"IULIU HAŢIEGANU" UNIVERSITY OF MEDICINE AND PHARMACY CLUJ-NAPOCA
THE DOCTORAL SCHOOL

UNIVERSITAT DE VALÈNCIA
PROGRAMA DE DOCTORAT AMB MENCIÓ CAP A L'EXCEL•LENCIA EN CIÈNCIES DE L'ALIMENTACIÓ

International co-supervised PhD THESIS

Mycotoxin contamination of grains and grain derivatives in Romania

PhD Student **Oana-Maria Stanciu**

Scientific supervisors **Prof. Felicia Loghin, PhD**
Prof. Jordi Mañes Vinuesa, Dr.HC

September, 2017

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Prof. Felicia Loghin, PhD, Professor in the Department of Toxicology (Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania) and **Prof. Jordi Mañes Vinuesa**, **Dr.HC,** Professor in the Department of Nutrition and Bromatology (Faculty of Pharmacy, University of Valencia, Spain),

CERTIFY THAT:

Ms. Oana-Maria Stanciu, graduated in Pharmacy, has fully completed the work in the doctoral thesis entitled **Mycotoxin contamination of grains and grain derivatives in Romania *(Contaminarea cerealelor şi derivatelor de cereale cu micotoxine în România / Contaminación de los cereales y los derivados de cereales por micotoxinas en Rumania),*** and authorize its public defense in order to obtain the Doctoral degree at the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca and University of Valencia.

And for this, the present certificate was signed.

Cluj-Napoca, Romania — Valencia, Spain

September, 2017

Loghin F., PhD — Mañes J., Dr.HC

"Work is the law of the modern world, which has no place for lazy people."
Mihai Eminescu
(Romanian poet, 1850-1889)

"Unfortunately, nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity."
Santiago Ramón y Cajal
(Spanish pathologist, Nobel laureate, 1852-1934)

"After all, science is essentially international, and it is only through lack of the historical sense that national qualities have been attributed to it."
Marie Skłodowska Curie
(French-Polish physicist, Double Nobel laureate, 1867-1934)

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LIST OF PUBLICATIONS

Articles published *in extenso* as a result of the doctoral research

1. **Stanciu O**, Banc R, Cozma A, Filip L, Miere D, Mañes J, Loghin F. Occurence of *Fusarium* mycotoxins in wheat from Europe. Acta Universitatis Cibiniensis. Series E: Food Technology. 2015;19(1):35-60. *BDI (publication included in the Review of the literature).*

2. **Stanciu O**, Juan C, Miere D, Loghin F, Mañes J. Occurrence and co-occurrence of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania. Food Control. 2017;73:147-155. *ISI - Impact Factor 3.496 (publication included in Study 1).*

3. **Stanciu O**, Juan C, Miere D, Dumitrescu A, Bodoki E, Loghin F, Mañes J. Climatic conditions influence emerging mycotoxin presence in wheat grown in Romania – A 2-year survey. Crop Protection. 2017; 100:124-133. *ISI - Impact Factor 1.834 (publication included in Study 2).*

4. **Stanciu O**, Juan C, Miere D, Loghin F, Mañes J. Presence of enniatins and beauvericin in Romanian wheat samples: from raw material to products for direct human consumption. Toxins (Basel). 2017;9:189. *ISI - Impact Factor 3.03 (publication included in Study 3 and Study 5).*

5. **Stanciu O**, Juan C, Miere D, Berrada H, Loghin F, Mañes J. Study on trichothecene and zearalenone presence in Romanian whet relative to weather conditions during 2015. Food Control. 2017. Under review. *ISI - Impact Factor 3.496 (publication included in Study 4).*

6. **Stanciu O**, Juan C, Miere D, Loghin F, Mañes J. Analysis of enniatins and beauvericin by LC-MS/MS in wheat-based products. Cyta J Food. 2017; 15(3):433-440. *ISI - Impact Factor 1.18 (publication included in Study 5).*

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ABBREVIATIONS USED IN THE TEXT

3AcDON	3-Acetyldeoxynivalenol
15AcDON	15-Acetyldeoxynivalenol
ADI	Acceptable Daily Intake
AFLAs	Aflatoxins
ASE	Accelerated Solvent Extraction
bw	Body Weight
BEA	Beauvericin
BSA	N,O-bis(trimethylsilyl)acetamide
C_{18}	Octadecylsilica
CAC	Codex Alimentarius Commission
CE	Collision Energy
CEP	Collision Cell Entrance Potential
CHO-K1	Chinase Hamster Ovary Cells
CONTAM	European Food Safety Authority Panel on Contaminants in Food Chain
CXP	Collision Cell Exit Potential
CYP	Cytochrome P450
d-SPE	Dispersive Solid-Phase Extraction
DAS	Diacetoxyscirpenol
DLLME	Dispersive Liquid-Liquid Micro-Extraction
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
DP	Declustering Potential
Dt	Dwell Time
EC	European Commission
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ENA	Enniatin A
ENA1	Enniatin A1
ENB	Enniatin B

ENB1	Enniatin B1
ENs	Enniatins
EP	Entrance Potential
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Statistic Division of Food and Agriculture Organization of the United Nations
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
FID	Flame Ionization Detector
FUMOs	Fumonisins
FP	Fusaproliferin
FUS-X	Fusarenon-X
GAC	Green Analytical Chemistry
GAP	Good Agricultural Practice
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled with Mass Spectrometry
GC-MS/MS	Gas Chromatography coupled to tandem Mass Spectrometry
GC-QqQ-MS/MS	Gas Chromatography coupled with Triple Quadrupole Mass Spectrometry
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points
HepG2	Liver Hepatocellular Carcinoma Cells
HPLC	High Performance Liquid Chromatography
HPLC-MS/MS	High Performance Liquid Chromatography tandem Mass Spectrometry
HRMS	High Resolution Mass Spectrometry
HT-2	HT-2 toxin
IAC	Immunoaffinity Column
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee in Food Additives
LB	Lower Bound
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LC-QqQ-MS/MS	Liquid Chromatography coupled with Triple Quadrupole Mass Spectrometry

LD	Limit of Detection
LQ	Limit of Quantification
mRNA	Messenger Ribonucleic Acid
MAE	Microwave-Assisted Extraction
MAPK	Mitogen Activated Protein Kinase
ME	Matrix Effect
MIP	Molecularly Imprinted Polymer
ML	Maximum Level
MON	Moniliformin
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSPD	Matrix Solid Phase Dispersion
NCR	National Research Council
NEO	Neosolaniol
NIV	Nivalenol
NOAEL	No Observed Adverse Effect Level
OTA	Ochratoxin A
PC1	First Principal Component
PC2	Second Principal Component
PCA	Principal Component Analysis
PMTDI	Provisional Maximum Tolerable Daily Intake
PTFE	Polytetrafluoroethyl
QqQ	Triple Quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RASFF	Rapid Alert System for Food and Feed
RNA	Ribonucleic Acid
RSD	Relative Standard Deviation
S/N	Signal-to-Noise
SCF	Scientific Committee on Food
SFE	Supercritical Fluid Extraction
SLE	Solid-Liquid Extraction
SPE	Solid-Phase Extraction
SPME	Solid-Phase Micro-Extraction
SRM	Selected Reaction Monitoring
T-2	T-2 toxin
TDI	Tolerable Daily Intake
TLC	Thin Layer Chromatography
TMCS	Trimethylchlorosilane
TMSI	N-trimethylsilyimidazole
UB	Upper Bound
WHO	World Health Organization

ZEA Zearalenone

ABSTRACT

Mycotoxins are among the most frequent contaminants of wheat and wheat-based products. The most important mycotoxins in wheat are mainly *Fusarium* toxins, such as deoxynivalenol (DON), zearalenone (ZEA), nivalenol, HT-2 toxin (HT-2), T-2 toxin, and, recently studied, the emerging mycotoxins, particularly enniatins (ENs) and beauvericin. Agriculture and especially wheat cultivation are important parts of the Romanian economy. Despite its high producer and consumer potential, only sporadic data concerning mycotoxin presence in wheat foodstuffs in Romania is registered.

The aim of this thesis was to survey the natural presence of both regulated and unregulated *Fusarium* mycotoxins in wheat and wheat-based products from Romania, the research being divided into four blocks: validation of analytical methods; surveillance of natural occurrence of *Fusarium* mycotoxins in wheat and wheat-based products from Romania; influence of various factors on mycotoxin production; risk assessment studies for the Romanian population.

In this thesis, different multi-component qualitative and quantitative analytical methods have been validated in order to investigate the occurrence of various *Fusarium* mycotoxins, including old discovered mycotoxins, but also emerging mycotoxins, in wheat and wheat-based food commodities. Sample preparation consisted particularly in solid-liquid extractions, presenting the advantage of being rapid, and user-friendly. Analysis methods were different depending on the mycotoxins evaluated, liquid or gas chromatography coupled to tandem mass spectrometry, both presenting satisfactory results for linearity, sensitivity, accuracy, matrix effect and repeatability.

Concerning natural mycotoxin occurrence in Romanian wheat and wheat-based products, DON and ENs showed the highest frequencies. With respect to the legislated mycotoxins, DON was the most detected in Romanian wheat (60/102, 59%), and wheat-based products (114/181, 63%). The highest frequency of DON was observed for pasta (34/40, 85%), whereas the highest levels of DON (mean value of 353 μg kg^{-1}) were found for wheat flour. From the emerging mycotoxins, ENB was the most

prevalent in both wheat samples (55/133, 41%), and wheat-based products (35/111, 32%).

With respect to the correlation between *Fusarium* mycotoxin levels in Romanian wheat and weather conditions during the grain-growing season, a complex 2-year survey was presented. For the emerging mycotoxins, after monitoring three different counties, using univariate data analysis and multivariate approach, the results indicated that high precipitation (with at least 20 mm more than long-term normal monthly precipitation) during the months of May and July promote high moisture in wheat kernels, increasing EN occurrence, co-occurrence and mean concentrations, particularly ENA1, ENB, and ENB1. Regarding the other *Fusarium* mycotoxins, the results suggested that a prolonged rainy weather during earing phase, anthesis, dough formation and filling (months of May and June) influence fungi development and mycotoxin production, particularly DON, HT-2 and ZEA.

Comparing the occurrence data on emerging mycotoxins in Romanian organic and conventional wheat, higher values for incidences, mean levels, and number of mycotoxins found simultaneously were registered for organic wheat samples, but, interestingly, the maximum values found corresponded to conventional wheat samples. Concerning the influence of the wheat cultivar on emerging mycotoxin presence, the highest means and levels were for Arieșan, Balaton, and Izvor varieties.

Finnaly, the estimated daily intake (EDI) of *Fusarium* mycotoxins through wheat-based product consumption by the Romanian adult population was calculated. The highest EDI values were registered for the sum of DON, 3AcDON and 15 AcDON (689.6 ng kg^{-1} bw day^{-1} at an overestimation), even so being lower than the tolerable daily intake established by the European regulations.

REZUMAT

Micotoxinele sunt una dintre cele mai frecvente categorii de contaminanţi ai grâului şi produselor pe bază de grâu. Cele mai importante micotoxine din grâu sunt în principal produse de specii ale genului *Fusarium*, incluzând deoxinivalenolul (DON), zearalenona (ZEA), nivalenolul, toxina HT-2 (HT-2), toxina T-2, şi, mai recent studiate, micotoxinele emergente, în special eniatinele (ENs) şi beauvericina. Agricultura şi cu precădere cultivarea grâului sunt ramuri importante ale economiei românești. În ciuda potenţialului ridicat de producţie şi consum de grâu al României, sunt înregistrate doar date sporadice referitoare la prezenţa micotoxinelor în produsele alimentare pe bază de grâu din România.

Scopul acestei teze a fost acela de a monitoriza prezenţa naturală atât a micotoxinelor legiferate, cât şi a celor nelegiferate, produse de specii ale genului *Fusarium* în grâu şi produse pe bază de grâu din România, cercetarea fiind împărţită în patru direcţii: validarea metodelor analitice; studierea apariţiei naturale a micotoxinelor genului *Fusarium* în grâu şi produse pe bază de grâu din România; evaluarea influenţei diverşilor factori asupra producţiei micotoxinelor; evaluarea riscului legat de prezenţa micotoxinelor în lanţul alimentar pentru populaţia din România.

În această teză, diverse metode analitice multi-component au fost validate pentru a evalua calitativ şi cantitativ apariţia diferitelor micotoxine produse de specii ale genului *Fusarium* în grâu şi produse alimentare pe bază de grâu, incluzând atât micotoxinele clasice, cât şi emergente. În general, pregătirea probelor a constat în extracţie solid-lichid, aceasta prezentând avantajul de a fi rapidă şi uşor de utilizat. Metodele de analiză au variat în funcţie de micotoxinele evaluate, fiind folosite cromatografia de lichide sau cea de gaze cuplate cu spectrometria de masă în tandem, ambele cu rezultate satisfăcătoare corespunzătare parametrilor liniarităţii, sensibilităţii, preciziei, efectului de matrice şi repetabilităţii.

În ceea ce priveşte prezenţa naturală a micotoxinelor în grâul românesc şi produsele pe bază de grâu, DON şi ENs au înregistrat cele mai mari incidenţe. Referitor la micotoxinele legiferate, DON a fost cel mai frecvent detectat în grâul românesc

(60/102, 59%), şi produsele pe bază de grâu (114/181, 63%). Cea mai mare incidenţă a DON a fost observată în pastele făinoase (34/40, 85%), iar cele mai ridicate nivele de DON au fost găsite în făina de grâu (valoare medie de 353 μg kg^{-1}). Dintre micotoxinele emergente, ENB a fost cea mai frecvent detectată în probele de grâu (55/133, 41%), dar şi în produsele pe bază de grâu (35/111, 32%).

În privinţa corelării între nivelele de micotoxine din grâu şi condiţiile meteorologice din România în timpul sezonului de cultivare a cerealelor, a fost prezentat un studiu complex desfăşurat pe durata a 2 ani. Pentru micotoxinele emergente, după monitorizarea a trei judeţe diferite şi folosind analiza univariată a datelor, dar şi o abordare cu variabile multiple, rezultatele au indicat că precipitaţiile ridicate (cu cel puţin 20 mm mai mult decât precipitaţii lunare normale pe termen lung) în lunile mai şi iulie promovează o umiditate ridicată a boabelor de grâu, favorizând dezvoltarea, apariţia concomitentă şi concentraţii ridicate de micotoxine emergente, în special ENA1, ENB, şi ENB1. Referitor la celelalte micotoxine incluse în studiu, rezultatele au sugerat că o vreme ploioasă prelungită în timpul fazei de înspicare, înflorire, formare a bobului şi de umplere (lunile mai şi iunie) influenţează dezvoltarea mucegaiurilor şi producerea de micotoxine, în special DON, HT-2 şi ZEA.

Comparând datele privind apariţia micotoxinelor emergente în culturile de grâu organic şi convenţional din România, au fost înregistrate valori mai mari ale incidenţelor, nivelelor medii şi numărului de micotoxine detectate simultan pentru probele de grâu organic, dar, în mod interesant, valorile maxime constatate au corespuns unor probe de grâu cultivate în mod convenţional. Legat de influenţa soiului de grâu asupra prezenţei micotoxinelor emergente, cele mai mari nivele de micotoxine au fost înregistrate pentru soiurile Arieşan, Balaton şi Izvor.

În final, a fost calculat aportul zilnic estimat (EDI) de micotoxine ale genului *Fusarium* adus prin intermediul consumului de produse pe bază de grâu de către populaţia adultă din România. Cele mai mari valori ale EDI au fost înregistrate pentru suma dintre DON, 3AcDON şi 15 AcDON (689,6 ng kg^{-1} corp zi^{-1} la supraestimare), cu toate acestea valoarea fiind mai mică decât doza zilnică tolerabilă stabilită de reglementările europene.

RESUMEN

Las micotoxinas son los contaminantes más frecuentes en el trigo y los productos a base de trigo. Las micotoxinas más importantes encontradas en trigo son principalmente las toxinas producidas por el género *Fusarium*, como deoxinivalenol (DON), zearalenona (ZEA), nivalenol, toxina HT-2 (HT-2), toxina T-2, y, recientemente estudiadas, las micotoxinas emergentes, en particular eniatinas (ENs) y beauvericina. La agricultura y en especial el cultivo de trigo son partes importantes de la economía rumana. A pesar de su alto potencial productor y consumidor, Rumania tiene registrados pocos datos relativos a la presencia de micotoxinas en los productos alimenticios a base de trigo.

El objetivo de esta tesis fue estudiar la presencia natural de micotoxinas legisladas y no legisladas, producidas por el género *Fusarium* en trigo y los productos a base de trigo de Rumania. La investigación se ha dividido en cuatro bloques: la validación de los métodos analíticos; la vigilancia de copresencia natural de micotoxinas de *Fusarium* en trigo y productos de trigo de Rumania; la influencia de varios factores en la producción de micotoxinas; y el estudio de evaluación del riesgo para la población rumana.

