT-29), cervical (HeLa), lung (MRC-5) and liver (Hep-G2) cell lines [30, 65, 66]. Recently, Svingen *et al.* (2017) demonstrated hepatotoxicity of ENN B and BEA using a 'quadroprobe' hepatotoxicity assay. The study showed significant cytotoxicity toward human liver HepG2 cells at a concentration lower than that for aflatoxin B1 which is considered a liver-carcinogenic mycotoxin [30]. A comparative cytotoxicity study of ENNs on Caco-2 cells, HepG2 and HT-29 demonstrated that at low micromolar concentrations toxic activity was already observed after 24 h exposure. Moreover, the study revealed that ENN A1 appeared to be the most cytotoxic of the ENNs tested [66]. Dornetshuber *et al.* (2007) investigated different aspects of enniatin-induced apoptosis and cell cycle distribution in epidermal carcinoma-derived KB-3-1 cell line and reported that ENN exerts cytostatic and cytotoxic activities against several human cancer cell lines [67]. Further, the toxic potential of ENN A1, B and B1 was analyzed in H4IIE rat hepatoma, HepG2 human hepatoma and C6 rat glioma cells. Prominent toxicity was observed after 3 h in H4IIE rat hepatoma cells (with EC_{50} = 5-10 µM) determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Furthermore, the mycotoxins induced apoptotic cell death in H4IIE hepatoma cells accompanied by disruption of the extracellular signal-regulated kinase (ERK) pathway [68].

These cytostatic and cytotoxic effects against diverse human cancer cells might be useful as cancer treatments [67, 68]. For example, BEA inhibited migration of the metastatic prostate cancer (PC-3M) and breast cancer (MDA-MB-231) cells and showed anti-angiogenic activity in human umbilical vein endothelial (HUVEC-2) cells at sublethal concentrations [69]. Moreover, ENNs in low non-toxic doses have shown perspectives as clinical applications based on their antibiotic activity. In addition, by inhibiting ACAT, ENNs could be significant in the treatment of atherosclerosis and hypercholesterolemia. Despite the potential use as therapeutics, so far only a mixture of ENNs called fusafungine (Locabiotal®) has been used as local treatment of respiratory infections. However, due to

the risk of serious allergic reactions and insufficient evidence of benefit, fusafungine-containing medicines were recently withdrawn from the market [70].

Clearly, many toxic activities have been attributed to ENNs and BEA exposure but people are still in search for explanations to link the involvement of different pathways of cytotoxicity and apoptosis and to relate all the observed mechanisms to the basic ionophore action. The conclusion of the European Food Safety Authority (EFSA) report on exposure to BEA and ENNs was that there is no concern to human health regarding acute exposure. However, no conclusion regarding chronic exposure was currently possible due to the lack of toxicity data [71]. But many reports have indicated that ENNs are one of the most prevalent of the emerging mycotoxins, so the chronic exposure might pose a significant risk to human health such as cancer induction, kidney toxicity and immune suppression [6, 27].

There is *in vitro* evidence of diverse toxic effects of BEA and ENNs, as well as the widespread presence in several types of food products. Unfortunately, *in vivo* information is still lacking for the described toxins. Since *in vitro* cell culture is a significantly different environment, the translation from the *in vitro* results to the *in vivo* situation is difficult. In a preliminary *in vivo* study Wistar rats were fed during 28 days with repeated doses of ENN A. Data on the tissue distribution of ENN A were collected, but no adverse effects were observed [72]. Furthermore, another subacute toxicity study was done with Wistar rats, which were fed with ENN A-contaminated diets. After two weeks, significantly higher concentrations of ENN A were observed in serum while no detectable levels of ENN A were found in feces and urine. In addition, based on $CD4^+/CD8^+$ T cell ratio, immunotoxicity was assessed in the lymphocytes of peripheral blood. The observation of an impaired immune status might result in a decreased humoral and innate immunity in the case of infection with reduced lymphocytes. [73]. In another study with Wistar rats, the situation of acute toxicity was simulated by administration of a single oral dose of an ENNs-mixture (1.19, 2.16, 1.03 and 1.41 $mg.kg^{-1}$ body weight for ENN A, A1, B and B1). During an 8-h duration experiment, no acute adverse effects on the animals were observed. In serum and urine samples collected at different time points, no quantifiable levels of ENNs were found, probably due to fast absorption and extensive metabolization of these mycotoxins. In contrast, ENNs were detected in feces of the treated group since they were partially excreted unchanged in feces following oral administration [74]. To elucidate the toxic effects *in vivo*, a thorough investigation of the toxicokinetic characteristics of ENNs is

essential. Therefore, Devreese *et al.* (2014) studied the toxicokinetic properties of ENN B1 in pigs and revealed a rapid absorption after oral administration as well as a rapid distribution and elimination after both oral and intravenous administration [75]. In addition, a high absolute oral bioavailability of 91 % was recorded which indicates systemic exposure. In a subsequent study, ENN B1 metabolites found in the *in vivo* pig study and ENN B1 biotransformation products formed *in vitro* in pig liver microsomes were identified [76]. In contrast to the pig study, a toxicokinetic study of ENN B and ENN B1 performed in broiler chickens indicated low absolute oral bioavailabilities of 5 % and 11 %, respectively, resulting in a low systemic exposure [77]. These results correspond to the EFSA statement that adverse health effects from chronic exposure to BEA and ENNs are unlikely for poultry [71]. However, based on these toxicokinetic studies in different animal species, no firm conclusions on functional implications of BEA and ENNs could be drawn. Another in vivo study focused on the blood-brain barrier (BBB) permeation kinetics of BEA and ENNs in mice and reported a very high BBB-influx of these compounds, which can result in local central nervous system effects [63].

The limited *in vivo* studies indicate a low acute toxicity and so far no reports on adverse effects in humans caused by contaminated food have been described. In contrast, several authors describe significant *in vitro* toxicity in several mammalian cell lines. This *in vitro* - *in vivo* discrepancy can be attributed to the rapid metabolization and elimination of BEA and ENNs, although the exact metabolization pathways still remain unclear. *In vitro* experiments in rat, dog, and human liver microsomes suggested extensive phase I metabolization of ENN B, by members of cytochrome P450 family (mainly CYP3A4, CYP2C19, and CYP2A1) resulting in oxidation and N-demethylation [78, 79]. In contrast, no significant serum and brain metabolization was observed *in vitro* for ENNs [63]. Therefore, it is important to elaborate toxicokinetic studies to further elucidate phase I and II metabolization pathways and the toxicity of the metabolites. In addition, more relevant *in vivo* toxicity studies are required to perform risk assessment in humans and most livestock animal species, especially due to the observed species-specific differences.

Detection

Several methods have been described for determination of ENNs and BEA in a range of matrices with differential chemical compositions. Similar to other mycotoxin detection methods the sample preparation generally includes specific extraction of mycotoxins from the matrix using the

appropriate solvents, followed by a clean-up step in order to remove impurities and finally identification and quantification. For the analysis of BEA and ENNs several sample purification techniques have been described: defatting with hexane or other non-polar solvents, liquid-liquid partitioning with dichloromethane or chloroform, silica or C_{18} and C_8 solid phase extraction (SPE) columns [80-82]. Krska *et al.* (1996) reported that defatting the matrix by use of hexane greatly reduced the recovery of BEA [82]. In some studies, only a filtration step after extraction was used. Moreover, tandem mass spectrometry enabled minimization of the additional purification steps, which saved time, organic solvents and columns [30, 83].

Although BEA and ENNS have hydrophilic and hydrophobic properties, they are considered as lipophilic compounds, due to the apolar side chains directing out from the outer surface of the molecules. The following solvent mixtures have been used for the extraction: methanol-water, acetonitrile-water and acetonitrile-methanol-water [42]. Besides these traditional extraction methods, a supercritical fluid extraction (SFE) method with methanol as co-solvent was described for the extraction of BEA from maize. However, average recovery results (76.9 %) were comparable with these of conventional extraction protocols [84].

In search for improved methods to achieve the best recovery, adequate sensitivity and repeatability, liquid chromatography (LC) was coupled with diode-array detector (DAD), ultraviolet (UV) or massa spectrometry (MS) [82]. Currently, the combination with MS (and tandem MS) enables the simultaneous detection of low concentrations of different analytes using mainly electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) as interface. For MS detection, different mixtures of water, acetonitrile and/or methanol are typically used as mobile phase, whether or not supplemented with modifiers such as formic or acetic acid and ammonium buffers to promote the formation of ionophore-cation complexes or the ionization process. By adding ammonium acetate to the mobile phase, the formation of a $[M+NH_4]^+$ adduct as predominant signal is promoted [80, 83]. Moreover, by adjusting the declustering voltage, abundance of protonated molecules and ammonium adducts can be controlled leading to higher reliability of quantification [80]. Jestoi *et al.* (2005) introduced the use of triple-quadrupole mass spectrometry for the analysis of ENNs and BEA providing a sensitive method with low limits of detection of (0.1-0.7 $\mu g.kg^{-1}$) and limits of quantification (0.2-1.5 $\mu g.kg^{-1}$) values [80]. Many other authors used similar liquid chromatography-mass spectrometry (LC-MS) methods whether or not with some modifications,

especially for sample preparation, depending on the matrix and the other toxins included [29, 38, 63, 83, 85, 86]. No immunoassays have been described for BEA and ENNs since no suitable antibodies are available.

More generally, LC-MS instrumentation is used for the elucidation of degradation products of the toxins which might as well be potentially dangerous [87]. Another way of anticipating on potential risks is an untargeted approach, which allows the possibility of identifying unexpected contaminants, degradation products, impurities or untargeted toxic compounds. TOF techniques serve as an excellent non-specific detection tool for identification and confirmation of food contaminants by recording an accurate full-scan spectrum throughout the acquisition range.

Legislation

Despite the request of the European Commision (EC) to EFSA for a scientific opinion on the risk to human and animal health related to the presence of BEA and ENNs in food and feed, no risk assessment was possible for BEA and ENNs due to insufficient data on toxicity [71]. For a thorough evaluation of human risk, relevant toxicological endpoints and the toxicological relevance of BEA and ENNs should be considered. Regarding toxicology, additional *in vivo* data on the possible interactions within the related ENNs as well as with other toxins that can be produced by the same fungi are required. Moreover, with regard to the human risk assessment, exposure of the population to BEA and ENNs has to be estimated. Considering the animal risk assessment, species-specific exposure to BEA and ENNs has to be determined as well as the possible carry-over from feed to animal food products. In addition, the EC asked for identification of feed materials as possible sources of BEA and ENNs and characterization of the distribution of contamination levels herein. For both human and animal risk assessment, the co-occurrence of BEA and ENNs with other (*Fusarium*) toxins has to be evaluated. Hence, EFSA already suggested that for the assessment of ENNs, the sum of ENN A, A1, B and B1 should be considered since these compounds have similar structures and modes of action and are frequently co-occurring in food and feed [71]. This implies that the cumulative health risks should be assessed. Besides the oral intake of these mycotoxins, the possible exposure to mycotoxins via inhalation and dermal contact has to be taken into account [88]. Because of insufficient toxicity data for ENNs and BEA in experimental animals, EFSA´s Panel on Contaminants in the Food Chain (CONTAM Panel) could not establish an acute reference dose or a tolerable daily intake. Consequently, EFSA requested a subchronic oral toxicity study in rodents in which the standard

toxicological endpoints (nervous, immune and endocrine systems) and screening of the reproductive and developmental functions are included [89].

1.1.1.3 MONILIFORMIN

Chemical structure

Moniliformin (3-hydroxy-3-cyclobutene-1,2-dione, MON) is a small, water-soluble molecule with a low pKa value (<1.7), that occurs in nature as sodium or potassium salt which makes it soluble in polar solvents (Figure 1.3). At pH = 7, this molecule will be deprotonated which explains its low log P value. Due to the acid conditions in the stomach, MON will be protonated which allows passive diffusion through the cellular membranes.

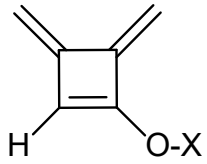


Figure 1.3 Chemical structure of MON, X = H (free acid, MW 98.057 g.mol^{-1}), Na (sodium salt) or K (potassium salt).

Biosynthesis

MON was first isolated by Cole *et al.* in 1973 from a *Fusarium* strain, initially called *F. moniliforme* [90]. However, taxonomically this fungus was eventually recognized as *F. subglutinans*. A large number of other *Fusarium* species including *F. avenaceum*, *F. proliferatum*, *F. tricinctum*, *F. fujikuroi*, *F. nygamai*, *F. pseudonygamai*, *F. temperatum* and *F. thapsinum* produce moniliformin. Recently, *Penicillium melanoconidium* appeared to be an additional moniliformin producer [91]. In general, *F. proliferatum*, *F. subglutinans*, *F. avenaceum* and *F. tricinctum* are most commonly associated with MON production [92, 93].

The biosynthesis of MON follows the polyketide pathway. This involves the condensation of two acetate moieties to gain one cyclobutadione molecule followed by oxidation and dehydration to form MON [94].

Occurrence in food and feed

MON is found worldwide in several cereals such as maize, rice, barley, wheat, oats, rye and triticale [6, 95]. Especially in maize, the MON contamination is higher than in other grains. Beside a wide variety of crops, MON has been found in different geographical areas. Analysis in Gambian and South African maize and maize-based products revealed levels up to 3.16 and 2.73 $mg.kg^{-1}$, respectively [96]. In South Europe, MON has been found in maize infected by *F. proliferatum* and *F. subglutinans*. In North-West Italy, maize samples (n=180), collected over four years (2008-2011) were analyzed for natural MON contamination. Each year considerably high levels of MON were found with average concentrations between 89 and 1127 $\mu g.kg^{-1}$ and overall, an average of 93 % of the samples were positive for MON [95]. In contrast, Danish maize samples showed low levels (1-12 $\mu g.kg^{-1}$), presumably because the examined samples were derived from the entire maize plant, whereas the high levels reported in other studies were mainly observed in maize kernels [97]. In Poland, an average concentration of 15900 $\mu g.kg^{-1}$ MON was reported in *Fusarium*-damaged wheat grains, while the average amount of MON in healthy looking kernels from the same sample was 420 $\mu g.kg^{-1}$ [98]. In Finish grain samples (harvested in 2001-2002), MON was found in 74 % of the samples with the highest level of 810 $\mu g.kg^{-1}$. Moreover, a positive correlation between the *F. avenaceum* contamination level and MON was found [28]. A study conducted in Norway, showed very similar results as MON was found in 45 % of the samples (oats, barley, wheat) with a maximum level of 950 $\mu g.kg^{-1}$ in wheat [99]. Environmental conditions such as rain and temperature varied according to the year of harvest and greatly affected the prevalence of MON. A study investigated the effect of environmental conditions on MON production by *F. avenaceum* and revealed that MON favored low water activity combined with elevated temperature [93]. At present, EFSA is preparing a scientific opinion on the risks to public health related to the presence of MON in feeds and food.

Because high concentrations of MON have been found together with ENNs, the question of possible interaction between MON and the concurrently produced BEA and ENNs has been raised. No synergistic effects were found *in vitro* on the contractility of vascular smooth muscles [100]. However, further studies are needed to evaluate whether the co-occurrence of MON, BEA, ENNs and also trichotechenes has synergistic effects.

Biological activity and toxicity

Despite the confirmed toxicity, *in vitro* and *in vivo*, the mechanism of toxicity is not well understood. Thiel (1978) suggested that the molecular mechanisms of the toxic action are based on the selective inhibition of mitochondrial pyruvate and α-ketoglutarate oxidations, which are intermediates in the tricarboxylic acid cycle [101].

Regarding *in vitro* toxicity, MON seemed to exert relatively low cytotoxicity based on cell viability or metabolic activity assays measured in several cell lines [6]. However, the cytotoxic effects on lymphocytes, skeletomyocytes and cardiomyocytes were more pronounced. This correlates well with the *in vivo* findings where the effects such as myocardial degeneration and muscular weakness are more severe. For example, feed containing a MON-producing isolate of *F. moniliforme* var. *sublgutinans* induced cardiotoxic effects with microscopic lesions in chicks, ducklings and turkey poults [102]. In addition, the role of pure toxin was confirmed by the death of birds injected with MON [103]. More toxic effects including intestinal problems and immunosuppression were described in other animals such as ducklings, turkey poults and rats [104-106]. The clinical symptoms of acute MON intoxication observed in ducklings were muscular weakness, respiratory stress and myocardial degeneration eventually resulting in death [104]. Studies on absorption, distribution, metabolism and excretion after oral administration of MON are scare. In rats, MON was rapidly excreted into urine but only 20-40% of the administered dose was found as the parent form. However, due to the very polar and hydrophilic nature of MON, accumulation in adipose tissue is unlikely [107].

Detection

Due to the high polarity of MON, it is highly soluble in water (10 $mg.mL^{-1}$). However, extraction with water may be accompanied with undesirable impurities. Therefore, most studies used 84-96 % acetonitrile in water as extraction solvent. Similar to ENNs and BEA, sample preparation requires clean-up of the cereal extract such as defatting with hexane, dichloromethane or chloroform, and the use of different purification columns such as strong anion exchange (SAX), C_{18} and MycoSep® 240 moniliformin clean-up columns [95]. Lim *et al.* (2015) developed a protein exclusion purification column known as ‘‘PHREE’’. In this way, sensitivity, specificity and recoveries improved for the quantitative detection of MON in cereals [108]. A capillary zone electrophoresis diode array detection (CZE-DAD) method developed by Maragos *et al.* (2004) was compared with high

performance liquid chromatography coupled to ultraviolet detection (HPLC-UV) using absorbance at 229 nm. Although, the limit of detection of the CZE-DAD method was 100 µg.kg^{-1} compared to 50 µg.kg^{-1} for the HPLC-UV method, the CZE-DAD detection required only 10 min [109]. Commonly used methods for the detection of MON are based on LC coupled to different detectors such as UV, DAD and MS [95, 97, 110, 111]. Furthermore, ion-pair chromatography has routinely been used because of the ionic nature of MON resulting in a weak retention retained by reversed-phase (RP) chromatography [96, 112]. The LOD and LOQ were respectively 40 and 120 µg.kg^{-1} when using ion pairing RP HPLC with DAD-UV detection [99]. However, in general LC-MS is preferred above UV detection for quantitative MON determination because lower LOD and LOQ can be achieved. A study reported that HPLC-UV analysis (with LOD = 48 µg.kg^{-1} and LOQ = 96 µg.kg^{-1}) indicated no MON contamination, while MON was successfully detected in 15 out of 28 samples by HPLC-MS; hence, in concentrations below the LOQ of 12 µg.kg^{-1} [97]. The application of LC–MS for determination of MON in maize allowed measuring levels down to 10 µg.kg^{-1} [113]. A sensitive LC-MS/MS multi-mycotoxin method including MON was developed for rice and reached an LOD of 0.08 µg.kg^{-1} and LOQ of 0.25 µg.kg^{-1}. Note that MON detection with mass spectrometric analyses using ESI was performed in the negative-ion mode. In tandem MS, the deprotonated molecule (m/z = 97.0) $[M–H]^-$ generates only one strong product ion (m/z = 41.0) [95]. Currently, no antibody-based techniques are available for detecting MON, probably due to the low molecular weight of the molecule [114].

1.1.2 BACTERIAL TOXINS

Foodborne diseases caused by bacteria pose a global public health threat. Among them, there is a distinction between foodborne infection, toxicoinfection and intoxication. A foodborne infection is caused by the consumption of food contaminated with pathogenic microorganism such as *Salmonella* spp., *Campylobacter jejuni/coli*, *Listeria monocytogenes* and *Escherichia coli*. The organism grows inside the intestinal tract and cause illness without the involvement of toxins. Besides these food infectans, certain types of bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum* are capable of producing bacterial toxins. A food intoxication occurs after consumption of these toxins that are formed in food by bacterial growth, rather than the bacterium itself. A toxicoinfection or toxin-mediated infection occurs when bacteria grow in the human intestinal tract where they release toxins that cause illness.

1.1.2.1 BACILLUS CEREUS FOOD POISONING

Bacillus cereus has been linked to two types of foodborne diseases, both related to toxigenesis (the ability of bacteria to produce toxins). *B. cereus* (*sensu stricto*) belongs to the *Bacillus cereus* group, also known as *Bacillus cereus* sensu lato with the other members being *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides*. Recently, *B. toyonensis*, *B. cytotoxicu* and several others have been recognized as plausible members of the group [115]. *B. cereus* is a rod-shaped, gram-positive, aerobic or facultative anaerobic, endospore-forming bacterium. Both the spores and vegetative forms are omnipresent in a wide variety of environments, including soil and plants and consequently in various food types such as cereals, cereal derivatives, milk and meat [116, 117]. The bacteria enter the environment due to the adhesive nature of the endospores who germinate and grow when in contact with organic materials, insects or animal hosts. As a result, *B. cereus* has been isolated from a diverse range of different foods [118]. Furthermore, the spores are very resistant against heat. As a consequence, they survive pasteurization and other production and processing conditions. After food processing, control of the cold chain during storage and transport is crucial because the spores of *B. cereus* may germinate into vegetative cells and produce food poisoning toxins in favorable conditions such as time and temperature abuse. *B. cereus* is known for two different types of toxin-related foodborne illness: the diarrheal and emetic (vomiting) syndrome. The toxin producing *B. cereus* strains are classified as mesophilic and psychotropic types. Low storage temperatures (<10 °C) should prevent growth of mesophilic *B. cereus* species. This indicates that even at mild temperature abuse the microorganism are able to multiply [119]. Therefore, the maintenance of the cold chain is an essential strategy to ensure the safety of the products. Nevertheless, even at refrigeration temperatures, the psychotropic strains are of concern since they can even grow at 4-6 °C [120]. For the consumers' safety, improper and prolonged storage needs to be avoided, especially in the case of the leftovers of previously heated food. To reduce *B. cereus* intoxications, leftovers should be refrigerated properly by cooling rapidly to 5 °C or below. For example, in order to ensure rapid cooling, leftover containers should not stacked close together to allow for adequate airflow around the food. However, CER that is known to be resistant towards heat will not be inactivated by food preparations such as heating and steaming. This emphasized the importance of prevention and cold chain maintenance [121].

The so-called diarrheal syndrome is characterized by abdominal pain and watery diarrhea 8-16 h after consumption of contaminated food. Symptoms generally resolve after 12-24 h. Diarrheal outbreaks have been linked to a variety of food including meat, milk, vegetables and fish. It is postulated that the diarrheal type is caused by heat-labile proteins (enterotoxins) that are formed inside the gastrointestinal tract after ingestion of food contaminated with *B. cereus* cells [122]. The enterotoxin hemolysin BL (HBL), the non-hemolytic enterotoxin (NHE) and the necrotic cytotoxin K (CytK) are considered as the causative agents of the diarrheal syndrome.

In contrast, the emetic form of food poisoning is the result of a heat stable peptide called cereulide (CER) which is pre-formed in food. The first reported emetic syndrome episodes have been associated with the consumption of fried rice from Chinese restaurants [123]. This toxin has usually been detected in temperature-abused farinaceous foods, such as pasta, noodles and rice [124-126]. These emetic-type outbreaks are characterized by its rapid onset (within 1-5 h) with vomiting and nausea as predominant symptoms, occasionally accompanied by diarrhea later on. Recovery usually occurs within 24 h. Nevertheless, fatal outcomes were reported after the ingestion of food contaminated with high amounts of CER. In the reported cases, fatal outcome was related to the consumption of rice and pasta dishes that were prepared some days before, improperly stored and then reheated [126-129].

Because of the wide distribution, the resistance of the spores and capacity of producing toxins, *B. cereus* is considered an important hazard for the food industry. An overview of the chemical structure, biosynthesis by *B. cereus*, occurrence in food, biological activity and toxicity and analytical detection methods of CER will be fully described.

1.1.2.2 CEREULIDE

Chemical structure

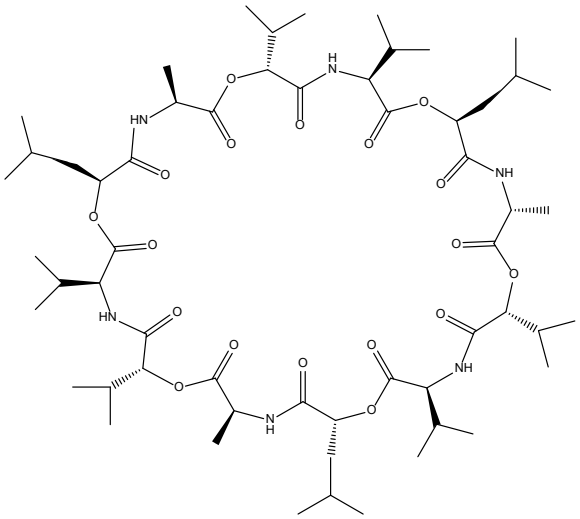


Figure 1.4 Structure of cereulide (MW 1153.4 g.mol^{-1}).

CER is a ring-structured dodecadepsipeptide of 1.2 kDa in which α-amino acid and α-hydroxy acid moieties are arranged in three repeating tetradepsipeptide units, namely (D-O-Leu-D-Ala-L-O-Val-L-Val)$_3$ (Figure 1.4) [130, 131]. Recently, a series of CER variants have been discovered after mass spectrometric screening of *B. cereus* strains, among which the isocereulides A-G. Remarkably, these isocereulides showed different cytotoxicity correlating with their ionophoric properties [132].

CER closely resembles the structure of valinomycin (VAL) [D-O-Val-D-Val-L-O-Ala-L-Val]$_3$, which is a natural antibiotic produced by *Streptomyces* spp. VAL shows high similarity with CER, being also a cyclic dodecadepsipeptide with a similar three repeating sequence and a known K^+-ion-selective ionophore. The only difference between these molecules are the side chains which are linked to a similar backbone. For that reason, VAL has often been used as (internal) standard or to express the content of CER as VAL equivalents [133, 134].

Biosynthesis

Similar to the other cyclic depsipetides BEA and ENNs, the biosynthesis of CER is performed by the large multi-domain NRPS, encoded by the CER synthase gene cluster (*ces*) [135]. This *ces* gene cluster is located on a mega plasmid [136]. Sequence analysis revealed that the *ces* gene cluster comprises 7

coding DNA sequences (*ces*H, *ces*P, *ces*T, *ces*A, *ces*B, *ces*C and *ces*D) with specific activities leading to the final construction of CER [137]. The regulation of *ces* gene expression involves central transcriptional regulators, such as CodY and AbrB [138]. The exact mechanism of CER biosynthesis by NRPS is far from understood. However, parallel to BEA and ENNs, a typical thiol template mechanism that involves cyclo-trimerization of tetrapeptide unit (D-O-Leu-D-Ala-L-O-Val-L-Val) carried out by one NRPS that is regenerated and repetitively used for synthesis, is proposed [137].

Production of the emetic *B. cereus* toxin CER is restricted to strains containing the plasmid with the *ces* gene. Besides the *B. cereus sensu stricto*, also *B. weihenstephanensis* isolates producing CER have been identified by means of LC-MS by Thorsen *et al.* (2006) [139]. Through polymerase chain reaction (PCR), Toh *et al.* (2004) amplified and identified specific DNA sequences of genes associated with the production of the toxin. In this way, potentially toxigenic strains of *B. cereus* strains capable of producing emetic toxin can be determined among isolates of *B. cereus* [135]. Moreover, the emetic strains are believed to have other preferred ecological niches compared to their non-emetic types which suggests that CER might be fundamental for the survival in specific habitats [138].

The conditions influencing CER production were investigated by Biesta-Peters *et al.* (2016) under laboratory conditions to understand CER production in food products. Addition of salt resulted in delayed production of CER [140]. In another study, the optimal temperature for CER production in rice culture was determined at 25-30 °C.

Occurrence in food

Only a subgroup of *B. cereus* carries the plasmid-encoded *ces* genes and can produce CER. For example, despite the frequent contamination of spores of *B. cereus* in raw milk, the presence of emetic strains of *B. cereus* in samples taken along the dairy production chain is rare and consequently dairy products have been seldom associated with human emetic illness [141]. Emetic *B. cereus* is presumably unable to grow and produce CER below 10 °C, or without oxygen [142]. Nevertheless, psychrotolerant species such as *B. weihenstephanensis* capable of producing CER at temperatures ≤ 8°C, can present a health risk if CER is produced during refrigerated storage [143, 144]. However, contradicting results on the amount of CER produced at low temperatures by these psychrotolerant *B. weihenstephanensis* are reported [143]. Generally, storage below 4 °C is assumed to be safe, even for psychrotolerant species. Hence, proper refrigeration (at ≤ 7 °C and preferably at

≤ 4 °C) is the main management option in order to avoid food spoilage and poisoning [145]. CER is known to be extremely resistant to heat, acidic conditions and proteolysis. As a result, it will not be inactivated by heat treatments during food processes and preparations and by gastric acid or proteases of stomach and intestine [121].

The majority of reported outbreaks were linked to reheated foods (mainly cooked pasta and rice dishes) and frequently occurred in restaurants and catering settings. As mentioned, pasta, rice and noodles were among the most common food vehicles implicated in emetic intoxications. As a consequence, these types of food matrices were most targeted regarding CER research. Nonetheless, CER has been reported in other types of foods as well such as vegetables, fruit products, sauces, soups, salads, cheese and meat products [146]. Based on the amount of food consumed, the emetic dose for humans was estimated around 8-10 $\mu g.kg^{-1}$ body weight. These concentrations are similar to the values obtained in studies with rhesus monkeys (9 to 10 $\mu g.kg^{-1}$ bw) and *Suncus murinus* (EC_{50} of 9.8-12.9 $\mu g.kg^{-1}$ bw) [147, 148]. However, further research is necessary to define the actual emetic dose for humans (in $\mu g.kg^{-1}$ bw). Leftover rice from takeaway dishes from an Asian restaurant implicated in a family outbreak, contained significant *B. cereus* counts ($2.8x10^5$ – $2.4x10^7$ $CFU.g^{-1}$ and up to 13.2 µg of CER per g food. The people involved in this family outbreak (age 14-80) reported vomiting episodes within 2 h and some diarrhea episodes, with hospitalization of the oldest person (80 years old) [125]. These concentrations were comparable to those found in a spaghetti meal (*B. cereus* counts: $9.5x10^7$ $CFU.g^{-1}$ and 14.8 µg $CER.g^{-1}$) which resulted in the death of a 20-year-old man. The leftover spaghetti with tomato sauce, which had been prepared five days before, was left at room temperature and reheated in the microwave [128]. Moderate levels of CER (up to 4.2 $\mu g.g^{-1}$) were found in rice-based meals after an emetic outbreak in a Belgian kindergarten. However, these levels provoked profuse-vomiting episodes in 20 toddlers (age 10-18 months) [149]. Agata *et al.* (2002) investigated food samples, mostly pasta and rice dishes, implicated in several emetic cases and found that the CER concentrations ranged from 0.01 to 1.28 $\mu g.g^{-1}$ [150].

When the natural occurrence of CER in food products is assessed in samples not implicated in foodborne outbreaks, the incidence and concentration levels of CER were low. A recent study evaluated the presence of *B. cereus* and CER in refrigerated processed foods of extended durability (REPFED), ready-to-heat (RTH) and ready-to-reheat (RTR) food products, containing either pasta or rice, from the Belgian market [151]. CER was not detected in any of the samples, which was

confirmed by the absence of the *ces* gene in the *B. cereus* strains. Between 2007 and 2010 various types of food samples (n=3008) available on the Dutch market were tested for the presence of CER [140]. The samples were divided in different categories based on the main component of the matrix (protein, starch, fat, multiple components, or other). Only two samples contained detectable levels of CER at 3.2 $\mu g.kg^{-1}$ (rice dish suspected to have caused food poisoning) and 5.4 $\mu g.kg^{-1}$ (fried rice).

The occurrence of CER has mostly been reported related to cases of emetic food poisoning outbreaks after consumption of (temperature-abused) farinaceous food products containing high levels of CER. In contrast, the natural occurrence of CER in samples available on the market that were not implicate in food poisoning is rather low. Sub-emetic concentrations however, might potentially be harmful in the long run. The effects of the repeated exposure to low concentration are more complex to identify because they are not easily recognized compared to clinical symptoms like vomiting [151-153]. In addition, CER is rarely routinely determined and most intoxications proceed mildly, which indicate presumable underreporting [126-128]. The incidence may be more common than suspected and the problem of underreporting hampers the unravelling of chronic exposure. Because most cases of emetic food poisoning are relatively mild and of short duration and symptoms mimic *S. aureus* food poisoning, significant underreporting seems likely. In addition, a proportion of reported foodborne outbreaks are of unknown cause and the exact incidence of CER is unknown.

Biological activity and toxicity

CER intoxication is generally self-limiting with mild symptoms and therefore not commonly reported. Nevertheless, there are several cases described with organ damage such as liver failure or brain edema [127, 129]. These toxic effects are attributed to the ionophoric properties of CER. This toxin acts as a channel-forming ionophore with a high affinity for alkali ions (K^+, Li^+ Na^+, Cs^+ or Rb^+) and $NH4^+$. The mode of action is analogous to that of the structurally related VAL which is also a potassium ionophore [154]. The formation of K^+ selective channels leads to an increased permeability of phospholipids bilayers of the cell membrane. This results in an ionophoric K^+ uptake with ejection of H^+ and subsequently, the inner mitochondrial membrane is depolarized. This will cause swelling of the mitochondria, reduction of respiratory function, inhibition of ATP synthesis and release of proapoptotic or necrotic factors [155, 156].

As mentioned before, VAL has a similar chemical structure and properties as CER. However, no emetic effects are induced and VAL appeared less toxic then CER towards Hep-2 cells (human larynx carcinoma) [133, 157]. Teplova *et al.* (2006) found that CER binds K^+ ions with a higher affinity and selectivity than VAL, at concentrations below 1 mM. As a consequence, CER exerts its toxic effects at low K^+ concentration which is close to the physiological K^+ level in the serum [158].

The results of Agata *et al.* (1995) indicate that the emetic effects of CER might be the result of binding with the serotoninergic 5-HT3 receptor which leads to stimulation of the vagal afferent neurons and subsequently to vomiting. This theory was supported by the observation that a 5-HT3 receptor antagonist completely abolished the emetic effect [148]. However, it is not clear whether there is direct or indirect binding to the 5-HT3 receptor which is a Ca^{2+} permeable ligand-gated ion channel [159].

In the aftermath of fulminant liver failure in a human case, the hepatic damage was confirmed with animal studies. The hepatocytes of mice injected with CER showed mitochondrial swelling, with loss of cristae and accumulation of small fatty droplets [160]. However, the toxic effects on liver tissue were reversible. The occasional complications of fulminate hepatic failure and brain edema are a consequence of the inhibition of mitochondrial fatty acid oxidation. By acting as a potassium ionophore, the fatty acid oxidation and the mitochondrial activity are affected which results in degradation of the cells. Even hepatic microvascular and extensive coagulation necrosis have been diagnosed in intoxicated patients [126, 127]. Moreover, *post mortem* analysis of a fatal case of *B. cereus* food poisoning caused by CER, demonstrated bioaccumulation in liver, bile, plasma and small intestines [127].

As mentioned, due to the ionophoric properties CER is considered a mitochondrial toxin. Mitochondrial swelling has been observed in the HEp-2 cell line and has been used as a tool for detection called 'HEp-2 vacuolisation assay' [130]. A number of cell lines have been used to evaluate the toxic effects such as HeLa (human cervical carcinoma) cells, Caco-2 (human epithelial colorectal adenocarcinoma) cells, Calu-3 (human lung adenocarcinoma) cells, Paju (human neuroblastoma) cells, Hep2 cells [161, 162]. Most of them focus on the threshold concentration that provokes mitochondrial damage and vacuolization. With the use of assays for mitochondrial activity (MTT) and changes in protein content (sulforhodamine B (SRB)), the impact of low (sub-emetic) doses of CER have been studied in differentiated Caco-2 cells. Concentrations between 0.125 and 1 $ng.mL^{-1}$

induced cell release for the differentiated monolayer, increased lactate presence in cell medium, and decreased energy managing and H_2O_2 detoxification proteins [153]. In addition, the ionophoric effects have caused mitochondrial swelling in natural killer cells which induced apoptosis of the NK cells and consequently inhibition of the cytokine production *in vitro*. [156]. Virtanen *et al.* (2008) observed necrotic cell death in porcine pancreatic Langerhans cells after exposure to CER. Within two days after exposure with 1 $ng.mL^{-1}$ CER, beta-cells were damaged which was indicated by the impaired insulin content [163]. The involvement of CER in beta-cell death has been investigated in type 2 diabetes related research [152].

Only a few *in vivo* studies have been performed regarding the acute toxicity or emetic potential of CER. In one study, the histopathological changes were examined in mice intraperitoneal injected with synthetic CER (5-20 µg per mouse). Within 6 h, dose-dependent liver damage was observed including lesions, small fatty droplets and degeneration of hepatocytes with mitochondrial swelling and loss of cristae. Additionally, general recovery after acute liver dysfunction was observed four weeks after the injection with CER [160]. The emetic activity of CER was confirmed in rhesus monkeys and *Suncus murinus* [147, 148]. Moreover, a toxicokinetic study after a single intravenous injection of 5 µg indicated fast elimination or metabolization of CER in rabbits. Furthermore, liver damage was evaluated by the concentration of aspartate aminotransferase (AST) in plasma. Increasing AST levels in the plasma after 0.5 h demonstrated fast transfer of CER from blood to liver. Again, liver function recovered with normal AST levels 32 h after administration [164].

Although the *in vivo* studies on CER are scarce, cases of human CER food poisoning can provide relevant information on CER toxicity. Reported clinical symptoms such as vomitus, fever, abdominal pain, liver failure and rhabdomyolysis give a first idea of the severity of CER toxicity. In addition, *post mortem* findings like diffuse brain, vacuolar degeneration of renal tubular epithelia and enlarged liver with diffuse microvesicular steatosis as well as the presence of CER in bile, liver, plasma and intestines support the assumption that CER can enter the portal and systemic circulation [127, 129]. Both *in vitro* and *in vivo* research should go on, especially for continuous exposure to low doses since data on chronic effects are lacking.

Detection

A range of different methods has been developed for detection and to assess the toxicity of CER (Table 1.1). First, experimental animal models with monkeys were used to demonstrate emetic activity [165]. Several cytotoxicity tests or *in vitro* tests using Hep-2 cells were based on mitochondrial swelling which appears as vacuolization in the cytoplasm [147, 166, 167]. However, the mitochondrial swelling as diagnostic marker for the presence of the toxin, might be considered as subjective and unreliable. Hence, this assay was improved in combination with MTT [167]. MTT is used as an indicator of cell viability and consequently as a tool to evaluate the cytotoxicity. The boar sperm bioassay relies on the toxicity of CER against mitochondria of boar spermatozoa. Because CER acts by uncoupling oxidative phosphorylation, the energy production which is required for the motility of the sperm cells will be disrupted [168, 169]. Rajkovic *et al.* (2006, 2007) modified this bioassay by implementing a computer assisted sperm analysis (CASA) to generate semi-quantitative results [170, 171]. Based on the mitochondrial toxicity, a quantitative detection method was developed in rat liver mitochondria [172]. These diagnostic methods were hampered because they were based on the toxic action of CER and give a toxicity titer rather than a direct measurement of the toxin itself.

In addition, several molecular PCR-based methods using different primers have been developed which detect the genes encoding for toxin production [135, 173, 174]. Moreover, Dommel *et al.* (2010) developed a bioluminescence reporter system to monitor CER synthetase promoter activity in different environments [175]. Again, these techniques do not provide an accurate concentration of CER. So far, no immunochemical assays for detection of the emetic toxin have been developed due to the poor antigenic properties of CER.

Although these techniques evolved to quantitative detection, none of them directly detects CER. For this reason, LC-MS has become the preferred technique since it offers a specific and sensitive detection of the toxin itself. Nonetheless, Häggblom *et al.* (2002) validated the boar spermatozoa motility assay against HPLC-MS analysis and the results corresponded with the CER concentrations found with HPLC-MS analysis [176]. In search for improved methods for extracting CER from foods, various sample preparations have been investigated combined with LC-MS detection [133, 134, 177]. Because the use of LC-MS/MS for the detection and quantification of CER is more specific compared to bio-assays, ISO standard ISO 18465:2017 for the quantitative determination of CER in products

intended for human consumption is based on LC-MS/MS analysis [178]. The use of chemical analysis as standard method within the scope of food intoxications related to food pathogens is an important evolution.

In many of these LC-MS based methods, VAL has been used as internal standard based on the structural resemblance. However, the production of $^{13}C_6$-labeled CER allowed the development of a stable isotope dilatation analysis (SIDA) to completely overcome matrix effects during LC-MS/MS [179].

Moreover, LC-MS/MS and LC-time of flight mass spectrometry (LC-TOF-MS) have also been used to detect CER [143, 179, 180]. An UPLC–TOF MS profiling method was developed to discriminate *B. cereus* strains into no/low, medium and high producers of the emetic toxin CER. The results were confirmed by absolute quantitation of CER in selected samples by means of HPLC–MS/MS. Hence, this combination provides identification of CER and quantitative comparison of the strains' toxin-producing activity [181].

Table 1.1 Overview of the different approaches for CER detection divided in indirect and direct detection.

Type of detection	Type of assay	Method	'Target'	Reference
Indirect	Whole-animal bioassay	Rhesus monkey	Clinical symptom: emesis	[147]
		Suncus murinus	Clinical symptom: emesis	[148]
	Cell-based bioassay	Boar semen	Mitochondrial toxicity: sperm motility	[133, 168, 170, 171, 176]
			Dissipation of the mitochondrial inner membrane transmembrane potential	[169]
		HEp-2	Mitochondrial toxicity: vacuole formation	[133, 166, 167, 181-183]
		HepG2	Mitochondrial toxicity: vacuole formation	[183]
	Molecular assays	PCR-based	Specific genes	[135, 137, 173-175, 184]
Direct	LC-MS	LC-MS	Cereulide	[133, 176]
		LC-MS/MS	Cereulide	[134, 179, 181, 185-187]

Legislation

A proper risk assessment on CER in food is hampered by the lack of scientific data on CER. Many variables determine the pathogenicity of emetic *B. cereus* strains such as the variety in the amount of CER produced by various emetic *B. cereus* strains and the influence of the immediate environment (the food composition and the gastro-intestinal tract) and environment conditions (temperature, substrates, etc.) on the toxin production.

Therefore, no clear safety margin or threshold value could be determined so far. Moreover, the possible impact of repeated exposure of CER and the long-term effects thereof are largely unknown and need to be clarified. Acquiring more toxicity data, occurrence and exposure will contribute to identifying the risk and reducing the incidence of foodborne disease.

1.2 CYCLIC DEPSIPEPTIDES

1.2.1 STRUCTURE AND OCCURRENCE

From a chemical point of view, both the *B. cereus* toxin CER and the *Fusarium* mycotoxins BEA and ENNs belong to the class of cyclic depsipeptides. Depsipeptides are a subcategory of peptides comprising amino- and hydroxy-acid residues, which are connected by amide and ester bonds. In cyclic depsipeptides, the residues are connected in a ring [188]. Due to the wide diversity of chemical structures of these cyclic depsipetides, a chemical classification system was proposed by Taevernier *et al.* (2017) including 1348 naturally occurring cyclic depsipeptides. The compounds categorized in the same groups are considered to be structurally related in contrast to traditionally formed cyclic depsipeptide subfamilies (*e.g.* dolastatins). Depending on the hydroxyl acid groups involved in the formation of the ester bonds of the cyclic depsipeptdie, six major groups are distinguished: α-hydroxy hydroxyl acid; β-hydroxy or longer chain hydroxyl acid; α + β-hydroxy acid; longer chain + α-hydroxy acid and longer chain + β-hyroxy acid. According to this classification, BEA, ENNs and CER belong to the α-hydroxy hydroxyl acid category, however, with a different number of building blocks (*i.e.* ring size) [189]. CER is a cyclic dodecadepsipeptide (12-membered), while BEA and ENNs are smaller cyclic 6-membered hexadepsipeptides.

Cyclic depsipeptides are widely distrusted in nature as they have been identified from fungi, bacteria, plants, and marine organisms. Many of secondary metabolites play a crucial role for the survival of microorganisms like fungi and bacteria. However, for CER, ENNs and BEA no ecological function has been assigned.

1.2.2 BIOSYNTHESIS

In general, these cyclic depsipeptides are synthesized by non-ribosomal peptide synthases (NRPS) or by hybrids formed by both NRPS and polyketide synthases (PKS). Each cyclic depsipeptide is composed by specific amino acid and carboxylic acid precursors. In the case of BEA, ENNs and CER,

the biosynthesis is catalyzed by NRPS according to a so-called thiol template mechanism pursuing stepwise condensation, where each module of the enzyme system accounts for the incorporation of a specific monomer. This is an intramolecular process in which the building blocks are condensed in an iterative manner by a single NRPS molecule followed by a final cyclization [24]. Additional variations of these cyclic depsipeptides are the result of modifications (*e.g.* epimerisation, heterocyclisation, oxidation, methylation, ketoreduction, dehydration and formylation). This creates an extremely diverse group of molecules with different number and nature of monomeric building blocks, molecular mass, lipophilicity, polarity, side chains and ring size [189].

1.2.3 DETECTION

Due to the great structural differences within the group of cyclic depsipeptides, no common approach for the detection of these compounds is proposed. In the case of the foodborne toxins CER, BEA and ENNs, so far no records have been published on their co-occurrence since no methods for their simultaneous detection have been reported. However, Hoornstra *et al.* (2003) investigated several mitochondria damaging toxins including CER and ENNs A, A1, B and B1 using the sperm motility inhibition assay. Exposure to these toxins resulted in similar effects such as dissipation of the mitochondrial inner membrane transmembrane potential and hyperpolarization of the plasma membrane [169]. For accurate determination of the toxins, the use of LC-MS/MS is most commonly preferred, especially with development of stable isotope-labeled internal standards ($^{13}C_6$-labeled CER and $^{15}N_3$-labeled ENNs and BEA).

In general, triple quadrupole (QqQ) coupled to tandem MS has emerged as the cornerstone technique for the accurate detection and characterization of food contaminants. QqQ-MS/MS provides high sensitivity and selectivity, which are required for the detection of *e.g.* mycotoxins as they can occur in low concentrations. The use of full-scan MS approaches such as LC–time-of-flight (TOF)-MS are less commonly used compared to LC-QqQ-MS/MS in the mycotoxin field. However, due to the improved sensitivity, LC-TOF-MS has increasingly been used in untargeted analyses. The introduction of LC–MS enabled the development of multi-toxin methods suitable for detection of a range of toxins in a single chromatographic run.

1.2.4 IMPORTANCE

The group of cyclic depsipeptides with a diversity of biological activities has attracted a great deal of attention for discovery of new drugs (*e.g.* antibiotics and anticancer drugs). However, there is a thin line between the toxicological risk and the potential therapeutic benefit that should not be overlooked. Cyclic depsipeptides are a class of natural products that possess diverse biological properties that may be useful in the development of new therapeutics.

An important feature of several cyclic depsipeptides such as CER, BEA and ENNs, is their ionophoric action. They can adopt a conformation with the apolar side chains pointing out from the outer surface of the molecules. In this way, the lipophilic molecules are able to incorporate into cellular membranes in which they create cation selective channels. This consequently results in disturbances in the physiological cation level in the cell. Consequently, a high cell membrane permeability with a high affinity for cations will influence the cellular homeostasis. These ionophoric properties are partly responsible for their therapeutic potential. However, the interactions with several cellular compartments and signal transduction pathways are the main reasons for their pharmacologically relevant actions [190]. Some known examples are romidepsin for injection (Istodax®), an Food and Drug Administration (FDA) approved medicine for the treatment of cutaneous T-cell lymphoma and emodepside has been used as part of the patented topical solution Profender® for veterinary use for controlling endoparasites. VAL, which closely resembles CER has been applied in Reflotron® K^+ for the quantitative determination of potassium in heparinized plasma or serum [191-193].

Among the cyclic depsipeptides, BEA and ENNs, which show different kinds of bioactivities, are potential drug candidates. This is mainly because of the reported cytotoxicity in different cancer cell lines, which supports their potential use as anticancer drugs. Moreover, the interaction of ENNs with the multidrug resistance protein Pdr5p in *Saccharomyces cerevisiae* proposed their clinical use in combination with chemotherapeutic drugs. In addition, the interaction of ENNs and BEA with the transport function of ABCB1 and ABCG2 suggested that their intake might influence the pharmacokinetics of other pharmaceutical compounds [194]. The effects of ENNs are less pronounced in non-malignant cells (fibroblasts and HUVEC cells) compared to cancer cells [67]. This feature might be useful in the future in terms of finding the adequate therapeutic window [195]. Only fusafungine (Locabiotal®), a mixture of ENNs, has been developed as a drug used as a topical treatment of upper respiratory tract infections by oral and/or nasal inhalation. However, this

medicine was recently withdrawn due to serious allergic reactions and limited evidence of beneficial effects.

The effect of BEA on Crohn's disease was examined by intrarectal delivery of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to mimic this disease in mice. The results indicated the potential of BEA for the treatment of inflammatory bowel disease development, mainly by inhibiting PI3K/Akt signaling in activated T-cells [196]. In search for new antifungal therapeutics, especially for immunocompromised patients with fungal infections, BEA has been studied as combination therapy. The antifungal miconazole-potentiating activity of BEA was demonstrated against pathogenic fungus *Candida albicans* as well as against fluconazole resistant strain [197]. Furthermore, BEA showed synergistic effects on antifungal activity when co-administered with ketoconazole (KCZ) in rats [198]. In mice, the combination of BEA and KCZ prolonged survival of the host infected with *Candida parapsilosis*, which was not achieved with ketoconazole alone [199]. The stability of a BEA-KCZ combination tablet was tested in order to further develop the combination drug product [200].

Further research should provide more insight in the mechanism of cytotoxicity and their feasible therapeutic potential. Especially because the impact on the central nervous system due to the ability of the cyclic depsipeptides BEA and ENNs to cross the BBB is still unclear [63].

1.3 RISK ASSESSMENT

1.3.1 INTRODUCTION TO RISK ANALYSIS

Although considerable efforts have been made to reduce and prevent foodborne disease, food safety remains a fundamental challenge in both developing and developed countries. In order to provide safer food, the concept of risk analysis has been introduced as a holistic approach towards food safety, where the entire food chain needs to be considered. According to the EC, a risk is defined as an *"undesirable consequence of a particular activity in relation to the likelihood that it may occur"*. Risk analysis is a structured model going from problem formulation to decision making for reduction of foodborne illness and improving food safety systems. It is a powerful tool for a science-based analysis to find consistent solutions for food safety problems. As depicted in Figure 1.5, the process of risk analysis constitutes of the interplay of three major multidisciplinary tasks: risk assessment, risk management and risk communication. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have played an important role in the development

of food safety risk analysis. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) elevated the scientific knowledge for assessing the risks of chemicals in food at regional and international levels [201].

Figure 1.5 Risk analysis.
The diagram depicts the three stages of risk analysis: risk assessment, risk management and risk communication.

Risk managers such as the EC, Member State authorities and the European Parliament, are accountable for making decisions or setting the legislation. For example, when a question is asked to the EC regarding scientific issues that impact human health and the environment, the EC relies on independent Scientific Committees such as the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) before making a legislative proposal. The role of these Scientific Committees and other Union risk assessment bodies such as the EFSA, the European Medicines Agency (EMA), the European Centre for Disease Prevention and Control (ECDC) and the European Chemicals Agency (ECHA) as risk assessors, involves collecting and evaluating existing research data to perform risk assessment and providing scientific advice to support decision-making by risk managers [202].

1.3.1.1 RISK ASSESSMENT

In general, foodborne hazards can be of biological, chemical or physical nature. A hazard is regarded as *"the capability of substance to cause an adverse effect"* in contrast to the risk which considered as *"the probability that the hazard will occur under specific exposure conditions"*. Another definition for risk is *"the chance that an adverse event will happen, multiplied by the extent of the effect"* [202].

The risk assessment process consists of four elements: hazard identification, hazard characterization, exposure assessment and risk characterization [203].

Hazard identification

During **hazard identification**, the agent(s) that can cause adverse health effects under specific conditions and that are present in food are identified. The aim is to evaluate whether the agent has the potential to cause adverse effects in humans. This is done by reviewing the available scientific data and developing an evidence weight for the characterization of the link between the adverse outcomes and the agent. Hazards identification can rely on animal toxicity studies, clinical studies, epidemiological studies, *in silico* methods and *in vitro* tests.

Although clinical trials with humans provide the most accurate information, human *in vivo* studies are often unavailable due to ethical reasons. Furthermore, there is a need to reduce animal testing, which is still commonly used in safety testing of chemicals. *In silico* approaches based on (quantitative) structure activity relationship or (Q)SAR models provide an alternative to otherwise time-consuming laboratory and clinical testing methods by mimicking the results of *in vivo* studies. Currently, QSAR models can be used to supplement experimental data, to help prioritization when no experimental data are available and to replace experimental animal testing methods [204].

Hazard characterization

The **hazard characterization** (or dose-response assessment) is closely related to hazard identification since the nature of the health effects associated with these hazards is studied. It concerns the actual toxicological research in order to estimate the possible adverse health effect. In this step, the relevance of different adverse health effects and the dose-response relation need to be considered. Besides dose-response evaluation, the threshold dose levels for the critical effects are identified such as tolerable daily intake (TDI) and acceptable daily intake (ADI). In general, ADI is used for substances intentionally added to food, while TDI is used for substances appearing in food but not intentionally added. These thresholds values derive from an important parameter called 'no adverse effect level' (NOAEL) which is the highest dose in human or animal studies which has been demonstrated not to cause toxicity. The NOAEL is divided by a safety or uncertainty factor reflecting the limitations of the used data. Solid epidemiological data are often lacking which prevents risk assessors to establish a reliable dose-response relationship for toxic effects. As a result, generally two major extrapolations

are required: from high experimental doses used in the experimental setting to low doses found in the environment and from animal to human dose. Another useful measure is the acute reference dose (ARfD) which is defined as the amount of a substance in food that can be consumed in the course of a day or during a single meal with no adverse effects. An alternative measure that may be useful to some risk managers is the margin of exposure (MOE) and is the magnitude by which the NOAEL of the critical toxic effect exceeds the estimated exposure dose.

Exposure assessment

Exposure assessment defines the human exposure levels. In this stage, the occurrence of the hazard in our food and other possible sources is evaluated. This also involves identification of exposure routes. In addition, an estimation is made to which extent these foods are consumed by specific consumer groups and how often the population is exposed. It is assessed by integration of the consumption and occurrence data and can be based on deterministic and probabilistic approaches.

Risk characterization

In **risk characterization**, the results from the previous three steps are integrated to provide an estimate of the probability that the identified adverse effects will occur in general or in specific population groups. This estimate takes the uncertainties associated with the *in vitro* - *in vivo* extrapolation and translation from animals to humans into account. For threshold chemicals, risk assessors draw conclusions on the level of risk based on guidance values (ADI and RfD), levels of exposure and the intake for which the risk is considered safe. If the long- and short-term exposure levels exceed the recommendations, there may be a safety concern for a given population.

1.3.1.2 RISK MANAGEMENT

Risk management and risk assessment are closely connected processes with a repeated interaction between risk managers, risk assessors and other participants. Based on scientific information gathered during risk assessment, the available options for managing a food safety issue are weighted, costs are compared to reduce the risk and the significance of the estimated risk is evaluated. Risk management includes the evaluation of risk management options. In light of these results, appropriate control options, including regulatory measures are selected and implemented. Within this step, a value-based decision may be reached on the desired level of public health

protection expressed as Appropriate Level of Protection (ALOP). The agreement regarding the Application of Sanitary and Phytosanitary Measures (SPS Agreement) by the World Trade Organization (WTO) defined the ALOP as follows: *"The level of protection deemed appropriate by the Member establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory"* [205]. Regarding food safety, this definition can be interpreted as the level of risk that is accepted by a country for a specific hazard in food [206].

Strategies for risk reduction include prevention of contamination, TDI evaluations and setting regulatory limits such as maximum residue limits (MRL) with the ultimate goal to achieve acceptable regulations on international terms. The outcome of the risk management process consists of the development of standards, guidelines and other recommendations for food safety.

1.3.1.3 RISK COMMUNICATION

During the course of food risk analysis, there is a constant interaction between risk managers, risk assessors and other interested parties (such as industry, consumers and researchers) within the framework of risk communication [207]. The goal is to provide information on the risk management findings and the risk management decisions in order to prevent, reduce or minimize the risk to acceptable levels.

1.3.2 RISK ASSESSMENT OF CHEMICAL MIXTURES

1.3.2.1 INTRODUCTION

Animals and humans are daily exposed to various biological, chemical and physical agents or stressors from various sources (*e.g.* food, consumer products and environment). The exposure to multiple agents can result in altered toxic effects compared to the toxic effects of the individual agents. The health risk associated with co-exposure depends on how the effects of the individual chemicals combine and can be categorized into additive, antagonistic (less than additive) or synergistic (more than additive) toxic effects. In addition, many evaluations only consider the oral ingestion as the route of exposure even though exposure through inhalation and dermal contact might potentially occur.

To move beyond the traditional chemical-by-chemical approach, the conventional methods need to be adapted and extended to handle specific challenges related to chemical mixtures. The complexity

of the problem of chemical mixtures poses a number of challenges to scientists, risk assessors and risk managers including the gaps in combined exposure and toxicity data and the lack of awareness that chemical exposure is mostly associated with mixtures and not with individual chemicals. Moreover, the process of risk assessment of mixtures becomes complex due to *e.g.* the exposure to a wide array of individuals and population with differing susceptibilities (such as infants, adults and elderly), the potential of numerous interactions between stressors and the occurrence of multiple chemicals ('chemical mixtures') in the environment from various sources [208]. As a result, traditional risk assessment does not routinely considered the potential combined effects from exposure to diverse stressors through various routes according to the realistic situation.

For this reason, adequate research on mixtures to evaluate potential 'cocktail effects' and implementation of the assessment methodologies are of genuine importance to address this complex issue.

1.3.2.2 MIXTURE TERMINOLOGY AND ASSESSMENT CONCEPTS

To unravel the interactions of different chemicals and to assess the influence on the overall exposure due to uptake via multiple routes and sources, the combined exposure assessment was introduced which focusses on more than one compound and/or route and pathway. Depending on the types of mixtures and pathways of exposure, cumulative and aggregate exposure represent the two major categories within combined exposure assessments (Figure 1.6). The aggregate exposure combines the exposure to a specific agent or stressor across multiple routes (*e.g.* dermal, inhalation and oral ingestion) and sources (*e.g.* consumer products and food). The cumulative exposures evaluates the combined exposure to multiple stressors via multiple exposure pathways that affect a single biological target (*e.g.* heart) [209, 210].

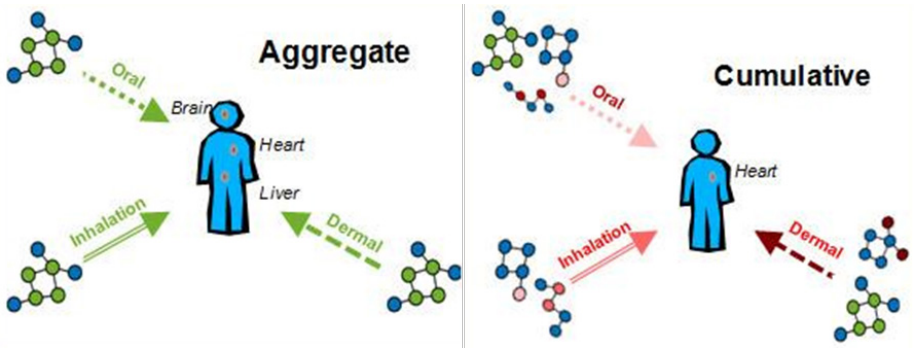


Figure 1.6 Cumulative and aggregate exposure (U.S. EPA, 2003).

Despite the difficulties, a number of approaches are available for characterization of aggregate exposures and cumulative risk assessment. In the classical whole mixture approach, the mixtures are tested in their totality following the same principle of single risk assessment. Other examples are based on the mixture toxicity concepts of Concentration Addition (CA) and Independent Action (IA). The CA approach assumes that the mixture components have the same mode of action, except that they differ in the concentrations to elicit a toxic effect, while according to the concept of IA, the substances that are simultaneously present act independently to contribute to a common biological endpoint [211].

1.3.2.3 INITIATIVES TOWARDS COMBINED RISK ASSESSMENT

The U.S. Environmental Protection Agency (EPA) started to develop a range of tools, guidelines, and other resources (including grant programs) for the evaluation of the cumulative and/or aggregate exposure and risk. In the case of pesticides, efforts were made towards a combined risk assessment. The Food Quality Protection Act (FQPA) of 1996 mandates that the EPA considers both aggregate and cumulative risks. As a result, the EPA's Office of Pesticide Programs (OPP) was required to modify its exposure and risk assessment methods to consider the exposure to a single pesticide by multiple pathways and routes of exposure (aggregate risk assessment) as well as the potential human health risks from all pathways of dietary and non-dietary exposures to more than one pesticide acting through a common mechanism of toxicity (cumulative risk assessment) [212, 213].

In parallel, EFSA launched initiatives such as 'MixTox' and 'Euromix' projects with the goal of developing a practical frameworks and methodologies to assess the combined toxicities of chemicals in different areas of risk assessments, which will help and support risk managers in their decision-making [214, 215]. As part of the MixTox project, EFSA launched in 2013 a cumulative risk

methodology to assess the risk posed by exposure to multiple pesticide residues. In this new approach, pesticides are classified into so-called cumulative assessment groups (CAGs) based on compounds with similar toxicological properties in a specific organ or system. The methodology consists of hazard identification/characterization, data collection and grouping of pesticides. During hazard identification the toxic effects that adversely affect an organ or system are identified. Hazard characterization defines the nature of these adverse effects to specific organs or systems. Next, all data on the indicators related to a specific toxic effect in an organ/system are gathered. In a final step, pesticides with a similar toxicological effect are categorized into CAGs by organ or system [216]. This methodology has been applied to define groups of pesticides, which are toxic to the thyroid and central nervous systems. However, the implementation of these cumulative risk assessment methodologies is still ongoing and further work is still needed to refine the methodologies and extend to other groups of substances (*e.g.* metals and other contaminants). The Euromix project aims to develop a tiered strategy for risk assessment of mixtures of multiple chemicals derived from multiple sources. An important concept of this new strategy is the identification and prioritization of the compounds relevant for the project based on their exposure and hazard identification. To categorize the chemicals into CAGs, *in silico* tools and computational models such as QSAR will be applied with the focus on four selected endpoints (liver, hormones, development and immunology). As a result, the projects provided an innovative platform of bioassays for mixture testing and for categorization of chemicals in CAGs. The goals is to provide a clear guidance on a tiered hazard and exposure test and risk assessment strategy that serves as a scientific basis for managing risks from chemical mixtures [215].

In addition, EFSA published a review of international frameworks for assessing the risk of exposure to chemical mixtures. This scientific report will support the introduction of harmonized terminology and methodologies for risk assessors [217]. Prior to performing risk assessment, the problem is formulated by identification of priority chemicals taking into account relevant hazard and exposure information. Next, the classical four steps of the risk assessment paradigm used for single chemicals hazard identification, hazard characterization, exposure assessment and risk characterization follow. In addition, tools for modelling combined toxicity are being developed since numerous combinations of chemicals are possible.