En la presente tesis, diferentes métodos analíticos multi micotoxinas han sido validados con el fin de investigar la presencia de micotoxinas de *Fusarium*, incluyendo también micotoxinas emergentes, en el trigo y derivados destinados a la alimentación humana. En general, la preparación de la muestra consistió en una extracción sólido-líquido, presentando la ventaja de ser rápida y fácil de manejar. Los métodos de análisis varían dependiendo de las micotoxinas evaluadas; la cromatografía de líquidos o de gases acoplada a espectrometría de masas en tándem se han utilizado, mostrando resultados satisfactorios para la linealidad, la sensibilidad, la precisión, el efecto matriz y la repetibilidad del método.

En cuanto a la presencia natural de micotoxinas en el trigo y los productos a base de trigo de Rumania, el DON y las ENs mostraron las frecuencias más elevadas. Respecto a las micotoxinas legisladas, el DON en el trigo (60/102, 59%), y los derivados (114/181, 63%) es la micotoxina mas prevalente; concretamente en pasta

(34/40, 85%) y en la harina de trigo (el valor medio más elevado, 353 μg kg^{-1}). Entre las micotoxinas emergentes, la ENB fue la micotoxina más prevalente en trigo (55/133, 41%) y productos a base de trigo (35/111, 32%).

Con respecto a la correlación entre los niveles de micotoxinas de *Fusarium* en trigo de Rumania y las condiciones climáticas durante la temporada de cultivo de cereales, se presentó un estudio de 2 años. Para las micotoxinas emergentes, tras la monitorización de tres regiones diferentes, el uso de datos de análisis estadístico univariante y multivariante, se observó que las precipitaciones elevadas (con al menos 20 mm más de precipitación que la media mensual normal a largo plazo) durante los meses de mayo y julio producen una alta humedad en los granos de trigo, aumentando la presencia, co-presencia y concentraciones de ENs, particularmente ENA1, ENB, y ENB1. En cuanto a las otras micotoxinas de *Fusarium*, los resultados sugirieron que un tiempo lluvioso prolongado durante la floración, y la formación del grano, hasta la cosecha (meses de mayo y junio) favorece el desarrollo de hongos y la producción de micotoxinas, en particular DON, HT-2 y ZEA.

Las incidencias más altas, los niveles medios mayores, y el número de micotoxinas encontrado simultáneamente se registraron en muestras de trigo orgánico, pero, curiosamente, los valores máximos encontrados correspondieron a unas muestras de trigo convencional. En cuanto a la influencia de la variedad de trigo en la presencia de micotoxinas emergentes, los niveles más altos correspondieron a las variedades Arieşan, Balaton, e Izvor.

Finalmente, se calculó la ingesta diaria estimada de micotoxinas de *Fusarium* a través del consumo de productos a base de trigo para la población rumana. La suma de DON, 3AcDON y 15 AcDON alcanzo valores de 689.6 ng/kg pc/día, pero aún así estos valores fueron inferiores a la ingesta diaria tolerable establecida por las normas europeas.

INTRODUCTION

Grains can be contaminated by different types of contaminants including: seeds of weeds and other crops, infected grains (e.g. ergot, moldy), defected grains, tainting materials, agricultural chemicals, foreign materials, insects or animals [1]. Mycotoxins are natural contaminants of grains, known as secondary metabolites produced by fungi, mostly belonging to the *Aspergillus*, *Penicillum* and *Fusarium* genus [2]. Wheat, a member of the cereal family, is unique among the food grains, due to its composition and its major contribution at the daily human diet and human health. Wheat has many food usage forms, starting with the wheat flour and bran, and continuing with bread, breakfast cereals, noodles, pasta, pastry, donuts, cakes, biscuits and baby foods [3]. The most important mycotoxins in wheat are mainly *Fusarium* toxins, such as deoxynivalenol (DON), zearalenone (ZEA), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2) and, recently studied, the emerging mycotoxins, particularly enniatins (ENs) [4].

The formation of mycotoxins in wheat seeds and the transfer through the trophic chain to humans is stimulated by several factors. These factors of influence are related to the field mycotic flora (e.g. soil tillage, fertilizers, fungicides, weather parameters, crop rotations, culture placement, variety) and to the storage houses mycotic flora (e.g. seeds and rough material storage, temperature, moisture, duration, preservation) [5]. Due to the high demand of organic products in the past years and the climate changes registered all over the world, agriculture practices and climate are considered the most important factors of influence on mycotoxin development in wheat during the preharvest period [6–8].

Mycotoxins are of public health concern due to their worldwide prevalence that may lead to negative effects, notably in chronic exposure [9]. Humans are exposed to mycotoxins by oral, inhalation and dermal routes, the oral pathway being predominant [10]. Generally, *Fusarium* mycotoxins possess carcinogenic, cytotoxic, neurotoxic, immunosuppressive, estrogenic, teratogenic, hepatotoxic, and nephrotoxic properties, proved by *in vitro* or *in vivo* studies [11–14].

In the context of food safety, to protect the consumer from the harmful effects of these toxins, regulations for those mycotoxins with high biological implications have been established in more than 100 countries [4,15]. The European Union (EU) harmonized the regulations, maximum levels (ML) permitted in foodstuffs and tolerable daily intake (TDI) values or provisional maximum tolerable daily intake (PMTDI) values being set. In order to evaluate the risk, exposure of the population to mycotoxins is compared with the correspondant legislated values [16]. As simultaneous mycotoxin presence is the most frequent type of contamination, the multi-mycotoxin analyses have become the most popular and the most used in the recent years [17–20].

Despite Romania's high potential for wheat production [21], data on the occurrence of both legislated and non-legislated *Fusarium* mycotoxins in wheat and wheat by-products from Romania are quite limited [22]. Concerning the factors of influence on the presence of mycotoxins in wheat harvested in Romania, only the impact of climate conditions and type of agriculture on DON occurrence was investigated. Moreover, no comprehensive study concerning the exposure of the Romanian population to mycotoxins was published until now. All these aspects underline the interest of investigating *Fusarium* mycotoxins in unprocessed wheat and wheat products from Romania.

Starting with these remarks, the present work presents a complex approach of the occurrence of *Fusarium* mycotoxins, including trichothecenes, ZEA and emerging mycotoxins, in wheat harvested in Romania and wheat products commercialized in this country. In the first step, the influence of different factors (geographic position, weather parameters, type of agriculture, wheat cultivar) on mycotoxin incidence in wheat was evaluated. Then, the presence of mycotoxins in a wide range of wheat-based foodstuffs was assessed, and finally the exposure of the Romanian population to mycotoxins was estimated.

REVIEW OF THE LITERATURE

1. *Fusarium* mycotoxins as wheat contaminants

Cereal-based foodstuffs are by far the major source of food, energy, protein, vitamin B, and minerals for the current world population. First, cereal grains are considered as caloric or starchy foods and, more recently, foods from whole grains are considered a rich source of dietary fiber. Commonly, cereals are milled and processed, and then refined milled products are manufactured [23]. Cereals and cereal-based products are associated with health-promoting effects and they have an important role in human nutrition, being included in all food based dietary guidelines [24]. On the other hand, cereals are exposed to various biotic and abiotic stress factors, from cultivation and throughout their life cycle to processing. Consequently, whole grain cereals and cereal-based foodstuffs may also contribute with anti-nutrients, contaminants or toxic compounds to the total human daily diet [25]. Among the most important risks linked with cereal consumption are mycotoxins, heavy metals, pesticides residues, and alkaloids [4].

1.1. Wheat

According to the Food and Agriculture Organization of the United Nations (FAO), rice, maize, and wheat are staple foods for world population, wheat (*Triticum aestivum* L.) being the main strategic crop worldwide [26,27].

1.1.1. Wheat production

Recent global data reports for wheat a total area harvested of 224.7 million hectares and an annual production around 734 million metric tones. The EU is the world's largest wheat producer, last data of the Statistic Division of FAO (FAOSTAT) reporting a total area harvested of 26.71 million hectares cultivated and an annual production around 157.4 million metric tones [28].

Romania is the largest country in the southeastern Europe, with an agricultural land cover of 62% [29]. In Romania, wheat has a special contribution to traditional agriculture. Romania is the fifth biggest producer of wheat in the EU, after France, Germany, Poland, and Spain, with an annual production between 5 and 8 millions of metric tones in the last years [30], more than half of it being exported to different countries around the world. The evolution of wheat production and area for cultivation and harvest of wheat in Romania during the last five years is presented in Fig. 1. The

main production area for wheat is the Danube plane in the South of the country. Other important wheat growing areas are Transylvania, the northern part of Moldova (in the northeastern part of Romania), and the Banat region in the South-West [31].

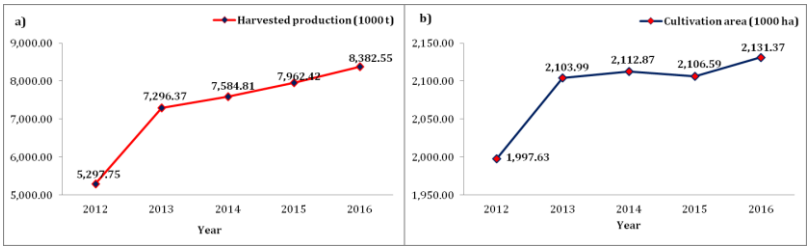

Fig. 1. **a)** Wheat and spelt harvested production (1000 t) in Romania during 2012-2016. **b)** Wheat and spelt cultivated area (1000 ha) in Romania during 2012-2016 [32].

1.1.2. Wheat consumption

For Romanian population, a high consumption of wheat and wheat products has been recorded (133.09 kg/capita/year), more than the European annual average (102.86 kg/capita/year) and the double of global average (65.26 kg/capita/year). Moreover, Romania is the the third highest consumer of wheat and wheat products (Fig. 2), after Italy and Malta [33].

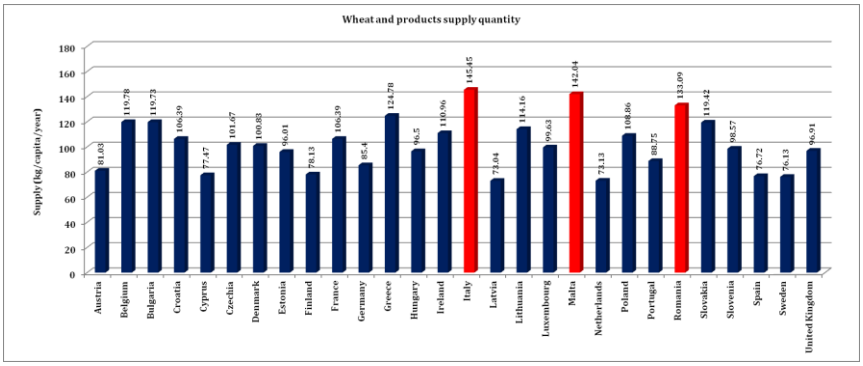

Fig. 2. Consumption of wheat and products in the European Union [33].

1.2. Classification of *Fusarium* mycotoxins

The term "mycotoxins" defines secondary fungal metabolites (metabolites not essential to the normal growth and reproduction of the fungus) that cause biochemical, physiologic, and/or pathologic changes in other species, including vertebrates, other animal groups, plants, and other microbes [2]. Even if more than 400 compounds have been classified as mycotoxins, only approximately 50 have been studied in detail [34]. The class of mycotoxins constitutes a heterogeneous class from toxicological and chemical points of view. The most common classification of mycotoxins is according to the origin genera. The major mycotoxin-producing fungal genera are *Fusarium*,

Aspergillus, and *Penicillium*, but mycotoxins can also be produced by *Cladosporium*, *Claviceps*, *Alternaria* and *Helminthosporium* genera [10,35]. The most important mycotoxins in wheat are mainly *Fusarium* mycoxotins [4].

Fusarium genera is widespread, both in soil and organic substrates, it contains over 70 phytopathogenic species, occurring in natural conditions in different regions of the world and affecting mainly cereals, other vegetables and fruits [36]. *Fusarium* spp produce three of the most important classes of mycotoxins with respect to animal and human health: fumonisins (FUMOs), ZEA and trichothecenes. *Fusarium* genera also produce emerging mycotoxins, such as fusaproliferin (FP), beauvericin (BEA), ENs and moniliformin (MON), or fusaric acid, fusarin A-D, gliotoxin, butenolite which are recently discovered and less studied [2,14]. Modified mycotoxins represent another emerging topic. Plant metabolites have been identified so far for DON, NIV, fusarenon-X (FUS-X), T-2, HT-2, ZEA, fusaric acid, and FUMOs, particularly in wheat and other cereal commodities [37,38]. The most common examples related to modified mycotoxins, the acetylated derivatives of DON, 3-acetyldeoxynivalenol (3AcDON) and 15-acetyldeoxynivalenol (15AcDON), are frequently detected in DON contaminated grains [39].

An important aspect for *Fusarium* genera is that the same mycotoxin can be produced by different *Fusarium* spp and one fungus can produce various mycotoxins at the same time (Table I), so in the same substrate more than one metabolite can be found [40,41]. This aspect is a particular issue in the context of possible synergistic, additive or antagonistic effects of mycotoxins [42–44].

Table I. Species of *Fusarium* infecting wheat and selected mycotoxins produced [45,46].

SPECIES	MYCOTOXINS
F. acuminatum	enniatins, moniliformin, beauvericin
F. avenaceum	moniliformin, beauvericin
F. cerealis	nivalenol, fusarenone, zearalenone
F. culmorum	deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenon-X, moniliformin, zearalenone
F. equiseti	fusarochromanone, zearalenone, diacetoxyscirpenol, beauvericin
F. graminearum	deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenon-X, zearalenone
F. oxysporum	moniliformin, fusaric acid
F. poae	T-2 toxin, HT-2-toxin, nivalenol, diacetoxyscirpenol, fusarenon-X
F. proliferatum	fumonisins, moniliformin, beauvericin, enniatins, fusarin C
F. sporotrichioides	T-2 toxin, HT-2 toxin, neosolaniol, diacetoxyscirpenol, fusarenon-X, enniatins, beauvericin, moniliformin, zearalenone
F. tricinctum	moniliformin
F. verticillioides	fumonisins, moniliformin, fusarin C

1.3. Mycotoxins in the food chain

Mycotoxins commonly enter the food chain through contaminated food and feed crops, mainly cereals. FAO estimated that approximately 25% of cereals produced in

the world are contaminated by mycotoxins, but perhaps this value is closer to 50%, if one takes into account emerging mycotoxins, studied intensively only in the last five years. Even if mycotoxins are specific contaminants for cereals, they can be ingested by humans consuming various foodstuffs. From cereals, mycotoxins can be transferred directly to food products with vegetal origin, or they can be found in food products with animal origin, cereals being used as feed for animals that are vectors for mycotoxins [22].

The accumulation of mycotoxins in foods represents a major threat for human health as they are responsible for various toxicities, and also it has a high economic significance [47]. The presence of mycotoxins in the food chain is considered a human public health issue [9]. Acute exposure to high levels of mycotoxins is not very common, but the adverse effects in chronic exposure continue to attract worldwide attention because of their impact on human health [48].

The impacts and the risks associated with the presence of mycotoxins in the food chain are specific for each level: plants (costs of quality control and monitoring), animals (animal health, decreased animal productivity), vegetal and animal food products (costs of quality control and monitoring, economic losses accruing from condemned foods, serious impact on internationally traded commodities), humans (public health) [22].

1.4. Factors affecting mycotoxin development in wheat

Factors influencing the occurrence of diseases produced by *Fusarium* spp are linked to both substrat composition and environment conditions [49]. Fungi can invade, colonize and produce mycotoxins during either preharvest or postharvest stages [4].

There are many factors that influence the occurrence of mycotoxins in wheat, including: plant substrate (composition, pH, water activity), environmental and climatic factors (rainfall, relative air humidity, temperature, moisture availability, mechanical injury, insect/bird damage), topographic factors (relief position, topographic wetness index), biological factors (susceptible crop, compatible toxigenic fungus), crop system and management factors (tillage, preceding crop, type of agriculture), harvesting (crop maturity, temperature, moisture, handling, detection/ diversion), storage (structure, conditions, moisture, and temperature), handling and processing [7,8,50,51].

Whereas there are many factors involved in mycotoxin infection, climatic and topographic parameters possess the highest influence [7,8,52,53]. The temperature, relative humidity and moisture content of the grain are critical factors in fungal growth and mycotoxin production. In general, fungi grow at a temperature between 10°C and 40.5°C, above 70% relative humidity and a pH ranging from 4 to 8 [51]. Variations in climate parameters may lead to notable alterations in the quality of wheat crops; climate changes might influence crop yield and the degree to which the crops are

contaminated with mycotoxins or could increase the development of fungi not identified previously within a given area [54,55].

Additionally, secondary infections of wheat with mycotoxins can occur during cleaning, milling, grading or packaging processes [25]. Furthermore, mycotoxins can be transferred to final products for human consumption, frequently these compounds being stable at high temperature [36]. Thus, monitoring studies are recommended continuously.