Considering the ability of fungi to produce several mycotoxins on the same crops and their food products, the concepts of risk assessment of aggregate exposure and cumulative risk assessment may be relevant for mycotoxins. Hence, there is an increasing trend towards reliable and sensitive multi-toxin detection. Despite these efforts, risk assessments and established regulations focus on the individual mycotoxin exposure without considering the combined effects of mycotoxins. To address the issues of co-occurrence and co-exposure, combined toxicity and cumulative risk assessment, several research projects have been initiated in the mycotoxin field [218]. An example is the 'MycoMix' project in which the risk assessment of multiple mycotoxins present in breakfast cereals consumed by children (1–3 years old) from Lisbon region, Portugal was performed. The results indicated that for the aflatoxin group (including AFB1, AFB2, AFG1 and AFM1) a potential health concern is associated with the consumption of breakfast cereals [219]. Another study applied the CA approach for the assessment of the cumulative risk associated with dietary intake of multiple mycotoxins (DON and its acetyl derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON)) in maize and wheat originating from Shanghai, China [220]. Currently, the EU research project entitled 'EuroMix' aims to develop new, efficient and validated test strategies for the toxicity of mixtures of multiple chemicals (including mycotoxins) derived from multiple sources. Within this project, key mixtures will be tested and the results will be used in future aggregate and cumulative exposure assessments. Due to the societal need to reduce animal testing, *in silico* methods and *in vitro* bioassays for mixture testing will be explored and validated against *in vivo* animal tests. In turn, the results of *in silico* methods and *in vitro* bioassays need to be extrapolated to humans. In the future, the developed tools and models will be applied for performing realistic assessment of chemical mixtures [215]. For combined risk assessment, more research is required before conclusions can be drawn regarding the impact of mycotoxin mixtures.

1.3.2.4 BEA, ENNS AND CER

The emerging *Fusarium* mycotoxins BEA and ENNs are classified as cyclic depsipeptides based on their similar chemical structures. Within this large group of cyclic depsipeptides, CER is also considered a foodborne toxin. These cyclic depsipeptides exert a common mechanism of toxicity based on their ionophoric properties, which potentially can lead to the complex scenario of aggregate and cumulative exposure. The FQPA defined cumulative effects as substances that have a 'common mechanism of toxicity', which is the case for BEA, ENNs and CER. The combination of those substances can affect the overall level of toxicity. Previous *in vitro* studies have demonstrated

additive and synergetic effects of ENNs depending on the concentrations and on the combination of mycotoxins [221, 222]. Regarding occurrence, more and more studies have considered the total ENNs instead of the individual toxins, due to the similar structure as well as high potential to occur simultaneously. Based on incidence data, the sum of ENNs can reach alarming levels that might pose concerns for chronic exposure to humans and animals, particularly because of their similar toxicological actions [71]. In addition, BEA and ENNs are likely to co-occur with other *Fusarium* mycotoxins such as the trichotechenes. Therefore, the multi-toxin approach should be extended to toxicity studies that focus on the interactions between multiple mycotoxins.

1.4 OBJECTIVES

1.4.1 BACKGROUND

This research was initiated due to the recognition that BEA, ENNs and CER are all foodborne microbial toxins with a cyclic depsipeptide structure. The invasion by toxigenic fungi and bacteria and the associated contamination with toxins is a worldwide problem leading to substantial amounts of food waste and economic loss. Moreover, due to acute and chronic effects associated with their exposure, the toxins have been recognized as major threats to human and animal health. This thesis focusses on detection, occurrence and cytotoxicity to attribute to the first steps of risk assessment. The foundation of this thesis is a reliable detection method that can be applied or adapted within to scope of a certain question.

1.4.2 MAIN OBJECTIVES

An imperative step in order to assess the impact of these toxins on food safety is the development of fast and reliable tools for quantitative detection. Based on the structural resemblance, a common approach towards an LC-MS/MS can be applied. So far, no methods combine the cyclic depsipeptides of fungal and bacterial origin. Therefore, the **first objective** was the development and validation of a methodology for the specific detection and quantification of BEA, the related ENNs and CER in cereals and cereal-based food products (Chapter 2). The goal was to provide a method that can be adapted according to the research question (*e.g.* different types of food and toxins). This analytical method was applied in Chapter 3 and modified in Chapter 4 in order to fit within the scope of the scientific problem or question.

The **second objective** of this research was to provide quantitative data on the occurrence of CER, BEA, and ENNs in Belgium (Chapter 3). Therefore, the analytical method described in Chapter 2 was applied to Belgian wheat samples. During a two-year study, the incidence of BEA, ENNs and CER was evaluated. In turn, the occurrence data served as basis for correlation studies regarding fungicide treatment, wheat genotypes, several *Fusarium* species and weather conditions.

A **third objective** was to gain inside into the toxicogenic capacity of *F. avenaceum* and to assess the possible link between virulence of *F. avenaceum* and mycotoxin production. In view of this, Chapter 4 described the development and validation of an LC-MS/MS method for leek samples based on the analytical method described in Chapter 2. This method was developed after the discovery of *F. avenaceum* on leek, which is one of the most important vegetables cultivated outdoors in Belgium. For this reason, the method was focusing on relevant *F. avenaceum* toxins BEA, ENNs and MON. The detection of CER was not relevant in this case and therefore omitted.

It is known that the foodborne cyclic depsipeptides CER, BEA and ENNs act as mitochondrial toxins due to their ionophoric properties. Therefore, a **fourth objective** was to investigate the *in vitro* toxicity of these toxins, using CER as model. Concerning the toxicity of CER, the acute effects associated with CER food poisoning have been reported. However, the impact of repeated exposure to low doses of CER is still unclear. In view of this, an *in vitro* study was conducted to study the long-term impact of repeated exposure to low doses of CER (Chapter 5).

The objectives are summarized per chapter in Table 1.2. For each chapter, an overview of the type and number of samples and the target toxins are given.

Table 1.2. Overview of the different experimental chapters with the type and number of samples, target toxin and purpose.

Chapter	Samples: type and number		Toxins	Purpose
2	Maize, wheat, pasta and rice: 59		CER - BEA - ENNs	Method application
3	Growing 2014-2015	Growing 2015-2016	CER - BEA - ENNs	Occurrence
	-	Wheat: 140	BEA - ENNs	Relation with *Fusarium* spp., wheat genotype and fungicides
	Wheat: 32	Wheat: 66	BEA - ENNs	Inter-season variability and impact of weather
4	Leek: 76		MON - BEA - ENNs	Relation with virulence of *F. avenaceum*
5	-		CER	Cytotoxicity

Chapter 2

Development and validation of UPLC-MS/MS methods for the simultaneous determination of BEA, ENNs A, A1, B, B1 and CER in maize, wheat, pasta and rice

This chapter was redrafted from:

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Development and validation of UPLC-MS/MS methods for the simultaneous determination of BEA, ENNs A, A1, B and B1 and CER in maize, wheat, pasta and rice

2.1 INTRODUCTION

Contamination of food and feed with toxins is one of the main concerns in the food industry. Both bacteria and fungi are capable of producing microbial metabolites in food and feed under the appropriate environmental conditions. These toxins can enter the food chain directly through contaminated food or indirectly through the presence of contaminants in food of animals, which were fed with contaminated grains. Even though several pre- and post-harvest efforts such as sorting, kernel and hand sorting are made in order to prevent and control bacteria and fungi, the produced toxins can remain active even after very harsh treatments [223]. In addition, the toxins are stable under the most common conditions used in food processing and can consequently be found in the prepared products [45, 121]. Contamination with toxins of fungal and bacterial origin may lead to acute poisoning or have long-term negative consequences on the health of both human and animals [224]. Besides the health risk, contaminated food and feed cause financial losses with enormous economic impact all over the world. Therefore, an assessment of the presence and impact of these harmful toxins is imperative and starts with developing methods for their detection and quantification.

The most prevalent mycotoxins such as zearalenone, aflatoxins, ochratoxin A and trichothecenes (DON) have been frequently studied. Unfortunately, there is limited data on the toxicity and occurrence of the so-called 'emerging' mycotoxins. These mycotoxins are neither routinely determined, nor legislatively regulated. Examples are BEA and the related ENNs A, A1, B, B1, both produced by several *Fusarium* species. Their presence has been reported in cereals from several countries and in human biological fluids [36, 42, 86]. Recently, EFSA published an opinion on the presence of ENNs and BEA in food and feed, but the lack of relevant toxicity data prevented a risk assessment [71].

In addition to mycotoxins, bacterial toxins are of global concern, mainly related to foodborne illnesses. The latest report of EFSA on zoonoses, zoonotic agents and foodborne outbreaks revealed that bacterial toxins encounter for 19.5 % of all reported foodborne outbreaks caused by microbial contamination. This figure shows an increase of almost 50 % over a period of five years [225]. Foodborne bacterial pathogens that are well known as toxin producers are *Staphylococcus aureus*, *Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus*. The most resistant toxin produced by these pathogens is the emetic toxin CER. *Bacillus cereus* is a gram-positive spore-forming pathogen that causes two types of food poisoning syndromes: an emetic (vomiting) intoxication and a diarrheal infection. The emetic syndrome, which is inducted by the toxin CER, results in vomiting a few hours after ingestion of the contaminated food [150]. Although *B. cereus* can be present in various food products, most reported food poisoning cases were associated with rice and pasta dishes. This emetic toxin is often related to acute food poisoning, occasionally even with a fatal outcome [128, 149]. CER is characterized by its resistance to extreme pH and heat conditions, and resistance to digestion enzymes like pepsin and trypsin [121]. Consequently, it survives food processing and preparation, and retains activity during gastrointestinal passage [121, 182]. This illustrates the high importance of a rapid identification and detection of the emetic toxin.

BEA, ENNs and CER are all cyclic depsipeptides with ionophoric properties. The chemical structures are depicted in Figure 1.1 and Figure 1.4 (Chapter 1). Both the bacterial toxin CER and the fungal toxins BEA and ENNs are regarded as emerging health hazards and their striking similarities should allow a common approach towards the development of a detection technique. The possible co-occurrence of the different toxic compounds in one matrix implies a potential risk for additive, synergic or antagonist toxic effects. Considering the risks to human and animal health, the determination of the occurrence of these medium-sized cyclic depsipeptides in food and feed is imperative. Their potential presence at low levels is of special relevance to food safety [153, 226].

The risk associated with the presence of these toxins initiated the search for more sensitive analytical methods applicable in various matrices. Santini *et al.* (2009) published a review that summarizes techniques used for extraction and quantification of BEA and fusaproliferin in food matrices [40]. It became clear that in the search for low detection levels, mass spectrometry has been increasingly used to achieve this goal. The commonly exerted steps regarding the sample preparation are extraction with solvents sometimes followed by an extra clean-up with different types of columns

and/or a filtration step. Over the past few year, several methods have been developed for BEA and/or ENNs using mainly acetonitrile, chloroform, methanol or a mixture with water as extraction solvent [6, 80, 81, 113, 227]. Alternately, Ambrosino *et al.* (2004) optimized a sample preparation involving supercritical fluid extraction (SFE) with supercritical CO_2. SFE with methanol as modifier provided similar extraction yields compared to conventional extraction protocols [84]. Although this procedure required less organic solvent, it has not been used regularly. Most studies focused on the detection in cereal (based) samples, but only a few reported method development for biological samples like hen eggs and pig plasma [81, 228, 229]. Sample preparation time and detection levels significantly improved from 1-50 $mg.kg^{-1}$ to trace analysis at low $\mu g.kg^{-1}$ levels by switching from HPLC with UV or DAD detection to UPLC with (tandem) MS detection [82, 113]. Concerning CER, the use of LC-MS is preferred over the HEp-2 cell assay and the boar sperm motility bioassay. Parallel to BEA and ENNs, improved sample preparation is essential for an accurate quantification. Methods developed for determination of CER revealed similar sample preparation involving liquid extraction followed by a filtering and/or centrifuging step. Among the increasing number of studies focusing on the determination of the emerging *Fusarium* mycotoxins, none of the studies included CER as target compound. Nevertheless, these toxins have been reported in similar kinds of food matrices, more specifically cereals and cereal-based food products.

The goal was to develop and validate simple sample preparations with a minimum of additional clean- up steps for the simultaneous analysis with LC-MS/MS. The selection of the matrices was based on relevance of the matrix with respect to (myco)toxin contamination. Since food poisoning caused by CER is often associated with rice and pasta dishes, these matrices were included. Concerning BEA and ENNs, mainly grains such as wheat and maize are reported and therefore added. The selected matrices are relevant sources of contamination, which might give insight into co-occurrence of CER and BEA and the related ENNs. Such approach will foster efforts of studies of mixture toxicities, which is one of the primary targets in current regulatory toxicology.

2.2 MATERIALS AND METHODS

2.2.1 REAGENTS AND CHEMICALS

Methanol (absolute, LC-MS grade), acetonitrile (HPLC grade and LC-MS grade) and glacial formic acid (99 %, ULC-MS) were purchased from BioSolve BV (Valkenswaard, the Netherlands). Methanol

(HiPerSolv Chromanorm HPLC grade) was obtained from VWR International (Zaventem, Belgium). Ammonium acetate was supplied by Merck (Darmstadt, Germany). Water was purified on a Milli-Q® SP Reagent water system from Millipore Corp (Brussels, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 µm) were obtained from Millipore (Bredford, MA, USA).

2.2.2 STANDARD SOLUTIONS

BEA, ENN A, ENN A1, ENN B, ENN B1 (1 mg, solid standard) and VAL (10 mg, solid standard) were purchased from Sigma-Aldrich (Diegem, Belgium), while CER (1 mg, solid standard) was supplied by Chiralix (Nijmegen, The Netherlands). Primary stock solutions were prepared by dissolving the solid standard in acetonitrile (1 $mg.ml^{-1}$). All stock solutions were stored at −20 °C, except VAL was stored at 4 °C. Working solutions of 10 $\mu g.mL^{-1}$ were prepared in acetonitrile, stored at 4 °C and renewed monthly. Mixture solutions (BEA, ENNs and CER) were prepared prior to each experiment by diluting the working solution in acetonitrile.

2.2.3 NATURALLY CONTAMINATED SAMPLES

A total of 59 food and feed samples were randomly collected in Belgium. White rice (n = 12) and white pasta (n = 12) samples were collected from Belgian supermarkets in 2015. Wheat (n = 10) and maize (n = 25) samples were randomly collected from several European and African countries such as Nigeria, Zimbabwe, Poland, Italy, Spain and Hungary. The samples were collected after harvest and immediately stored at room temperature until analysis. The samples were quantified with matrix-matched calibration curves using blank samples. Both the unknown samples as well as the spiked samples of the calibration curve were treated as described below (2.3.2).

2.2.4 OPTIMIZATION OF LC-MS/MS PARAMETERS

The method development was initiated by optimization of MS/MS parameters by introducing a constant flow (10 $\mu L.min^{-1}$) of the individual analyte (10 $ng.\mu L^{-1}$) into the ion source using a syringe infusion pump. In order to optimize the MS parameters, tuning solutions of each compound (10 $ng.\mu L^{-1}$) were directly infused (flow rate of 10 $\mu L.min^{-1}$) into the mass spectrometer. Ideal fragmentation conditions were accomplished by varying the cone voltage and collision energies.

For chromatographic separation, three columns were compared Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm X 50 mm), Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm X 100 mm) and Symmetry C_{18} (5 µm, 2.1 mm x 150 mm). Furthermore, various mixtures of solvents such as methanol, acetonitrile, and methanol-acetonitrile as mobile phase were tested. Further, the use of modifiers (*e.g.* formic or acetic acid) and ammonium buffers (*e.g.* ammonium acetate and formic acid) was considered in function of the MS signals. In addition, different column temperatures (25 to 40 °C) and flow rates (0.2 $mL.min^{-1}$ and 0.3 $mL.min^{-1}$) were evaluated. The gradient eluent program was optimized to achieved high separation within a short time frame.

2.2.5 OPTIMIZATION OF SAMPLE PREPARATION

During the optimization of the extraction procedure, the performance of the extraction was evaluated by extraction yield experiments. Therefore, blank samples were spiked in triplicate at one concentration level before and after extraction. Calculations were performed by comparing mean peak areas of the toxin in samples spiked before and after extraction which provides the extraction recovery (%). Based on literature and overall physicochemical properties of the target toxins different proportions of acetonitrile/water and methanol/water were investigated in order to achieve acceptable extraction recoveries [29, 83, 187, 230]. For further clean-up, the use of SPE cartridges (Oasis HLB™), membrane filters (Filter Paper Circles MN 617 11 cm diameter, Whatman™ glass microfiber filters circles 21 mm diameter), centrifugal filter devices (Millipore Ultrafree®-MC centrifugal filter devices 0.22 µm) and an n-hexane defatting step was investigated.

2.2.6 METHOD VALIDATION

2.2.6.1 *VALIDATION DESIGN*

For validation study, Commission Decision 2002/657/EC, Commission Regulation 401/2006/EC and International Conference on Harmonisation (ICH) guidelines were used as guidance [231-233]. Since no reference material was available, spiked blank samples of the corresponding matrix were used for validation of the multi-method for wheat, maize, rice and pasta. During method validation the performance characteristics of the method were evaluated by a set of parameters: linearity, apparent recovery (R_{app}), repeatability (intra-day RSD_r), intermediate precision (inter-day RSD_R) and measurement uncertainty [231]. Determination of limit of detection (LOD) and limit of quantification

(LOQ) was based on ICH guidelines [233]. All validation parameters were calculated using the response (ratio of peak area of analyte to peak area of internal standard valinomycin). Calibration curves were obtained by plotting the response of each analyte against the spiked concentration levels. For confirmatory methods, four identification points should simultaneously be fulfilled to assure appropriate certainty in identification: one precursor and at least two products ions should be monitored, both with a signal-to-noise (S/N) ratio more than three, the relative intensities of the detected ions should correspond with those of the calibration within accepted deviations and the relative retention time (with regard to the internal standard) of the detected ions must range within a margin of 2.5 % [231].

2.2.6.2 *LOD, LOQ AND LINEARITY*

Limit of detection (LOD) and limit of quantification (LOQ) were experimentally determined according to the ICH guidelines [233]. Therefore, blank samples were spiked with decreasing concentrations of the toxins of interest and treated as described in 2.2.5. For this purpose, the selected concentration range was close to the expected LOD and LOQ levels determined during method optimization. This experiment was conducted in three independent replicates for each matrix.

Subsequently, a calibration curve was constructed and LOD and LOQ were calculated based on the standard deviation of the response (σ) and the slope (S) [233]:

$$\mathrm{LOD} = \frac{3.3\sigma}{\mathrm{S}}$$

$$\mathrm{LOQ} = \frac{10\sigma}{\mathrm{S}}$$

Since the linear range of most analytical instruments is known to be limited, the linearity should be assessed. The calibration curve starts around the calculated LOQ and covers a concentration range based on experimental data obtained during method development as no legal limits exist for CER, BEA and ENNs. The linearity of the calibration curves was expressed using the coefficient of determination (R^2) and confirmed by means of the lack-of-fit test (SPSS) [234].

2.2.6.3 *ACCURACY AND MEASUREMENT UNCERTAINTY*

For accuracy and measurement uncertainty, blank samples of each matrix were spiked in triplicate

on low, medium and high concentration levels with the different toxins. For each toxins, these three concentrations levels can be found in Table 2.3. The procedure was executed on three consecutive days. Accuracy is studied as two components: trueness and precision. Trueness can be expressed as bias (%) or as apparent recovery (%). Since no certified reference material was available, the apparent recovery (R_{app}) was assessed by addition of known amounts of the analytes to a blank matrix. The apparent recovery (%) is defined as the ratio of the observed concentration for the spiked sample, calculated from the matrix-matched calibration curves, divided by the reference or spiked concentration. For precision, repeatability (intra-day precision) and intermediate precision (inter-day precision) were evaluated by calculating the relative standard deviation (RSD), respectively RSD_r and RSD_R using One-Way ANOVA. To report analytical results with respect to their measurement uncertainty, three concentration levels (low, medium, high) were determined and the measurement uncertainty was estimated at that level. This uncertainty is the range within the analytical result is likely to fall and depends on the inherent "trueness" and precision of the analytical method. The combined standard uncertainty (u_c) is equal to the positive square root of the intermediate precision (RSD_R) and the bias of the analytical method, which is associated with the uncertainty of the purity of the standards ($U[C_{ref}]$), the accuracy of the bias (S_{bias}) and the root mean square of the bias (RMS_{bias}). Measurement uncertainty was expressed as the combined expanded measurement uncertainty (U), by using a coverage factor k = 2 providing a level of confidence of approximately 95 %.

$$U = 2 \cdot u_c = 2 \cdot \sqrt{RSD_R^2 + U[C_{ref}]^2 + S_{bias}^2 + RMS_{bias}^2}$$

2.2.7 STATISTICAL ANALYSIS

Data processing and calculations were performed using Microsoft Office Excel 2010, IBM SPSS Statistics 22 and GraphPad Prism 6.

2.3 RESULTS AND DISCUSSION

2.3.1 OPTIMIZED LC-MS/MS PARAMETERS

LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Waters Quattro Premier XE™ Mass Spectrometer (Waters, Milford, MA, USA) equipped with an electrospray

interface (ESI). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used.

The MS analyses were carried out using multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI+). The two most abundant product ions were selected. Ideal fragmentation conditions were accomplished by varying the cone voltage and collision energies for each compound and can be found in Table 2.1. The product ion with the highest intensity and S/N ratio was selected for validation and quantification, whereas the second production ion was used for confirmation.

Promoting the formation of $[M+NH_4]^+$ adducts led to higher signal intensities, hence ammonium adducts were chosen as precursor ions. Initially, the three most abundant product ions (including the $[M+H]^+$ ion) for each compound were selected. After optimization of the sample preparation, the two most intense transitions were further used for quantitative and qualitative purposes.

Table 2.1 Optimized ESI^+ MS/MS parameters for CER, BEA and ENNs including VAL (internal standard).
*The underlined product ion is the most abundant and thus used for quantification purposes.

Compound	Precursor ion (m/z)	Molecular ion	Product ion (m/z)*	Cone (V)	Collision (eV)
CER	1170.7	$[M + NH4]^+$	<u>172.3</u>	70	76
			314.2	70	62
BEA	801.3	$[M + NH4]^+$	<u>244.3</u>	38	47
			262.4	38	47
ENN A	699.2	$[M + NH4]^+$	210.3	20	43
			<u>682.3</u>	20	17
ENN A1	685.4	$[M + NH4]^+$	<u>210.3</u>	38	32
			228.3	38	36
ENN B	657.3	$[M + NH4]^+$	196.3	40	30
			<u>640.2</u>	40	17
ENN B1	671.2	$[M + NH4]^+$	196.3	32	32
			<u>654.0</u>	32	18
VAL	1128.6	$[M + NH4]^+$	<u>343.5</u>	66	62
			713.5	66	44

Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 50 mm) with a flow rate of 0.3 $mL.min^{-1}$. This stationary phase provided a good separation and

shortened the analysis time. The column and auto sampler temperature were set at 30 °C and 20 °C, respectively. Furthermore, based on peak intensity, shape and resolution, a mixture of ACN and MeOH (80/20, v/v) was used as organic solvent of the mobile phase. Preliminary experiments indicated that the use of ammonium acetate and formic acid improved the efficiency of the MS ionization of the toxins. The final mobile phase consisted of eluent A (water/organic solvent, 95/5, v/v) and eluent B (organic solvent/water, 98/2, v/v) both containing 1 mM ammonium acetate and 0.3 % formic acid. Gradient elution allowed separation in 7 min. A gradient eluent program at a flow rate of 0.3 $mL.min^{-1}$ and a column temperature of 30 °C resulted in a better separation and peak symmetry. The gradient elution program initiated with 70 % eluent B that was linearly increased to 100 % in 3 min. From 3 to 5 min an isocratic phase of 100 % eluent B was maintained. In 0.1 min the gradient switched again to 70 % eluent B and was maintained for 2 min to equilibrate the column. The dwell volume to allow method transfer across different instrument platforms was not experiment determined. However, a dwell volume of 0.073 mL was proposed for an Acquitiy UPLC I system with a binary pump system by Waters. The antibiotic valinomycin (VAL) was selected as internal standard based on the structurally resemblance with CER [18, 31]. Total ion chromatograms of a spiked rice sample which was achieved with the optimized method, are shown in Figure 2.1.

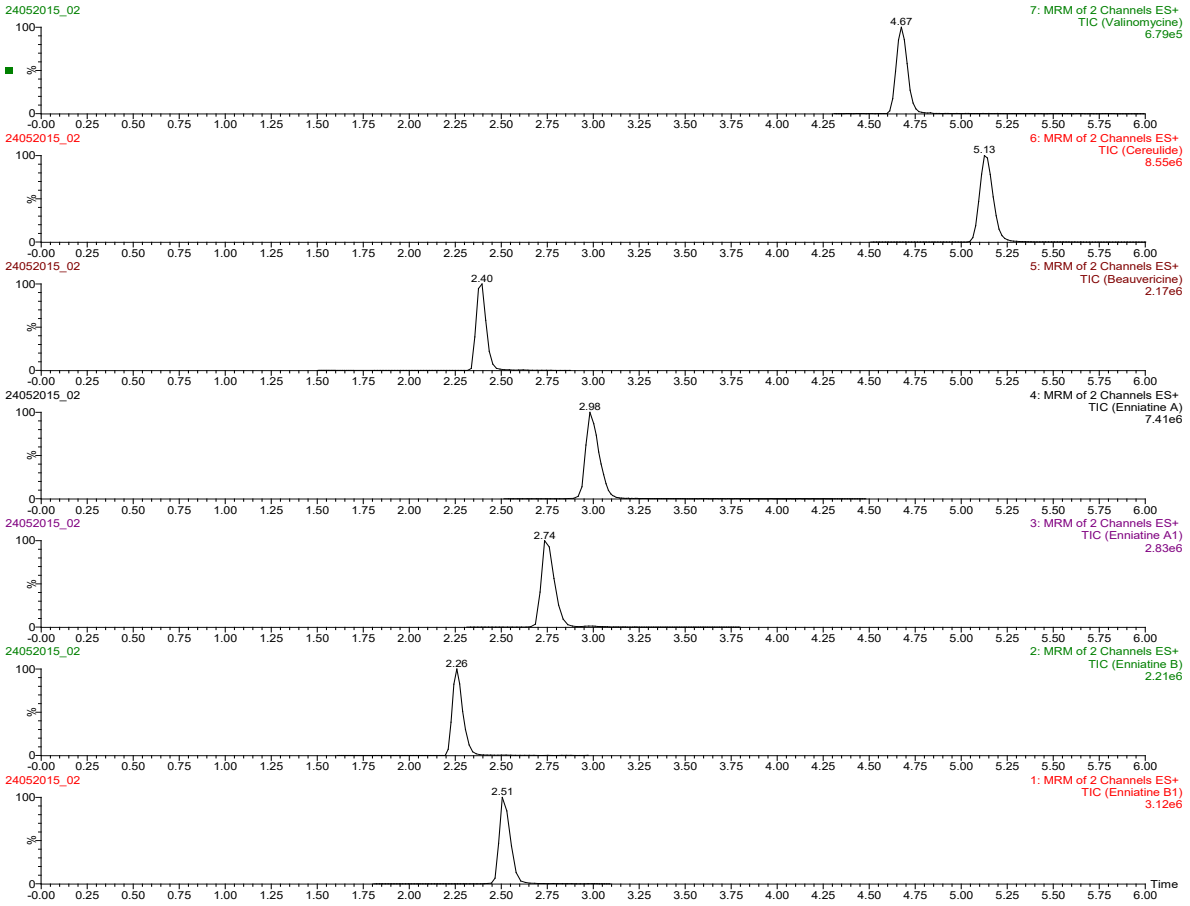

Figure 2.1 Total ion chromatograms (TIC) of the analytes in a spiked rice sample at 100 µg.kg^{-1}.

2.3.2 OPTIMIZED SAMPLE PREPARATION

In this study the best compromise for the simultaneous extraction, based on extraction recovery was achieved by using 100 % MeOH for rice, while for the other matrices ACN/H_20 (84/16, v/v) gave the best recovery results.

The performance of the additional clean-up step was again evaluated by extraction recovery experiments. Only the use of centrifugal filters prior to LC-MS/MS analysis gave cleaner sample extracts with a comparable recovery (results not shown). Clean-up procedures using n-hexane, membrane filters and SPE resulted in lower or comparable recoveries (data not shown). Since a simple liquid extraction is less time-consuming and allows reaching similar recovery results, the clean-up steps with n-hexane and SPE were omitted. Recovery data for the different matrices and the different toxins extracted with the selected solvents are given in Figure 2.2. Using these

extraction solvent, the extraction recoveries of all toxins from the four tested matrices were close to 100 % (ranging between 84 and 106 %), with low SD values.

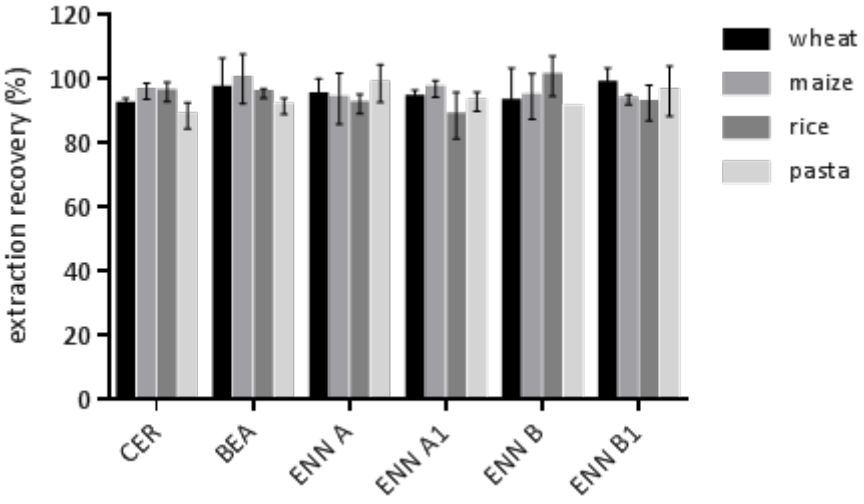

Figure 2.2 Mean extraction recovery ± SD of CER, BEA and ENNs (%) (n=3) in wheat, maize, rice and pasta.

Finally, the best conditions were selected for a simple and fast sample preparation. Initially, the food and grain samples were homogenized and ground using a M20-grinder (Ika Werke, Staufen, Germany). Then, 2.000 g ± 0.005 g portions of the homogenized samples were transferred into 50 mL extraction tubes. Each sample was fortified (spiked) with a fixed concentration (10 µg.kg^{-1}) of VAL internal standard and mixed with a vortex for 0.5 min. After leaving the samples 30 min for equilibration, 10 mL of extraction solvent was added. For rice, this extraction solvent was 100 % MeOH, while for the other matrices ACN/$H_2$0 (84/16, v/v) was selected. Samples were extracted for 20 min using an overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France) and subsequently centrifuged for 10 min at 4000 x g. An aliquot of 8 mL supernatant was transferred and evaporated to dryness under a gentle stream of nitrogen using a Turbovap LV Evaporator (Biotage, Charlotte, USA). After solvent evaporation, the extract was reconstituted with 300 µL of the injection solvent (eluent A/eluent B, 20/80, v/v), vigorously vortexed for 1 min and filtered through an Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) prior to injection in the LC-MS/MS system.

2.3.3 METHOD VALIDATION

2.3.3.1 *LOD, LOQ AND LINEARITY*

For each matrix, calibration curves were constructed in triplicate by spiking blank samples with increasing concentrations around the expected LOD. Based on these calibration curves the LOD and LOQ values were calculated. Consequently, the mean recoveries and the associated repeatability was verified for the calculated LOQ. Only LOQ values with mean recoveries within the range 70–110 % and an associated repeatability $RSD_r \leq 20$ % were accepted [231]. The LODs ranged from 0.1 to 1.0 $\mu g.kg^{-1}$ and the LOQs from 0.3 to 2.9 $\mu g.kg^{-1}$.

Based on preliminary experiments during method development and data found in literature, concentration ranges were selected for the different toxins and the different matrices. The level of linearity of the calibration curve is crucial for the quality of a method. Therefore an appropriate regression model should be selected, preferably a linear regression model [234]. According to the coefficients of determination (R^2), with the lowest observed value being 0.978 for ENN B1 in wheat, calibration curves revealed good linearity within the selected range for all analytes. Furthermore, a lack-of-fit test was carried out to assess if the regression model fits the data. *p*-values above 0.05 demonstrated no lack of fit of the linear model within the selected range. These results ascertain the linearity for the compound within the selected ranges [234]. In addition to the lack-of-fit test, an evaluation of the residual plot was done. If individual residuals deviate by more than ±20 % from the calibration curve, weighted linear regression ($1/x^2$) was used [235]. An overview of the linearity data of the matrix-matched calibration curves is shown in Table 2.2. By lowering the highest concentration of ENN A in wheat, maize and rice from 400 to 200 $\mu g.kg^{-1}$, the linearity improved remarkably, especially when preforming a lack-of-fit test. For pasta, the concentration ranges are smaller compared to the other matrices. This adjustment increased both trueness and linearity while still covering the relevant concentration range for dry pasta samples.