Recently, a new field studying the possibilities to reduce mycotoxins in wheat has gained significant interest. To manage fungal growth and mycotoxin development in wheat, two directions are followed: control fungi proliferation in field (preharvest), and mitigate the infection by *Fusarium* fungi during storage and processing (postharvest) [56–59]. It is considered that growing cereal cultivars with reduced susceptibility to mycotoxin development is the most promising strategy [60].

Over the past years, the scientific community has proposed good agricultural practices (GAP), followed by implementation of good manufacturing practices (GMP), and hazard analysis and critical control points (HACCP) during food processing as an essential measure in addressing the problems posed by fungi and mycotoxins in the food system [61]. The possibilities to mitigate mycotoxin accumulation during preharvest period are: usage of resistant crops (breeding and transgenic approach) and agronomic practices (crop selection and crop rotation; tillage; crop planting time; plan physiological stage of plants; avoid drought stress; conventional *vs*. organic; chemical-control - fungicides, insecticides, aromatic plant essential oils; bio-control - enzymes and microorganisms). Harvest should be done at low moisture or water activity, reducing mechanical damage of seeds. In the postharvest period, mitigation strategies are related to: humidity and temperature during storage; physical decontamination (sorting, cleaning, dehulling, debranning, milling, irradiation, thermal processes, or combined approaches, inorganic or organic mycotoxin binders); chemical preservation (use of ammonia, calcium hydroxide); bio-control (biocompetition, antibiosis, parasitism, induced systemic resistance, plant growth-promoting rhizobateria/fungi); additives for gastrointestinal preservation; wheat processing [4,61–63].

2. *Fusarium* mycotoxins in wheat and wheat derivatives

The occurrence of *Fusarium* mycotoxins in wheat grains and wheat-based products is of great concern because of their toxic effects in humans and animals [13,64]. In the past decade, the interest of researchers from different European countries concerning the presence of *Fusarium* mycotoxins, especially the major mycotoxins (trichothecenes and ZEA), in wheat and wheat-based products, and the human dietary exposure to these mycotoxins has increased [22,61,65–70]. Additionally, the occurrence of emerging mycotoxins and mask mycotoxins became an attractive field for researchers, even if the toxic potential of these compounds has not been fully elucidated [37,38,71,72]. Until now, only few studies were done in Romania, most analyzing DON or ZEA by enzyme-linked immunosorbent assay (ELISA), and founding that DON is the most frequent myocotoxin in wheat from Romania [73–75].

Literature presents a considerable variety of results [22] on mycotoxin presence in wheat and products, the discrepancy of mycotoxin levels in different studies being explained by the large number of factors of influence on mycotoxin development, and the variability in reporting results, analytical methods used, sensitivities obtained, and statistical analyses applied. On the other hand, multi-mycotoxin methods were optimized and validated, with the purpose to analyze more mycotoxins simultaneously, with a high sensitivity and precision [66,76]. These methods and fungal studies have demonstrated the co-presence of more than one mycotoxin in the same food matrix, indicating the importance of mycotoxin co-occurrence evaluation [77,78].

2.1. Trichothecenes

Tricothecenes (Fig. 3) are a family of tetracyclic sesquiterpenoid substances (12,13-epoxytrichothec-9-ene skeleton) comprising over 200 compounds of widely varying toxicity [13,62]. They possess a double bond between C-9 and C-10, an epoxide between carbon atoms C-12 and C-13 (considered essential for toxicity [79]), and a variable number of hydroxyl and acetoxy groups in the molecule. Trichothecenes can be divided into two groups, macrocyclic and non-macrocyclic trichothecenes, based on the presence or absence of a macrocyclic ring linking C-4 and C-15. Trichothecenes with the major economic importance in agriculture are non-macrocyclic mycotoxins [2,80]. Most frequently, trichothecenes are divided into four types, named A, B, C, D,

according to their functional groups present in the molecule, types A and B being the most common [81–85]. Type A trichothecenes include HT-2, T-2, diacetoxyscirpenol (DAS), and neosolaniol (NEO), and differ from type B trichothecenes, such as DON, NIV and their acetylderivatives, respectively 3AcDON, 15AcDON and FUS-X, by the absence of a carbonyl group at the C-8 position [61,84,85].

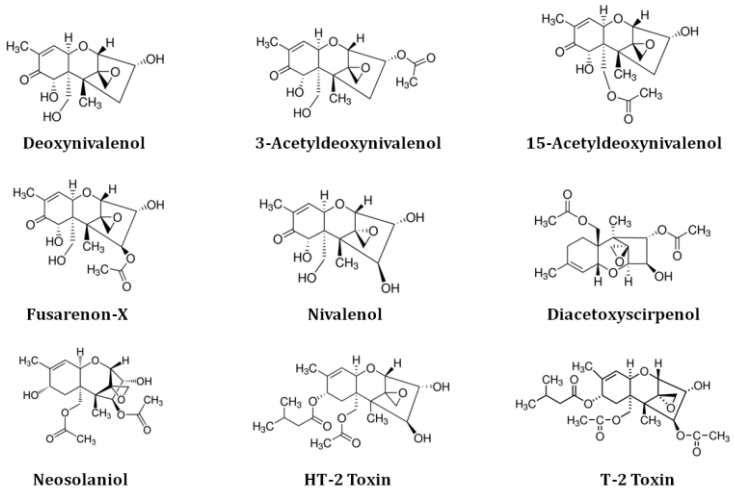

Fig. 3. Chemical structures of trichothecenes.

2.1.1. Toxicology

Trichothecenes are small, amphiphilic molecules that can move passively across cell membranes and can be absorbed through the gastrointestinal and respiratory tracts, as well as skin [2,83]. Trichothecenes can undergo the four basic reactions in xenobiotic metabolism: phase I (hydrolysis, oxidation, reduction), and phase II (glucuronidation). The ability to remove the epoxide oxygen (deepoxidation) is an important step in the detoxification of trichothecenes. Metabolic pathways vary, metabolites produced being often differt among species. The majority of these reactions occurs in tissues and result in reduced toxicity; however some metabolites may be more toxic than the parent mycotoxin. For example, HT-2 contributes to T-2 exposure, as HT-2 is a major metabolite of T-2. Excretion is via the biliary system and urine. Enterohepatic recirculation may occur, resulting in delayed excretion and, ultimately, increased toxicity [2,80].

Trichothecenes target the 60S ribosomal subunit, suggesting that the major mechanism of toxicity is translational inhibition. Trichothecenes have multiple effects on eukaryotic cells, the most important being the inhibition of protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis. The mechanism of DNA synthesis

inhibition has not yet been clarified, however, it may be a secondary effect of the inhibition of the protein synthesis or of the apoptotic effect of trichothecenes [84,86]. In addition, they can produce: alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis, activation of cytokines and chemokines, activation of mitogen activated protein kinases (MAPKs), modulation of immune responses or alteration at neurotransmitter levels [2,14,36,80,87,88]. Of all the trichothecenes, clinical data from animal studies suggest that T-2 and DAS are more potent [84]. Because of their effects on the immune system, the exposure to trichothecenes could predispose humans and animals to infectious disease, particularly in sensitive populations (examples for humans: young children, immuno-depressed people and old people) [14].

DON, also know as vomitoxin, has a potential to cause chronic effects such as reduced growth and anorexia, as well as neuroendocrine changes, immunosuppression, and exacerbation of infections [41,48,80,83,87,88]. Besides, DON can induce apoptosis of various cells, causing neurotoxicity, cytotoxicity, immunotoxicity, genotoxicity, teratogenicity, carcinogenicity, and embriotoxicity [89–92]. New findings indicate that DON could affect also epithelial cells through various mechanisms, targeting mucus and microbiota and proceeding to alterations at gastrointestinal level [93]. With respect to the harmfulness of toxins, NIV is more toxic than DON towards animals, while DON is more toxic against plants [94]. Similarly to DON, NIV has been shown to exert clinical effects such as hematotoxicity and immunotoxicity in mammals [61].

2.1.2. Occurrence

In Europe, type B trichethecenes seem to be the most dominant in wheat [95]. DON is probably the most important trichothecene because it is commonly detected in wheat grains, followed by T-2 and HT-2 toxins [2,80]. Also, NIV is usually found associated with DON and it has been intensively studied [48,83,85]. As mentioned, DON, NIV, and T-2 are commonly found together, this topic being important because simultaneous exposure to multiple mycotoxins could synergistically interact for toxicity, heightening concerns about health risks [44,51,96,97]. Hence, multi-mycotoxin analyses are useful in terms of *Fusarium* mycotoxin evaluation exposure. Across Europe, a high number of studies have been conducted, with the aim to identify and quantify trichothecenes. In the Tables II, III, IV and V, a summary of the occurrence data for the most important trichothecenes (DON, NIV, HT-2, and T-2, respectively) in wheat or wheat-based products from selected European countries is presented.

The highest concentrations of DON (20333 μg kg^{-1}), and HT-2 (486 μg kg^{-1}) were observed in wheat samples from United Kingdom and Italy, respectively, while the highest levels of NIV (590 μg kg^{-1}), and T-2 (495 μg kg^{-1}) were registered in wheat samples from Hungary and Serbia, respectively. Generally, lower levels of trichothecenes were registered for wheat-based products compared to those for wheat

samples, probably due to the steps in the food chain from unprocessed wheat to wheat-based products for direct human consumption.

Table II. Occurrence and levels of contamination in wheat commodities in selected European countries relative to deoxynivalenol.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Belgium	wheat	66.66	4/6	max. 150	[98]
Bulgaria	wheat	67.0	94/140	max. 1800	[99]
Croatia	wheat	65	33/51	max. 278	[100]
	wheat	58	30/52	27.1 - 1220	[101]
	wheat flour	60	9/15	27.1 - 126	[101]
Czech Republic	wheat	96.7	175/181	6.8 - 2265.2	[102]
Denmark	wheat flour	89	108/120	10 - 2591	[103]
Finland	wheat	29.9	101/338	max. 5865	[69]
Germany	wheat flour	100	28/28	15 - 965	[104]
	wheat flour	92	12/13	38 - 756	[104]
	whole grain wheat flour	100	19/19	15 - 1379	[104]
	wheat flour	11.11	2/18	max. 177	[105]
Hungary	wheat	78.2	287/367	70 - 1560	[106]
	wheat	72	21/29	230 - 1880	[107]
Italy	wheat	28	16/57	9.6 - 99.6	[108]
	wheat	62.8	27/43	13 - 1230	[109]
	wheat	16	12/74	48 - 2267	[66]
Lithuania	wheat	98.4	61/62	max. 642	[110]
	wheat	94.3	83/88	max. 1121	[110]
	wheat	100	48/48	max. 223	[111]
	wheat	94.11	32/34	max. 445	[111]
The Netherlands	wheat	71.4	671/940	max. 10000	[69]
Norway	wheat	14	24/169	max. 350	[112]
	wheat	29.4	245/832	max. 890	[69]
Poland	wheat	59.4	19/32	max. 997	[113]
	wheat	89	17/19	max. 455	[114]
	wheat	80	12/15	max. 341	[114]
Portugal	wheat products	40	4/10	333 - 1821	[115]
	wheat flour	80	8/10	20 - 77	[116]
	wheat flour	43	3/7	205 - 434	[117]
Romania	wheat	100	25/25	max. 5600	[118]
	wheat	83.3	10/12	6.1 - 154.3	[75]
	wheat	42.5	17/40	max. 95.7	[74]
	wheat	73.08	19/26	294 - 3390	[73]
	wheat	19.23	5/26	254 - 1440	[73]
	wheat	90	38/42	21 - 3395	[119]
Serbia	wheat	85.7	24/28	52 - 3306	[120]
	wheat	93.3	70/75	50 - 1090	[120]
	wheat	50	2/4	0.63 - 1.84	[65]
	wheat	34.5	19/55	0.057 - 0.42	[65]
	wheat	27.78	15/54	41 - 309	[121]
	wheat flour	86.7	13/15	17.5 - 976	[122]
Slovakia	wheat	76.6	229/299	max. 7880	[68]
	wheat	78	145/186	200 - 2940	[123]
Slovenia	wheat and wheat products	68.8	55/80	max. 3070	[124]
Spain	wheat products	79.8	95/119	max. 83.2	[125]
	wheat semolina	100	15/15	5.8 - 55.4	[126]
Sweden	wheat	20.6	114/554	max. 890	[69]
	winter wheat	72	46/64	max. 1394	[127]
	spring wheat	92	56/61	max. 6460	[127]
	wheat	90	26/29	max. 3230	[128]

Table II. Occurrence and levels of contamination in wheat commodities in selected European countries relative to deoxynivalenol *(continued)*.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
United Kingdom	wheat	86	1396/1624	max. 20333	[129]
Germany, Austria & Slovakia	wheat	100	23/23	203 - 4130	[130]
Europe	wheat and wheat flour	61	3891/6350	max. 3600	[20]
Europe	flour	50	51/103	20 - 2270	[131]
Europe and Mediterranean region	wheat	62	157/254	max. 5510	[132]

Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

Table III. Occurrence and levels of contamination in wheat commodities in selected European countries relative to nivalenol.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Czech Republic	wheat	78	32/41	15.4 - 25.9	[102]
Denmark	wheat flour	47.5	57/120	10 - 234	[103]
Germany	wheat flour	4	1/28	max. 25	[104]
	wheat flour	8	1/13	max. 25	[104]
	whole grain wheat flour	26	5/19	max. 25	[104]
Hungary	wheat	9	33/367	50 - 590	[106]
Italy	wheat	19.3	11/57	12 - 106	[108]
	wheat	3	2/74	50 - 197	[66]
The Netherlands	wheat	0	0/134	-	[69]
Norway	wheat	0	0/169	-	[112]
Poland	wheat	44	14/32	max. 80	[113]
	wheat	53	8/15	max. 23	[114]
	wheat	84	16/19	max. 18	[114]
Romania	wheat	2	1/42	max. 30	[119]
Serbia	wheat	0	0/54	-	[121]
Spain	wheat products	13.4	16/119	max. 53.6	[125]
	wheat semolina	20	3/15	8.8 - 13.6	[126]
Sweden	wheat	0	0/75	-	[69]
	winter wheat	94	29/31	max. 111	[127]
	winter wheat	33	11/33	max. 39	[127]
	spring wheat	50	14/28	max. 39	[127]
	spring wheat	27	9/33	max. 50	[127]
United Kingdom	wheat	67	1088/1624	max. 430	[129]

Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

Table IV. Occurrence and levels of contamination in wheat commodities in selected European countries relative to HT-2 toxin.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Belgium	wheat	33.33	2/6	max. 14	[98]
Czech Republic	wheat	14.6	6/41	12.7 - 18.3	[102]
Denmark	wheat flour	16.7	6/36	max. 33	[103]
Germany	wheat flour	0	0/28	-	[104]
	wheat flour	8	1/13	max. 12	[104]
	whole grain wheat flour	16	3/19	max. 4	[104]
	wheat products	94	122/130	max. 22	[133]
Italy	wheat	0	0/20	-	[134]
	wheat	29	6/20	max. 13.7	[134]
	wheat	5.3	3/57	6.78 - 60.1	[108]
	wheat	8	6/74	115 - 486	[66]
The Netherlands	wheat	0	0/134	-	[69]
	wheat	5	4/85	max. 38	[134]
Poland	wheat	22	7/32	max. 66	[113]
	wheat	7	1/15	max. 9	[114]
	wheat	26	5/19	max. 2	[114]
Romania	wheat	50	21/42	3 - 18	[119]
Serbia	wheat	5.56	3/54	128 - 129	[121]
	wheat flour	0	0/15	-	[122]
Spain	wheat products	16.8	20/119	max. 28.2	[125]
	wheat semolina	33.3	5/15	6.7 - 15.2	[126]
Sweden	wheat	10	3/29	max. 13	[128]
Norway	wheat	1.2	2/169	max. 20	[112]
United Kingdom	wheat	31	503/1624	max. 193	[129]
	wheat	20	12/60	max. 49	[135]

Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

Table V. Occurrence and levels of contamination in wheat commodities in selected European countries relative to T-2 toxin.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Belgium	wheat	0	0/6	-	[98]
Bulgaria	wheat	0.7	1/140	55	[99]
Croatia	wheat	25	13/51	max. 18	[100]
Czech Republic	wheat	39	16/41	5.7 - 8.2	[102]
Denmark	wheat flour	29	11/38	max. 153	[103]
Finland	wheat	0	0/338	-	[69]
Germany	wheat flour	0	0/28	-	[104]
	wheat flour	0	0/13	-	[104]
	whole grain wheat flour	16	1/19	max. 4	[104]
	wheat products	85	110/130	max. 1.9	[133]
Hungary	wheat	6.5	24/367	80 - 370	[106]
	wheat	31	9/29	54 - 87	[107]
Italy	wheat	8	2/20	max. 1.4	[134]
	wheat	29	6/20	max. 4.9	[134]
	wheat	3.5	2/57	7.14 - 17.8	[108]
	wheat	8	6/74	10 - 149	[66]
Lithuania	wheat	69	33/48	max. 18.8	[111]
	wheat	100	34/34	max. 23	[111]
The Netherlands	wheat	0	0/159	-	[69]
	wheat	13	11/85	max. 7	[134]

Table V. Occurrence and levels of contamination in wheat commodities in selected European countries relative to T-2 toxin *(continued)*.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Norway	wheat	0.6	1/169	max. 20	[112]
Poland	wheat	0	0/32	-	[113]
	wheat	0	0/15	-	[114]
	wheat	16	3/19	max. 4	[114]
Romania	wheat	24	6/25	max. 63	[118]
	wheat	100	2/2	0.8 - 1	[136]
	wheat	2	1/42	max. 7	[119]
Serbia	wheat	75	21/28	60 - 495	[120]
	wheat	60	45/75	86 - 200	[120]
	wheat	0	0/54	-	[121]
	wheat flour	26.7	4/15	9.8 - 26.9	[122]
Spain	wheat products	0.8	1/119	max. 13.7	[125]
Sweden	wheat	7	2/29	max. 12	[128]
United Kingdom	wheat	16	260/1624	max. 52	[129]
	wheat	5.3	3/57	max. 13	[135]

Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

2.2. Zearalenone

ZEA (Fig. 4) is a non-steroidal estrogenic mycotoxin, which is prevalent in temperate and warm countries. Chemically, ZEA is 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-beta-resorcylic acid lactone [87].

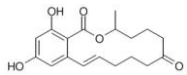

Fig. 4. Chemical structure of zearalenone.

2.2.1. Toxicology

ZEA is readly and rapidly absorbed from gastrointestinal tract [2] and undergoes both phase I and phase II metabolism reactions with the involvement of different enzymes catalyzing the first biotransformation step [80]. ZEA is reduced to α- and β-isomers in mammalian tissues. ZEA and its metabolite zearalenol (as a combination of free and conjugated forms) are excreted relatively rapidly in feces, urine, and to a small extent in milk [137].

ZEA and its derivatives are the only known mycotoxins with primarily estrogenic effects, thus they are considered mycoestrogens, a subset of naturally occurring estrogenic compounds or xenoestrogens, and they are classified as endocrine disrupting chemicals. Also, ZEA can act on the hypothalamic-hypophysial axis with release of prolactin and luteinizing hormone, and it can activate the pregnane X receptor, the constitutive androstane receptor, the aryl hydrocarbon receptor messenger ribonucleic acid (mRNA) levels, as well as a number of CYP enzymes in human hepatocyte cultures [2].

The predominant adverse effects are related to the estrogenic activity of ZEA and its metabolites: alterations in the reproductive tract, uterus enlargement, decreased fertility, increased embryolethal resorptions, reduced litter size, and changes in the serum levels of progesterone and estradiol [137]. ZEA is of major interest because, despite its low acute toxicity, it has proven to be also hepatotoxic, immunotoxic, carcinogenic to a number of mammalian species, acting as a enhancer of lipid peroxidation [80,137,138]. The consumption of cereals spoiled by ZEA can cause genital and fertility problems, decrease of hepatic function, and negative effects on the hematological parameters [62,87]. In addition, literature reported also a possible link between ZEA and the incidence of human cervical cancer or esophageal cancer [61]. According to the available toxicological data, ZEA is classified by the International Agency for Research on Cancer (IARC) as Group 3 [139].

2.2.2. Occurrence

ZEA was ussully found to co-occurr with other mycotoxins including DON, 3AcDON, 15AcDON, NIV, and FUS-X because of the ability of the producing fungi to synthesize more than one mycotoxin which often results in synergistic and/or additive effects on the host organism, the co-exposure with other mycotoxins presenting a probable health risk [18,64,140]. ZEA has a worldwide distribution with differences in the frequency percentages, which are generally lower compared with the most representative trichothecenes (e.g. DON) [84]. In the Table VI, the occurrence data for ZEA in wheat or products from selected European countries is summarized.

Table VI. Occurrence and levels of contamination in wheat commodities in selected European countries relative to zearalenone.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Bulgaria	wheat	69	97/140	max. 120	[99]
	wheat	1.9	1/54	max. 10	[141]
Croatia	wheat	66.7	4/6	13 - 50	[142]
	wheat	69	35/51	max. 107	[100]
	wheat	58	30/52	4.7 - 115	[101]
	wheat flour	33.3	5/15	4.1 - 10.1	[101]
Denmark	wheat flour	33.3	10/30	max. 2	[103]
Germany	wheat	92	22/24	11 - 860	[143]
	wheat flour	11	3/28	1 - 2	[104]
	wheat flour	31	4/13	1 - 8	[104]
	whole grain wheat flour	79	15/19	2 - 24	[104]
Hungary	wheat	58.6	215/367	50 - 890	[106]
	wheat	17	5/29	50 - 98	[107]
Lithuania	wheat	31.4	16/51	max. 95.6	[110]
	wheat	32.6	16/49	max. 33.4	[110]
	wheat	69	33/48	max. 28.1	[111]
	wheat	97	33/34	max. 45.8	[111]
Italy	wheat	8.8	5/57	2.35 - 27.15	[108]
The Netherlands	wheat	8.7	27/312	max. 310	[69]
	wheat flour	100	2/2	12.4 - 13.7	[144]
	mainly wheat flour	50	2/4	19.8 - 37.2	[144]
Portugal	wheat based products	50	2/4	11 - 15	[115]
	wheat flour	14	1/7	max. 27	[117]

Table VI. Occurrence and levels of contamination in wheat commodities in selected European countries relative to zearalenone *(continued)*.

REGION	COMMODITY	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Portugal	wheat flour	23.5	4/17	7.4 - 15.3	[144]
	mainly wheat flour	30.8	4/13	5.4 - 39.4	[144]
Romania	wheat	100	25/25	max. 170	[118]
	wheat	10	2/20	0.88 - 3.57	[145]
	wheat flour	31.25	5/16	0.41 - 41.8	[145]
	wheat bran	100	1/1	max. 0.42	[145]
	wheat	50	6/12	36.7 - 67.3	[75]
	wheat	10	4/40	max. 5.52	[74]
	wheat	69.23	18/26	37.6 - 1000	[73]
	wheat	76.92	20/26	28 - 105.6	[73]
	wheat	5	17/336	max. 80	[146]
Serbia	wheat	88.6	22/28	10 - 143	[120]
	wheat	94.6	71/75	16 - 201	[120]
	wheat	0	0/54	-	[121]
	wheat flour	33.33	5/15	1.9 - 21.1	[122]
Slovenia	wheat and wheat products	23.8	19/80	max. 113	[124]
Spain	wheat products	0	0/119	-	[125]
	bread	65	52/80	27 - 905	[147]
Sweden	wheat	0	0/51	-	[69]
	winter wheat	53	34/64	max. 86	[127]
	spring wheat	38	23/61	max. 678	[127]
	wheat	34.5	10/29	max. 116	[128]
United Kingdom	wheat	19	309/1624	max. 1292	[129]
Europe	wheat milling products	14	432/3088	max. 507	[87]
Europe and Mediterranean region	wheat	92	44/48	max. 921	[132]

Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

2.3. Emerging mycotoxins

The emerging mycotoxins are represented by FP, BEA, ENs (ENA, ENA1, ENB, and ENB1) and MON. ENs and BEA (Fig. 5) are six-membered cyclic depsipeptides, that present a high interest in the past years [14,71,148]. The occurrence and toxicity of emerging mycotoxins are currently under evaluation by the European Food Safety Authority Panel on Contaminants in Food Chain (CONTAM).

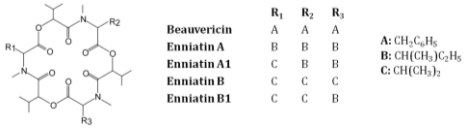

Fig. 5. Chemical structures of emerging mycotoxins (enniatins and beauvericin).

2.3.1. Toxicology

The toxicity of ENs and BEA is based on their ionophoric properties, disrupting normal concentrations of ions like K^+ and Ca^{2+} across membranes, which affects cell homeostasis [14,71,94]. ENs and BEA possess several potential properties: antimicrobial,

anthelmintic, insecticidal, antifungal, herbicidal, phytotoxic, immunosuppressive, and cytotoxic [149,150]. Due to their similar chemical structures, ENs and BEA can present additive or synergistic cytotoxic effects, as it has been demonstrated by *in vitro* studies [42,43,151].

A special focus on type B ENs was observed, with ENB1 presenting the highest cytotoxicity on chinase hamster ovary cells cells (CHO-K1) and producing major disturbances on liver hepatocellular carcinoma cell (HepG2) cycle [42,149]. The combination ENB1+ENA1 is presented as the most cytotoxic combination of emerging mycotoxins on CHO-K1 cells, followed by ENA+ENB, ENA+ENA1+ENB, and ENA+ENA1+ENB1 [42]. This information has particular importance in the context of the high occurrence of ENB and ENB1, and their co-occurrence with other mycotoxins.

2.3.2. Occurrence

The interest in emerging mycotoxin presence in foodstuffs started with the first published study that clearly defined these compounds [152]. Several authors reported high incidences for ENs and BEA in wheat and products like pasta, infant formulas, breakfast cereals, and biscuits, with incidences between 40% and 90% [108,147,152–155]. Regarding the simultaneous presence of ENs and BEA or the co-occurrence with other mycotoxins in wheat or wheat-based products, there is a lack of information in the literature [156]. Some recent results related to the presence of emerging mycotoxins in wheat or its products are presented in Table VII.

Table VII. Occurrence and levels of contamination in wheat commodities in selected European countries relative to emerging mycotoxins.

REGION	COMMODITY	MYCOTOXIN	FREQUENCY (%)	INCIDENCE	RANGE (μg kg^{-1})	REF.
Italy	Wheat	BEA	14	6/43	1.8 - 5.1	[109]
		ENA	14	6/43	3.1 - 18.1	
		ENA1	26	11/43	4.5 - 40.4	
		ENB	42	18/43	3.1 - 87.2	
		ENB1	26	11/43	1.5 - 69.8	
Portugal	Wheat products	BEA	2	1/61	max. 3.2	[153]
		ENA	16	10/61	2.6 - 71	
		ENA1	52	32/61	3.4 - 789	
		ENB	49	30/61	1.6 - 491	
		ENB1	44	27/61	2.8 - 369	
Spain	Bread	BEA	0	0/80	-	[147]
		ENA	0	0/80	-	
		ENA1	17.5	14/80	2.2 - 2.6	
		ENB	96	77/80	0.4 - 54	
		ENB1	79	63/80	0.2 - 14.8	
Spain	Pasta	BEA	18	20/114	0.1 - 21	[157]
		ENA	77	88/114	0.5 - 42	
		ENA1	76	87/114	0.25 - 22	
		ENB	80	91/114	0.5 - 122	
		ENB1	71	81/114	0.5 - 980	

BEA: Beauvericin; ENA: Enniatin A; ENA1: Enniatin A1; ENB: Enniatin B; ENB1: Enniatin B1; Frequency: the percentage of samples ≥ limit of detection (LD) / total samples analyzed; Incidence: number of samples ≥ LD / total samples analyzed.

3. Legislation of *Fusarium* mycotoxins

World Health Organization (WHO) and FAO are the principal stuctures involved in ensuring food security. At international level, WHO and FAO established the Joint FAO/WHO Food Standards Programme with Codex Alimentarius Commission (CAC) as principal group of work on legislation on food and feedstuff [158]. At regional or national levels, specific regulations are set by authoritative bodies, for example, European Commission (EC), Food and Drug Administration of United States, Public Health Agency of Canada, Health Surveillance Agency of Brazil, Food and Drug Administration of China, or the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing [94]. Moreover, European Food Safety Authority (EFSA) was established by the Council and European Parliament in 2002 to provide scientific advice and technical support in all areas impacting on food safety [159].

Mycotoxins are among the most important risks associated with the consumption of cereals in general, and wheat in particular, this group of compounds being classified as individual hazard category by the Rapid Alert System for Food and Feed (RASFF) [160]. Due to the diversity of commodities produced and consumed, the European regulations on mycotoxins are probably the most complete, comprising the majority of contaminant toxins with mechanism of toxicity elucidated.

3.1. Maximum permitted levels

Taking into account toxicity data, consumption frequency for each food category and vulnerabiliy of various groups of population (e.g. babies, children), maximum or recommended levels of mycotoxins in food are proposed. ML is designed to prevent the occurrence of each mycotoxin at levels considered to be harmful to human and/or animal health. Selected examples of maximum values for some *Fusarium* mycotoxins (DON, HT-2, T-2, and ZEA) in wheat or wheat-by products are given in Table VIII. The levels of DON and ZEA are set by Decision of the EC [161], while the values for the sum of HT-2 and T-2 are included in a Recommendation of the EC [162]. The MLs for DON vary between 200 and 1750 μg kg^{-1}, whereas the MLs for ZEA and the sum of HT-2 and T-2 are lower, varying from 20 to 100 μg kg^{-1}, and from 15 to100 μg kg^{-1}, respectively.

Table VIII. Maximum levels of certain *Fusarium* mycotoxins in wheat food commodities.

DEOXYNIVALENOL IN FOOD [161]	
COMMODITY	**Maximum Level (μg kg^{-1})**
Unprocessed durum wheat and oats	1750
Cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ	750
Pasta (dry)	750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based foods and baby foods for infants and young children	200
HT-2 and T-2 TOXINS IN FOOD [162]	
COMMODITY	**Maximum Level Sum HT-2+T-2 (μg kg^{-1})**
Unprocessed wheat	100
Cereals intended for direct human consumption, except oats and maize	50
Cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100
Other cereal milling products	50
Breakfast cereals including formed cereal flakes	75
Bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25
Cereal-based foods for infants and young children	15
ZEARALENONE IN FOOD [161]	
COMMODITY	**Maximum Level (μg kg^{-1})**
Unprocessed cereals other than maize	100
Cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ	75
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	50
Processed cereal-based foods and baby foods for infants and young children	20

3.2. Tolerable daily intakes

The TDI is an estimation of the amount of a contaminant in air, food and drinking water that can be taken in daily over a lifetime without notable health risk. Based on the toxicological experiments, and taking into account the total human diet and the possibilities for mycotoxin intake, legislation concerning TDI of mycotoxins has been published. The Joint FAO/WHO Expert Committee in Food Additives (JECFA), the Scientific Committee on Food (SCF), and the EFSA have proposed TDI values for DON (1000 ng kg^{-1} body weight day^{-1}) [163] and ZEA (200 ng kg^{-1} body weight day^{-1}) [164], and provisional maximum TDIs (PMTDI) for NIV (700 ng kg^{-1} body weight day^{-1}) [163], the sum of DON, 3AcDON and 15AcDON (1000 ng kg^{-1} body weight day^{-1}) [165], and the sum of T-2 and HT-2 (100 ng kg^{-1} body weight day^{-1}) [166]. However, for emerging mycotoxins no data is available until now regarding the TDI levels.

To date, it can be observed that TDIs have been set normally for individual mycotoxins. In the past years, a new scenario has been issued, reconsidering these values in light of the proved increase of toxicity during co-occurrence of mycotoxins in comparison with the one derived from the individual toxins (e.g. the sum of DON, 3AcDON and 15AcDON [165], and the sum of T-2 and HT-2 [166]).

4. Mycotoxin evaluation in wheat and wheat derivatives

Evaluating mycotoxin presence in food is a complex process, due to the high variety and complexity of food composition. Mycotoxins are present in food commodities at very low concentrations (from ng kg^{-1} to mg kg^{-1}), thus development of satisfactory methods for identification and quantification of mycotoxins is a key step in research, surveillance, and regulation of these compounds.

The concomitant presence of more than one mycotoxin in food products may represent a real risk due to potential additive, antagonistic, or synergistic toxic effects, sometimes underestimated. Hence, a new topic represented by developing validated multi-mycotoxin analytical methods became important in the last years, with the goal to obtain an accurante assessment of human exposure to mycotoxins [167,168]. To analyze mycotoxins in wheat and its products, several steps are required: sampling, homogenizing, storage, preservation, weighing, extraction, purification (if necessary), separation, derivatisation (if necessary), detection and quantification [17,169].

4.1. Sampling

Sampling is essential to obtain true and reliable results of an analysis concerning mycotoxin levels in food. Only having a perfect sampling process the levels obtained can be used to confirm compliance with regulatory values. In the case of mycotoxins, sampling is often a difficult task due to the high heterogeneity of the distribution of producing toxigenic fungi and corresponding mycotoxins in contaminated raw, unprocessed and processed foods. This is particularly important for raw cereals, because some mycotoxins (e.g. DON) are mainly found in the pericarp of the grain [170]. To guarantee the truthfulness of the results, the EU has established protocols in Regulation (EC) No 401/2006 for sampling some foodstuffs for the analysis of mycotoxins [171].