Table 2.2 Concentration range (µg.kg^{-1}) and R^2 values of the matrix-matched calibration curves in wheat, maize, rice and pasta with the corresponding LOD and LOQ (µg.kg^{-1}) for CER, BEA and ENNs.

	Wheat				Maize				Rice				Pasta			
	Range (µg.kg^{-1})	R^2	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	Range (µg.kg^{-1})	R^2	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	Range (µg.kg^{-1})	R^2	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	Range (µg.kg^{-1})	R^2	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})
CER	2-400	0.995	0.97	2.94	2-400	0.996	0.11	0.32	2-400	0.997	0.11	0.32	1-100	0.989	0.22	0.66
BEA	2-400	0.983	0.64	1.94	2-400	0.988	0.09	0.27	2-400	0.996	0.23	0.70	1-100	0.986	0.34	1.03
ENN A	2-200	0.983	0.72	2.19	2-200	0.984	0.47	0.75	2-200	0.990	0.87	0.38	1-100	0.989	0.20	0.60
ENN A1	2-400	0.998	0.51	1.54	2-400	0.994	0.47	1.41	2-400	0.995	0.87	2.63	1-100	0.995	0.45	1.37
ENN B	2-400	0.980	0.65	1.95	2-400	0.997	0.21	0.64	2-400	0.995	0.21	0.65	1-100	0.983	0.33	1.01
ENN B1	2-400	0.978	0.78	2.38	2-400	0.992	0.49	1.49	2-400	0.988	0.29	0.88	1-100	0.980	0.38	1.16

2.3.3.2 *ACCURACY AND MEASUREMENT UNCERTAINTY*

The trueness was evaluated by recovery experiments and results were reported as apparent recovery (%). Note the difference between the terms 'recovery' and 'apparent recovery'. Recovery is related to the yield of the extraction stage and therefore named extraction recovery in this study, whereas apparent recovery (R_{app}) is used to denote ratio of the observed value for the spiked sample, obtained via a calibration graph, divided by the reference value [236]. Hence, blank samples were spiked with increasing concentration of the toxin standards prior to extraction and analyzed by the method described above. All values for R_{app} varied from 84 to 109 % and are thus in good agreement with the guideline ranges (80–110 %) of 2002/657/EC [231]. Results are summarized in Table 2.3.

Validation of analytical methods for quantitative determination includes an investigation of precision. Precision was considered at two levels: repeatability (intra-day) and intermediate precision (inter-day). The reported RSD_r-values for repeatability are based on three determinations for low, medium and high concentration levels within one day. To express variations between different days (intermediate precision), the procedure was repeated on three days. Repeatability (RSD_r) and intermediate precision (RSD_R) ranged from 1.7 to 20.1 % and 2.2 % to 21.3 %, respectively. Consequently, the Horwitz equation ($RSD_r=2/3(2^{[1-0.5 \log C]})$; $RSD_R= 2^{[1-0.5 \log C]}$, where C is the concentration expressed as a mass fraction) was used to evaluate these RSD values. As described in commission decision 2002/657/EC, the Horwitz equation gives unacceptable high values for concentrations lower than 100 µg.kg^{-1}. Therefore, the RSD for concentrations lower than 100 µg.kg^{-1} shall be as low as possible [231]. Overall, the RSD values never exceeded the level calculated by the Horwitz equation and thus the method appears to be both repeatable and accurate for all matrices.

Next, the expanded uncertainty *U*, expressed as percentage (*U* %) was calculated to express the uncertainty of the measured results. *U* was determined for each toxin on three concentration levels. If no certificate of analysis of the reference standards is available, an arbitrary level of $\sqrt{3}$ is chosen for the uncertainty related to the reference standard $U[C_{ref}]$. This high $U[C_{ref}]$ term in the calculation leads to higher values for *U*. Additionally, a high intermediate precision resulted in high *U* values. All *U* values ranged from 5.6 to 48.5 % (Table 2.3). In general, the highest values for *U* were found for maize.

Based on this detailed validation, the procedures are suitable for the simultaneous determination of the target toxins. The sample preparation was minimized to a simple one-step liquid extraction, which enables the preparation of a high number of samples in a relatively short time. The similar structure and behavior of the target toxins, avoided loss of sensitivity that often comes with multi-toxin methods. All molecules undergoing ionization in the positive ion mode, formed abundant $[M+NH_4]^+$ adducts when adding ammonium acetate to the mobile phase. As the modifiers (ammonium acetate and formic acid) influence the target molecules in the same positive way, no compromises had to be made. Similarly, the total analysis time could be reduced due to a short toxin extraction and an efficient LC separation, which contributes to the potential to rapidly screen samples. The results show that the LC-MS/MS method is very efficient, sensitive and rapid for the quantification of the target toxins. Furthermore, the methodology enabled detection at low detection limits without the need for additional clean-up. As proof of principle, 59 samples were tested.

Table 2.3 Results for trueness expressed as apparent recovery (R_{app}), repeatability (RSD_r), intermediate precision (RSD_R), and expanded measurement uncertainty (U) for CER, BEA and ENNs on low, medium and high concentration level in wheat, maize, rice and pasta.

	Wheat					Maize					Rice					Pasta				
	Conc (µg.kg^{-1})	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	Conc (µg.kg^{-1})	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	Conc (µg.kg^{-1})	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)	Conc (µg.kg^{-1})	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)
CER	2	101	7.0	13.6	37.3	2	100	8.2	12.9	25.9	2	92	9.8	13.7	27.4	1	101	19.1	19.1	38.2
	200	97	2.5	2.2	10.0	200	103	6.0	8.1	16.1	200	103	5.6	9.1	18.2	50	102	7.1	10.2	20.4
	400	101	3.1	4.9	13.8	400	106	4.8	5.6	11.1	400	105	8.0	8.0	16.0	100	103	6.8	6.8	13.6
BEA	2	99	9.1	12.5	31.5	2	101	14.5	14.5	29.1	2	98	16.6	16.6	33.3	1	92	13.5	18.9	37.7
	200	93	3.9	6.6	22.7	200	104	6.8	6.8	13.7	200	104	3.1	9.4	18.8	50	103	7.1	7.1	14.1
	400	85	2.4	2.4	10.6	400	94	7.0	9.2	18.4	400	91	5.3	8.2	16.5	100	108	5.4	5.5	11.0
ENN A	2	97	6.2	7.6	21.8	2	98	18.7	18.7	37.4	2	93	9.1	9.7	19.5	1	98	8.5	8.9	17.9
	100	94	6.8	11.1	34.2	100	100	5.6	7.2	14.4	100	84	10.3	12.3	24.7	50	104	5.6	5.6	11.2
	200	92	4.4	7.2	24.7	200	99	7.7	8.2	16.4	200	99	12.2	12.2	24.3	100	109	4.9	4.9	9.8
ENN A1	2	101	16.8	17.5	48.5	2	99	9.0	13.4	26.7	2	87	11.8	12.0	24.0	1	105	13.8	14.6	29.1
	200	99	4.5	8.7	23.7	200	108	5.3	5.3	10.6	200	101	3.7	8.7	17.4	50	108	5.3	5.3	10.5
	400	100	4.8	12.8	34.7	400	108	3.6	4.7	9.3	400	100	7.2	12.7	25.4	100	108	2.5	4.4	8.7
ENN B	2	95	14.6	21.3	46.7	2	98	9.1	19.6	39.1	2	102	15.2	16.4	32.7	1	99	5.3	16.6	33.3
	200	100	4.4	6.7	18.6	200	104	5.5	6.6	13.2	200	101	15.3	15.8	31.6	50	93	7.8	7.9	15.9
	400	102	6.0	7.4	23.5	400	108	2.6	5.2	10.3	400	104	6.0	7.3	14.6	100	93	9.1	10.7	21.5
ENN B1	2	97	4.2	6.0	36.2	2	108	20.1	20.1	40.1	2	97	9.8	13.3	26.5	1	103	16.8	18.4	36.9
	200	104	3.6	6.2	21.9	200	109	4.5	8.6	17.1	200	108	5.1	11.4	22.8	50	101	6.9	9.1	18.3
	400	90	6.7	8.7	38.6	400	98	1.7	2.8	5.6	400	94	4.4	5.9	11.8	100	105	5.7	6.5	13.0

2.3.4 ANALYSIS OF NATURALLY CONTAMINATED SAMPLES

The suitability of the optimized and validated methods was finally tested by analyzing 59 naturally contaminated samples The samples were quantified against matrix matched standards. The results are reported in the form 'x ± U' where 'x' is the best estimate of the true value of the concentration (the analytical result) and 'U' is the expanded uncertainty. Results from the occurrence of CER, BEA and ENNs in the analyzed samples are given in Table 2.4. No CER was detected in any of the samples, which could be expected since the occurrence of CER is usually related to cooked dishes or leftovers [149, 237]. Generally, the level of contamination was low especially for BEA, ENN A and ENN A1, except for maize where in 68 % of the samples BEA was detected up to 209.0 ± 28.5 $\mu g.kg^{-1}$. No ENN A was detected in the samples and only traces of ENN A1 (<14.6 ± 3.9 $\mu g.kg^{-1}$) were found in maize and wheat. ENN B and ENN B1 were the mycotoxins most found and levels ranged from 2.8 ± 1.3 to 195.5 ± 25.8 $\mu g.kg^{-1}$ and 1.9 ± 0.7 to 42.5 ± 15.4 $\mu g.kg^{-1}$, respectively. Samples containing ENN B were generally also contaminated with ENN B1. The highest levels for each individual toxin recorded were 209.0 ± 28.5 $\mu g.kg^{-1}$ (BEA), 14.6 ± 3.9 $\mu g.kg^{-1}$ (ENN A1), 195.5 ± 25.8 $\mu g.kg^{-1}$ (ENN B) and 42.5 ± 15.4 $\mu g.kg^{-1}$ (ENN B1). The methods were considered to be suitable for use since the measured concentrations are within the validated linear concentration ranges. No toxins were found in the rice samples. A possible explanation for the absent of toxins can be that in this study only white rice was included. This type of rice is achieved after milling and often also polishing of the rice kernel. The process of milling and polishing results in the removal of husk, bran, and germ where most mycotoxins are present. The same reasoning can explain the low occurrence of mycotoxins in pasta since the pasta in this study was not white pasta where the germ and the bran are removed, instead of whole grain pasta.

Although no general conclusions can be drawn concerning the occurrence, the preliminary data of 59 samples tested in the current study suggested that ENN B, B1 and BEA are more abundant contaminants than ENN A and A1 in the selected matrices. These results suggested that in the future our method could be used in the screening of BEA, ENNs (A, A1, B, B1) and CER in cereals and cereal-based products.

Table 2.4 Presence of CER, BEA and ENNs in wheat, maize, rice and pasta.
The percentage of positive samples for each toxin is given together with the maximal concentrations found. Maximal concentration is reported as 'concentration ± expanded uncertainty', both expressed in $\mu g.kg^{-1}$. (n.d. = not detected).

Samples	CER		BEA		ENN A		ENN A1		ENN B		ENN B1	
	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)	Positive (%)	Maximal conc. ($\mu g.kg^{-1}$)
Wheat (n=10)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20	14.6 ± 3.9	70	89.2 ± 16.5	70	42.5 ± 15.4
Maize (n=25)	n.d.	n.d.	68	209.0 ± 28.5	n.d.	n.d.	8	10.7 ± 2.9	16	195.5 ± 25.8	8	40.7 ± 16.3
Rice (n=12)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pasta (n = 12)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	75	99.2 ± 38.8	50	21.0 ± 7.8

2.4 CONCLUSION

Quantitative LC-MS/MS methods applicable for the simultaneous determination of CER, BEA and ENNs in maize, wheat, pasta and rice have been developed. Extensive validation of the method was done for the target toxins in different matrices. Good values for extraction recovery (higher than 80 %) and precision (RSD less than 20.1 %) were obtained. The major strengths of the proposed methods are being rapid and simple for all target toxins. Finally, 59 commercially available cereal and cereal-based food samples were analyzed with the developed method proving suitability for the intended use. No CER was detected in the analyzed samples, which is not surprising as CER is more likely to occur in leftovers of rice and pasta dishes upon active growth of *B. cereus* during improper holding and storage. 32 % (19/59) of the samples were contaminated with ENN B. In 56 % (33/59) of the samples at least one of the mycotoxins was detected. None of the commercially available rice samples were contaminated with the detectable levels of the target toxins. Since it is likely that more than one toxin is present, a multi-toxin analysis suitable for various matrices helps to monitor the contamination risk. In the future, these methods can provide information on the occurrence of these toxic metabolites.

Chapter 3

Occurrence and correlation of *Fusarium* species and BEA and ENNs in Belgian wheat

This chapter was redrafted from:

Decleer, M., Landschoot S., De Saeger S., Rajkovic A. and Audenaert K. (2017). Occurrence and correlation of *Fusarium* species and beauvericin and enniatins in wheat *(in preparation)*.

Occurrence and correlation of *Fusarium* species and BEA and ENNs in Belgian wheat

Besides the availability of analytical methods for the accurate detection and quantification of the toxins, gathering occurrence data is an important part of risk assessment. Therefore, the developed and validated LC-MS/MS method described in Chapter 2 has been applied to Belgian wheat samples. The following study was accomplished in collaboration with the laboratory of Prof. dr. Kris Audenaert.

3.1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important small-grain cereals worldwide with a production amounting to 730 million tonnes in 2014 [238]. Wheat crops can be infected by several *Fusarium* species all causing the same disease called Fusarium head blight (FHB) which affects the yield and quality of the grains. However, the main concern of FHB in cereals are the concomitant toxic secondary metabolites called mycotoxins, which accumulate during the infection and contaminate the final product.

The occurrence of mycotoxins in wheat fluctuates from year to year, as it is a multifactorial problem influenced by weather conditions (such as temperature and moisture conditions), geography and agricultural practices during various stages of production (*e.g.* tillage, previous crop, pesticide treatment and storage conditions). The impact of these factors has been investigated in detail for common *Fusarium* mycotoxins such as trichothecenes, fumonisins and zearalenone [223, 239, 240]. However, contrary to the wealth of information on these well-known mycotoxins, detailed information on so-called emerging mycotoxins is rather scarce.

Regarding *Fusarium* species, FUS, fusaric acid, BEA, ENNs and MON are considered emerging mycotoxins. In 2014, EFSA has evaluated the risks to human and animal health related to the presence of BEA and ENNs in food and feed. They concluded that there is no indication for human health risk following acute exposure, although there may be a concern about chronic exposure [71]. Hence, more data are required in order to evaluate the potential long-term risk. A recent proof of concept study using a human-relevant high-content hepatotoxicity assay showed that ENN B and BEA showed significant cytotoxicity at a concentration lower than that for aflatoxin B1, which is the

archetypal acute hepatotoxic and liver-carcinogenic mycotoxin [30]. These new toxicological insights subscribe the emerging nature of these cyclic depsipeptide mycotoxins.

Worldwide, many studies have shown that ENNs and BEA are omnipresent in agricultural food commodities. In recent years, they have been frequently reported in different countries as contaminants in especially wheat, rye, oats, barley, rice and sorghum [36, 42, 85, 241, 242]. The occurrence data showed that generally the contamination levels of ENN A and ENN A1 were lower than the levels of ENN B and ENN B1. Although there are many studies on the occurrence of ENNs and BEA, linking their presence with specific *Fusarium* species is often lacking. This would provide a more profound understanding of the pathophysiology of these fungi. For 'traditional' trichothecenes such as DON, these studies are available, but there is still a huge lack of knowledge on their co-occurrence with BEA and ENNs [243, 244].

In the present study, a quantitative inventory of the toxins CER, BEA, ENN A, ENN A1, ENN B and ENN B1 was conducted on wheat samples collected from trial field in Belgium during growing season 2014-2015 (1 location) and 2015-2016 (3 locations). All samples were subjected to UPLC–MS/MS to detect the selected toxins. Moreover, these samples served for two additional objectives: (1) to investigate the causal relationships between these emerging mycotoxins BEA and ENNs and the residing *Fusarium* population, the wheat genotype and pesticide treatments and (2) to assess the impact of weather conditions on the levels of mycotoxins. For the first objective, species-specific qPCR was conducted on the samples harvested in 2016 (3 locations) to assess the presence of *F. avenaceum*, *F. poae*, *F. graminearum* and *F. culmorum*. In a second approach, the impact of weather conditions on the levels of mycotoxins found in one location was investigated during two growing seasons (2014-2015 and 2015-2016). DON was included as a reference mycotoxin and was detected using ELISA.

3.2 Materials and Methods

3.2.1 Field trials and collection of samples

During a two-year study (growing seasons 2014-2015 and 2015-2016), wheat samples were monitored in Flanders, Belgium (Figure 3.1). The collected samples were analyzed for different objectives.

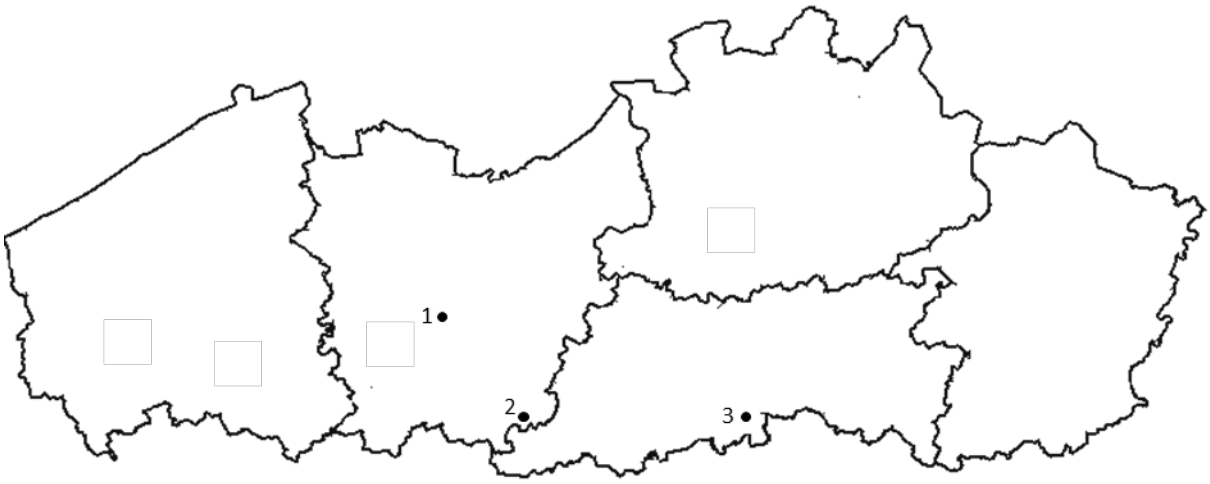

Figure 3.1 Origin of wheat samples collected from trial field in Flanders, Belgium.
1= Bottelare; 2=Nieuwenhove; 3=Huldenberg.

First, to investigate the impact of fungicides on the *Fusarium* population and mycotoxins, a fungicide trial was performed in the growing season 2015-2016 wheat samples from the three locations (Bottelare, Huldenberg and Nieuwenhove). A total of 19 commonly used wheat genotypes were sown in experimental field trials at sowing density of 350 seeds per m^2 (Figure S3.1). The experimental design was a completely randomized block design for all locations with at least 3 replications. The surface of each elementary plot was about 15 m^2. Normal crop husbandry measures were taken including three nitrogen fertilizations according to the N-index established by *Bodemkundige Dienst België* and pre- and post-emergence herbicide applications. The plant growth regulator mepiquatchloride + prohexadion (300 $g.ha^{-1}$ + 50 $g.ha^{-1}$), was applied to reinforce the antilodging effect [245]. The fungicide application comprised three application. First, at growth stage 39 (Zadok) cyproconazole + propiconazole (50 $g.ha^{-1}$ + 125 $g.ha^{-1}$) was applied. At stage 59, boscalid + epoxyconazole was applied (238 $g.ha^{-1}$ + 85 $g.ha^{-1}$) and finally at growth stage 65, epoxyconazole + fluxapyroxad + pyraclostrobin (72 $g.ha^{-1}$ + 72 $g.ha^{-1}$ + 116 $g.ha^{-1}$) was applied. Control plants were left untreated. All plots were harvested mechanically in August according to the ripening stage in each location (water content <15%). In total, 354 samples were collected after the harvest of 2016 for further analysis.

Second, in order to assess the impact of the weather conditions on the presence of *Fusarium* spp. and mycotoxin levels, samples of two growing season were considered. All samples were treated as

described in the field experiment above, however, only one location (Bottelare) was considered. During both growing seasons (2014-2015 and 2015-2016), relative humidity, temperature and precipitation were measured and these parameters were used to assess the impact on the emerging cyclodepsipeptides and *Fusarium* species.

At harvest, all wheat plots were harvested individually and a homogenous sample was ground using a Retsch GmbH grinding station (Mill: SM100, DR 100/75, divider: PK1000 Haan, Germany).

3.2.2 MYCOTOXIN ANALYSIS

The same reagents and chemicals and standard solutions were used as described in Chapter 2 in sections 2.2.1 and 2.2.2. The UPLC–MS/MS conditions and extraction procedure to determine the levels of CER, BEA and ENNs A, A1, B and B1 in the wheat samples are described in Chapter 2 in sections 2.3.1 and 2.3.2.

In addition, all samples were analyzed in duplicate for DON presence in all individual plots by ELISA using the AgraQuant® Deoxynivalenol Assay 0.25/5.0 (Romer Labs, Butzbach, Germany). One hundred mL of water was added to 20 g of ground sample and shaken for 30 min at room temperature. The suspension was filtrated (Rundfilter, Schleichger and Schueull), the flow through was diluted 1:4 and subsequently analyzed for DON presence according to the manufacturer's instructions. The lower limit of detection was 0.2 $mg.kg^{-1}$. A standard calibration curve was established using 0, 0.25, 1, 2 and 5 $mg.kg^{-1}$ DON.

3.2.3 IDENTIFICATION OF *FUSARIUM* SPECIES

DNA was extracted from all samples collected in 2016. DNA extraction was performed using the Invisorb Spin Plant mini kit (Invitek, Germany), as per the manufacturer's instructions. qPCR primers for fungal DNA based on elongation factor-1α (EF-1α) sequences were taken from literature and have been described as discriminating for ten FHB-causing Fusarium species most frequently encountered in Europe [16]. The qPCR reactions were performed in a total reaction volume of 12.5 µL, consisting of 6.25 µL GoTaq qPCR Master Mix (Promega), 0.208 µL of CXR reference dye (Promega), 250 nM of each primer and 2.5 µL of DNA. Analysis was performed on an ABI 7000 Sequence Detection System with the following cycle settings: 50 °C during 2 min; 95 °C during 10 min; 40 cycles of 95 °C during 15 s and 63.5 °C during 1 min; followed by dissociation curve analysis at 62.5 °C. Fungal DNA for every

species was normalized on the amount of plant DNA. All primers were tested and confirmed for specificity among the 10 Fusarium species investigated in this study according to Nicolaisen *et al*. (2009) [246]. This validation was performed with at least one MUCL (Mycothèque Université Catholique de Louvain) reference isolate per species (data not shown).

3.2.4 DATA ANALYSIS

For statistical evaluation, the R software package (R core Team 2014) version 3.1.1 was used. Since normality and homoscedasticity assumptions of parametric tests were not met, a non-parametric Kruskal-Wallis test was used to test if there were significant differences between groups of data. In case there was a significant difference (p-value < 0.05), a pairwise comparison of the groups using a Dunn's test was performed. The data are represented as box plots showing the median, quartiles, maximum and minimum of the data. For the assessment of relationship between the measured toxins and the presence of *Fusarium* species, the Pearson correlation coefficients were calculated.

3.3 RESULTS AND DISCUSSION

3.3.1 OCCURRENCE OF CER, BEA, ENNS AND DON IN WHEAT IN GROWING SEASON 2015-2016

In growing season 2015-2016, all samples were positive for DON, ENN A1, ENN B and ENN B1, considering the three locations. The concentration levels escalated especially for DON, ENN B and ENN B1 with maximum contamination levels of 3691.0 µg.kg^{-1} for ENN A1, 2168.0 µg.kg^{-1} for ENN B and 776.7 µg.kg^{-1} for ENN B1 (Table 3.1). The total amount of BEA and ENNs ranged from 21.9 to 2785.5 µg.kg^{-1}. In addition, 54 % of the samples were contaminated with at least four cyclic depsipeptides and 9 % of the samples contained even all five cyclic depsipeptides. This high incidence is in line with other European studies conducted in Romania, Italy, Poland, Finland, Denmark and Norway [28, 30, 38, 83, 247-249]. As suggested by Uhlig *et al.* (2006), the order of ENNs regarding incidence and concentration levels was ENN B > ENN B1 > ENN A1 > ENN A [27]. Furthermore, high concentrations were observed for DON with a median concentration of 710 µg.kg^{-1} and a maximum level of 3691 µg.kg^{-1} with 14 % of the samples exceeding the European threshold value of 1250 µg.kg^{-1} for human consumption [4]. As expected, CER was not found in any of the samples and therefore, it was not included further in the study.

Table 3.1 Presence of DON, BEA and ENNs in wheat samples in the growing season 2015-2016 (n=140). Cumulative concentrations are represented of both fungicide treated and non-treated samples from Bottelare, Huldenberg and Nieuwenhove).

Mycotoxin	Positive samples (%)	Mean (µg.kg^{-1})	Median (µg.kg^{-1})	Max (µg.kg^{-1})
DON	140 (100)	846.0	710.2	3691.0
BEA	50 (36)	4.8	3.9	13.5
ENN A	49 (35)	3.2	2.4	15.6
ENN A1	140 (100)	28.2	22.2	165.0
ENN B	140 (100)	735.3	667.6	2168.0
ENN B1	140 (100)	110.9	91.4	776.7

Moreover, for ENN A1 (p = 0.024), ENN B (p = 0.041) and ENN B1 (p = 0.027), a clear impact of the genotype on the mycotoxin levels was observed (Figure S3.1). For all other mycotoxins (BEA, DON and ENN A), no significant impact of the genotype was observed (data not shown). A previous study by Bryla *et al.* (2016) showed no impact of the genotype on total ENN levels, but this study did not provide details on the different ENN forms so a correlation might have been overlooked [38].

3.3.2 IMPACT OF FUNGICIDES ON DON, BEA AND ENNS CONCENTRATIONS IN GROWING SEASON 2015-2016

The results presented in Figure 3.2 clearly show that the application of fungicides did not result in a reduced concentration of ENN A1, ENN B and ENN B1 since no significant differences in concentration were found between fungicide treated and non-treated fields. For ENN A and BEA, results were slightly different and in one of the three locations, application of fungicides resulted in lower levels of ENN A and BEA compared to the untreated control fields. However, it is important to mention that the levels of ENN A and BEA were low. However, no significant differences does not imply that fungicide treatment should be discarded. For example, in the case of DON, the use of fungicides resulted in a reduction of DON especially for the DON concentrations exceeding 1500 µg.kg^{-1} (Figure S3.2). This result shows that the efficacy of fungicides to reduce the levels of DON in the field depends on the concentration of DON.

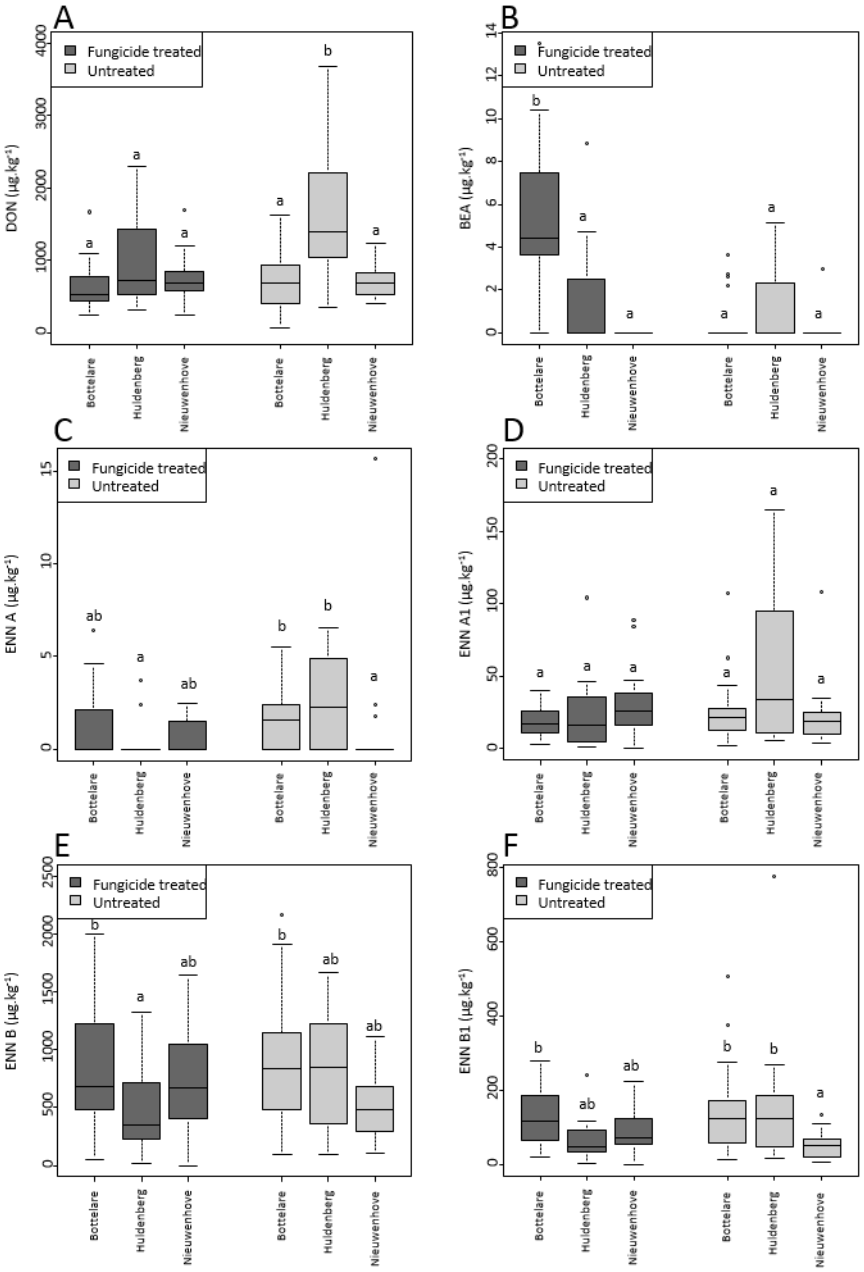

Figure 3.2 Impact of fungicide treatment on the presence of DON (A), BEA (B), ENN A (C), ENN A1 (D), ENN B (E), ENN B1 (F) in three locations.

Significant differences after analysis with a Kruskal-Wallis and *post hoc* Dunn's test are marked with different letters above box plots.

3.3.3 IMPACT OF FUNGICIDES ON THE FUNGAL POPULATION

To investigate the effect of the fungicide application on the presence of FHB *Fusarium* species, the four predominant *Fusarium* species were quantitatively determined through qPCR analysis (Figure 3.3, *F. avenaceum* (A)*, F. culmorum* (B)*, F. poae* (C) and *F. graminearum* (D)). Results from the field trial clearly subscribe that *F. poae* significantly accumulated compared to the control fields in one out of three locations (Figure 3.3C).