4.2. Extraction and purification

Over the time, the development of efficient extraction/clean-up techniques has been an important issue, having the aim to obtain an extract as clean as possible and concentrated in the compounds of interest [169]. Most analytical methods involve

solvent extraction to separate target mycotoxins and clean-up steps, depending on mycotoxin chemical structure and number of compounds analyzed. Organic solvents mixtures are normaly used in various ratios (with or without water) for extraction of mycotoxins. Sometimes, the acidified solvents are used, favoring mycotoxin extraction. Purification or clean-up of initial extracts is frequently used, increasing analytical sensitivity by reducing background noise [9,17].

The conventional extraction procedure is represented by the solid-liquid extraction (SLE), having the advantage of long-term use and cheap, sometimes presenting the disadvantage of being labor-intensive [172]. The most used organic solvents for SLE of mycotoxins are acetonitrile and methanol, followed by acetone, chloroform, dichloromethane, and ethyl acetate. The addition of water or acidified water (e.g. water with formic acid, acetic acid or citric acid, respectively) usually improves the extraction efficiency [173]. Additionally to the importance of the solvent or mixture of solvents used for extraction, there are also other critic parameters during SLE, like the ratio between sample and extractive solvent, temperature and time of extraction [17].

Currently, a mixture of acetonitrile/water (84/16, *v/v*) is the most widely reliable solvent for mycotoxin extraction in wheat commodities, especially for multi-class analysis. This mixture leads to good yields for most of the analytes, contributes to the good recoveries and decreases the matrix effects [77]. Even if the mixtures of acetonitrile and water are still the preferred extraction solvent in mycotoxin analysis, in the past years the concept of "Green Analytical Chemistry" (GAC) was rapidly developed, with the ambition to make chemical analysis environmentally friendlier, reducing chemical waste and using safer solvents. In this direction, the use of ethyl acetate was promoted for mycotoxin extraction [174].

Recently, new preparation techniques were developed, including supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), or microwave-assisted extraction (MAE). Concerning clean-up, certain techniques are available: solid-phase extraction (SPE), that uses cartridges or columns filled with different solid sorbents; immunoaffinity columns (IAC), composed by an activated solid phase support binded to specific antibodies for a given mycotoxin or group of mycotoxins; Mycosep/Multisep columns, filled by adsorbents packed into a plastic tube between two filter discs; molecularly imprinted polymers (MIPs) [17].

To design user-friendly techniques, extractive/clean-up procedures were combined. The most popular method is the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method, involving a micro-scale extraction with specific solvent coupled to a clean-up based on a dispersive solid-phase extraction (d-SPE) [17]. To obtain satisfactory analytical performances, QuEChERS-like methods were optimized during the last two years, e.g. QuEChERS type extraction without d-SPE clean-up, or

QuEChERS extraction performed by an acidified solvent followed by mixed-mode reversed phase-anion exchange SPE [168]. Another combined extractive/ clean-up technique useful for mycotoxin extraction from cereal products is the matrix solid phase dispersion (MSPD) [175]. Also, there are some extractive techniques that do not demand any additional clean-up step, because the procedure allows a clean and concentrated extract, ready to be analized, for example the solid-phase micro-extraction (SPME), or the dispersive liquid-liquid micro-extraction (DLLME) [17].

4.3. Detection and quantification

Due to its simplicity and low costs, ELISA is a routine method for the analysis of mycotoxins in food. While immunochemical methods rely on specific antibodies for each mycotoxin, chromatographic procedures can separate a higher number of analytes. The methods based on chromatographic separation used for mycotoxin detection and qualification include thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC) coupled with various types of detectors, e.g. flame ionization detector (FID), mass spectrometry (MS), or tandem mass spectrometry (MS/MS) [9,17,173]. High performance liquid chromatography tandem MS (HPLC-MS/MS) has become the most rising analytical tool for the determination of mycotoxins and their metabolites [176].

It is known that selectivity and sensitivity are classified as good for ELISA analysis, high for LC, and very high for LC coupled with MS (LC-MS), GC coupled with MS (GC-MS), LC coupled to tandem MS (LC-MS/MS), and GC coupled to tandem MS (GC-MS/MS) [9]. In mycotoxin analysis, modern generation LC-MS(/MS) approaches are more and more used, presenting some advantages: higher number of analytes determined per analysis from the same sample, decreasing of chemical waste, possibility to be used for multi-mycotoxin analysis, high sensitivity and selectivity, fast data acquisition features, avoiding sample extract clean-up which allows simplified sample preparation, and preference for lower flow rates. Moreover, the interest in applications of high resolution mass spectrometry (HRMS) techniques for quantitative mycotoxin analysis is disclosed, particularly for multi-class methods [174].

In the past years, to determine various mycotoxins, several articles reported significant advances in emerging technologies including: nanosensors, biosensors, aptamers, molecular imprimed polymers, nanobodies, electronic tongue instruments, optical methods (e.g. Fourier Transform mid-infrared spectroscopy; near-infrared transmittance spectroscopy) [9,17,168].

4.4. Risk assessment

Risks associated with mycotoxins depend on both hazard and exposure. It is indispensable from a public health perspective to develop acceptable methods to assess human risk associated to the presence of mycotoxins in food. Risk analysis is a scientific approach applied to food safety including three sections: risk assessment (a detailed analysis of the incidence of known or potential adverse health effects from human or animal exposure to food hazards), risk management (a process of scaling policy alternatives in light of risk assessment and strategies of surveillance and regulatory responses), and risk communication (a transfer of information regarding risk management options and actions among risk managers, consumers and other interested subjects) [177].

According to the National Research Council (NCR) of United States of America, risk assessment is a systematic process having the goal to describe the potential of adverse effects produced by exposure to hazardous agents. Human health risk assessment is based on a process consisting in four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization [178]. An exposure assessment requires the estimation of the frequency, intensity, and duration of ingestion of a mycotoxin, whereas risk characterization supposes to compare the results of the exposure assessment with hazard characterization to identify the level of concern. Human helath risk assessment uses toxicity and exposure data from both animal and human studies. In the traditional approach, TDI or acceptable daily intake (ADI) for human is derived from the no-observed-adverse-effect level (NOAEL) determined by animal studies [9].

To assess the exposure to a mycotoxin, its intake level in one day is calculated, normally named estimated daily intake (EDI), and often being expressed in ng kg^{-1} body weight day^{-1}. To calculate the EDI, it is necessary to know the mycotoxin occurrence in food or in a group of food commodities, and the dietary habits of the population in the region studied. To assess food consumption, various types of data can be used: food supply data, data from household consumption surveys, data from food consumption surveys, total diet studies, or duplicate diet collection. The current exposure assessment designs are largely deterministic and uncertainty and/or variability issues are accounted for by means of cautionary measures which are implicitly embedded in calculation designs [179,180]. More recently, probabilistic methods as Monte Carlo simulations have been developed to quantify the sources of uncertainty and variability of human exposure [180,181].

Mycotoxin exposure can be evaluated also by specific and suitable biomarkers of exposure for the human body (biomonitoring). Biomonitoring has an important role in estimating human exposure to mycotoxins and quantitative risk assessment. Because biomarkers are used to quantify exposure to a toxin and measure the extent of any

toxicity, they must be related to the biochemical mechanism pathway, react at realistic doses, be specific and sensitive, quantifiable and easily measurable [9].The process of biomonitoring includes the detection of the parent compound (mycotoxins) and/or its main phase I and phase II metabolites (e.g. glucuronide conjugates), measured in accessible body fluids (blood, urine) or body specimen (like hair) [182]. The newest strategy for human mycotoxin biomonitoring with future perspectives is represented by the use of breast milk in analyses [168].

PERSONAL CONTRIBUTION

1. Aims

Cereals are exposed to various biotic and abiotic stress factors, from cultivation and throughout their life cycle to processing, thus different types of contaminants can occur in grain cereals and cereal-based foodstuffs. One of the most important risks linked with wheat consumption is represented by mycotoxins. Despite of the high potential of Romania for wheat production and consumption, data on the occurrence of both regulated and unregulated *Fusarium* mycotoxins in wheat and wheat-based products from Romania are quite limited. Moreover, no comprehensive study concerning the exposure of the Romanian population to mycotoxins was published until now.

Given all these aspects, the **main objective** of the present thesis was to survey the natural presence of legislated and non-legislated *Fusarium* mycotoxins in wheat and wheat-based products from Romania.

To reach the major objective, the following **specific objectives** were proposed:

1. To validate specific and sensitive methods for multi-mycotoxin qualitative and quantitative analysis in wheat and wheat-based matrices.

2. To survey the natural occurrence of *Fusarium* mycotoxins in wheat and wheat-based products from Romania.

3. To evaluate the correlation between *Fusarium* mycotoxin levels in Romanian wheat and the weather conditions during the grain-growing season.

4. To assess the influence of the agricultural practice on the occurrence and concentration levels of emerging mycotoxins in wheat.

5. To estimate the daily intake of *Fusarium* mycotoxins through wheat-based product consumption for the Romanian population.

1. Obiective

Cerealele sunt expuse permanent la diverşi factori de stres biotici şi abiotici, de la cultivare şi pe parcursul întregului ciclu tehnologic până la procesare, astfel că, atât în cereale, cât şi în produsele pe bază de cereale, se pot regăsi diferite tipuri de contaminanţi. Unul dintre cele mai importante riscuri relaţionate cu consumul de grâu este reprezentat de micotoxine. În ciuda potenţialului ridicat de producţie şi consum de grâu al României, datele referitoare la incidenţa micotoxinelor legiferate şi nelegiferate produse de specii ale genului *Fusarium* în grâu neprocesat şi produse pe bază de grâu sunt destul de reduse. Mai mult decât atât, până în prezent nu a fost publicat niciun studiu complex referitor la expunerea populaţiei din România la micotoxine.

Luând în considerare aceste aspecte, **obiectivul principal** al acestei teze a fost realizarea unei studiu complex cu privirea la prezenţa naturală a micotoxinelor legiferate şi nelegiferate produse de specii ale genului *Fusarium* în grâu neprocesat şi produse pe bază de grâu din România.

Pentru a atinge obiectivul principal, următoarele **obiective specifice** au fost popuse:

1. Validarea de metode specifice şi sensibile pentru multi-analiza calitativă şi cantitativă a micotoxinelor în grâu şi produse pe bază de grâu.

2. Monitorizarea prezenţei naturale a micotoxinelor produse de specii ale genului *Fusarium* în grâu neprocesat şi produse pe bază de grâu din România.

3. Evaluarea corelaţiei între nivelurile de micotoxine produse de specii ale genului *Fusarium* în grâu din România şi condiţiile climatice în perioada de cultivare.

4. Evaluarea influenţei practicii în agricultură asupra frecvenţei şi nivelurilor micotoxinelor emergente în grâu.

5. Estimarea aportului zilnic de micotoxine produse de specii ale genului *Fusarium* al populaţiei din România prin intermediul consumului de produse pe bază de grâu.

1. Objetivos

Los cereales están expuestos a diferentes factores bióticos y abióticos de estrés, desde el cultivo y durante todo su ciclo de vida hasta el procesamiento, de manera que varios tipos de contaminantes fúngicos se pueden encontrar en los cereales y los productos alimenticios a base de cereales. Uno de los riesgos más importantes vinculados con el consumo de trigo está representado por micotoxinas. A pesar del elevado potencial de Rumania para la producción y el consumo de trigo, los datos sobre la presencia de micotoxinas de *Fusarium*, legisladas y no legisladas, en trigo y los productos a base de trigo de Rumania son bastante limitados. Y, ningún estudio exhaustivo sobre la exposición de la población rumana a las micotoxinas ha sido publicado hasta ahora.

Teniendo en cuenta todos estos aspectos, el **objetivo principal** de la presente tesis fue estudiar la presencia de micotoxinas de *Fusarium*, legisladas y no legisladas, en el trigo y los productos a base de trigo de Rumania.

Para lograr el objetivo principal, se han planteado los siguientes **objetivos específicos**:

1. Validar métodos selectivos y sensibles para el análisis cualitativo y cuantitativo de múlti micotoxinas en trigo y matrices a base de trigo.

2. Evaluar la presencia de micotoxinas de *Fusarium* en trigo y los productos a base de trigo de Rumania.

3. Correlacionar los contenidos de micotoxinas de *Fusarium* presentes en trigo procedente de Rumania y las condiciones climáticas durante la temporada de cultivo.

4. Estudiar la influencia de la práctica agrícola en los niveles de micotoxinas emergentes en trigo de Rumania.

5. Estimar la ingesta diaria de micotoxinas de *Fusarium* a través del consumo de productos a base de trigo para la población rumana.

2. General methodology

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were supplied by PanReac AppliChem (Castellar del Vallés, Spain), LC-MS/MS-grade methanol (≥99.9% purity) was supplied by VWR International Eurolab (Barcelona, Spain), and hexane was supplied by Merck KGaA (Darmstadt, Germany).

Ammonium formate (99%) and formic acid (≥98%) were obtained from Sigma Aldrich (St. Louis, USA). Ammonium acetate (>97%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain), and acetic acid (100%) was obtained from Merck KGaA (Darmstadt, Germany). Deionized water (<10 MΩ cm^{-1} resistivity) was manufactured in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA).

Anhydrous magnesium sulfate (99.5% purity) powder was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck KGaA (Darmstadt, Germany); C_{18} was purchased from Phenomenex (Torrance, CA, USA).

The derivatisation reagent BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from PanReac AppliChem (Castellar del Vallés, Spain).

Whatman No. 4 filter papers (Maidstone, UK) were used to filter the extract samples. Polypropylene syringes (2 mL) and nylon filters (13 mm diameter, 0.22 μm pore size) were purchased from Análisis Vínicos S.L. (Tomelloso, Spain).

The certified standards of ZEA, NIV, DON, 3AcDON, 15AcDON, DAS, NEO, HT-2, T-2, ENs (A, A1, B, and B1) and BEA were purchased from Sigma Aldrich (Madrid, Spain). The individual stock solutions were prepared in acetonitrile at 500 μg mL^{-1} for ENs and BEA, and 1000 μg mL^{-1} for ZEA, NIV, DON, 3AcDON, 15AcDON, DAS, NEO, HT-2, T-2. Also, a working mixed standard solution in methanol, at concentrations between 0.2 and 10 μg mL^{-1} was prepared by diluting the individual stock solutions. These solutions were used to construct the calibration curves, matrix matched

calibration curves, and for recovery and repeatability studies. Matrix matched calibration curves were used for both method validation and mycotoxin quantification in real samples. The solutions were stored in glass-stoppered bottles and in darkness under safe conditions at −20°C.

2.2. Multi-mycotoxin analysis

2.2.1. LC-QqQ-MS/MS

Mycotoxins were analyzed using a LC-MS/MS system, consisting of a LC Agilent 1200 using a binary pump and an automatic injector and coupled to a 3200 QTRAP ® AB SCIEX (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo-V™ source interface. Chromatographic separation of compounds was performed at 24 ± 1°C on a C_{18} reverse phase analytical column Gemini® (3 μM; 150 x 2 mm ID) and a C_{18} guard-column (3 μM; 4 x 2 mm ID) from Phenomenex (Madrid, Spain). Mobile phase was a time programmed gradient using methanol (acidified with 0.1% acid and 5mM ammonium salt) as phase A, and water (acidified with 0.1% acid and 5mM ammonium salt) as phase B. The following gradient was used: equilibration for 2 min at 90% B at 0.25 mL min^{-1}, 90-20 % B in 3 min at 0.25 mL min^{-1}, 20% B for 1 min at 0.25 mL min^{-1}, 20-10% B in 2 min at 0.25 mL min^{-1}, 10% B for 6 min at 0.25 mL min^{-1}, 10-0% B in 3 min at 0.25 mL min^{-1}, 100% A for 1 min at 0.25 mL min^{-1}, 100-50% A in 3 min at 0.25 mL min^{-1}, return to initial conditions in 2 min and maintain during 2 min at 0.25 mL min^{-1}.. The injection volume was 20 μL.

The QTRAP System was set in the selected reaction monitoring (SRM) mode and a triple quadrupole (QqQ) mass spectrometry detector (MS/MS) was used. The Turbo-V™ source was used in electrospray ionization (ESI) positive mode. The source/gas parameters have been set as following: vacuum gauge (10e^{-5} Torr): 3.1; curtain gas: 20; ionspray voltage: 5500; source temperature: 450°C; ion source gas 1 and gas 2: 50. The precursor ions (Q1), product ions (Q3), collision energies (CE), collision cell exit potential (CXP), declustering potential (DP) and collision cell entrance potential (CEP), are shown in the Table IX. The entrance potential (EP) was the same for all analytes, 10 V. Acquisition and processing data were performed using Analyst® software, version 1.5.2 (AB SCIEX, Concord, Ontario, Canada).

Table IX. Mass spectrometry parameters for mycotoxin detection using LC-QqQ-MS/MS.

ANALYTE	M_w	PRECURSON ION	PRODUCT IONS	DP	CEP	CE	CXP
	(g/mol)	(m/z)			(V)		
NIV	312.32	313.4	115.1^{Q}	50	18	80	3
		$[M+H]^+$	229.0^{q}			22	3
DON	296.32	297.1	203.1^{Q}	40	17	20	4
		$[M+H]^+$	231.0^{q}			20	4
3AcDON	338.35	339.2	231.1^{Q}	44	18	20	3
		$[M+H]^+$	203.1^{q}			20	3
15AcDON	338.35	339.2	137.0^{Q}	50	18	20	3
		$[M+H]^+$	261.1^{q}			20	3

Table IX. Mass spectrometry parameters for mycotoxin detection using LC-QqQ-MS/MS (*continued*).

ANALYTE	M_w	PRECURSOR ION	PRODUCT IONS	DP	CEP	CE	CXP
	(g/mol)	(m/z)			(V)		
DAS	366.41	384.0	307.2 *Q*	66	20	15	16
		[M+NH4]$^+$	105.0 *q*			63	12
NEO	382.40	400.2	185.0 *Q*	46	20	29	14
		[M+NH4]$^+$	215.0 *q*			25	12
HT-2	424.48	442.2	262.8 *Q*	21	22	19	4
		[M+NH4]$^+$	215.4 *q*			19	8
T-2	466.52	484.3	185.1 *Q*	21	23	22	4
		[M+NH4]$^+$	215.1 *q*			29	4
ZEA	318.36	319.0	301.0 *Q*	26	18	15	10
		[M+H]$^+$	282.9 *q*			19	4
BEA	783.95	801.2	784.1 *Q*	116	33	27	10
		[M+NH4]$^+$	244.1 *q*			39	6
ENA	681.90	699.4	210.1 *Q*	76	30	35	14
		[M+NH4]$^+$	228.2 *q*			59	16
ENA1	667.87	685.4	210.2 *Q*	66	29	37	8
		[M+NH4]$^+$	214.2 *q*			59	10
ENB	639.82	657.3	196.1 *Q*	51	28	39	8
		[M+NH4]$^+$	214.0 *q*			59	10
ENB1	653.85	671.2	214.1 *Q*	66	29	61	10
		[M+NH4]$^+$	228.1 *q*			57	12

CE: collision energy; CEP: collision cell entrance potential; CXP: collision cell exit potential; DP: declustering potential; Mw: Molecular weight; Q: quantification transition; q: qualification transition.

2.2.2. GC-QqQ-MS/MS

The analysis was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA). Quantitative data were acquired at SRM mode and mass spectrometer was operated in electron ionization (70 eV). The transfer line and source temperatures were 280 and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen (1.5 mL min^{-1}), and helium was used as the quenching gas (2.25 mL min^{-1}), both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using the Agilent Masshunter version B.04.00 software (Agilent Technologies, Palo Alto, CA, USA).

Analytes were separated on a HP-5MS 30 m × 0.25 mm × 0.25 μm capillary column. A total of 1 μL of extract was injected in splitless mode in programmable temperature vaporization inlet (150°C for 0.1 min then 600°C min^{-1} to 250°C for 5 min) employing helium as the carrier gas at a fixed pressure of 20.3 psi. The oven temperature programme was initially 80°C, and the temperature was increased to 245°C at 60°C min^{-1}. After a 3 minutes hold time, the temperature was increased to 260°C at 3°C min^{-1} and finally to 270°C at 10°C min^{-1} and then held for 10 min.

For identification of compounds, two MS/MS transitions were acquired: one for quantitation (*Q*), and one for confirmation (*q*). Table X shows the optimized GC-QqQ-MS/MS parameters of the selected mycotoxins.

Table X. Mass spectrometry parameters for mycotoxin detection using GC-QqQ-MS/MS.

ANALYTE	SRM transitions (m/z)	CE (eV)	Dt (ms)	ANALYTE	SRM transitions (m/z)	CE (eV)	Dt (ms)
NIV	289 > 73 Q	15	35	**FUS-X**	450 > 26 Q	10	35
	379 > 73 q	15	35		450 > 245 q	20	35
DON	392 > 259 Q	10	25	**NEO**	252 > 195 Q	10	25
	407 > 197 q	10	25		252 > 167 q	15	35
3AcDON	392 > 287 Q	10	25	**HT-2**	347 > 185 Q	10	25
	467 > 147 q	5	35		347 > 157 q	10	25
15AcDON	392 > 217 Q	20	35	**T-2**	350 > 229 Q	10	25
	392 > 184 q	20	35		350 > 259 q	15	35
DAS	350 > 229 Q	15	35	**ZEA**	462 > 151 Q	20	25
	378 > 124 q	10	25		462 > 333 q	20	25

CE: collision energy; Dt: dwell time; Q: quantitative transition; q: confirmation transition.

3. Study 1. Occurrence of *Fusarium* mycotoxins in wheat and flour from Romania

Study included in the article entitled "Occurrence and co-occurrence of Fusarium mycotoxins in whet grains and wheat flour from Romania"

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Abstract

In this study, the presence of fourteen Fusarium mycotoxins, legislated by the European Union - deoxynivalenol, zearalenone, HT-2 and T-2 toxins (EC/1881/2006; 2013/165/EU), or non-legislated (five trichothecenes and five emerging mycotoxins), was evaluated in 31 whole unprocessed wheat samples and 35 white wheat flour samples from different areas of Romania. For this purpose, a validated multi-mycotoxins liquid chromatography tandem mass spectrometry method was applied. Seventy three percent of the analyzed samples contained at least one mycotoxin. The highest occurrence was for enniatin B, 71% of the analyzed samples being positive (21-407 μg kg^{-1}). Regarding the legislated mycotoxins, deoxynivalenol was detected in 14% (111-1787 μg kg^{-1}) of the samples, while zearalenone was detected in 9% (51-1135 μg kg^{-1}). Only one sample was positive for neosolaniol. Concerning co-occurrence, 42% of the samples were contaminated with two to five mycotoxins, the most frequent being the binary or tertiary combinations of enniatins. This is the first study applied to Romanian wheat grains and flour samples using a high sensitive multi-mycotoxins method, and which included also "emerging" mycotoxins.

3.1. Introduction

Mycotoxins can be present in vegetable foods which can serve as a substrate for the growth of filamentous fungi of the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. There are many factors that predispose to mycotoxin production by fungi including substrate type and availability, climate conditions, storage and processing conditions [78,183,184]. Cereal contamination is decisive for mycotoxin occurrence, particularly for wheat, maize and rice which are of major importance [17]. The RASFF of the EU reports mycotoxins on the third position according to the total number of hazard notifications [160].

Wheat is considered the main strategic crop in the world with a global production of 729 million tons in 2014. The EU is the world's largest wheat producer. Romania is one of the five biggest wheat producers in the EU with a harvested area of 2,107,813 hectares and an annual production of 7,584,814 metric tons in 2014 [185]. More than half of Romanian annual wheat production is exported in different countries

around the world [186]. On the other hand, for Romanian population a high wheat and wheat product consumption is registered [33].

Growing wheat in Romania is a traditional and important part for the national agriculture. Main production area for wheat is Danube plain in the South of the country. Other important wheat growing areas are Transylvania, northern part of Moldova in north-eastern Romania, and Banat region in the South-West [31].

Presence of various moulds and their specific mycotoxins may be different according to climatic conditions. *Fusarium* species are the most prevalent toxin-producing fungi in cereals from temperate regions of America, Europe and Asia [187]. Romania has a temperate-continental climate with proper temperatures, rainfall and humidity for wheat growing. In the last twenty years, extreme meteorological events have been registered in this area, such as excessive dryness, substantial rainfall, tropical days, or high humidity [186,188].

Species of *Fusarium* genera are able to produce in wheat three of the most important classes of mycotoxins: FUMOs: FB1, FB2, FB3; ZEA; trichothecenes: HT-2, T-2, DAS, NEO, NIV, DON, 3AcDON, 15AcDON and FUS-X. They can also produce emerging mycotoxins like FP, BEA, ENs (ENA, ENA1, ENB, ENB1) and MON, or other mycotoxins as fusaric acid, fusarin A-D, gliotoxin, butenolite, which are recently studied [22].

In vitro and *in vivo* studies have demonstrated for trichothecenes, and ZEA their nephrotoxic, hepatotoxic, carcinogenic, immunosuppressive and mutagenic properties [14]. Moreover, in the last years, toxicological studies about the co-presence of mycotoxins have been stated their synergistic toxic effects [189].

3.2. Aims

The aim of this study was to evaluate the presence and the co-occurrence of 14 mycotoxins (NIV, DON, 3AcDON, 15AcDON, DAS, NEO, HT-2, T-2, ZEA, BEA, ENA, ENA1, ENB, and ENB1) in 66 wheat and flour samples from Romania. For this, a validated multi-mycotoxin method using LC-QqQ-MS/MS was applied. This is the first study which evaluates simultaneously a broad spectrum of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania using a highly sensitive method.

3.3. Materials and methods

3.3.1. Sampling

A total of 66 wheat samples, including whole unprocessed wheat (31) and white wheat flour (35), were analyzed in order to investigate the presence of mycotoxins. Whole wheat samples were collected during 2014 harvest season from four different Romanian areas: Bihor (2) – in the North-West of the country; Braşov (2), Dâmboviţa (7) – in central Romania; Teleorman (20) – in the South of the country. Information

about growing area (county and city) was collected. White wheat flour samples were purchased from different markets located in Târgoviște (Dâmbovița county, Romania), between January and March 2015.

Sampling was performed according to the EU guidelines [171], for the official control of legislated mycotoxins for lots of cereals and cereal products less than 50 tons. For both wheat and wheat flour samples, three incremental samples of 1 kg were collected or purchased, obtaining an aggregate sample of 3 kg total weight. After homogenization, samples were packed in plastic bags and kept at -20°C in a dark and dry place until analysis. Before the analysis, for all samples, subsamples of 300 g were milled with a blender and divided into three bulks of 100 g each one.

3.3.2. Extraction

Sample extractions were performed according to the method of Juan et al [108]. Representative sub-samples of each sample were weighed (2 g) and placed into 50 mL polytetrafluoroethyl (PTFE) centrifuge tubes, followed by the addition of 10 mL acetonitrile/water (84:16, *v/v*). The tubes were stirred for 1 hour at 300 shakes min^{-1} using a horizontal shaking device (IKA KS260 basic Stirrer, Staufen, Germany), and then centrifuged for 5 min at 5°C and 4500 rpm using an Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany), and filtered with Whatman filter paper. 5 mL of the supernatant were placed in 15 mL PTFE centrifuge tubes and were evaporated to dryness at 35°C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, MA, USA). The residue was reconstituted to a final volume of 1 mL with methanol/water (70/30, *v/v*) and filtered through a syringe nylon filter.

3.3.3. Mycotoxin analysis

The analysis of the fourteen mycotoxins was performed with a LC-QqQ-MS/MS system, using the method presented in the section 2.2.1. Mobile phases were methanol (0.1% formic acid and 5mM ammonium formate) as phase A, and water (0.1% formic acid and 5mM ammonium formate) as phase B. Statistical analysis was performed using SPSS software, version 22.0 (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA) with a statistical significance set at 95% ($p = 0.05$).

3.3.4. Method validation

Validation of the method was performed for linearity, accuracy, repeatability (intraday and interday precision) and sensitivity, according to previous studies [77], following the EU Commission Decision, 2002/657/EC [190]. External standard calibration was used in the validation of the analytical method.

The criteria for confirmation of positive findings were: comparing the peak area ratios between the quantification (Q) and confirmation (q) transitions with ion-ratios

obtained from the reference standard; the peak ratio of the confirmation transition against quantification one; the agreement with the retention times.

Matrix effect was assessed by employing matrix-matched standards and calculating the area ratio, which is defined as the absolute matrix effect (ME).

For linearity evaluation, matrix-matched calibration curves were constructed at concentration levels between 0.2 and 0.003 μg g^{-1} for mycotoxins with high sensitivity (ENs, BEA, NEO), and between 5 and 0.05 μg g^{-1} for mycotoxins with less sensitivity like ZEA and other trichothecenes except NEO.

Sensitivity was evaluated by limit of detection (LD) and limit of quantification (LQ). LDs were estimated using an extract of a blank of wheat (previous analyzed and negative for the mycotoxins included in this study), fortified with decreasing concentrations of the analytes, where the response of the qualifier ion was at least 3 times the response of the blank extract (n = 9). The LQs were estimated in the same way as the LDs, but using the criterion of S/N ≥ 10 for the qualifier ion.

Accuracy was evaluated through recovery studies (n = 6) which were carried out by spiking blank wheat at LQ, 2 times LQ, and 10 times LQ concentration levels. The spiked samples were left to stand for 3h at room temperature before the extraction to establish equilibration between mycotoxins and the matrix. Three replicates were prepared for each spiking level. Intraday and interday precision (repeatability) of the method were carried out by spiking wheat at the three levels previously indicated. Method precision was estimated by calculating the relative standard deviation (RSD) using the results obtained during the same day (intraday), and on three different days (interday) by the repeated analysis (n = 9) at the three spiked levels.

3.4. Results and discussion

3.4.1. Method validation and performance

Fig. 6 shows the chromatographic separation of the 14 mycotoxins. The parameters taken into account for method validation were: instrumental linearity, ME, sensitivity (LD and LQ), accuracy (recovery), and repeatability (intraday and interday precision).

To evaluate possible matrix interference on chromatographic response, external matrix matched calibration was used. Matrix interference is defined as area ratio between areas of matrix matched standard (blank wheat extract resolved in a standard solution with the same analyte concentration of the standard solution as a comparison) and standard solutions. ME is defined as the area ratio (B/A * 100), where "A" corresponds to the MS/MS area of the standard solution and "B" to the MS/MS area of the matrix-matched standard at the same concentration as "A". A value of 100% indicates that there is no ME, values higher than 100% reflect the enhancement of the

signal, while values below 100% reflect the suppression of the signal. Ion suppression was observed for all the mycotoxins analyzed, except for ENB, ENB1 and BEA where ion enhancement was present (Table XI). Furthermore, for the effective quantification in wheat and flour samples, matrix-matched calibration curves were used.

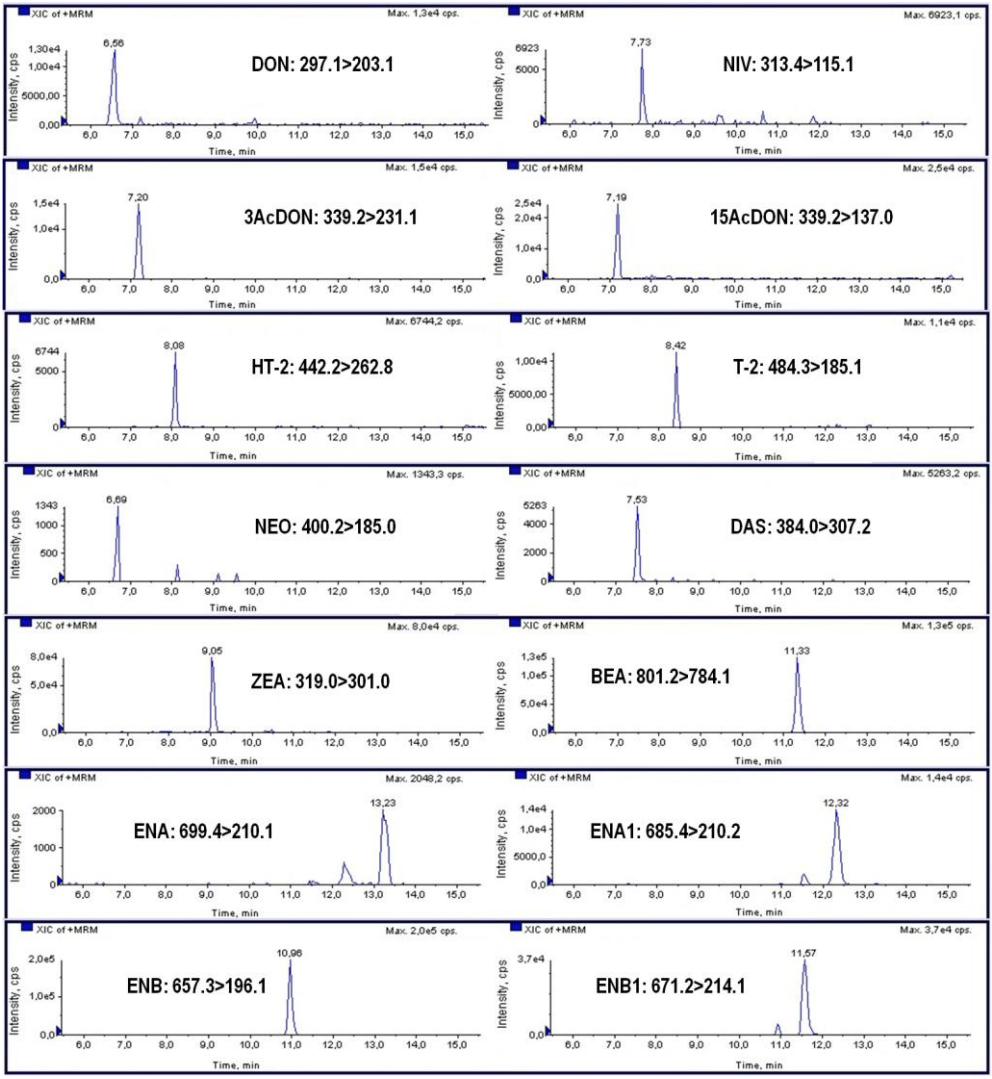


Fig. 6. SRM Chromatograms of matrix-matched standard solution at 1250 ng mL^{-1} (NIV, DON, 3AcDON, 15AcDON, HT-2, T-2, and ZEA), 125 ng mL^{-1} (BEA), and 100 ng mL^{-1} (NEO, DAS, ENA, ENA1, ENB, and ENB1).