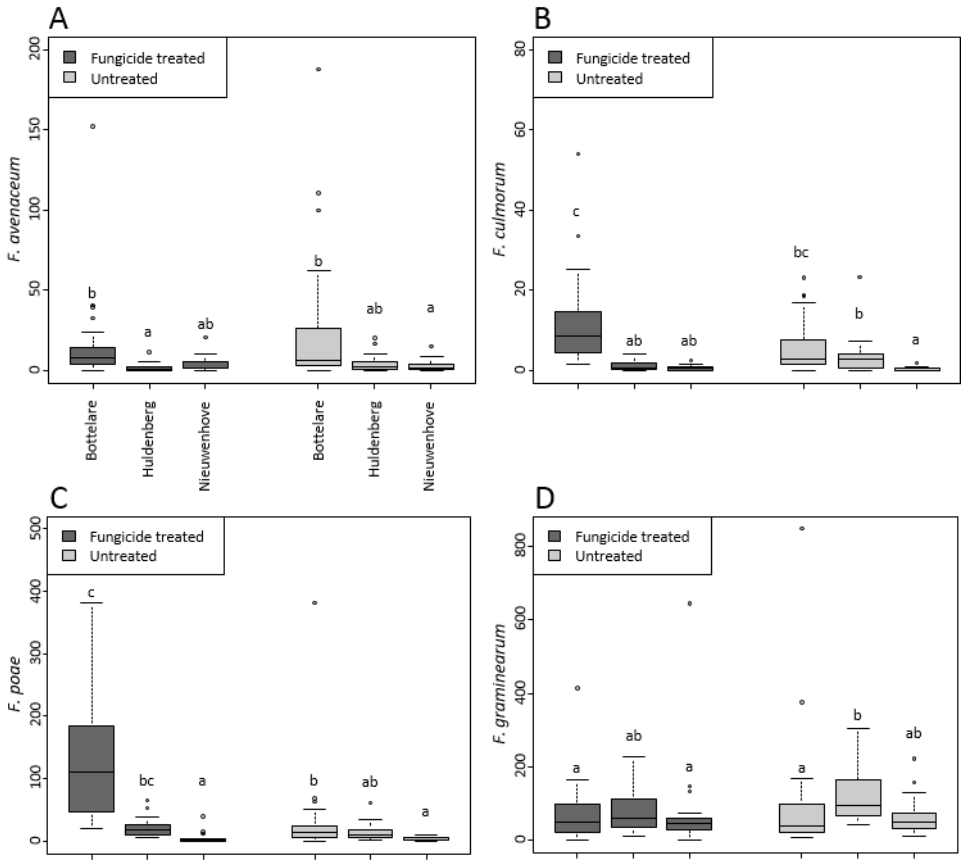

Figure 3.3 Impact of fungicide treatment on the presence of *F. avenaceum* (A), *F. culmorum* (B), *F. poae* (C) and *F. graminearum* (D) in three locations of wheat fields in 2015-2016.

3.3.4 *FUSARIUM* POPULATION AND MYCOTOXIN LEVELS

The relationship between the analyzed mycotoxins and the presence of several *Fusarium* species was assessed. Therefore, the LC-MS/MS and qPCR results were gathered in a correlation study and presented in a correlogram (Figure 3.4). The relationship between the mycotoxins and four *Fusarium* species was expressed with the Pearson correlation coefficients.

The correlogram (Figure 3.4) illustrates the high positive correlation between all ENNs, especially between ENN B and ENN B1 (r = 0.94). Remarkably, a significant negative correlation was present between BEA and ENN A1 (r = -0.27). Finally, all cyclodepsipeptides were significantly correlated with the trichothecene DON. For all ENNs this was a positive correlation, while for BEA this correlation was negative.

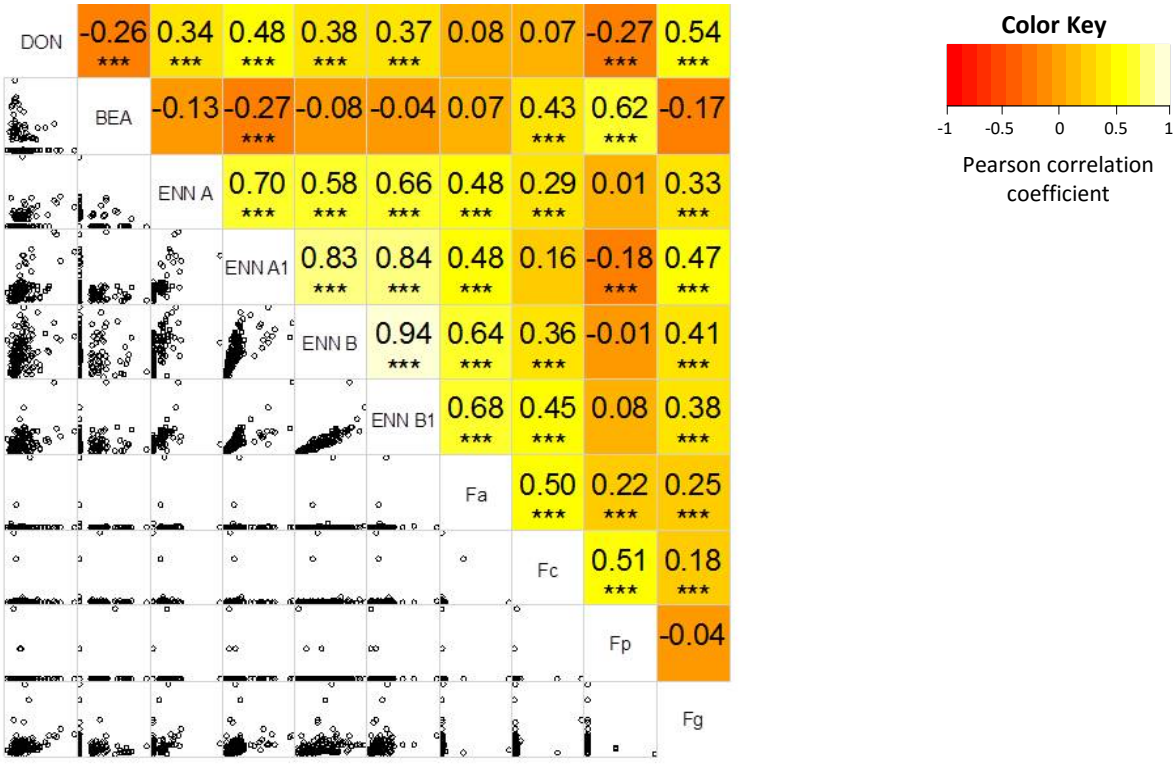

Figure 3.4 Scatterplot (lower panel) and correlogram (upper panel) with the Pearson correlations between the different measured toxins and *Fusarium* species (DON, BEA, ENN A, ENN A1, ENN B, ENN B1, *F. avenaceum* (Fa), *F. culmorum* (Fc), *F. poae* (Fp), *F. graminearum* (Fg) of the wheat samples of 2015-2016.
Correlations marked with *** are significant at α = 0.05. A lighter color designates a higher correlation.

Furthermore, positive correlations were observed between all *Fusarium* species except for *F. poae* and *F. graminearum*, where the correlation was not significant and was almost zero. This result was

in concordance with the lack of correlation between *F. graminearum* and BEA, which is known to be produced by *F. poae* (r = 0.62). As expected, a positive correlation between presence of *F. poae* and BEA is illustrated in the correlogram (Figure 3.4). The capacity of *F. poae* to produce BEA has been previously shown under laboratory conditions [19, 20]. Regarding the correlations with ENNs, high positive correlations were found between ENNs and *F. avenaceum*. It has been demonstrated in the past that *F. avenaceum* is one of the most important *Fusarium* species producing especially ENN B and ENN B1, which was demonstrated in the current study with the highest correlation.

Similar results were obtained in other parts of Belgium (Wallonia) where the diversity of *Fusarium* species occurring on wheat ears was monitored during a time period of four years (2010-13) and correlated to the presence of *Fusarium*-related mycotoxins in wheat fields. Mycotoxin analyses revealed that ENN A and ENN A1 were not detected, while BEA was present in 50 % of the samples. Regarding ENN B and ENN B1 concentrations, a strong correlation was observed and the concentration levels reached up to 891 $\mu g.kg^{-1}$ and 160 $\mu g.kg^{-1}$, respectively. In addition, strong correlations were observed between ENN B, ENN B1 and the number of strains of *F. avenaceum*. The BEA concentration was associated with the number of *F. tricinctum* and *F. poae* strains [250].

3.3.5 INTER-SEASON VARIABILITY IN MYCOTOXIN LEVELS

To assess the inter-season variability of the mycotoxin concentrations, DON, BEA and ENNs concentrations were measured in one location (Bottelare) during two growing seasons. During the first growing season, none of the 32 analyzed wheat samples were positive for ENN A, ENN A1 and ENN B1 which might hamper interpretation of the results. These results were compared to 66 wheat samples collected in 2016 on the same location (Bottelare).

In Figure 3.5, the results of both growing seasons are presented in boxplots. It should be noticed that results are separated for fungicide treatment and untreated wheat. Based on the non-parametric Kruskal-Wallis test, significant differences were observed between groups of data (p-value < 0.05). For each mycotoxin, a pairwise comparison was performed between the two growing seasons using a *post hoc* Dunn's test. This indicated that concentration levels of all tested mycotoxins significantly increased in the growing season 2015-2016 compared to 2014-2015. Moreover, in general the percentage of positive samples for all the tested mycotoxins was remarkably higher in the last

growing season. This observation was clear for both fungicide treated and untreated field trials, except for BEA.

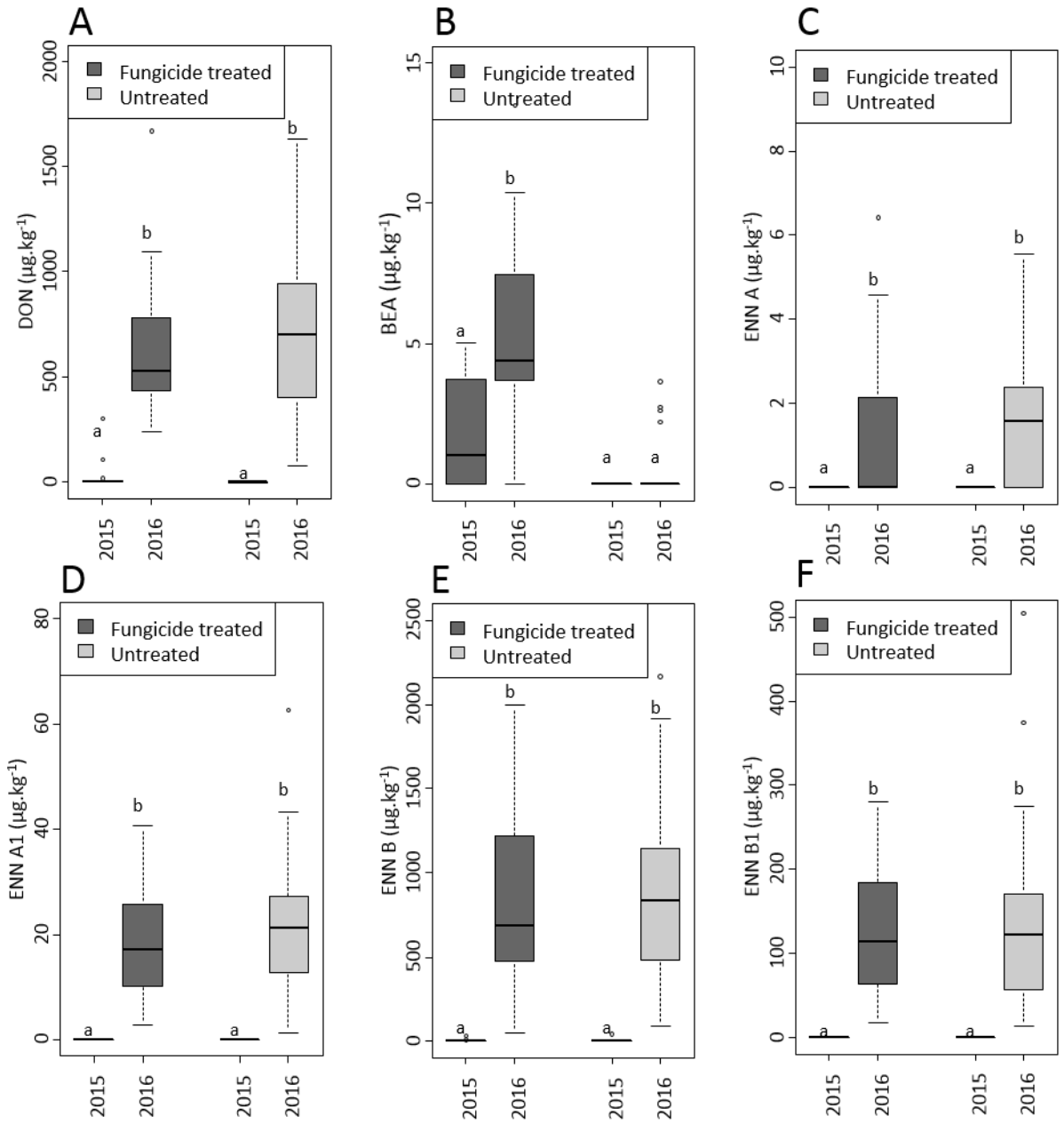

Figure 3.5 Inter-seasonal comparison of impact of fungicide treatment on the presence of (A) DON, (B) BEA, (C) ENN A, (D) ENN A1, (E) ENN B and (F) ENN B1 for all locations.

In search for a possible explanation for this large inter-season variability, the weather conditions (relative humidity, temperature and precipitation) were monitored and linked to the mycotoxin

levels. The total amount of precipitation, average relative humidity and average temperature during 15-day periods were calculated (Table 3.2) and correlated to the mycotoxin levels (Figure 3.6, 3.7 and 3.8).

Table 3.2 The total amount of precipitation, average relative humidity and average temperature during 15-day periods for 2015 and 2016 (Bottelare).

	Period	Total amount of precipitation (mm)	Average relative humidity (%)	Average temperature (°C)
2015	May 1-15	44.00	75.60	13.68
	May 16-31	29.40	77.12	12.43
	Jun 1-15	3.80	69.86	16.33
	Jun 16-30	30.60	75.80	16.73
	Jul 1-15	3.20	74.24	20.08
	Jul 16-31	30.40	78.36	17.44
	Aug 1-15	27.60	76.30	19.74
2016	May 1-15	12.80	69.62	15.00
	May 16-31	92.40	81.91	14.75
	Jun 1-15	35.80	87.60	16.81
	Jun 16-30	152.40	88.04	17.03
	Jul 1-15	31.80	80.33	17.84
	Jul 16-31	3.60	80.14	20.88
	Aug 1-15	48.00	84.88	17.36

Concerning the effect of precipitation on mycotoxin levels, it can be seen that starting from May 16 all correlations were positive, except during the period July 16-31. Moreover, correlations with these weather parameters were negative for BEA (Figure 3.6). The precipitation during July 1-15 seemed the most important period explaining the differences in DON values between both growing seasons. For other mycotoxins, except for BEA, the influence of precipitation was significant only earlier in the season. According to these observations, more precipitation recorded in the last season possibly resulted in higher mycotoxin levels. However, since this assumption is based on data of only two years, the results should be interpreted with caution. For example, a negative correlation was found between precipitation during the period July 16-31, meaning that a lot of precipitation during this period would result in low mycotoxin levels. In 2015 (with low toxin levels) there was more rainfall in July 16-31 compared to the same period in 2016. However, this thus not automatically mean that this lower amount of rainfall is responsible for the higher toxin contents in 2016.

Regarding the correlations between toxin levels and the average relative humidity during 15-day periods, it can also be concluded that, in general, a higher relative humidity leads to a higher mycotoxin level (Figure 3.7). However, the periods most influencing the toxin levels of a specific toxin are different. For example, for DON the relative humidity during the period June 1-15 was most important. Also, the correlations between temperature and mycotoxin levels were in most cases positive, except during the periods July 1-15 and August 1-15 (Figure 3.8). The negative correlation between temperature and toxin levels in the harvest period (August 1-15) can be explained by the fact that cooler average temperatures (17.36 °C in 2016) result in a slower grain drying and thus a possible augmented toxin accumulation.

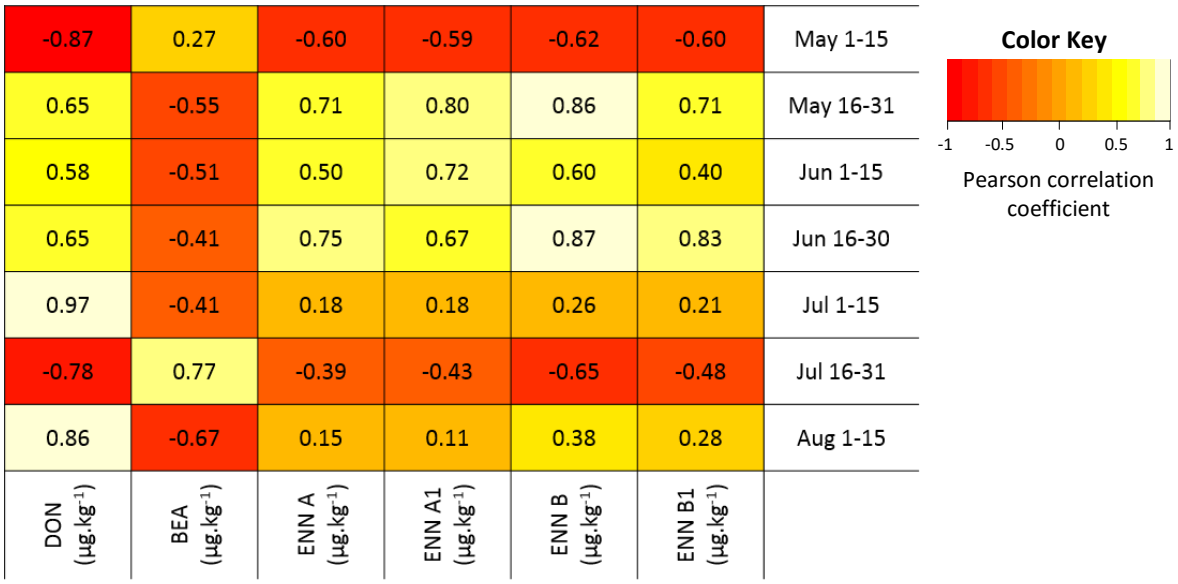

Figure 3.6 Pearson correlation between the amount of precipitation and mycotoxin concentrations during 15-day periods starting from May 1 until Aug 15.

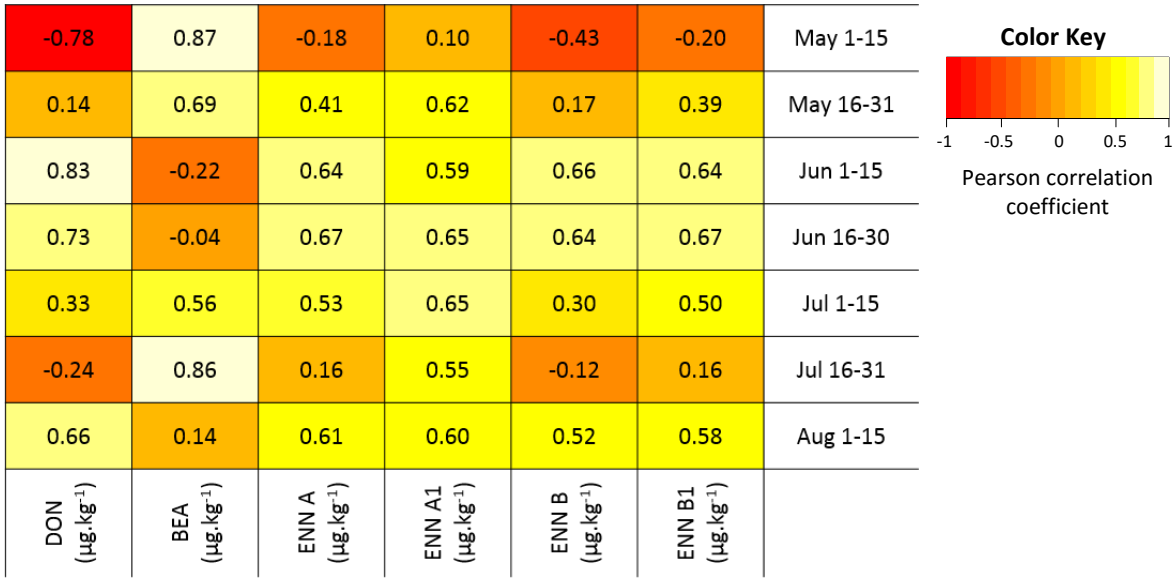

Figure 3.7 Pearson correlation between the humidity and mycotoxin concentrations during 15-day periods starting from May 1 until Aug 15.

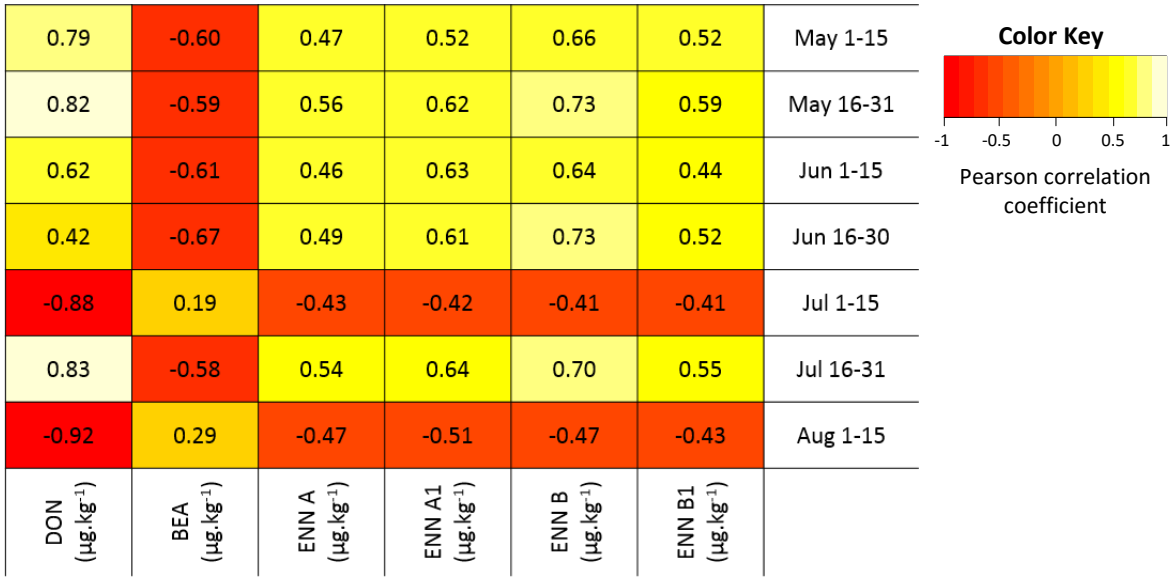

Figure 3.8 Pearson correlation between the temperature and mycotoxin concentrations during 15-day periods starting from May 1 until Aug 15.

3.4 CONCLUSION

Driven by the increasing interest in emerging mycotoxins, a two-year occurrence study was carried out for Belgian wheat. This study demonstrated that ENN B and ENN B1 are the most abundant mycotoxins occurring in the highest concentrations compared to BEA and the other ENNs. A large inter-season variability was observed with a significant increase in concentrations in growing season 2015-2016 for all tested mycotoxins (DON, BEA and ENNs). Moreover, besides the high prevalence of ENN B, the average concentration and maximum concentration reached 735.3 $\mu g.kg^{-1}$ and 2168 $\mu g.kg^{-1}$, respectively. Furthermore, in the last growing season, all samples were positive for ENN A1, ENN B and ENN B1. The presence of several ENNs in the same sample should thus be considered for their potential cumulative health risks in view of their similar toxicity [30].

In this study, the impact of fungicide applications on the occurrence and levels of cyclodepsipeptides was assessed. In addition, this was linked to the occurrence of the four predominant FHB species. The results demonstrated that *F. poae* tends to accumulate in the population upon application of fungicides at different growth stages. In the current study, we describe for the first time that fungicide application does not only cause a shift in the population towards a *F. poae* dominated population, but also an accumulation of BEA is pointed out. To the best of our knowledge, this is the first study that delves into the impact of fungicides on cyclodepsipeptide concentrations. Although no significant reduction in ENN A1, ENN B and ENN B1 concentrations were found after application of fungicides, this does not imply that fungicide treatment should be discarded.

In search for the producers of these cyclic depsipeptides, the highest correlations were found between BEA and *F. poae* and between ENNs and *F. avenaceum,* suggesting that these species are responsible for the respective mycotoxins. Despite the fact that *F. poae* is a predominant species in Belgian wheat, the concentrations of BEA were low. Therfore, the biological role of BEA in the infection or colonization of wheat by *F. poae* is not clear. With regard to ENNs, the highest correlation was observed with *F. avenaceum*, which is a known potent producer of ENNs and is present in the Belgian FHB population.

Based on the two-year sampling, a positive correlation was observed between all ENNs and the precipitation in the months of May, June and the beginning of July, while a negative correlation was found for all ENNs with precipitation and humidity in the second half of July. This observation might

imply that more precipitation during the beginning of the season leads to higher mycotoxin levels. Remarkably, reciprocal results were obtained for the mycotoxin BEA. This might point to the fact that weather conditions affect its producer (*F. poae*). For these results, it is important to highlight that the number of positive BEA samples was rather low, which might hamper a good interpretation of these correlation results. Nonetheless, since this correlation analysis is based on data of only two years the results should be interpreted with caution. For further interpretation of the impact of weather conditions and other cofounding factors, multi regression studies should be undertaken.

In conclusion, results of the current study shed a new light on the occurrence and concentration levels of *Fusarium* cyclodepsipeptide mycotoxins in wheat, under the impact of fungicides and seasonal variability in temperature and rainfall. The large year-to-year variability emphasized the importance of gathering occurrence data during different years and locations. It seems that within the tested cyclodepsipeptides, BEA and ENNs are affected by different parameters and behave differently upon application of fungicides.

ACKNOWLEDGEMENTS

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Chapter 4

Mycotoxin production by *Fusarium avenaceum* field isolates during basal rot infections in leek (*Allium porrum* L.)

This chapter was redrafted from:

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Mycotoxin production by *Fusarium avenaceum* field isolates during basal rot infections in leek (*Allium porrum* L.)

In parallel with Chapter 2, the development and validation of a similar LC-MS/MS method is described. In this method CER was not included as target since this method was developed as part of a study concerning the relation between *F. avenaceum* found on Belgian leek samples and its emerging mycotoxins. For this reason, MON was included in the method. The following study was accomplished in collaboration with the laboratory of Prof. dr. Kris Audenaert.

4.1 INTRODUCTION

Leek (*Allium porrum* L.) is a cultivated autotetraploid form of a wide, outbreeding, polyploid species complex (*Allium ampeloprasum* L.) [251]. Worldwide, the leek production amounts to 2M tonnes with Indonesia (584631 tonnes), Turkey (223303 tonnes) and Belgium (190000 tonnes) as leading leek producers in 2014 [252]. As leek has to meet high visual quality standards, any deficiency due to plant pathogens is pernicious for price making. White tip is one of the most important diseases on leek in Europe and is caused by the soil-borne oomycete pathogen *Phytophthora porri* Foister. Another devastating disease is rust caused by the fungus *Puccinia allii* Rud [251, 253-255]. Besides these well-established diseases of leek, *Fusarium* basal rot has been increasingly reported in the last decennium. In Italy and Spain, this disease has been associated with *F. culmorum*, while in France and Germany the presence of *F. oxysporum, F. solani* and *F. proliferatum* was described [256-259]. Beyond European borders, the occurrence of *F. avenaceum* in leek have been reported in California (USA) and in South-Australia [260, 261]. The symptoms associated with *Fusarium* infection include a brown/yellow or pink discoloration on roots and malformed emerging leaves from the leaf sheaths (Figure 4.2) [260]. However, the information on species that might cause *Fusarium* basal rot on leek is scarce. Therefore, research on onion (*Allium cepa*), which is related to leek, was used as an example in search for the causal agents of *Fusarium* basal rot. *F. oxysporum*, *F. proliferatum* and *F. solani* have been associated with symptoms of basal rot in onion in Turkey, Serbia, The Netherlands,

Italy and Germany [259, 262-266]. In addition, the presence of other species such as *F. equiseti*, *F. tricinctum* and *F. culmorum* was occasionally recorded in these studies.

Since the presence of *Fusarium* in crops often entails mycotoxins, the impact of these toxic secondary metabolites has to be considered. Studies tackling the role of *Fusarium* mycotoxins have mainly focused on trichothecenes and fumonisins in other crops. In this light, fumonisins have shown to be involved in the infection process of *F. verticillioides* in maize seedlings [267]. The trichothecene DON has been depicted as virulence factor in the interaction of *Fusarium* spp. with wheat [268], while a pivotal role for another trichothecene nivalenol and several structurally non-related mycotoxins have been attributed with respect to oxidative stress responses in the fungus [269, 270]. However, for the majority of the emerging *Fusarium* mycotoxins information on functionality is lacking.

The objectives of this study were: (1) to point out the causal agents of *Fusarium* basal rot in Belgian leek, (2) to unravel their toxigenic capacity and (3) to establish an artificial infection assay to assess the link between virulence and mycotoxin production.

4.2 MATERIALS AND METHODS

4.2.1 *FUSARIUM* SPECIES IDENTIFICATION IN BELGIAN LEEK

Basal rot of leek has frequently been observed in leek of Flanders (Belgium), which is an important leek producing and consuming region. In pursuit of the causal agents of Fusarium basal rot in Belgian leek, leek plants showing typical Fusarium basal rot symptoms were collected from 5 locations in Flanders matching up with the major leek growing areas in Flanders (Figure 4.1). In total, 47 samples were gathered. Symptomatic leek pieces were surface sterilized with 4 % sodium hypochlorite for 1 minute and subsequently rinsed threefold in sterile milli-Q water (Milli-Q® SP Reagent water system from Millipore Corp., Brussels, Belgium). Subsequently, leek pieces were placed on Potato dextrose agar (PDA, company, city, country) medium at 22 °C. Monosporic isolates were obtained by a dilution method obtained from Dr. Susanne Vogelgsang (personal communication).

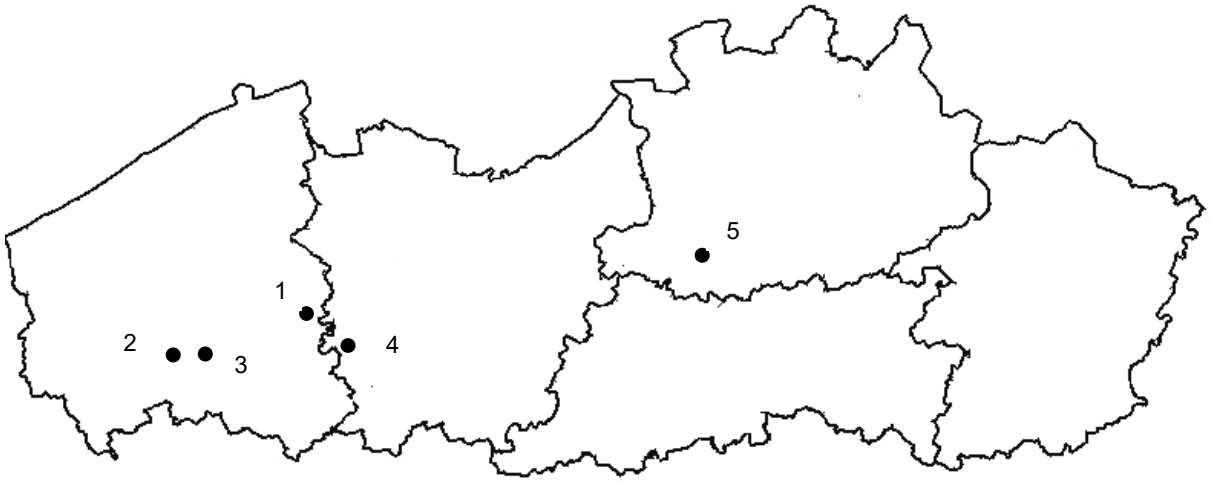

Figure 4.1 Origin of *Fusarium* isolates collected from leek samples in Flanders, Belgium.
1= Dentergem; 2=Passendale; 3=Beitem; 4=Kruishoutem; 5=Sint-Katelijne-Waver.

For identification and characterization, the monosporic *Fusarium* isolates were grown on a mineral medium for five days, lyophilized, ground and DNA was extracted as described by Saghai-Maroof *et al.* (1984) [271, 272]. Species were identified by sequencing the 5' portions of translation elongation factor (EF) 1α coding region and introns were amplified with primers EF-1 and EF-2 [273]. *F. avenaceum* and *F. culmorum* were PCR-confirmed using species specific primers for both species [274, 275]. The trichothecene chemotype of *F. culmorum* isolates was determined as described by Waalwijk *et al.* (2003) [276]. The cyclodepsipeptide chemotype of *F. avenaceum* isolates was determined as described by Stępień and Waśkiewicz (2003) [277].

4.2.2 *FUSARIUM* INFECTION ASSAY IN LEEK

To assess the virulence of the *Fusarium* isolates, an infection assay was performed on six weeks old leek plantlets (cultivar Harston). Therefore, a representative subset of isolates was selected based on their geographic origin and species. Leek seeds were germinated in bulk in small trays and after three weeks transplanted into 5 cm diameter pots containing potting soil. The design was a completely randomized design with six replications per isolate. Isolates were grown to sporulation on Mung Bean Broth for seven days and subsequently the suspension was filtered with miracloth. Conidia were counted and diluted to a concentration of 10^6 conidia per mL. One mL of spore suspension was administered at the base of each plantlet. After four weeks, the infection was evaluated using an ordinal scoring system of 1 to 5 (Figure 4.2). The infection experiment was repeated twice in time.

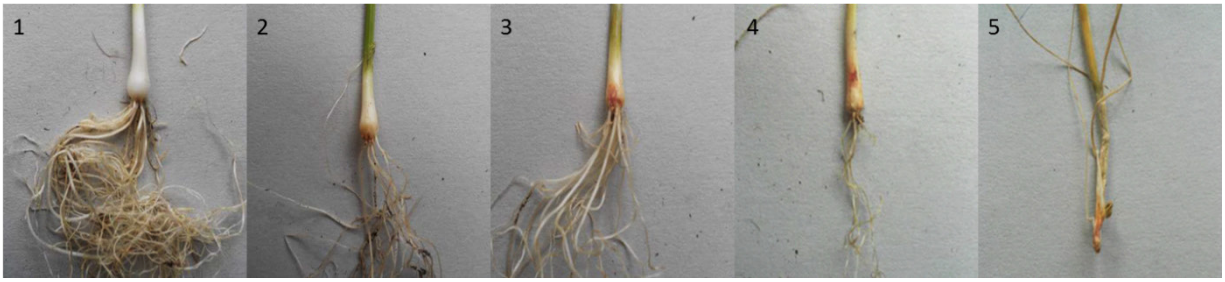

Figure 4.2 Ordinal scoring system to assess the *Fusarium* spp. symptoms on leek plants.
Scores vary from 1 (healthy, no visible symptoms) to 5 (plant is completely dead).

4.2.1 REAGENTS AND CHEMICALS

Methanol (absolute, LC-MS grade), acetonitrile (LC-MS grade) and glacial acetic acid (99%, ULC-MS) were purchased from BioSolve BV (Valkenswaard, The Netherlands). Ammonium acetate was obtained from Merck (Darmstadt, Germany). Water was purified on a Milli-Q® SP Reagent water system from Millipore Corp (Brussels, Belgium).

4.2.2 MYCOTOXIN ANALYSIS

4.2.2.1 STANDARD SOLUTIONS

BEA, ENN A, ENN A1, ENN B and ENN B1 (1 mg, solid standard) were purchased from Sigma-Aldrich (Diegem, Belgium). Stock solutions were prepared by dissolving 1 mg of solid standard in 1 ml of ACN, obtaining a 1 $mg.mL^{-1}$ solution. MON was purchased as sodium salt (1 mg, solid standard) and a 1 $mg.mL^{-1}$ stock solution of MON in acetonitrile-water (84/16, v/v) was prepared. All stock solutions were stored in darkness at 4 °C. A standard mycotoxin mixture of 100 $ng.ml^{-1}$ was prepared on the day of analysis.

4.2.2.2 SAMPLES

For method optimization and validation, frozen leek samples that were free (blank) of the six target mycotoxins were used.

4.2.2.3 OPTIMIZATION OF LC-MS/MS PARAMETERS

The MS/MS parameters were optimized on a MS triple quadrupole system (Waters) via direct infusion of the individual mycotoxin standards (1 $ng.\mu L^{-1}$, dissolved in methanol/ultrapure water

(50/50; v/v, 0.3 % formic acid, 5 mM ammonium acetate) into the MS source using a syringe injection at a flow rate of 10 µL.min^{-1}. For each analyte, ideal cone voltages and collision energies and two product ions (except for moniliformin) were chosen.

For chromatographic separation of the target toxins, the performance of an HPLC Symmetry C_{18} (5 µm, 2.1 mm x 150 mm), an Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm X 50 mm) and an Acquity UPLC CSH Fluoro-Phenyl (1.7 µm, 2.1 mm X 150 mm) column were tested. Since the mobile phase composition has a significant influence on the retention of the analytes on the column and on the peak shapes, different mixtures of water, methanol and acetonitrile were compared. In addition, modification of the mobile phase with volatile acids (formic acid and acetic acid) and salts (ammonium formate and ammonium acetate) were evaluated.

4.2.2.4 OPTIMIZATION OF SAMPLE PREPARATION

The goal was to develop a reliable multi-toxin analytical method with a specific extraction to obtain the best recovery and sensitivity. In order to save time and solvents, the sample preparation steps were minimized. Various sample preparation techniques may be considered including SPE, liquid–liquid extraction (LLE) and the QuEChERS method ("quick, easy, cheap, effective, rugged, and safe"). This latter technique was originally developed for pesticide residue analysis but the scope of this applicability can be extended to other natural contaminants, drugs, mycotoxins, etc. in high aqueous content samples like fruits and vegetables [278]. The QuEChERS sample preparation consists of extraction of the sample with a solvent, salt-out partitioning of water with salts like $MgSO_4$ and NaCl, followed by dispersive solid-phase extraction (dSPE) in order to remove matrix components. First, the influence of extraction solvent and extraction time was examined. Four commonly used extraction solvents were evaluated based on the extraction recovery: MeOH, ACN, MeOH/ACN (20/80, v/v) and ACN/acetic acid (99/1, v/v). For all toxins, 100 % ACN as extraction solvent provided the best recoveries. Subsequently, three procedures (liquid extraction, QuEChers and QuEChERS followed by dSPE) using ACN as extraction solvent, were compared. For this purpose, blank leek samples were spiked in increasing concentration to construct calibration curves and consequently subjected to the different procedures. Extraction efficiency was evaluated using the slopes of the calibration curves.

4.2.2.5 *METHOD VALIDATION*

The same procedure for the validation study was used as described in Chapter 2 (2.2.6.1 Validation design). Commission Decision 2002/657/EC, Commission Regulation 401/2006/EC and International Conference on Harmonisation (ICH) guidelines were used as guidance [231-233]. Since no reference material was available, spiked blank leek samples were spiked and used for the validation of the multi-method. Validation of the method for the simultaneous quantification of MON, BEA and ENNs in leek has been based on the following performance characteristics: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability (RSD_r), intermediate precision (RSD_R) and apparent recovery by spiking experiments using mycotoxin-free leek samples.

4.2.3 STATISTICAL ANALYSIS

Data processing and calculations were performed using Microsoft Office Excel 2010, IBM SPSS Statistics 22 and GraphPad Prism 6.

4.3 RESULTS AND DISCUSSION

4.3.1 *FUSARIUM* SPECIES DETERMINATION AND ISOLATE CHARACTERIZATION

The two species that were recovered from diseased leek plants in Flanders (Belgium) were *F. avenaceum* and *F. culmorum* but both species were never recovered on the same individual plant. Further genetic characterization of the 28 *F. avencaum* isolates revealed that these isolates were genetically of the ENN B type according to the methodology described in Stępień and Waśkiewicz (2013) [277]. For the first time, *F. avenaceum* has been associated with basal rot of leek in Europe, which has been previously reported as a generalist pathogen [279].

4.3.2 PATHOGENICITY OF *FUSARIUM* SPECIES ON LEEK PLANTS

Thirteen *F. avenaceum* isolates were included in an infection trial to test the *in planta* pathogenicity. These *F. avenaceum* isolates showed low virulence on leek since no significant symptom development was observed (Figure 4.3). Only one *F. avenaceum* isolate (isolate 44) was very aggressive on leek and showed comparable virulence towards leek as observed with *F. culmorum*,

which is also known as a causal agent of pink root rot in leek (Figure 4.3). *F. avenaceum* has been described as an endophyte [280, 281]. The latter feature might explain why all but one isolate of *F. avenaceum* caused very mild symptoms.

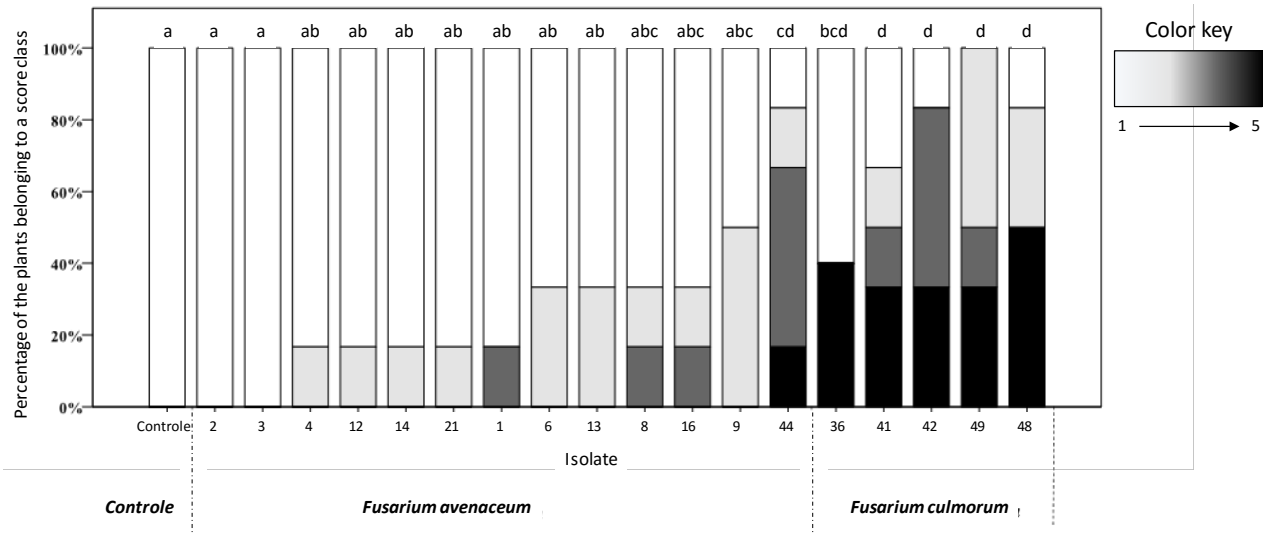

Figure 4.3 Aggressiveness of *F. avenaceum* and *F. culmorum* isolates on six weeks old leek seedlings.
Aggressiveness was assessed using an ordinal scoring system from 1 (healthy, white bar) to 5 (dead plant, black bar) as depicted in Figure 4.2. Experiment was evaluated after four weeks. Data are the results of two independent experiments with six plants per experiment and per treatment. Non-parametric Kruskal-Wallis test was used to test if there were significant differences between groups of data. In case there was a significant difference (p-value < 0.05), a pairwise comparison of the groups using a Dunn's test was performed. The letter codes distinguish groups for which significant differences were observed.

4.3.3 DETECTION OF MON, BEA AND ENNS IN LEEK

Because *F. avenaceum* was recovered from diseased leek plants, a multi-mycotoxin method for MON, BEA and ENNs was developed and validated in leek using UPLC-MS/MS.

4.3.3.1 OPTIMIZED LC-MS/MS PARAMETERS

Detection and quantification were performed with a Waters Acquity UPLC apparatus coupled to a Xevo TQ-S MS triple quadrupole system (Waters, Milford, MA, USA). For data acquisition and processing, Masslynx and Quanlynx software 4.0 (Waters) were used.

The mass spectrometric conditions were optimized by tuning the different analytes. For each analyte, ideal cone voltages and collision energies and two product ions (except for moniliformin) were chosen. The MS was operating in ESI- for MON and in ESI+ mode for BEA and ENNs. The data

acquisition was carried out in the MRM mode. The optimized MS/MS conditions can be found in Table 4.1

For chromatographic separation, the Acquity UPLC CSH Fluoro-Phenyl (1.7 µm, 2.1 mm X 150 mm) proved to be suitable for the simultaneous determination of the target mycotoxins. Column temperature was set at 30 °C. Solvent A (ultrapure water/methanol/acetic acid (94/5/1, v/v/v)) and solvent B (methanol/ultrapure water/acetic acid (97/2/1, v/v/v)) were both supplemented with 1 $mmol.L^{-1}$ ammonium formate and used as mobile phases. The gradient program at a flow rate of 0.3 $mL.min^{-1}$ was applied. During the first minute, a constant flow with 70 % mobile phase A was kept. In 0.10 min the gradient switched to 35 % A and this was maintained for 3 min. Afterwards the gradient slowly decreased to 25 % A in 5 min and then returned to the initial column conditions of 30 % A resulting in a total run time of 12 min.

Table 4.1 Optimized $ESI^{+/-}$ MS/MS parameters for MON, BEA and ENNs.
*The underlined product ion is the most abundant and thus used for quantification purposes.

Compound	Precursor ion (m/z)	Molecular ion	Product ion (m/z)	Cone (V)	Collision (eV)
MON	97.0	$[M–H]^-$	41.0	24	14
BEA	801.3	$[M + NH4]^+$	<u>244.3</u>	40	47
			262.4	40	47
ENN A	699.2	$[M + NH4]^+$	210.3	50	43
			<u>228.4</u>	50	43
ENN A1	685.4	$[M + NH4]^+$	<u>210.3</u>	38	36
			228.1	38	32
ENN B	657.3	$[M + NH4]^+$	196.3	40	32
			<u>214.4</u>	40	30
ENN B1	671.2	$[M + NH4]^+$	196.3	32	32
			<u>214.0</u>	32	32

4.3.3.2 OPTIMIZED SAMPLE PREPARATION

For none of the toxins, the use of QuEChERS - with or without dSPE - significantly improved the absolute peak areas. On the contrary, the QuEChERS approach combined with dSPE even decreased the efficiency of the extraction. Since the extraction efficiency is evaluated in terms of recovery and time, the liquid extraction with ACN without further sample preparation was selected as the most suitable procedure for the extraction of MON, BEA and ENNs. To obtain clear extracts, an additional

centrifugation step with filters was included prior to LC-MS/MS analysis. The mean recoveries obtained by spiking blank leek samples at level of 50 ng.mL^{-1} before (n=3) and after (n=3) the extraction ranged from 81 to 91 % (data not shown).

After optimization, the following sample preparation was selected. All samples were grounded using liquid nitrogen to ensure the desired homogeneity of the sample. For the calibration curve, blank leek samples of 1.000 g (± 0.005 g) were accurately weighed. Extraction was carried out with 5 mL acetonitrile for 30 min on an overhead shaker, followed by centrifugation for 10 minutes at 4000 x g. Next, 4 mL of the clear extract was transferred into a clean tube and evaporated at 60 °C to dryness under a gentle stream of nitrogen. The residue was reconstituted in 300 µL of mobile phase (65 % B) by vortexing for 1 min. Finally, the reconstituted extract was centrifuged for 10 min at 10000 x g, prior to the LC-MS/MS analysis.

4.3.3.3 METHOD VALIDATION

LOD, LOQ and linearity

Twenty representative blank leek samples were analysed to determine the specificity. No interfering peaks (S/N ≥ 3) were detected at the expected retention time of the target toxins. Analyses for linearity, accuracy (precision and apparent recovery) and measurement uncertainty were carried out on three different days as a combined experiment. On each day four calibration curves with five calibration points were constructed for all mycotoxins by spiking mycotoxin-free (blank) samples with increasing concentrations of mycotoxin standard mixture creating matrix-matched calibration curves. Because no suitable internal standard was available, the calibration curves were constructed by plotting the concentration against the absolute peak area.

The matrix-matched calibration curves showed good linearity with coefficient of determination (R^2) above 0.96. The linearity was confirmed with a lack-of-fit test in the selected concentration range. A *p*-value greater than 0.05 demonstrated no lack of fit of the model in the selected concentration range. Results of linearity and the concentration range of each analyte are shown in Table 4.2.

Table 4.2 Concentration range (µg.kg^{-1}) and R^2 values of the matrix-matched calibration curves in leek with the corresponding LOD and LOQ (µg.kg^{-1}) for MON, BEA and ENNs.

	Range (µg.kg^{-1})	R^2	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})
MON	4-200	0.99	1.4	4.2
BEA	4-400	0.98	1.0	2.9
ENN A	4-200	0.98	1.2	3.7
ENN A1	4-100	1.00	1.0	2.9
ENN B	2-400	1.00	0.3	0.8
ENN B1	4-400	0.96	1.0	3.0

LOD and LOQ were estimated by preparing specific calibration curves using samples spiked with the standard mixture of the analytes in the range around the expected LOD [233]. Table 4.2 shows the method LOD and LOQ for MON, BEA and ENNS in leek samples. Values for LOD were in the range of 0.3 to 1.4 µg.kg^{-1}. Thus, the method proved to be sensitive and results were lower or comparable with those obtained in other developed methods involving MON, BEA and/or ENNs [95, 108].

Accuracy and measurement uncertainty

Precision of the method was expressed as repeatability and intermediate precision. For repeatability, the intra-day relative standard deviations (RSD_r) were calculated, while for the intermediate precision, the inter-day relative standard deviations (RSD_R) were used. The results at low, medium and high concentration level are summarized in Table 4.3. RSD values ranged between 2.4 and 14.0 % for the intra-day precision, and between 4.2 and 14.8 % for the inter-day precision. Thus, all RSD-values did not exceed the level calculated by the Horwitz equation. Since no certified reference materials were available, trueness was expressed in terms of apparent recovery. The mean recoveries for all the tested mycotoxins were in the range of 81–107 %, which is in the recommended range required for other mycotoxins (70-110 %) described in commission regulation (EC) No 401/2006 (Table 4.3) [232].

The expanded measurement uncertainty *U* was calculated at low, medium and high concentration level for all the mycotoxins. As no certificates of analysis were available, the uncertainty of the standard $U(C_{ref})$ was covered by an arbitrary level of $\sqrt{3}$. The results ranged from 18.0 to 42.9 %.

Table 4.3 Accuracy of the validated method at low, medium and high concentration level.

	Conc (µg.kg^{-1})	R_{app} (%)	RSD_r (%)	RSD_R (%)	U (%)
MON	4	105	6.8	7.9	24.5
	100	103	11.6	11.6	32.9
	200	107	3.0	9.6	30.5
BEA	4	95	9.4	9.4	25.9
	200	96	5.8	7.8	22.1
	400	99	4.2	4.2	19.9
ENN A	4	99	6.1	7.5	20.4
	100	102	14.0	14.2	42.9
	200	92	7.0	7.0	24.7
ENN A1	4	101	6.7	6.7	19.6
	50	93	11.2	14.8	41.1
	100	81	11.0	12.7	39.8
ENN B	2	105	3.3	7.5	25.9
	200	88	4.6	5.4	23.2
	400	82	4.8	6.3	18.0
ENN B1	4	103	4.3	11.9	33.4
	200	82	12.8	12.8	36.4
	400	82	2.4	7.9	18.6

4.3.4 PRESENCE OF MON, BEA AND ENNS DURING INFECTION OF LEEK BY *F. AVENACEUM*

Leek samples (n=76) artificially infected with *F. avenaceum* were analyzed for the presence of MON, BEA and ENNs with the developed and analyzed LC-MS/MS method. Although molecular Esyn1 gene analysis showed that all isolates had Esyn1 genes of ENN-type sequence [249], the blend of mycotoxins present in leek was complex comprising all analyzed cyclodepsipeptides and MON. Samples were considered positive if the concentration was above the determined LOD. MON was only detected in two samples at concentrations below LOQ. For all ENNs the incidence was rather low: 7 % for ENN A, 8 % for ENN A1, 13 % for ENN B and 12 % for ENN B1. The most prevalent mycotoxin was BEA, which was found in 20 % of the samples. Mean and median values were calculated with concentrations > LOQ. As indicated in in Table 4.4, the mean levels for BEA and ENNs varied between 10.5 and 28.1 µg.kg^{-1}. In addition, the highest concentration observed was 87.2 µg.kg^{-1} BEA.

Since there was only one isolate (isolate 44) which caused clear disease symptoms (Figure 4.3), no correlations between the *F. avenaceum* mycotoxins and disease symptoms were calculated.

Table 4.4 Presence of MON, BEA and ENNs in leek samples artificially infected with *F. avenaceum* (n=76).

Mycotoxin	Positive samples (%)	Mean (µg.kg^{-1})	Median (µg.kg^{-1})	Max (µg.kg^{-1})
MON	1	-	-	-
BEA	20	28.1	16.3	87.2
ENN A	7	10.5	5.9	26.4
ENN A1	8	17.2	7.8	59.9
ENN B	13	24.6	13.7	69.9
ENN B1	12	15.1	8.5	40.9

4.4 CONCLUSION

This is the first European report where *F. avenaceum* is associated with *Fusarium* basal rot in leek.

In addition, a new reliable and highly sensitive method based on UPLC-MS/MS with electrospray source working in both positive and negative mode, has been developed and validated for the determination of BEA, ENNs and MON in leek. With a straightforward sample preparation using 100 % ACN as extraction solvent, extraction recoveries between 81 and 91 % were achieved. The LOQ of the target analytes ranged from 0.8 to 4.2 µg.kg^{-1}. Apparent recoveries ranged from 81-107 %, which is within the acceptable range of 70-110 % for other mycotoxins according to Commission Regulation (EC) No 401/2006. The linearity of calibration curves expressed as coefficient of determination (R^2) were in the range of 0.96-1.00 and repeatabilities expressed as RSD_r ranged between 2.4-14.0 %, while the RSD_R for intermediate precision ranged between 4.2 and 14.8 %.

In order to elucidate the toxicogenic capacity of *F. avenaceum* on leek and to assess the link between virulence of *F. avenaceum* and mycotoxin production, the developed method has been applied to 76 artificially leek samples. Remarkably, despite no visual symptoms on the artificially infected leek samples, low concentrations of ENNs were found. Which is important since leek is a plant which has to meet very high visual standards in order to make it to the market and all symptomatic plants are

removed during sorting after harvest. Although the Esyn1 gene of all *F. avenaceum* isolates were of the ENN-type as previously described by Stepien and Waskiewicz (2013), a complex blend of mycotoxins was observed in several isolates comprising both ENNs and BEA. This complex chemotype has been previously reported [277]. For all *F. avenaceum* isolates, mycotoxins were low both in incidence as in concentration. None of the BEA, ENNs or MON exceeded the level of 87.2 $\mu g.kg^{-1}$ of fresh weight. Therefore, we assume that BEA, ENNs and MON are most likely not involved in the interaction of *F. avenaceum* with its host leek. The physiological role of these emerging mycotoxins remains therefore unknown.

ACKNOWLEDGEMENTS

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Chapter 5

Impact of low doses of the cyclic depsipeptide CER on the mitochondrial function of Caco-2 and HepG2 cells

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Impact of low doses of the cyclic depsipeptide CER on the mitochondrial function of Caco-2 and HepG2 cells

Besides toxicity tests using laboratory animals, *in vitro* studies can also be used to identify or to further characterize a toxic hazard. Using human-relevant cells or tissues, *in vitro* methods are used to evaluate changes in normal cellular signaling pathways. For this reason, the toxicity of the toxins of interest was studied *in vitro*. CER was selected as a model, but the following strategy can be extended to other toxins like BEA and ENNs and mixtures.

5.1 INTRODUCTION

CER is a heat-stable emetic toxin known as the causative agent of an acute emetic food intoxication [118, 126, 128]. The emetic syndrome is usually mild and self-limiting, however in some cases the food poisoning leads to severe clinical conditions including fulminating liver failure and rhabdomyolysis [127]. CER is a cyclic and lipophilic dodecadepsipeptide (1.2 kDa) that acts as a potassium ionophore on the mitochondrial membrane and is structurally related to the macrolide antibiotic valinomycin [130]. It is formed in food and is highly resistant towards heat (up to 121 °C for 2 h), pH (pH range from 2 to 11), and proteolytic enzymes such as pepsin and trypsin [121, 182]. As a consequence, food preparations or reheating of the cooked foods will not destroy CER and the intact toxin will pass the stomach and reach intestines without the loss of toxic activity. Hence, the emetic syndrome is characterized by nausea, vomiting and malaise 0.5-5 h after ingestion of the contaminated food. Most of the reported cases implicated leftovers or takeaway pasta and rice dishes that were improperly stored allowing survival and growth of *B. cereus* with production of CER [125, 126]. Moreover, studies generally focused on high-carb food matrices (rice and pasta) linked with foodborne outbreaks, while prevalence of CER in other food categories is less described [282]. The acute intoxication dose provoking clinical manifestations was estimated at ca. 8-10 µg CER per kg of body weight [147, 148, 162]. Prevalence data on CER in samples not related to foodborne outbreaks are less reported and generally indicated distribution of low doses CER [140, 151]. A Belgian study conducted on rice dishes collected in Chinese-style restaurants revealed that CER was found in 7.4 % of the samples with an average CER concentration of 4 $\mu g.kg^{-1}$ food [125]. This

concentration is low compared to the levels found in food incriminated in foodborne outbreaks. However, Ronning *et al.* (2015) unexpectedly found considerable amounts of CER (up to 593 µg.kg^{-1}) in pasta and rice samples that were considered blank [237]. Nevertheless, besides the acute effects associated with food poisoning, a repeated exposure to low doses of CER present in food from restaurants or households that was improperly stored might be potently harmful [152, 153, 226].

Although *B. cereus* is ubiquitously distributed in the environment, it is known that only emetic strains of *B. cereus* that possess the *ces* gene have the potential to produce CER. Messelhausser *et al.* (2014) investigated the prevalence of emetic strains in foods of different origin (n=3654) and reported that about 1 % contain the emetic strain [146]. Remarkably, the emetic strains were not only detected in the expected farinaceous foods but also in vegetables, fruit products, sauces, soups, and salads and in cheese and meat products. A higher prevalence of emetic *B. cereus* strains was found in ice cream samples (4.7 %) [283]. A study conducted on different foods on the Dutch market revealed that even 16.8 % of the tested isolates produced CER and/or contained the *ces* gene [140]. The reason for the low prevalence of CER might be partly explained by the fact that the emetic strains of *B. cereus* are less commonly found.

Since CER causes emesis and the main exposure route is via food, the gut is most likely the first place in the human body exposed to the toxin. In view of this, it is of great importance to investigate the effects of various food-relevant CER concentrations on the models of intestinal epithelium [153]. To mimic the contact of CER in food with the human digestive tract, the Caco-2 cell line (human colorectal adenocarcinoma) was selected as model for the intestinal epithelial barrier in *in vitro* cultures. In addition, the CER intoxication is occasionally implicated in life-threatening conditions including acute liver failure, and hence HepG2 cells were included in the study as a model for human hepatocytes. Considering that CER acts on mitochondria by depolarization of the mitochondrial inner membrane and impairs the adenosine triphosphate (ATP) synthesis, the mitochondrial damage of CER was evaluated with the extracellular flux analysis of oxygen consumption by means of Seahorse XF technology. The Seahorse technology allows detection of mitochondrial function and cellular respiration by real time measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Seahorse XF Cell Mitochondrial Stress Test provides quantitative information on basal respiration, proton leak, maximum respiration rate, and non-mitochondrial respiration by sequential exposure of CER-treated cells to chemical modulators targeting specific key components of the

electron transport chain (ETC). In this study, the Seahorse XF Cell Mito Stress Test was used as a tool to evaluate the effects of repeated exposure of Caco-2 and HepG2 cells to low doses of CER.

5.2 MATERIALS AND METHODS

5.2.1 REAGENTS AND CHEMICALS

Dulbecco's Modified Eagle's Medium (DMEM), GlutaMAX™, penicillin/streptomycin and non-essential amino acids were purchased at Life Technologies (Merelbeke, Belgium), whereas foetal bovine serum was from Greiner Bio-One (Vilvoorde, Belgium). Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, was obtained from Westburg (Leusden, Netherlands) and trypsin-EDTA 0.05 % from ThermoFisher scientific (Merelbeke, Belgium).

5.2.2 CELL CULTURE

Caco-2 (human colorectal adenocarcinoma) and HepG2 (human hepatocellular carcinoma) cells were obtained from ATCC and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX™, 4.5 g/L D-glucose and pyruvate supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids and 1 % penicillin/streptomycin. The passage number of the cells used in this study was maintained between 12 and 25. Cells were grown in T25 (25 cm^2) polystyrene cell culture flasks in a humidified chamber with 5 % CO_2 at 37 °C and 95 % air atmosphere at constant humidity. The culture medium of the cells was changed every other day and cell morphology was daily checked by visual inspection with phase-contrast microscopy (Leica DMIC, Leica Microsystem GMbH, Wetzlar, Germany). Caco-2 and HepG2 cells with a degree of confluence of approximately 80 % were subcultured to maintain their undifferentiated character and therefore the rapid growth of the cells. At 80 % confluence, cells were gently rinsed with PBS and subsequently detached from the flask with trypsin-EDTA 0.05 % and seeded in a new T25 flask (ratio 1:5).

5.2.3 EXPOSURE OF CELLS TO CEREULIDE

The 1 $mg.mL^{-1}$ solution of CER (Chiralix, Nijmegen, The Netherlands) was diluted to working solutions (ranging from 0.05 to 10 nM) in cell culture medium to obtain the 'exposure' medium. The cells were subjected to CER treatment once the cells reached 80 % confluence. The medium was removed from the cells and replaced with exposure medium. During the ten days exposure period, cells were

allowed to grow to 80 % confluence before splitting. First, the non-exposed cells were counted with a Bürker counting chamber according to the Trypan blue staining method and the number of cells.mL^{-1} of non-exposed cells was obtained. Afterwards, the cells exposed to different CER concentrations were counted and seeded at a cell density equal to that of the non-exposed cells. In this way, after each splitting, cells were seeded equally to obtain the same number of cells for each condition which allowed comparison of the different concentrations.

5.2.4 CELL VIABILITY ASSAY

Two widely established assays, MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) and SRB (sulforhodamine B), were used for characterization of the cell viability after CER exposure to Caco-2 and HepG2 cells [284, 285]. Caco-2 and HepG2 were seeded to each well of the 96-well plates at a density of respectively, 20000 and 30000 cells per well. Subsequently, the cells continued to grow in exposure medium for 24 h at 37 °C in an incubator with a humidified atmosphere of 5 % CO_2. Mitochondrial activity of the exposed cells was measured after 24 h with the MTT assay. Briefly, 20 µL of MTT dissolved in PBS at 5 mg.mL^{-1} was added to all wells of the assay, and plates were incubated at 37 °C for 2 h to convert MTT to formazan. After incubation, medium was discarded and the purple formazan crystals were solubilized in dimethylsulfoxide (DMSO). Absorbance was recorded at the wavelength of 570 nm by SpectraMax M2 multimode plate reader (Molecular Devices, California, USA). In parallel, the SRB assay was used for cellular protein content measurement. The cell monolayers were fixed with 50 µL of 50 % trichloroacetate (TCA) in milliQ-water and stained for 1 h. After washing, cells were stained with SRB solution for 30 min. The excess was removed by washing repeatedly with 1 % glacial acetic acid and the protein-bound dye was dissolved in 10 mM Tris buffer. Absorbance was determined at 490 nm with SpectraMax M2 multimode plate reader. For each cell line and CER concentration, twelve repeats were made. Both assays were used for the preliminary determination of the sub-acute concentrations after 1 and 3 days, and as additional tests after a prolonged exposure of 10 days with low doses of CER.

5.2.5 SEAHORSE XF CELL MITO STRESS TEST

To elucidate the influence of chronic CER exposure on the mitochondrial function, Seahorse XF Cell Mito Stress Test provided by Seahorse XF technology was applied to both undifferentiated Caco-2 cells and HepG2 cells. The OCR was determined by the XF24 extracellular flux analyzer (Seahorse

Bioscience, North Billerica, USA). One day prior to the assay, cells were harvested from the T25 plates in which the cells were exposed to CER. The cells were seeded at 40000 cells per well in a Seahorse 24-well XF Cell Culture microplate in 250 µL of the appropriate exposure medium and were allowed to adhere and grow for 24 h in a 37 °C humidified incubator with 5 % CO_2. In addition, the Seahorse XF Sensor Cartridge was hydrated the day before running the XF Assay by filling each well of the XF Utility Plate with 1 mL of Seahorse XF Calibrant Solution. The hydrated cartridge was kept in a non-CO_2 37°C incubator for 24 h. On the day of analysis, unbuffered XF Assay Media was used for extracellular flux measurements. For this reason, the cells were washed three times with non-buffered DMEM supplemented with 10 mM glucose, 2 mM sodium pyruvate and 2 mM glutamine (adjusted to pH 7.4) and then maintained in 450 µL.well^{-1} of XF assay media at 37 °C in a non-CO_2 incubator for 1 h to allow pre-equilibrate with the XF Assay Medium. Mitochondrial function of the cells was analyzed by sequential injections of oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and mix of rotenone/antimycin A. These compounds were suspended in pre-warmed XF Assay Medium and loaded into the designated injection ports of the hydrated sensor cartridge corresponding to the order of injection.