The linear regression coefficients of all calibration curves demonstrated a good linearity, with corresponding correlation coefficients (r^2) higher than 0.996. LDs and LQs of the mycotoxins analyzed presented a high variability, ranging between 1 and

300 μg kg^{-1}. Recovery values were between 60 and 133%. Regarding precision values evaluated from the RSD of intraday and interday precision, these were lower than 12% and 26%, respectively (Table XI).

Table XI. Parameters of the LC-QqQ-MS/MS method for wheat analysis: limits of detection (LDs), limits of quantification (LQs), recovery, interday relative standard deviation (RSD_R), matrix effect (ME), and linearity expressed as correlation coefficient (r^2).

ANALYTE	LD (μg kg^{-1})	LQ (μg kg^{-1})	RECOVERY (RSD_R) (%)						ME (%)	Linearity (r^2)
			LQ		2 LQ		10 LQ			
NIV	150	300	60	(1)	80	(4)	69	(1)	65	0.999
DON	20	40	92	(7)	101	(7)	104	(1)	69	0.997
3AcDON	20	40	60	(9)	84	(1)	79	(9)	64	0.999
15AcDON	150	300	76	(17)	93	(7)	91	(13)	65	0.997
DAS	30	60	89	(2)	92	(11)	85	(16)	97	0.998
NEO	7	15	116	(3)	86	(4)	100	(1)	88	0.997
HT-2	50	100	102	(19)	67	(1)	85	(1)	72	0.998
T-2	75	150	63	(4)	92	(10)	92	(3)	78	0.999
ZEA	20	40	60	(7)	84	(4)	80	(2)	78	0.997
BEA	4	8	75	(7)	76	(19)	74	(21)	105	0.996
ENA	6	12	117	(8)	92	(6)	107	(13)	69	0.998
ENA1	3	6	84	(6)	86	(5)	81	(9)	82	0.999
ENB	1	2	133	(14)	107	(12)	86	(17)	130	0.998
ENB1	1	2	96	(26)	80	(13)	73	(11)	103	0.998

Taking into account our results for the validation of the method, this analysis is sensitive, precise and reproducible. Moreover, it presents the advantage of using LC-MS/MS which became the most frequent procedure for mycotoxins analysis in the past years [17]. The performance of the method is reflected in the LQs, which are similar to the values obtained in other studies, especially for DON, ZEA, and ENs, and the recoveries rates which are similar or better than other previous studies [68,109].

3.4.2. *Fusarium* mycotoxin occurrence data

The validated method was applied to analyze 14 mycotoxins in whole unprocessed wheat (n = 31) and white wheat flour (n = 35). Mycotoxin presence was assumed at levels exceeding the LDs. Analytical results showed that 73% of samples presented detectable levels of DON, ZEA, NEO or ENs (A, A1, B, B1), 48 samples being positive for at least one of the mycotoxins mentioned before. Levels between LDs and LQs were found in five wheat flour samples (two for ENA, two for ENA1, one for ENA+ENA1), and two wheat samples were in the same situation for ENA. Table XII presents the occurrence, range, and mean levels for the mycotoxins detected in the samples analyzed, and Table XIII summarizes the results for wheat samples according to the geographical origin.

Table XII. Mycotoxin levels found in Romanian wheat and wheat flour samples.

ANALYTE	WHEAT (*n* = 31)						FLOUR (*n* = 35)					
	INCIDENCE (FREQUENCY)	LD-LQ	ML	MEAN#	MEAN§	RANGE	INCIDENCE (FREQUENCY)	LD-LQ	ML	MEAN#	MEAN§	RANGE
				(μg kg^{-1})						(μg kg^{-1})		
DON	8 (26)	0	1750	748	193	110-1787	1 (3)	0	750	190	5	190
NEO	0 (0)	0		n.q.	n.q.	n.q.	1 (3)	0		38	1	38
ZEA	4 (13)	0	100	669	87	327-1135	2 (6)	0	75	62	4	51-73
ENA	6 (19)	2		70	9	17-140	3 (9)	3		n.q.	n.q.	n.q.
ENA1	10 (32)	0		113	36	14-356	4 (11)	3		7	0.2	7
ENB	19 (61)	0		128	78	21-407	28 (80)	0		19	15	3-60
ENB1	14 (45)	0		168	76	3-510	6 (17)	0		11	2	7-15

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; LD-LQ: number of samples ≥ LD and ≤ limit of quantification (LQ); ML: maximum levels set by the EU regulations [161]; n.q.: not quantified; #: mean value for samples ≥ LQ; §: mean value for all samples, assuming a zero value for samples ≤ LQ.

Table XIII. Summary of mycotoxin levels found in Romanian wheat samples distributed by growing area during 2014 harvest year.

GROWING AREA	PARAMETERS	MYCOTOXIN						OVERALL INCIDENCE (FREQUENCY)
		DON	ZEA	ENA	ENA1	ENB	ENB1	
Teleorman	Incidence (Frequency)	0 (0)	1 (5)	6 (30)	9 (45)	10 (50)	10 (50)	11 (55)
(n = 20)	Mean# (μg kg^{-1})	n.q.	638	70	123	159	216	
	Mean§ (μg kg^{-1})	n.q.	32	14	55	80	108	
	Range (μg kg^{-1})	n.q.	638	17 - 140	14 - 356	22 - 407	3 - 510	
Dâmbovița	Incidence (Frequency)	6 (86)	1 (14)	0 (0)	1 (14)	7 (100)	3 (43)	7 (100)
(n = 7)	Mean# (μg kg^{-1})	681	327	n.q.	26	97	52	
	Mean§ (μg kg^{-1})	584	47	n.q.	4	97	22	
	Range (μg kg^{-1})	281 - 1060	327	n.q.	26	35 - 196	33 - 82	
Bihor	Incidence (Frequency)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
(n = 2)	Mean# (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	
	Mean§ (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	
	Range (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	
Brașov	Incidence (Frequency)	2 (100)	2 (100)	0 (0)	0 (0)	2 (100)	1 (50)	2 (100)
(n = 2)	Mean# (μg kg^{-1})	949	856	n.q.	n.q.	76	36	
	Mean§ (μg kg^{-1})	949	856	n.q.	n.q.	76	18	
	Range (μg kg^{-1})	110 - 1787	576 - 1135	n.q.	n.q.	21 - 131	36	

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; n.q.: not quantified; #: mean value for samples ≥ limit of quantification (LQ); §: mean value for all samples, assuming a zero value for samples ≤ LQ.

3.4.2.1. Trichothecenes

DON, which is the most studied trichothecene in cereals, was detected in 14% (111-1787 μg kg^{-1}) of analyzed samples. DON presented the highest concentration found (1787 μg kg^{-1}) of all mycotoxins analyzed, being also higher than the ML of DON in unprocessed wheat, 1750 μg kg^{-1} [161]. Regarding the other trichothecenes, one wheat flour sample was positive for NEO (38 μg kg^{-1}), and all samples were negative for 3AcDON, 15AcDON, NIV, DAS, HT-2, and T-2.

Comparing our results for wheat and flour samples with others presented in the literature, DON revealed lower values for incidence (26%, and 3% respectively), maximum concentration detected (1787 μg kg^{-1}, and 190 μg kg^{-1}, respectively), and mean level (748 μg kg^{-1}, and 190 μg kg^{-1}, respectively) than other studies. Alexa et al [73] analyzed samples from Romania and found that DON was present in 73% (max. 3390 μg kg^{-1}) of wheat samples, similar results with another study which analyzed Slovakian wheat samples and DON was found in 77% (max. 7880 μg kg^{-1}) of samples [68]; in a Croatian study performed by Pleadin et al [100], the incidence of DON in wheat samples was 65% (max. 278 μg kg^{-1}), a study from Poland reported that 46% of wheat samples contained DON (max. 2975 μg kg^{-1}) [76], while in a Spanish research, DON was found in 30% of wheat samples (max. 2330 μg kg^{-1}) [191].

Regarding other studies on wheat flour, the incidences found were also higher than our results. That was in Serbia where DON was found in 87% of wheat flour samples (max. 976 μg kg^{-1}) [122], and similar results were calculated in Danish [103] and Portuguese [116] samples, with an incidence of 89% (max. 2591 μg kg^{-1}), and 80% (max. 77 μg kg^{-1}), respectively.

All wheat and wheat flour samples analyzed in our study were negative for NIV, HT-2 and T-2, similar to another study that included wheat samples from Finland, Sweden, and The Netherlands [69].

3.4.2.2. Zearalenone

ZEA was detected in 9% (51-1135 μg kg^{-1}) of analyzed samples (Table XII). All ZEA positive wheat samples exceeded the 100 μg kg^{-1} set as ML for unprocessed wheat [161].

The incidences (13% for wheat, 6% for flour) of ZEA in our study were lower than the levels found in other studies, but the values for maximum concentration found (1135 μg kg^{-1}, and 73 μg kg^{-1}, respectively), and mean (669 μg kg^{-1}, and 62 μg kg^{-1}, respectively) were higher than other values from literature. In published articles about mycotoxin presence in wheat samples, such as the study elaborated by Alexa et al [73] in Romania, ZEA was found in 69% of wheat samples (max. 1000 μg kg^{-1}), and Pleadin et al [100] found ZEA in 69% of 51 Croatian wheat samples analyzed, at levels between 7 and 107 μg kg^{-1}. Also, Bryła et al [76] observed that 47% of wheat samples from Poland contained ZEA (max. 100 μg kg^{-1}). On the other hand, studies about wheat flour

samples reported modest values for maximum concentration of ZEA, as 21.1 μg kg^{-1} 37.2 μg kg^{-1}, and 39.4 μg kg^{-1} in Serbian, Dutch, and Portuguese wheat flour samples, respectively [122,144].

3.4.2.3. Emerging mycotoxins

This study is the first that analyze emerging mycotoxins in Romanian wheat and wheat flour. The findings (Table XII) showed that ENs are the most frequent (73%) mycotoxins in both matrices, ENB being the most detected (71%). Even if the frequency of ENB for wheat flour samples (80%) was higher than the frequency for wheat samples (61%), the mean (19 μg kg^{-1}) and highest concentration found (60 μg kg^{-1}) for wheat flour were lower than the correspondent values for wheat (128 μg kg^{-1}, and 407 μg kg^{-1}, respectively), milling process being an element with an important influence on these values [192]. In terms of occurrence, the second mycotoxin was ENB1, followed by ENA1 and ENA. All samples were negative for BEA.

Other studies from Italy, Spain, and Finland reported also that the most frequent emerging mycotoxin in wheat was ENB, with incidences between 42% and 78% [66,109,193]. The principal factor influencing ENB frequency in wheat is represented by the species of fungus developed in the growing area of wheat, which are correlated with the climatic conditions until harvest; this was observed for trichothecenes, ZEA, and emerging mycotoxins [67,78,193]. Similar results were obtained for other wheat products, including pasta (ENB incidence of 80%, max. 122.13 μg kg^{-1}) and baby food (ENB incidence of 70%, max. 133.6 μg kg^{-1}) [154,157].

3.4.3. *Fusarium* mycotoxin co-occurrence

With regard to mycotoxin co-occurrence in our study, this was found for 42% of samples (90% of wheat positive samples being contaminated with two to five mycotoxins, and 36% of wheat flour positive samples being contaminated with two or three mycotoxins). Results for co-occurrence are detailed in Table XIV. For wheat, the most frequent was the tertiary contamination, while for wheat flour samples the most frequent was the co-occurrence of two mycotoxins. It was observed that the most frequent was the combination ENA1+ENB+ENB1 (15% of the positive samples), followed by the combination ENB+ENB1 (13% of the positive samples).

Fusarium spp can produce simultaneously different mycotoxins, thus co-occurrence became in the last years an important issue for risk assessment [66,108,109], even if most studies have focused on the occurrence and toxicology of a single mycotoxin. For cereals and products, more than 127 mycotoxin combinations are described in literature. However, only few studies specified the number of co-occurring mycotoxins with the percentage of the co-contaminated samples, as well as the groups found. Research on mycotoxin toxicology suggests that mycotoxins can have antagonist, additive or synergic effects [43,194], therefore, investigation on co-occurrence should be considered a special topic for exposure assessment studies [156].

Table XIV. Mycotoxin co-occurrence data for Romanian wheat and flour positive samples (*n**).

CO-OCCURRENCE	NUMBER OF SAMPLES (FREQUENCY)	
	Wheat (*n = 20)**	**Wheat flour (*n** = 28)**
2 Mycotoxins		
DON+ENB	2 (10)	1 (3.5)
ZEA+ENB	-	2 (7)
NEO+ENB	-	1 (3.5)
ENB+ENB1	1 (5)	5 (18)
3 Mycotoxins		
DON+ENB+ENB1	2 (10)	-
DON+ENB+ZEA	2 (10)	-
ENA1+ENB+ENB1	6 (10)	1 (3.5)
4 Mycotoxins		
DON+ENA1+ENB+ENB1	1 (5)	-
DON+ENB+ENB1+ZEA	1 (5)	-
ENA+ENA1+ENB+ENB1	2 (10)	-
5 Mycotoxins		
ZEA+ENA+ENA1+ENB+ENB1	1 (5)	-

Frequency: % of samples ≥ limit of detection / total positive samples (n*);

In 2012, a research including foodstuffs from Mediterranean area (Marocco, Spain, Italy, Tunisia) concluded that the most frequent is the contamination with one mycotoxin, but 14% of the positive samples were contaminated with two mycotoxins and 18% of the positive samples were contaminated with at least three mycotoxins simultaneously [175].

The results obtained by Rodríguez-Carrasco et al [126] which analyzed ZEA, eight trichothecenes and patulin in fifteen Spanish wheat semolina samples suggest that the most common co-occurrence is the presence of two or three mycotoxins (27%) in different combinations, and only one sample was contaminated with four mycotoxins simultaneously (DON+3AcDON+NIV+HT-2).

Similar values with our results regarding co-occurrence were found by Juan et al [66] when evaluating 26 mycotoxins in durum wheat samples from Italy, they found that 20% of the positive samples were contaminated with two mycotoxins at the same time, while the most frequent co-occurrence was the presence of three mycotoxins (35%).

3.4.4. Geographical distribution

Applying ANOVA Single Factor Test for analysis of variance between mycotoxin levels in the wheat samples retrieved and investigated regions, the results revealed statistically significant difference ($p<0.05$) for DON and ZEA, and no statistically differences ($p>0.05$) were observed for ENA, ENA1, ENB, and ENB1.

Eloquently, the maximum concentrations of ENB1, ENA1, and ENA (510 μg kg^{-1}, 356 μg kg^{-1}, and 140 μg kg^{-1}, respectively) were found in the same wheat sample from Teleorman county (southern Romania), while the highest levels for DON and ZEA (1787 μg kg^{-1}, and 1135 μg kg^{-1}, respectively) were found in the same wheat sample from Braşov region (central Romania). Due to the limited number of wheat samples, to

confirm these ideas a further monitoring research including more samples and more regions for analysis should be considered. Our results confirm those of several previous surveys for trichothecenes and ZEA [68,100,109], while for ENs there is a lack of information concerning their presence in wheat kernels and the regional distribution.

Various studies performed worldwide have described the correlation between mycotoxin contamination and the presence of one or more toxigenic *Fusarium* species, which is highly linked with the environmental conditions. In our study, the frequency of DON and ZEA and the correlation with the region of origin of wheat samples can be explained by microflora composition. Studies done on cereal samples from western and southeastern Romania have demonstrated that *F. graminearum* and *F. culmorum,* which mostly produce DON and ZEA, are the most spread species [75].

Studying wheat samples from different areas and countries [68,100,109] or using logistic models to predict the contamination during wheat anthesis and preharvest period [195], the conclusion of researchers has been that weather parameters have a high influence on mycotoxin content, as it was observed in the present study also. Weather conditions during wheat growth in Braşov and Dâmboviţa counties, in particular precipitations and temperatures during anthesis and harvest, can have a major influence on DON and ZEA levels and frequency. For the two regions mentioned before, the Romanian National Meteorological Administration reported a monthly precipitation deviation against the multiannual mean between 51-75% in May and July 2014, while for the other two regions included in our study (Bihor and Teleorman), this value was normal or it did not exceeded 25%. Moreover, for Braşov and Dâmboviţa counties, mean temperatures for May and July 2014 were with 2 to 4°C less than normal, coupled to very wet days and high humidity [196].