Subsequently, the loaded XF Sensor Cartridge with the XF Utility Plate was placed into the XF24 Analyzer and calibrated. After calibration, the XF Utility Plate with the calibration fluid was replaced with the plate containing cells. Each measurement cycle consisted of 3 min of mixing, 2 min of waiting, and 3 min of OCR measurements. First, three basal OCR measurements were performed before addition of modulators, followed by the sequential addition of oligomycin, FCCP, and rotenone/antimycin A. Measurement cycles were performed after each addition of given compounds. The final concentrations in each well after injection were as follows: 1.0 µM oligomycin, 0.25 µM FCCP and 0.5 µM rotenone/antimycin A.

5.2.6 DATA ANALYSIS

Statistical analyses were performed using the GraphPad Prism V6.01 software package (GraphPad Software, Inc., San Diego, CA). Comparisons between the control and CER treatments were done by performing One-Way ANOVA tests followed by *post hoc* analysis with Dunnett's multiple-comparison test. Differences were considered significant at $p < 0.05$. Data are presented as mean ± SD.

5.3 RESULTS AND DISCUSSION

5.3.1 DETERMINATION OF SUBACUTE CONCENTRATIONS

In preliminary experiments, the so-called subacute concentrations were determined after one and three days exposure with CER. After one day, results of the MTT assay indicated a significant decrease in cell viability of more than 50 % starting at 1 nM CER (Figure 5.1A). A similar decrease was observed for SRB, however less pronounced. Hence, higher concentrations that already gave a significant decrease in cell viability after one day were omitted in the three days exposure. According to the results of the MTT assay depicted in Figure 5.1B, three days of CER treatment significantly decreased cell viability with 21 % and 43 % compared to the untreated cells after exposure with 0.25 and 0.50 nM CER, respectively. CER induced significant reduction in protein content in the SRB assay at ≥ 0.25 nM (Figure 5.1 B). The selected treatments (0.05-0.50 nM) induced toxicity, but the majority of the cells survived the exposure. Based on these findings, the same concentration range was used for the subsequent study of ten days exposure.

In parallel, similar experiments were performed on undifferentiated HepG2 cells. As shown in Figure 5.2A, CER concentrations of 2 nM and 5 nM inhibited 30 % and 35 % of cell viability after one day calculated with the MTT assay. The toxicity was more pronounced based on the SRB results with reductions of 44 % for 2 nM and 52 % for 5 nM. However, at these concentrations cells continued to grow and thus the same concentrations were used for an exposure of three days. Clearly, the three days treatment significantly reduced the cell viability compared to the one day treatment. At 1 nM of CER and higher, viability was severely compromised in HepG2 cells with about 50 % as indicated by the MTT assay (Figure 5.2B). Results for SRB were even more drastic with a reduction of about 75 %, indicating the impact on both metabolic activity of cytoplasmic enzymes and cells and total protein amount. At these concentrations (≥ 1 nM) cells continue to grow. However, CER induced significant toxicity and cellular alternations. To ensure sufficient amount of living cells after ten days, the maximum exposure concentration was reduced to 0.50 nM CER for the following experiments.

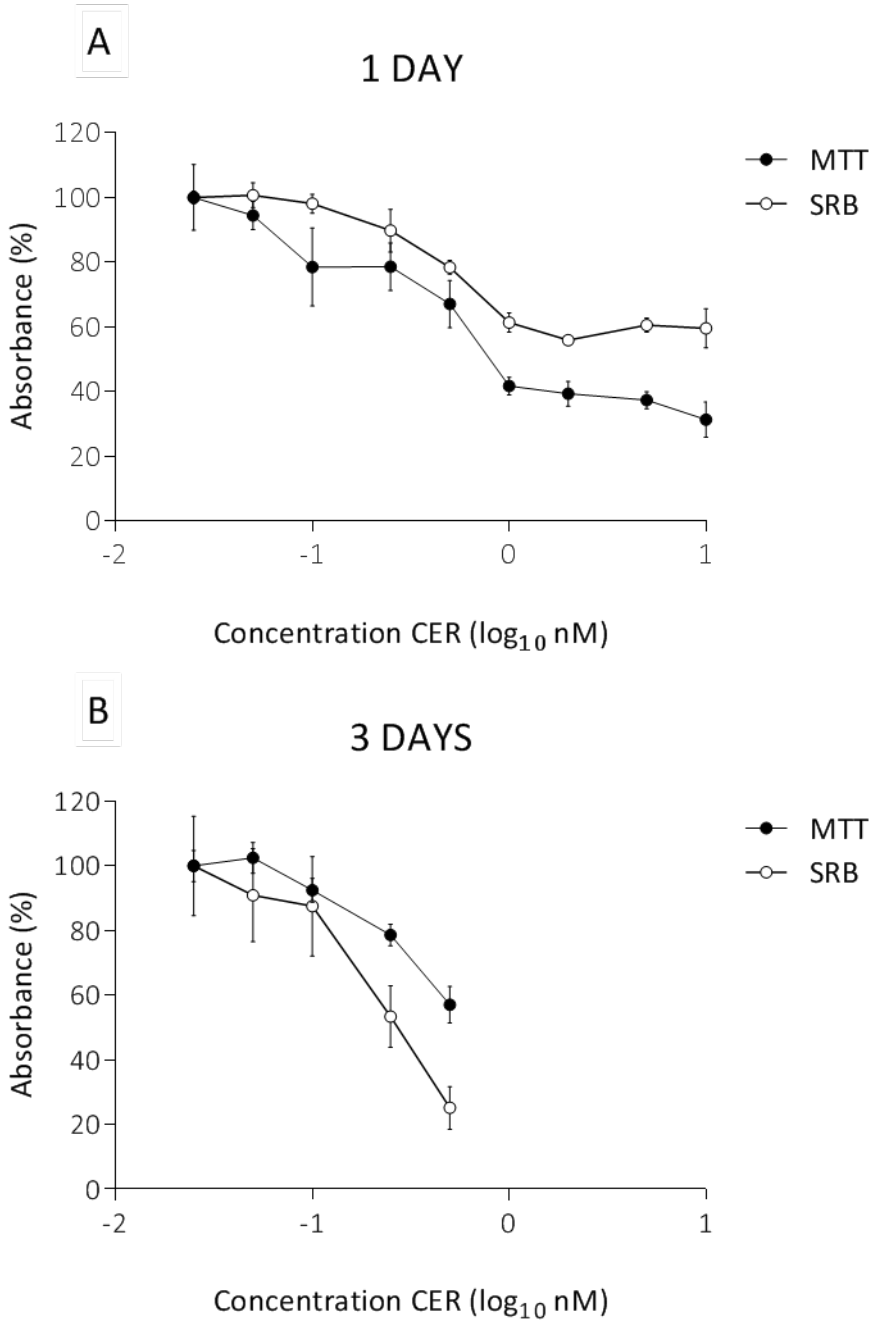

Figure 5.1 MTT assay and SRB assay after 1 day (A) and 3 days (B) treatment with CER in undifferentiated Caco-2 cells. The absorbance is expressed as a percentage of the control values (0 nM CER). Data (n=12) are presented as mean with error bar indicating standard deviation. * indicated a significant difference at $p < 0.05$.

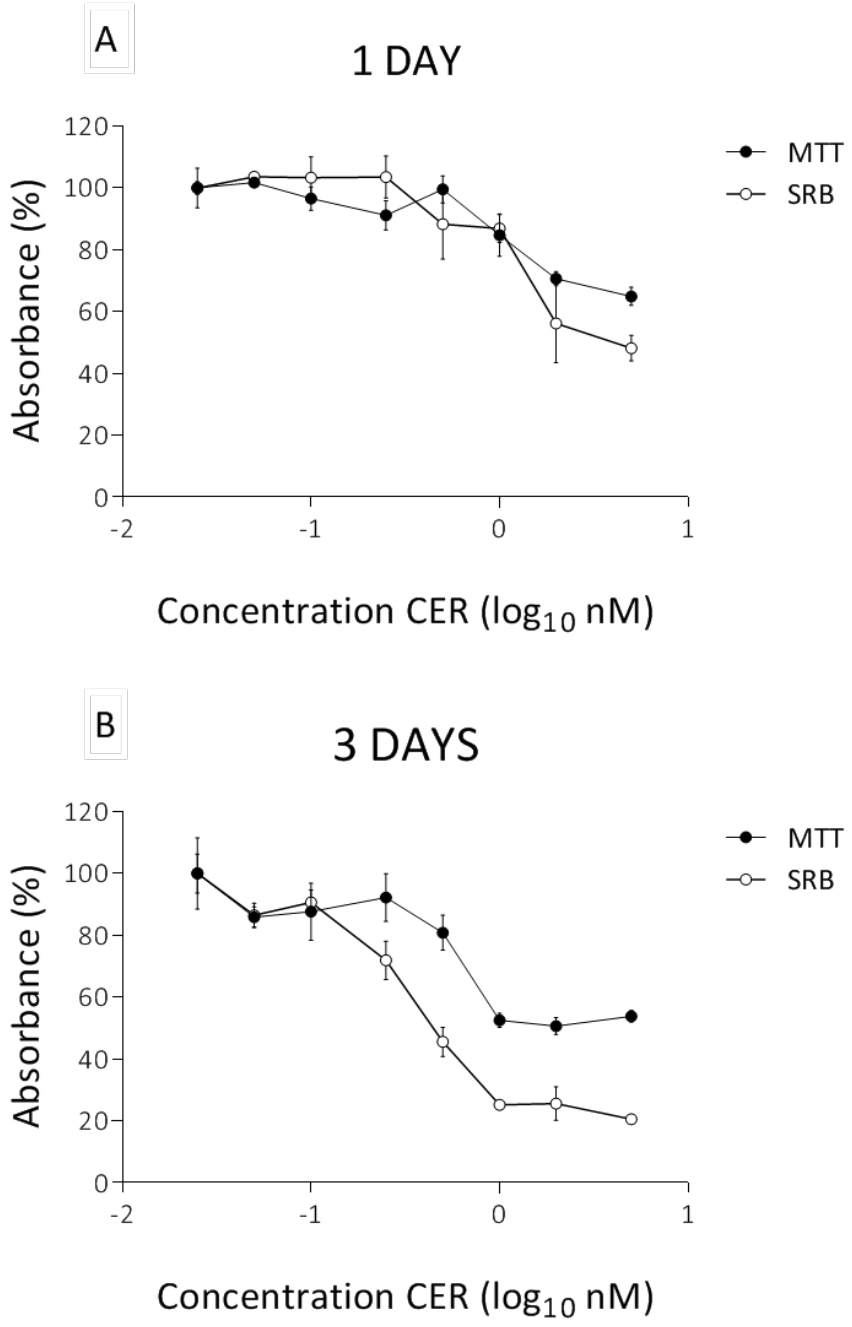

Figure 5.2 MTT assay and SRB assay after 1 day (A) and 3 days (B) treatment with CER in undifferentiated HepG2 cells. The absorbance is expressed as a percentage of the control values (0 nM CER). Data (n=12) are presented as mean with error bar indicating standard deviation. * indicated a significant difference at $p < 0.05$.

5.3.2 LONG-TERM EFFECTS OF CER ON MITOCHONDRIAL RESPIRATION OF CACO-2 AND HEPG2

The effects on the mitochondrial respiration after ten days exposure with varying doses of CER were determined using the Seahorse XF Cell Mito Stress Test kit. The basal respiration and respiration after sequential injection of oligomycin, FCCP and rotenone + antimycin A are depicted in Figure 5.3 and Figure 5.4 for undifferentiated Caco-2 cells and HepG2 cells, respectively.

As shown in Figure 5.3, mitochondrial functions of the undifferentiated Caco-2 cells were compromised starting at 0.25 nM CER. The toxic effects of CER were most reflected in a decreased non-mitochondrial respiration, ATP-linked respiration and especially in maximal respiration. Maximal respiratory capacity was estimated by an FCCP-stimulated respiration and a decrease is a strong indicator of potential mitochondrial dysfunction. Treatments with 0.05 nM CER had small effects on respiration, with the exception of a significant reduction in maximum respiration that could not be observed with MTT and SRB assays (Figure 5.5). Treatments up to 0.10 nM CER did not affect the basal respiration of the Caco-2 cells, but produced a reduction in the maximum respiration and ATP turnover. Higher concentrations of CER (0.25 and 0.50 nM) decreased mitochondrial respiration of cells even at the basal state, and their metabolic activity has been largely decreased so that no effects of modulators were observed. Note that the MTT/SRB results did not indicate significant reduction after treatment with 0.50 nM CER and even for higher concentrations only moderate toxicity was observed (Figure 5.5). The use of electron transport chain modulators is important in order to obtain sensitive information on mitochondrial and energetic status of the cell.

Figure 5.4 depicts OCR traces of undifferentiated HepG2-cells exposed to CER (0.05-0.50 nM). Similar effects have been seen with a significant reduction of ATP-linked respiration and maximal respiration starting at 0.05 nM CER (Figure 5.4). After exposure to 0.50 nM CER, all mitochondrial functions of the cells were severely compromised, whereas in Caco-2 cells comparable effects started at 0.25 nM CER. Maximal respiration significantly decreased with 50 % following 0.25 nM CER treatment, while a 98 % decrease was observed after 0.50 nM CER exposure compared with the control. In parallel, the mitochondrial ATP production declined to 58 % (0.05 nM CER), 34 % (0.25 nM CER) and 6 % (0.50 nM) of the untreated group. The experiments conducted in both cell lines indicated that various CER treatments caused perturbations in the mitochondrial respiration, although the effects were more

pronounced in Caco-2 cells. In both cell lines, the maximal respiration decreased in a dose-dependent manner (Figure 5.3 and Figure 5.4).

The generation of ATP oxidative phosphorylation is one of the predominant physiological functions of mitochondria. Since CER acts on mitochondria by uncoupling of oxidative phosphorylation, the results confirmed the expected decrease in ATP production with increasing CER concentration. Both maximal respiration and ATP-linked respiration proved to be sensitive parameters for determination of the mitochondrial dysfunction especially when compared to MTT and SRB assays. As a consequence, also cell spare respiratory capacity (SRC) was impaired indicating an affected ability of the exposed cells to cope with sudden increased need for ATP. The mitochondrial SRC is regarded as an important aspect of the mitochondrial function and is given by the difference between maximal and basal cellular OCR. When cells are subjected to stress, energy demand increases, with more ATP required to maintain cellular functions. A cell with a larger spare respiratory capacity can produce more ATP and overcome more stress, which indicates that this is an estimative of the cell's ability to cope with large increases in ATP turnover. Consequently, CER exposure that is negatively affecting the mitochondrial function possibly exerts negative effects on the ability of cells to cope with other stresses. This is in agreement with expected impact of CER on mitochondria by depolarization of the mitochondrial inner membrane and impairment of ATP synthesis [286].

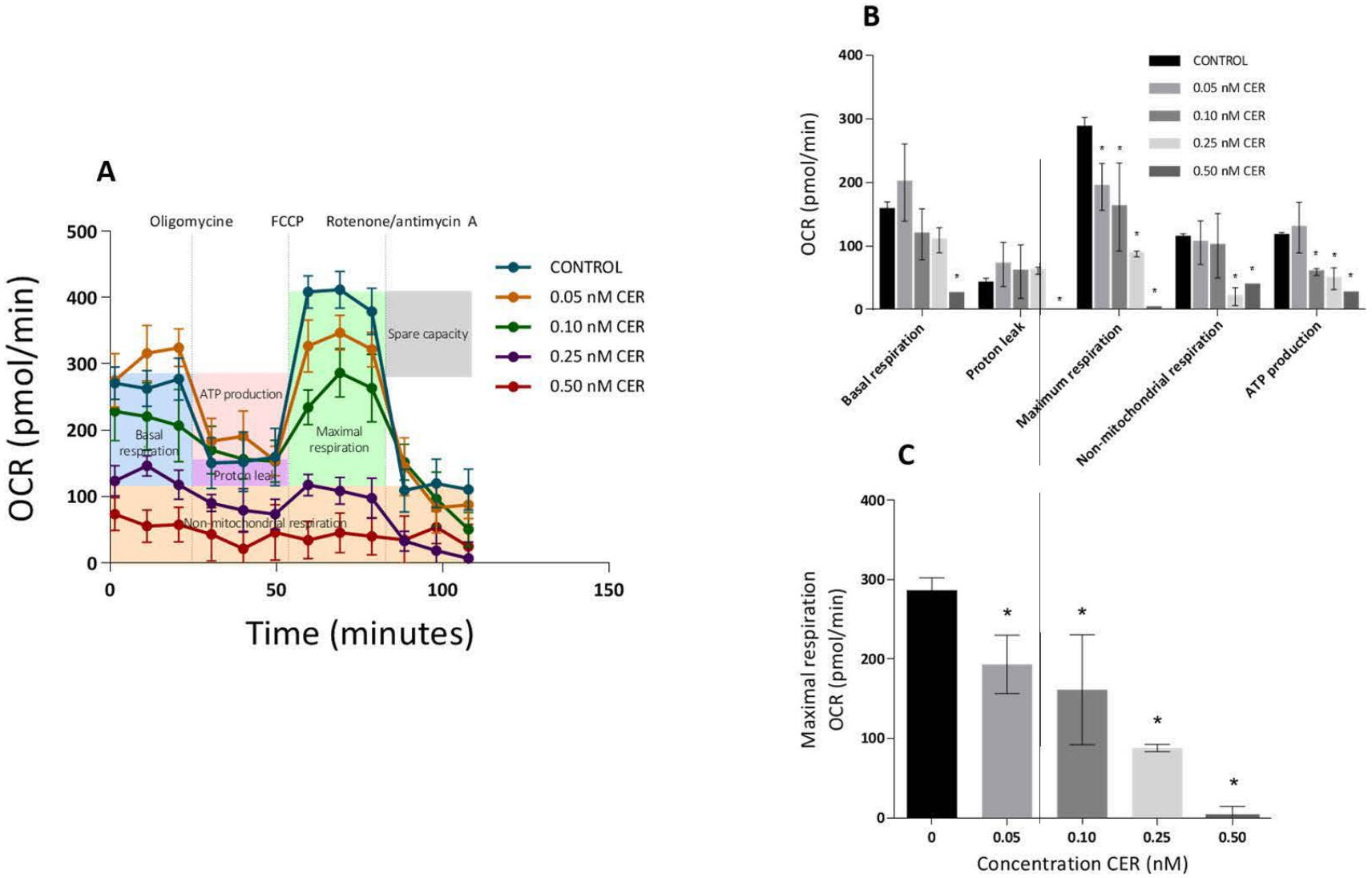

Figure 5.3 Effects of 10-day CER treatments on mitochondrial respiration of Caco-2 cells. (**A**) Mitochondrial profile (**B**) Mitochondrial parameters (**C**) Maximal respiration. Data (n = 4) are expressed as mean ±SD. * indicated a significant difference at $p < 0.05$.

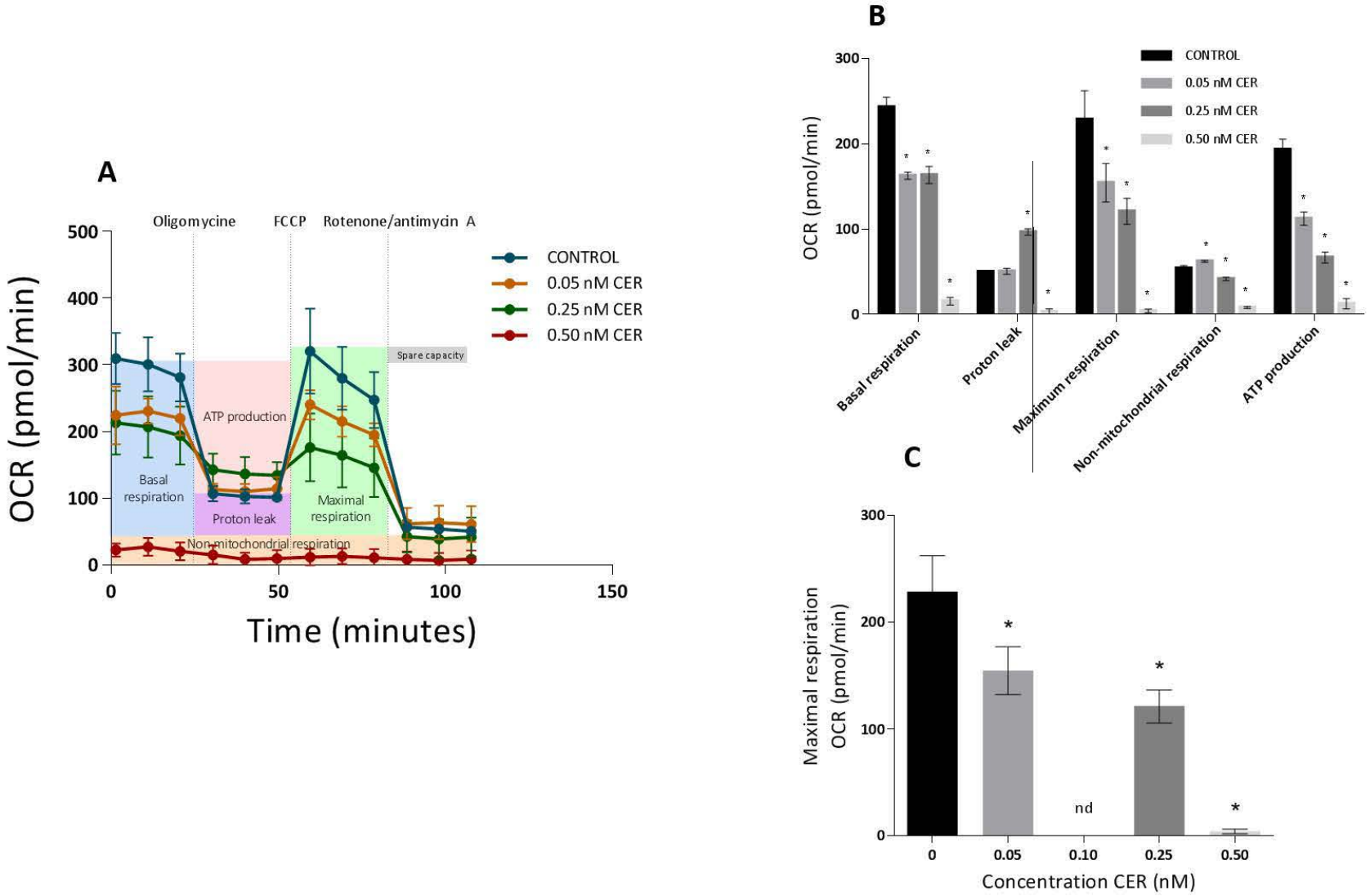

Figure 5.4 Effects of 10-day CER treatments on mitochondrial respiration of HepG2 cells. (**A**) Mitochondrial profile (**B**) Mitochondrial parameters (**C**) Maximal respiration. Data (n = 4) are expressed as mean ±SD. * indicated a significant difference at $p < 0.05$.

The impact of CER on mitochondrial ATP synthase that also translocates protons across the inner membrane has been best observed after the addition of oligomycin. The presence of oligomycin during the estimation of maximal respiration is important to prevent the reverse activity of ATP synthase with rapid intracellular ATP depletion, which may lead to cellular metabolic dysfunction and death. Hence, recent data showed that the presence of oligomycin significantly underestimates FCCP-induced maximal OCR and SRC [287]. Finally, the addition of a potent respiratory chain inhibitor, such as antimycin A, allows estimation of non-mitochondrial OCR. The mitochondrial respiratory chain in the inner membrane is translocating protons: H^+ ions are pumped out of the matrix space when electrons are transported along the chain. Considerable impact of CER on the metabolic fate of exposed cells is based on the fact that mitochondria carry out most cellular oxidations and produce the bulk of the cell's ATP. Large amounts of NADH (and $FADH_2$) are produced by the oxidation reactions. The energy available from combining oxygen with the reactive electrons carried by NADH and $FADH_2$ is harnessed by an electron-transport chain. This respiratory chain pumps H^+ ions out of the matrix to create a transmembrane electrochemical proton gradient. In turn, the transmembrane gradient is used both to synthesize ATP and to drive the active transport of selected metabolites across the mitochondrial inner membrane [288].

Additionally, the Seahorse XF Cell Mito Stress Test findings were confirmed by MTT and SRB assays (Figure 5.5). Hence, the reduction in cell viability was rather low compared to the findings of the Seahorse XF Cell Mito Stress Test. In both cell lines, cell viability started to decrease significantly at 0.25 nM CER with a small reduction around 25 % and 20 % for Caco-2 cells and HepG2-cells, respectively. Especially for the highest concentration (0.50 nM CER), the effects were more pronounced according to maximal OCR. Exposure to 0.50 nM resulted in a critically compromised OCR profile, while based on the SRB assay, 56 % and 50 % of the Caco-2 and HepG2cells respectively were still viable. The findings indicated that the MTT/SRB assays proved to be less sensitive than the Seahorse XF Cell Mito Stress Test.

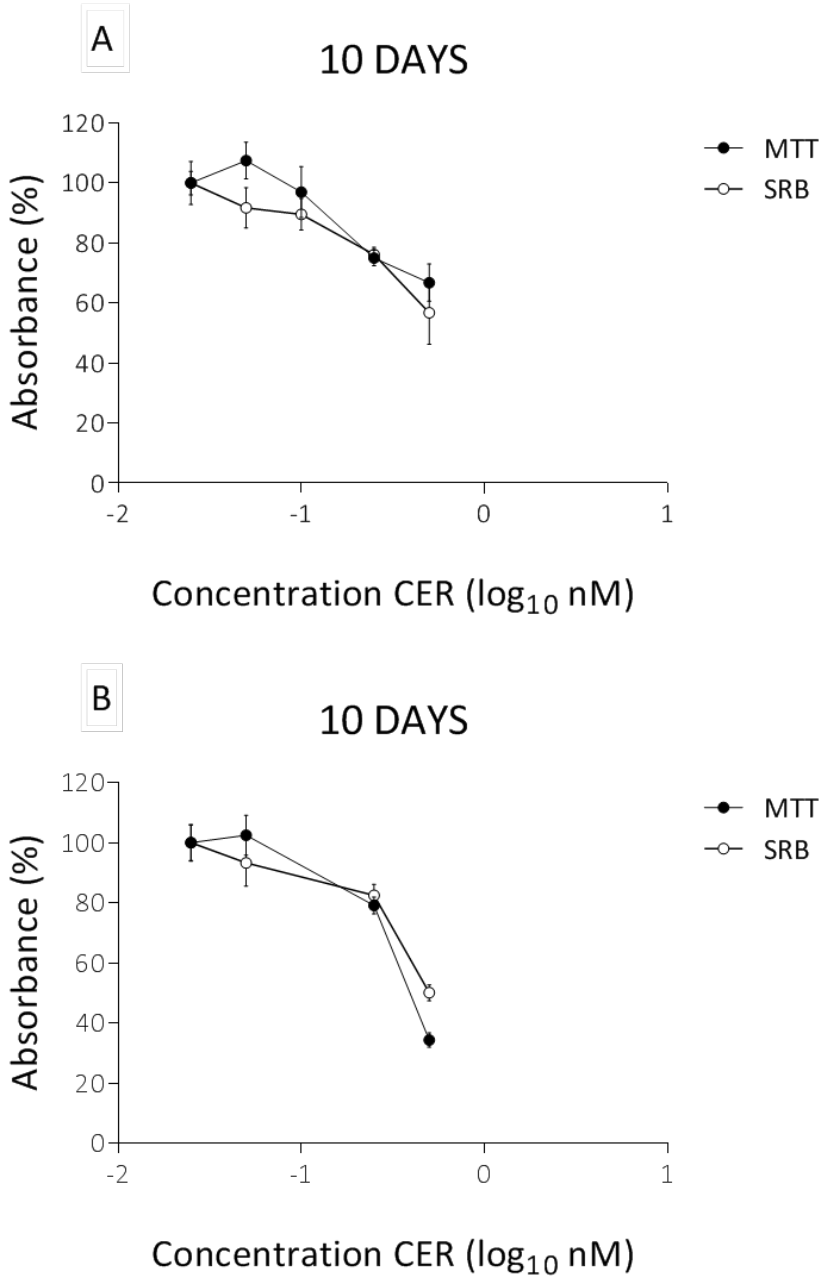

Figure 5.5 MTT assay and SRB assay at the end of the treatment (10 days) with CER in undifferentiated Caco-2 cells (A) and HepG2 cells (B).
The absorbance is expressed as a percentage of the control values (0 nM CER). Data (n=12) are presented as mean with error bar indicating standard deviation.* indicated a significant difference at $p < 0.05$.

Other *in vitro* toxicity studies indicated that at extremely low doses CER was toxic towards porcine fetal Langerhans islets and beta cells. Within two days, 1 ng.ml^{-1} of CER caused necrotic cell death of the islet cells hampering their insulin content [163]. In whole mouse islets apoptotic cell death was observed in 75 % of the islets after 72 h exposure with 0.25 ng.ml^{-1} CER [152]. These results indicated

that beta cells are even more sensitive to CER exposure than HepG2 cells. Nonetheless, similar to our study, very low doses of CER impaired the mitochondrial activity of the cells. In the case of beta cells this leads to impaired insulin secretion and cell death which both play key roles in the regulation of the diabetes [289].

Examination of the cell morphology was analyzed by phase-contrast microscopy supporting the observations found with Seahorse XF Cell Mito Stress Test. The exposure to CER slowed down the cell growth starting from a concentration of 0.10 nM CER. Vacuolization in the cells was observed starting at 0.10 nM CER. In parallel, the reduction in basal respiration observed using the Seahorse XF Technology corresponded to the decrease in cell number counted according to the Trypan blue staining method. Exposure with 0.50 nM CER induced cytotoxicity as the cells were reduced in number as well as in size. Hence, based on the results of MTT and SRB assays, the remaining cells appeared to be alive and active. In contrast, at this concentration mitochondrial respiration could barely be observed in the Seahorse XF Cell Mito Stress Test. This suggested that the microscopic observations corresponded to the data found with Seahorse XF Cell Mito Stress Test. At the end of the HepG2 treatment period with 0.50 nM CER, the cell morphology was severely impaired which was reflected in the number and size of cells. The remaining cells were barely viable as indicated by the MTT and Seahorse experiments.

5.4 CONCLUSION

The use of Seahorse XF Cell Mito Stress Test is a suitable model for the identification of mitochondrial toxicity when used alongside HepG2 cells and Caco-2 cells. When no significant reduction in cell viability was observed based on MTT and SRB results, The Seahorse XF Technology was able to detect a reduced maximal respiration, which suggested that toxic impact might be underestimated or go unnoticed based on MTT/SRB alone. Moreover, Seahorse XF Cell Mito Stress Test allowed assessment of critical information about the key parameters of mitochondrial functions, which could not be evident in basal respiration alone. Especially the maximal OCR appeared to be more sensitive to the presence of CER than other currently used assays for CER detection such as *in vitro* boar semen bio-assay where the threshold concentration provoking visible mitochondrial damage was 2 $ng.mL^{-1}$ [162].

Mitochondrial damage has been proposed as the mechanism underlying the induced hepato- and intestinal damage associated with CER intoxication. Therefore, human intestinal epithelial and hepatocellular cells were used as a starting point to assess the impact of chronic exposure. Our *in vitro* studies have shown that both cell types are highly susceptible for the toxic effect of very low CER concentrations. A long-term exposure of ten days with 0.25 nM CER seemed to be a critical concentration in both cell lines. However, the effects were even more pronounced in HepG2 cells. This might suggest that chronic low exposure of CER might induce damaging effects at liver cell level. As indicated in this *in vitro* study, CER exerts harmful effects to liver and epithelial colon cells at very low doses that were not observed after repeated exposure. Literature indicated that low doses of CER are found in foods with no intoxication history, supporting the hypothesis of possible hidden exposure [125]. Taken together, chronic or repeated exposure to low doses of CER that are too low to cause acute effects is a potential threat for human health and awareness of these issue is imperative.

The emetic dose for CER has been estimated around 8-10 $\mu g.kg^{-1}$ body weight. This value was based on concentrations found in leftovers from food intoxications and on studies conducted on rhesus monkeys and *Suncus murinus* [147, 148]. However, currently no extrapolation studies have been conducted with CER to translate the observations found in animal models to humans. Cell culture assays have the advantage to detect the biological active toxin in contrast to chemical assays and immunoassay that cannot distinguish the active from the non-active forms. During risk assessment, dose-response data from *in vivo* studies in animals or humans are preferred, but *in vitro* studies allow the investigation of mode of action of the toxin.

It is difficult to translate the external dose, which is found in the food, to the internal dose that is absorbed in the body and to the eventual target dose at the level of tissue/organ. This means that the concentrations found in the food are not necessary the dose that will cause the toxicity. In order to know the exact ratios between different doses, the concentration of the food, the amount of the food that is consumed and the concentration in the organ (*e.g.* liver) should be determined. However, so far no such studies have been done for CER. The internal dose is the fraction of the external dose that is absorbed and enters the general circulation. This concentration is depended of absorption, distribution, metabolism and excretion and can be derived from suitable toxicokinetic studies. It is expected that the metabolization of CER is limited since CER is considered as a stable

toxin and based on case reports, non-metabolized forms were found in *e.g.* intestinal cells, body fluids and liver cells etc. The tissue dose is the amount that is distributed to and present in a specific tissue of interest (*e.g.* liver in our case). In the case of Caco-2 cells, which are an intestinal model, the concentration will be close to the external concentration. Ideally, physiologically based toxicokinetic (PBTK) models, which are computational tools that can simulate the absorption, distribution, metabolism, and excretion of chemicals, should be used to link the target dose to the administered dose.

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Chapter 6

Broader international context, relevance and future perspectives

Broader international context, relevance and future perspectives

6.1 FOOD SAFETY

Food safety related scandals and their global mass media coverage have been recently demonstrated across the European Union in the case of the fraudulent presence of fipronil in eggs in Belgium, The Netherlands, Germany, etc., which rapidly ballooned into an EU-wide crisis. In the summer of 2017, millions of eggs were recalled from shops and warehouses and/or were blocked from sale after some were found to contain high levels of fipronil. Fipronil is an effective and widely used insecticide on crops and pets. Although within the EU its use is not allowed in the vicinity of food-producing animals, it is sometimes fraudulently used in poultry farms as disinfectant and in treatments against lice, fleas and ticks. It can last for months in animals and soil, and through the chickens, fipronil reaches the eggs. Fipronil is considered moderately toxic when ingested in large quantities over time and can cause kidney, liver or thyroid damage. In their attempt to manage the crisis, authorities have frantically tested eggs, manure and hens' blood for contamination. EFSA has identified an acute reference dose (ARfD) for humans of 0.009 mg fipronil per kg bw, which corresponds to 0.72 mg of fipronil for an adult person (80 kg) and 0.225 mg for a child (25 kg). Above this dose health effects, might be observed when ingested during a short period of time (daily or during a meal). Only eggs containing fipronil above this value were being recalled [290]. Questions were raised on how this could happen and why this was not discovered or reported earlier. Nevertheless, the crisis seems to be blown out of the proportion making unnecessary damages to food industry and the public confidence to the food safety authorities. Besides a food safety and a health risk concern, the issue also appeared to be of an economic nature since fipronil was sold as a 'natural' product to poultry farms [291]. A crisis like this clearly shows the crucial role and responsibility of national food safety agencies to protect consumers. Moreover, it emphasizes the relevance of monitoring food safety and the importance of reliable analysis of contaminants.

The increasing focus in the media on nutrition and health, the high number of reports on food recalls and food fraud incidents (such as melamine in milk and horse meat substitution for beef) have led to negative perception regarding the safety and health of our food [292, 293]. This does not imply that our food is less safe, but rather that previously these issues might have flown under the radar.

Currently, food producers are often obliged to have different forms of food safety management systems. In addition, the number of countries in which authorities require that food companies have self-control systems in place is increasing. Moreover, food is routinely tested for a range of contaminants based on legislative requirements and initiatives like Europe's Rapid System for Food and Feed (RASFF) ensure the flow of information [294].

However, most of these incidents are related to abusive use of a product whereas the problem of cyclic depsipeptides is related to improper storage or production of food. Nevertheless, one cannot disagree with the fact that the complexity of the food chain, mass production and globalization add to the potential of food spoilage. Consequently, the implementation of early warning systems can avert an incident or even a crisis to a controlled issue by rapid actions. Still, protecting public health still requires a continuous monitoring and alertness for unknown and emerging risks. In case of non-regulated hazards, the information on prevalence and occurrence is key input for risk assessment and later risk management. To establish regulations, dose-response studies are required as well as quantitative information on the prevalence of hazards in food.

Although it is difficult to foresee and assess every single risk, the 'drivers' associated with emerging risks can be identified and can help to intervene. EFSA defined an emerging risk as "*a risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard*". Note that assessment of emerging risks differs from the assessment of risks in an emergency food/feed crisis [295]. To tackle the issue of early recognition of emerging risks, EFSA established monitoring procedures for systemic searching, collecting and analyzing information. A pilot study has been carried out to assess the practicable approach of different methodologies. Approaches for identification mainly focus on the exploitation of knowledge networks, and individual and institutional expertise as well as on web-scouting tools such as Digimind. However, big amount of data still need to be ordered and analyzed to detect abnormal trends to subsequently find the cause and to anticipate with the appropriate actions. In addition, another useful tool for identification of potential risks is called 'vulnerability assessment'. Understanding and mapping the vulnerability of the food chains will help to unravel the complexity of food supply networks and international food trade and reduce the possibility of food fraud [296]. Further efforts are needed to stimulate gathering and sharing of information and the consistent use of the early recognition tools [297].

6.2 CLIMATE CHANGE

The widely debated global warming is predicted to affect the entire agricultural system [298]. The consequences of the climate change are difficult to forecast and are still widely debated, but in general it is believed that warming of the eco-system involves increasing prevalence of harmful micro-organisms which unavoidably results in increased toxin accumulation in crops [298-300]. It is important to raise the awareness to all stakeholders that the changes might implicate a switch of the population of mycotoxin-producing fungi over a wide range of geographical areas and in various types of crops. Recently, the aflatoxin B1 contamination in maize and wheat crops was predicted under a +2 °C climate change scenario applying a modelling approach. In the +2 °C scenario, which is currently considered as the most probable situation for the next 100 years, aflatoxin B1 is predicted to become an emerging food safety issue in maize grown in Europe, whereas the predicted impact on contamination in wheat was negligible. The aflatoxin risk maps could be used as an efficient management tool for aflatoxin control and intervention strategies. Further, a similar approach could be extended to other crops and mycotoxins to predict future trends and anticipate potential control options [301].

Moreover, there is the hypothesis that climate change might attribute to the spread of crop pests and pathogens. The global redistribution of species occurs mainly through human activities like transportation. Because organisms track the optimal temperature, latitudinal range shifts. However, to which extent global warming is involved in this process, is largely unknown. Therefore, Bebber *et al*. (2013) made an analysis of latitudinal shifts in species distributions based on observations of 612 crop pests and pathogen. This indicated that on a global level pests and pathogens are moving towards the poles. In addition, the global food security is threatened in case climate change allows an easier spread of these organisms [302]. This suggests that climate change can have profound impacts on damage to food crops and to contamination by pathogens in the future.

ENNs and BEAs producing *Fusarium* spp. might be prone to these global-warming driving spread since they are found on the field. It is known that populations of *Fusarium* fluctuate based on climate conditions. This is reflected in the scattered distribution of *Fusarium* species in cereals in Europe, where different Fusarium species are predominant in North and South Europe. It is expected that

warmer and more humid conditions are favorable for Fusarium head blight infections on grains. This make the hosts of these fungi vulnerability to the impact of global warming. This implies different strategies to overcome the risks associated with food production, storage and distribution in order to maintain food safety [303].

The changes in temperature and aquatic enrichment will affect the biodiversity and thus the bacterial landscape. Studying the adaptations of *Bacillus cereus* in the changing environment might be interesting since this group consists of both mesophilic and psychrotrophic strains [301]. Currently, psychrotrophic types are more common in colder areas, whereas mesophiles are more found in moderate regions. Considering the CER producing strains, mainly mesophilic strains of *B. cereus* have been identified. Later, also one psychrotrophic isolate of *B. cereus* was discovered that could produce the emetic toxin. However, it was not specified whether this one emetic psychrotrophic *B. cereus* strain was a *B. weihenstephanensis* strain [304]. For the psychrotropic strains, only limited information is available regarding environmental conditions affecting CER production. The role in food poisoning is still unknown but the discovery suggests the possibility that psychrotrophic isolates *B. cereus* and *B. weihenstephanensis* could grow in refrigerated fresh foods and produce CER [143, 304]. While for mesophilic types, studies indicated that storage below 10 °C will not lead to emetic intoxications, substantial toxin levels however may be obtained even when moderate temperature abuse (12-15 °C) for several days is allowed [119, 305]. But the issue of global warming will not have a direct impact on the CER in food since the production process of food at risk is usually done in a controlled environment, which is not directly influenced by the climate change. However, global warming may influence the ecology of microbiota on specific plants, soils and environments and could promote the prevalence of mesophilic strains. Moreover, the climatic changes could impact artisanal productions that are produced in less controlled environments and following less stringent food safety management systems. Recently, Kranzler *et al.* (2016) investigated the growth temperature range of emetic *B. cereus* and revealed that temperature primarily determines the regulation of CER synthesis on post-transcriptional levels. Also, the formation of the recently identified CER isoforms with different cytotoxic properties is highly temperature dependent [306]. In order to detect changes in the epidemiology of *B. cereus* foodborne poisonings, continuous surveillance is needed. Nevertheless, the implication of climate change regarding CER-producing strains of *B. cereus* is rather minor compared to the predicted shift in the mycotoxins population.

Aside from the presence of microbial toxins in food, the ongoing global climate change is likely to cause an increase in the prevalence of water-damage of homes, schools, worksites and public indoor locations in many parts of the world where temperature and rainfall are expected to increase. In turn, moisture and water accumulation provide ideal circumstances for the development of microbiological growth in buildings. Subsequently, this will result in a range of adverse health effects, especially since many people resulting in typically may spend 80 % or more of time indoors resulting in the development of various - often diffuse - symptoms and allergies [307, 308]. Research aiming to find connections between microbial exposure and disease development has given diverging results. The development and application of improved analytical methods for toxins will clarify the grounds of the reported epidemiological findings of measurable human exposure. Since for the vast majority of these toxins, identification and characterization information is absent or limited the assessment of their health significance is complex.

6.3 GROWING POPULATION

The last twelve years approximately one billion people have been added to the world reaching a current world population of nearly 7.6 billion. According to the United Nations Department of Economic and Social Affairs, the world population is expected to exceed 9 billion people by 2050 (Figure 6.1) [309]. The growing population challenges the need for global food security. The rising population requires not only maintaining the current level of food production but also an impressive increase in food production. The increasing food production to scope with the high demand for food might poses a risk for the quality of the food. Because producers are in search for higher yields, food production standards might be neglected or declined. Managing food safety requires implementing strategies at all stages of the food chain to keep our food free of harmful constituents. However, the problem is more complex than only providing safe food. Moreover, the changing consumption patterns and the increasing scarcity of water and land affect the ability to produce food. Particular challenging will be the preservation the environment and natural resources while increasing the food production to sustain the population [310].

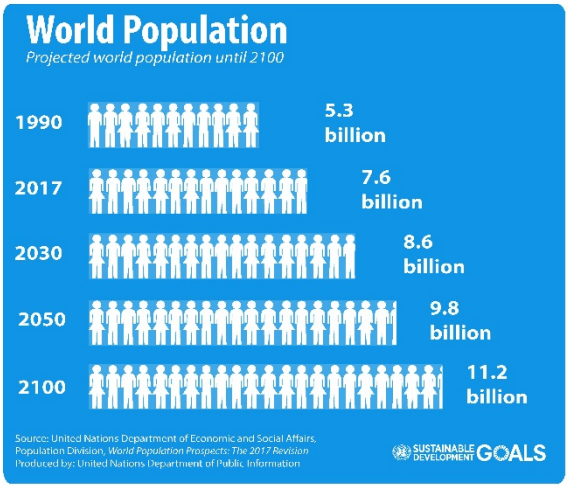

Figure 6.1 Projected world population until 2100 by the United Nations Department of Economic and Social Affairs.

6.4 IMPORTANCE OF (SUB)CHRONIC EXPOSURE TO LOW DOSES

Besides the acute toxicity, which is generally characterized by rapid onset and obvious toxic responses, possible effects due to chronic exposure at subclinical levels may be of great importance. Chronic toxicity is defined by low-dose exposure over a long time period, resulting in the so-called 'slow' and generally irreversible pathological conditions such as carcinogenic effects, immune suppression, mutagenicity, developmental toxicity, neurotoxicity and reproductive toxicity. Therefore, subtle exposure and chronic effects should be included in risk assessment and management. Because these effects are often overlooked, a more in-depth evaluation of the effects of multiple low-level exposures is required. Risk assessors are responsible to evaluate the question of how long it takes for a hazard to cause a toxic effect: acute (right away or within a few hours to a day), subchronic (weeks or months (for humans generally less than 10 % of their lifespan)) or chronic (a significant part of a lifetime or a lifetime (for humans at least seven years)) [311].

During hazard characterization, there is frequently a lack of dose-response data available for human subjects, especially for long-term effects. Even when human data are available, they often cover only a portion of the possible range of the dose-response analysis. This means that extrapolation must be done in order to extrapolate to lower dose levels than the range of data obtained from scientific studies. Moreover, doses that are used in animal studies are often at much higher doses than would be anticipated for humans. This involves a first extrapolation to lower doses and an additional

extrapolation from that animal species to humans, which introduces uncertainty into the dose-response relationship.

Regarding mycotoxins, the chronic effects have been studied in the case of aflatoxicosis, which results from the chronic exposure to low or moderate levels of aflatoxins. Chronic aflatoxicosis compromises immunity and is known to cause hepatocellular carcinoma, generally in association with hepatitis B virus [312]. So far, little is known about the potential chronic toxicity of BEA and ENNs. Nevertheless, several studies indicate a relatively high prevalence of ENN B in cereals products, which suggests an almost continuous exposure to low levels. In addition, ENN B has been found to be cytotoxic at low micromolar concentrations [65, 66]. As suggested by EFSA, there might be danger for humans and animals in chronic dietary exposure of ENNs and BEA and their impact should therefore be further investigated.

While it is known that CER is hazardous at high doses, uncertainties associated with low-dose exposure remain. In general, the relevance of the low doses is mainly underestimated since it does not involve the typical systemic symptoms of food poisoning. In contrast to the acute effects, the chronic symptoms easily go unnoticed because the involvement of the toxins is not recognized. In addition, it is hard to prove that cancer or other chronic conditions are caused by (myco)toxin exposure [313]. Chapter 5 illustrates the potential of CER to cause chronic toxicity due to repeated exposure, even at very low doses, which might go unnoticed. As postulated by Vangoitsenhoven *et al.* (2014) , low doses of CER might play a role in the pathophysiology of diabetes [152]. For most toxicological endpoints, a certain threshold below which no toxic effect occurs, is defined such as ADI and RfD. The levels are established after subchronic or chronic exposure during risk assessment. They represent the amount of a chemical to which a person can be exposed each day for a long time (usually lifetime) without suffering harmful effects [202]. The determination of these threshold levels might be useful with regard to CER. However, so far only limited *in vitro* results are available on chronic toxicity. Therefore, similar experiments should be conducted using *in vitro* bioassay and *in silico* methods.

Efforts should be taken to create awareness of the hidden danger of chronic exposure to low doses. Moreover, it is important to draw public attention to harmful consequences of improper handling and storage of food, especially in this era of prepackaged and take-away meals. Although it is difficult to investigate and follow up the activities in households, media campaigns can play a crucial role in

increasing the adoption of safe food handling practices. In the case of BEA and ENNs, it is even more difficult to create awareness as no acute mycotoxicoses have been reported. As a consequence, the danger of these mycotoxins is generally unknown to the public. Although it is yet hard to pin down the negative health effects of BEA and ENNs, there is sufficient evidence of their *in vitro* toxicity. EFSA's call already encouraged further research to enable risk assessment which can initiate regulations for these mycotoxins in food and feed [89].

6.5 MICROBIAL TOXINS: A HUMAN HEALTH THREAT

As indicated, an accurate assessment of the exposure to multiple microbial toxins (bacterial and fungal) via versatile vectors of food, environment and human activities is required to determine the level of threat posed by (re)emerging toxins to the public health. The latest report of EFSA and European Centre for Disease Prevention and Control (ECDC) on zoonoses, zoonotic agents and foodborne outbreaks revealed that about 19.5 % of the reported foodborne outbreaks in EU in 2015 were caused by bacterial toxins. This implies that the number of outbreaks caused by bacterial toxins in 2015 (n=825) was almost twice those reported in 2010 (n=453). All bacterial toxins were produced by *Bacillus* spp., *Clostridium* spp. and *Staphylococcus* spp. and were reported in all types of food. In all reported cases clinical concentrations were found resulting in an acute toxicity [225]. Currently, no information exists on the chronic exposure to sub-clinical concentrations with potentially (unknown) health consequences over a larger period.

Pre-requisite management of microbial toxins, which goes far beyond the management of microbes themselves, requires the establishment of safety levels in order to ensure adequate consumer protection. Special attention is needed when evaluating prevalence and exposure to more than one toxin at a time and over a period of time. Only limited information exists to evaluate the risk related to the exposure to multiple foodborne hazards. In the case of the combined exposure to multiple bacterial toxins, even less is known. Because for many of these pathogens the bacterial toxins are primarily virulence factors, further elaboration of this problem is needed.

Finally, this complete work offers a quantitative inventory and a strategy for toxicity evaluation of selected toxins. CER, BEA, ENNs (A, A1, B and B1) and MON were evaluated in natural mixtures taking into account several important parameters (season, pathogen presence, weather conditions).

Assessment of the toxicity of different toxins together (mixtures) as suggested throughout the present work is a step forward towards more precise evaluation of acute and chronic exposures.

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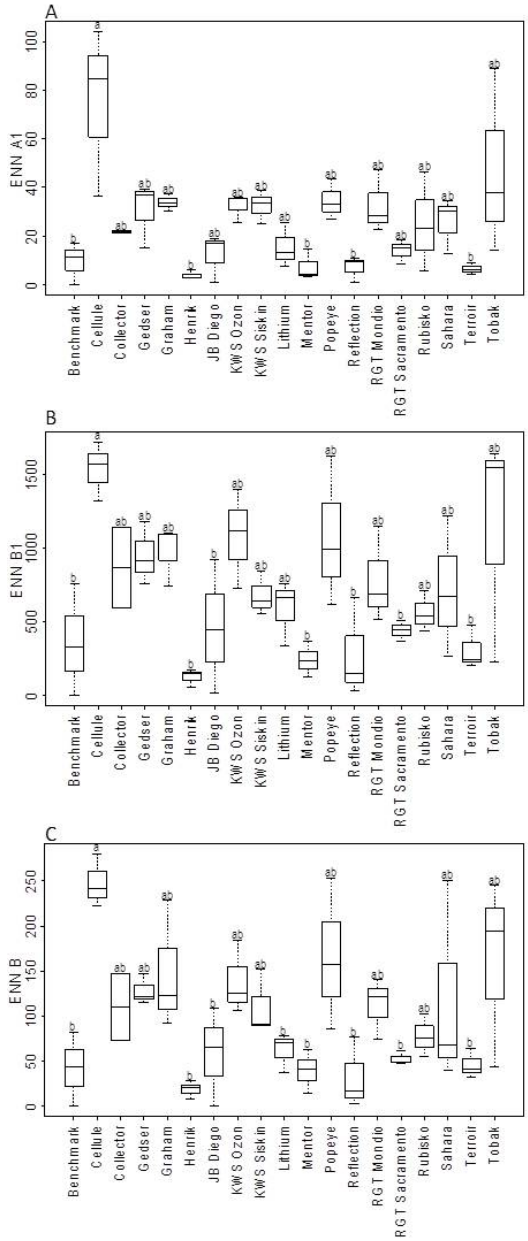
Supplementary File

Supplementary File

S.3.1 IMPACT OF WHEAT GENOTYPES ON MYCOTOXIN CONCENTRATIONS

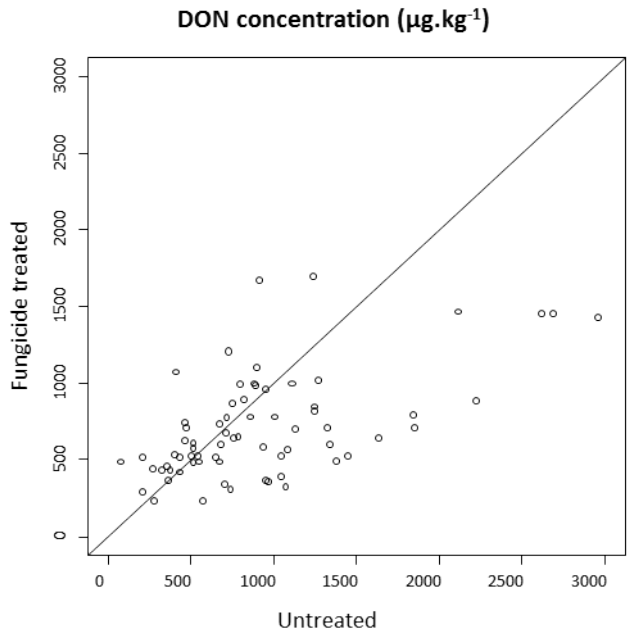
Figure S3.1 Concentration levels for ENN A1 (A), ENN B (B) and ENN B1 (C) during growing season 2015-2016 in 19 wheat genotypes.

Non-parametric Kruskal-Wallis test was used to test if there were significant differences between groups of data. In case there was a significant difference (*p*-value < 0.05), a pairwise comparison of the groups using a *post hoc* Dunn's test was performed. The letter codes distinguish groups for which significant differences were observed.



S.3.2 IMPACT OF FUNGICIDE TREATMENT ON DON CONCENTRATIONS

Figure S3.2 Scatterplot of DON levels in untreated plots (X-axis) and treated plots (Y-axis) which illustrated that the scatterplot diverges from the diagonal especially for DON levels higher than the European legislative limits.



This observation shows that at higher concentrations of DON, fungicides tend to lower the DON levels while being ineffective at lower DON levels.

Conclusions

Conclusions

Based on similar chemical structures and toxicity, the foodborne cyclic depsipeptides from fungal (BEA and ENNs) and bacterial (CER) origin have been examinated in this study. Accurate and specific methods for the detection are needed for the establishment of occurrence data, which is an important part for risk assessment. For this reason, a sensitive multi-mycotoxin LC-MS/MS method was developed and validated for simultaneous analysis of BEA, ENNs and CER in wheat, maize, rice and pasta. Although they are all classified as cyclic depsipeptides, this is the first method enabling their simultaneous quantitative detection. These sensitive (LOD ≤ 1.0 $\mu g.kg^{-1}$ and LOQ ≤ 2.9 $\mu g.kg^{-1}$) multi-toxin methods fit within the context of risk assessment of mixtures and risk assessment of chronic exposure to low doses. When these methods have been applied to different types of samples (maize, wheat, pasta and rice), no co-occurrence of BEA/ENNS with CER has been found. However, the setting in which these toxins are found is different. BEA and ENNs have been evidenced in these matrices by other authors while according to the occurrence data on CER, it is rather unlikely to find CER in these types of food. The reason is that the tested samples were dry, properly stored and uncooked in which the presence of CER is unlikely. Therefore, it might be worthwhile to assess the co-occurrence of CER with BEA and ENNs on other types of samples such as reheated, temperature-abused or take-away meals, which are more likely to contain CER.

The developed method has been applied to Belgian wheat samples from growing season 2014-2015 and 2015-2016 for a quantitative inventory of the mycotoxins BEA and ENNs. Occurrence data revealed vulnerability of wheat for the naturally occurring ENNs. A high variability in ENNs concentrations in Belgian wheat was observed during these two growing seasons. A significant increase in concentration levels was observed for ENN A1, ENN B, ENN B1 in growing season 2015-2016. In search for a possible explanation for the discrepancy between the growing seasons, the weather conditions (relative humidity, temperature and precipitation) were monitored. This difference resulted in higher *Fusarium* contamination levels as well as in high levels of BEA and ENNs, especially for ENN B in the samples harvested in 2016. However, this type of correlation study should be extended over a longer period covering more locations since so far only two years were studied on three locations. In both years, ENN B was the most prevalent mycotoxin occurring in 52 % of the wheat samples of growing season 2014-2015 and in 100 % of the samples of growing season 2015-2016. Although the method enables the simultaneous detection of CER, again none of the samples

were contaminated with the bacterial toxin CER. In addition, the samples from growing season 2015-2016 were related to the presence of *F. avenaceum*, *F. poae*, *F. graminearum* and *F. culmorum*. As expected, the presence of *F. poae* was positively correlated with BEA and the highest correlation for *F. avenaceum* was observed with the mycotoxins ENN B and ENN B1. Furthermore, all ENNs were positively correlated, especially for ENN A and its derivate ENN A1 and ENN B and ENN B1. Taking into account the regional and year-to-year variability as well as the remarkably high doses of ENN B, the collection of analytical data on occurrence of BEA and ENNs in relevant food and food commodities should continue in order to better asses the risk for humans and animals. Beside wheat, other cereals should be targeted for continuous monitoring. Based on the monitoring data collected, maximum levels for BEA and ENNs in certain food and feed have to be set and further risk management measures should be considered.

An additional LC-MS/MS method was developed for leek samples for the simultaneous detection of MON, BEA and ENNs after the observation that *F. avenaceum* was associated with basal rot of Belgian leek. The same approach was applied as for the development and validation of the LC-MS/MS methods in maize, wheat, rice and pasta. A reliable and sensitive method was successfully developed and applied to 76 leek samples that were artificially infected with *F. avenaceum*. The number of samples positive for the ENNs was low (≤ 13 %) and MON was only found in two samples at concentrations below the LOD. In the case of BEA, 20 % of the samples were positive and the highest concentration was 87 $\mu g.kg^{-1}$. Due to the low prevelance of the mycotoxins, it was not the possible to link the virulence of *F. avenaceum* and mycotoxin production.

Another aspect of foodborne toxins is the toxicity after chronic exposure. In the case of CER, emetic food poisoning after ingestion of contaminated food is associated with emesis, nausea and in severe cases with liver failure and even death. While acute effects have been studied in the aftermath of food poisoning, repeated exposure to low doses of CER might cause unnoticed damages to intestines and liver. Due to the increasing concern on the impact of repeated exposure to low doses of toxins, the effects of sub-emetic concentration of CER have been studied on Caco-2 and HepG2 cells using the Seahorse XF analyzer in addition to the cell viability (MTT/SRB) assays. After ten days of repeated exposure to 0.25 nM CER, measurable mitochondrial damage was observed in both cell lines which was mostly reflected in reduction of maximal cell respiration. These results corresponded with severe reduction in amount of cells and altered morphology observed by

microscopic examination of the cells. Seahorse XF Cell Mito Stress Test provided by the Seahorse XF technology allowed evaluation of the key parameters of mitochondrial functions by measuring the oxygen consumption rate during the sequential injection addition of different modulators of respiration that target components of the electron transport chain (ETC) in the mitochondria. This assay has proven to be a useful tool for the identification of mitochondrial toxicity of low doses because a reduction of maximal respiration could be observed even at low doses for which no signification effects were observed based on the MTT and SRB assays. The harmful effects towards liver and epithelial colon cells were observed at doses far below the concentrations associated with visual symptoms such as vomiting. The effects of CER were more pronounced in HepG2 cells suggesting that liver cell damage is slowly induced after repeated exposure to low exposure of CER. Although more data are required in order to evaluate the potential long-term risk, these experiments can serve as a starting point for further evaluation of other (mitochondrial) toxins as well as of the mixtures. Especially because even individually non-toxic concentrations might result in a significant toxicity when occurring as a chemical mixture.

The different aspects of this work fit very well within the context of the assessment of mixtures as well as towards chronic exposure.

Curriculum vitae

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EDUCATION

2011-2013	Master of Pharmaceutical Care, Ghent University - cum laude
Feb - Jun 2012	Erasmus Student Exchange program, Therapeutic Polymers Laboratory, Prince Felipe Research Centre, Valencia, Spain *Master thesis: Investigation of the mechanism of action of HPMA copolymer-DOX in MCF-7 cells. Promoters: Dr. M.J. Vicent & Prof. dr. S. De Smedt*
2008 - 2011	Bachelor of Pharmaceutical Sciences, Ghent University
2002 - 2008	Latin/Mathematics-Sciences, Sint-Pietersinstituut Ghent

TRAINING

Nov 2016	Introductory Course on Technology Transfer Skills
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Apr 2014	Medical and Scientific Writing Course

PROFESSONAL EXPERIENCE

Oct 2017 - Current	Production Engineer at Pfizer
Sept 2013 - Aug 2017	PhD position: Faculty of Pharmaceutical Sciences - Department of Bioanalysis & Faculty of Bioscience Engineering - Department of Food Safety and Food Quality, Ghent University *Promoters: Prof. dr. S. De Saeger & Prof. dr. A. Rajkovic* Additional tasks: - Scientific supervisor for bachelor's and master's theses - Communication tutor and mentor for bachelor's theses
Aug 2013	Pharmacist at COOP Apotheken, Ghent
2012 - 2013	26 weeks work placement in a public pharmacy *Mentor: Apr. Liesbet Vandermeersch, Kortrijksesteenweg 580, 9000 Ghent*

PUBLICATIONS

PUBLISHED

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PRESENTATIONS AT (INTER)NATIONAL CONFERENCES

POSTER PRESENTATIONS

Decleer M., Vakula A. & Rajkovic A. (2017). Influence of cereulide on the mitochondrial function of Caco-2 and HepG2 cells using extracellular flux analysis. 7th Congress of European Microbiologists (FEMS 2017) (July 2017), Valencia, Spain.

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A1, B, B1) and cereulide in maize, wheat, rice and pasta. Food Micro 2016 (July 2016), Dublin, Ireland. (Poster Prize).

Decleer M., Rajkovic A., Sas B., Madder A. & De Saeger S. (2016). Development of LC-MS/MS methods for the simultaneous determination of beauvericin, enniatins (A, A1, B, B1) and cereulide in maize, wheat, rice and pasta. 38th Mycotoxin Workshop (May 2016), Berlin, Germany.

Decleer M., Rajkovic A., Sas B., Madder A. & De Saeger S. (2016). Development and validation of an LC-MS/MS method for beauvericin, enniatins (A, A1, B, B1) and cereulide in wheat, pasta and rice. National Symposium on Applied Biological Sciences (Feb. 2016), Antwerp, Belgium.

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Decleer M., Rajkovic A., Sas B., Madder A. & De Saeger S. (2014). Development of and LC-MS/MS method for the simultaneous determination of beauvericin, enniatins (A, A1, B, B2) and cereulide. 36th Mycotoxin Workshop (June 2014), Göttingen, Germany.

ORAL PRESENTATIONS

Decleer M. & Rajkovic A. (2017). Unravelling the impact of the bacterial depsipeptide cereulide on the mitochondrial function of Caco-2 and HepG2 cells. International Union of Microbiological Societies IUMS (July 2017), Singapore.

Decleer M., Audenaert K., Rajkovic A. & De Saeger S. (2017). Emerging *Fusarium* mycotoxins in Belgian leek and wheat. MYTOX Happening (May 2017), Ghent, Belgium.

Decleer M. & Rajkovic A. (2017) Impact of chronic exposure of low concentrations of microbial depsipeptide cereulide on mitochondrial disruptions in Caco-2. IAFP's European Symposium (March 2017), Brussels, Belgium.

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