A Croatian survey from 2011 that evaluated DON, ZEA, FUMOs, and T-2 presence in four types of cereals (wheat, maize, barley, oat) from six regions, reported that for DON and ZEA there was no statistically significant difference by region, but the authors mentioned that the lower average contamination levels observed in their study could be explained by the fact that in 2011, throughout the study period, the investigated parts of Croatia had extremely warm and dry weather conditions. This is an important idea, given the fact that high mycotoxin concentrations are usually associated with climate changes, in particular humidity and temperature as the factors most critical for mould formation and mycotoxin production also [100].

Other study performed by Alkadri et al [109] found incidences and levels of DON and ZEA in Syrian samples lower than in Italian ones, and it is mentioned that climatic differences between Syria and Italy could explain this diversity. Regarding the regional distribution of the positive samples for each country, it was observed that the majority of positive samples were from central Italy and southern Syria, while the samples from

southern Italy and central-eastern Syria were negative for all *Fusarium* mycotoxins evaluated.

3.5. Conclusions

Fourteen *Fusarium* mycotoxins were determined in 66 samples of whole unprocessed wheat and white wheat flour from Romania. Detectable values were obtained for DON, ZEA, NEO and ENs. The detected mycotoxin incidences were lower than the values reported in the literature in the last years. The mycotoxin exposure risk in Romania through wheat consumption is low, the ML of DON (1750 μg kg^{-1}) and ZEA (100 μg kg^{-1}) in unprocessed wheat being exceeded in one sample, and four samples, respectively.

It was confirmed that the incidences and levels of mycotoxins are correlated with the region and probably with weather parameters. The wheat cultivated in central and southeastern part of Romania was more contaminated with DON and ZEA than that cultivated in western or southern regions, mostly as a result of weather conditions (high quantities of rainfall, low temperatures) during wheat anthesis and harvest periods. On the other hand, high levels of ENs were present in wheat samples cultivated in the South part of Romania, where normal rainfall and high temperatures were registered.

This research brings an important contribution in terms of occurrence of legislated and non-legislated mycotoxins evaluation in wheat from European countries.

4. Study 2. Climatic conditions influence emerging mycotoxin presence in wheat grown in Romania – A 2-year survey

Study included in the article entitled "Climatic conditions influence emerging mycotoxin presence in wheat grown in Romania – A 2-year survey"

Crop Protection 100 (2017) 124-133

Abstract

The correlation between the occurrence of four enniatins (ENA, ENA1, ENB, and ENB1) and beauvericin (BEA) and the weather parameters during anthesis and preharvest period was studied in 97 wheat samples collected in 2014 and 2015 across three counties from central and south Romania (Braşov, Dâmboviţa, and Teleorman). The highest mean values of ENA (16.1 μg kg^{-1}) and ENB (147.1 μg kg^{-1}) were measured in the samples from Braşov county in the harvest year 2015, whereas for ENA1 and ENB1 the highest means (55.2 μg kg^{-1}, and 108.0 μg kg^{-1}, respectively) were noted in samples from Teleorman county in 2014. Statistically significant differences ($p<0.05$) were identified between ENA1 and ENB1 and the harvest year, coupled with a strong correlation with the weather parameters (ENA1: $r_s=0.8745$ and $r_s=0.9326$; ENB1: $r_s=0.7814$ and $r_s=0.8909$, for temperature and precipitation, respectively). Principal component analysis revealed that the influence of weather parameters on emerging mycotoxin concentrations in wheat samples varied by region. This study showed that the presence and levels of emerging mycotoxins are related to weather parameters from the respective Romanian region.

4.1. Introduction

There are many factors that influence the occurrence of mycotoxins in cereals, e.g., plant substrate, management factors, topographic factors, but weather parameters represent the key determinants for fungal colonization and mycotoxin production [7,8]. In this context, climate changes might influence crop yield and the degree to which the crops are contaminated with mycotoxins or could increase the development of fungi not identified previously within a given area [55].

Romania is the largest country in southeastern Europe, with an agricultural land cover of 62% [29]. The main production area for wheat is the Danube plane in the South of the country, followed by other areas in Transylvania, the northern part of Moldova, and the Banat region [31].

Fusarium species are the most prevalent mycotoxin-producing fungi in cereals in the temperate regions of America, Asia, and Europe [187]. The most frequently identified species of *Fusarium* in Romania are *F. graminearum, F. culmorum, F. oxysporum, F. verticillioides,* and *F. poae*, the last one being recognized to produce emerging mycotoxins [75]. The Romanian climate is transitional temperate-continental, with oceanic influences from the West, Mediterranean modulations from the South-West, and excessive continental effects from the North-East. Climatic variations are modulated by geographical elements, such as the Carpathian Mountains chain and the location of the Black Sea [197].

Analysis of data from the last 50 years shows that there is a general warming signal over Romania, with the air temperature and the number of sunshine hours presenting significantly increasing trends. The precipitation amount remained rather stable. However, extreme meteorological events have been frequently registered, such as excessive dryness, tropical days, substantial rainfall and high humidity [188,198].

Researchers have studied wheat samples from different areas or they used logistic models to predict mycotoxin contamination during wheat anthesis and preharvest period and they concluded that fungi and their specific mycotoxins are climate-dependent; thus, when changes in normal weather occur, mycotoxins are affected [57]. These aspects were studied in particular for trichothecenes and ZEA, whereas for emerging mycotoxins there is a lack of information about their presence in wheat kernels and their regional distribution [53,195].

4.2. Aims

The aims of this work were to determine by LC-QqQ-MS/MS the levels of ENs and BEA in wheat cultivated in three different regions of Romania and two different harvest years and correlate the measured mycotoxin levels with weather conditions during the grain-growing season. To the best of our knowledge, this is the first survey on the presence of emerging mycotoxins in wheat produced in Romania correlated with environmental parameters.

4.3. Materials and methods

4.3.1. Sampling

A total of 97 whole unprocessed wheat samples were obtained to investigate the presence of mycotoxins. Wheat samples were collected during the 2014 (n = 29) and 2015 (n = 68) harvesting season from three Romanian counties: Braşov (BV, n = 8) from central Romania and Dâmboviţa (DB, n = 35) and Teleorman (TR, n = 54) from the South of the country (Fig. 7). The criterion used to include a sample in the study was that wheat must be grown in one of the three regions and it must be dedicated to human consumption. The number of samples per county was influenced by the

frequency of wheat cultivation in that area. Information about growing area (county and city), sowing and harvest periods were considered. Normally, in Romania, wheat is sown in the first ten days of October and it is harvested in the last ten days of July. If it is necessary, wheat is aerated and dried.

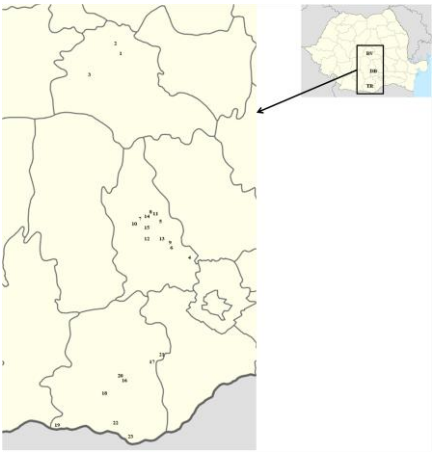


Fig. 7. Regions of Romania from which wheat samples in this study were obtained: **Braşov (BV)**: 1. Cuciulata, 2. Rupea, 3. Şona; **Dâmboviţa (DB)**: 4. Cojasca, 5. Comişani, 6. Dobra, 7. Dumbrava, 8. Matraca, 9. Mărceşti, 10. Mogoşeşti, 11. Nisipuri, 12. Pierşinari, 13. Racoviţa, 14. Ulmi, 15. Văcăreşti; **Teleorman (TR)**: 16. Alexandria, 17. Drăgăneşti-Vlaşca, 18. Furculeşti, 19. Islaz, 20. Nanov, 21. Prunaru, 22. Suhaia, 23. Zimnicea.

Sampling was performed according to the EU guidelines [171] for the official control of legislated mycotoxins for lots of cereals and cereal products of less than 50 tons. Three incremental samples of 1 kg of wheat were collected in the first seven days after harvesting, obtaining an aggregate sample of 3 kg. After homogenization, samples were packed in plastic bags and stored at -20°C in a dark and dry place until analysis. Before the analysis, for all the samples, subsamples of 300 g were milled with a blender and divided into three bulks of 100 g each one.

4.3.2. Extraction

Extraction was performed according to the procedure presented by Stanciu et al [199], using SLE as it was described in section 3.3.2. All experiments were performed in triplicate.

4.3.3. Mycotoxin analysis

The analysis of the five mycotoxins was performed with a LC-QqQ-MS/MS system, using the method presented in the section 2.2.1. Mobile phases were methanol (0.1% formic acid and 5mM ammonium formate) as phase A, and water (0.1% formic acid and 5mM ammonium formate) as phase B. The multi-mycotoxin method was previously validated by Stanciu et al [199].

4.3.4. Climate and weather conditions

For the registration of climatic parameters, all time series were extracted from the climatic database of Romanian National Meteorological Administration (Meteo Romania). Gridded data spatially interpolated at a resolution of 1 km x 1 km was used for the mean annual precipitation and mean annual temperature (1961-2000), and the actual mean precipitation and mean air temperature during anthesis (months of April and May) and preharvest (months of June and July) periods for each year of our study. Table XV presents the basic information about the regions included in our research. This information reveals the short-term humidity during anthesis and grain-maturing seasons across the studied areas as this has a superior impact on *Fusarium* infestation.

4.3.5. Statistical analyses

All statistical analyses were fulfilled to identify environmental variables that control emerging mycotoxin presence in wheat samples. All results were included for statistical analysis, assuming for negative samples a value of 0 μg kg^{-1}. Analysis of variance (ANOVA single factor test) was used to assess the significance of the differences between the determined mycotoxin concentrations. All *p*-values of < 0.05 (statistical significance of 95%) were considered to be statistically significant. Relationships between mycotoxin concentrations and environmental parameters (mean temperature from April to July and total precipitation from April to July, respectively) were calculated with the Spearman correlation. Statistical analysis was performed using SPSS software, version 22.0 (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA).

Principal component analysis (PCA) was performed on all available observations (N = 97) after performing unit variance scaling and mean centering of all variables (K = 20, sampling coordinates: village, county, year, GPS location; mycotoxin contamination levels; and local short- and long-term weather records) using Simca version 13.0.3.0 software (MKS Data Analytics Solutions, Sweden).

Table XV. Geographical coordinates and long term climatic data (1961-2000) of the wheat-producing localities studied.

COUNTY	LOCALITY	GEOGRAPHICAL COORD.		PRECIPITATION (mm)					TEMPERATURE (°C)				
		Lon, °E	Lat, °N	Annual	Monthly				Annual	Monthly			
					April	May	June	July		April	May	June	July
Braşov	Cuciulata	25.271875	45.943797	49.7	48.5	77.3	91.3	91.4	7.37	8.25	13.16	16.21	17.60
	Rupea	25.211767	46.032262	49.8	48.8	78.0	92.2	89.7	7.70	8.60	13.53	16.57	17.96
	Şona	25.041543	45.849336	50.4	49.7	79.1	93.5	95.3	7.76	8.71	13.69	16.78	18.22
Dâmboviţa	Cojasca	25.847129	44.726520	49.3	47.1	68.5	76.1	73.4	10.31	10.94	16.39	20.10	21.78
	Comişani	25.576043	44.869966	52.2	49.7	72.7	84.8	84.4	10.09	10.56	15.89	19.55	21.20
	Dobra	25.717644	44.789543	50.7	48.3	70.2	78.8	77.8	10.18	10.75	16.16	19.85	21.50
	Dumbrava	25.436676	44.889361	53.0	52.5	75.1	88.3	87.0	9.99	10.39	15.65	19.31	21.00
	Mărceşti	25.714372	44.804671	50.8	48.3	70.1	80.7	78.0	10.17	10.73	16.14	19.82	21.47
	Matraca	25.517046	44.896309	52.7	51.1	73.6	86.7	85.8	10.06	10.48	15.76	19.42	21.09
	Mogoşeşti	25.396163	44.882983	52.8	52.3	75.1	88.2	86.7	9.94	10.32	15.58	19.24	20.92
	Nisipuri	25.534244	44.910040	53.1	51.1	75.4	88.7	86.1	10.07	10.49	15.77	19.42	21.08
	Pierşinari	25.492368	44.799525	50.9	49.6	71.1	80.4	81.5	10.05	10.55	15.91	19.60	21.23
	Racoviţa	25.617344	44.843071	51.6	49.7	71.4	82.7	81.0	10.10	10.61	15.96	19.64	21.28
	Ulmi	25.498538	44.896070	52.6	51.1	73.5	86.7	85.7	10.04	10.46	15.74	19.40	21.07
	Văcăreşti	25.493359	44.854926	51.9	51.1	72.3	84.3	83.7	10.04	10.48	15.80	19.47	21.13
Teleorman	Alexandria	25.313278	43.984825	41.8	40.7	54.2	62.4	65.7	10.98	11.63	17.11	20.93	22.78
	Drăgăneşti-Vlaşca	25.597079	44.094176	43.9	44.2	56.9	63.2	60.8	10.88	11.56	17.05	20.82	22.63
	Furculeşti	25.149250	43.871337	42.4	40.9	54.8	61.1	63.3	10.97	11.73	17.17	20.90	22.71
	Islaz	24.767518	43.721333	42.2	39.1	54.2	56.2	57.0	11.33	12.29	17.70	21.33	23.09
	Nanov	25.291044	43.991133	41.6	40.8	52.9	62.5	65.6	10.99	11.65	17.13	20.94	22.80
	Prunaru	25.677218	44.124363	44.3	44.8	57.3	64.0	59.3	10.91	11.61	17.10	20.84	22.64
	Suhaia	25.238840	43.745610	42.7	41.2	54.9	59.9	62.6	11.14	11.98	17.39	21.08	22.83
	Zimnicea	25.370962	43.669306	43.0	41.8	54.9	59.2	61.0	11.31	12.19	17.59	21.27	22.98

4.4. Results and discussion

The purpose of the study was to investigate if climatic conditions in the three areas were related to fungal growth and mycotoxin production. Data structuring and inter-variable relationships (correlations) were identified by the obtained two-component PCA models. This study is the first to analyze emerging mycotoxins in Romanian wheat samples with the aim to correlate the results with regional and annual weather parameters. Table XVI summarizes the results of emerging mycotoxin analysis for wheat samples according to the geographical origin and harvest year.

Table XVI. Summary of the mycotoxin levels found in Romanian wheat samples distributed by harvesting area and year. *p*-Values of analysis of variance (ANOVA single factor test) between regions and harvesting period.

ANALYTE	PARAMETERS	REGION/YEAR (number of samples)						*p*-VALUE	
		Braşov		Dâmbovița		Teleorman			
		2014 (*n*=2)	2015 (*n*=6)	2014 (*n*=7)	2015 (*n*=28)	2014 (*n*=20)	2015 (*n*=34)	Region	Year
BEA	Incidence	0	1	0	0	0	0	-	-
	Frequency	0	17	0	0	0	0		
	Mean[#] (µg kg^{-1})	n.q.	9.1	n.q.	n.q.	n.q.	n.q.		
	Mean[§] (µg kg^{-1})	n.q.	1.5	n.q.	n.q.	n.q.	n.q.		
	Max. (µg kg^{-1})	n.q.	9.1	n.q.	n.q.	n.q.	n.q.		
ENA	Incidence	0	1	0	1	6	1	0.4593	0.1946
	Frequency	0	17	0	4	30	3		
	Mean[#] (µg kg^{-1})	n.q.	96.6	n.q.	81.6	69.6	55.2		
	Mean[§] (µg kg^{-1})	n.q.	16.1	n.q.	2.9	13.9	1.6		
	Max. (µg kg^{-1})	n.q.	96.6	n.q.	81.6	140	55.2		
ENA1	Incidence	0	3	1	3	9	7	0.2394	0.0077*
	Frequency	0	50	14	11	45	21		
	Mean[#] (µg kg^{-1})	n.q.	65	26.1	42.5	122.7	25.2		
	Mean[§] (µg kg^{-1})	n.q.	32.5	3.7	4.6	55.2	5.2		
	Max. (µg kg^{-1})	n.q.	150.4	26.1	94	356	68.3		
ENB	Incidence	2	6	7	12	10	7	0.2034	0.3162
	Frequency	100	100	100	43	50	21		
	Mean[#] (µg kg^{-1})	75.7	147.1	96.9	162.2	159.4	110.5		
	Mean[§] (µg kg^{-1})	75.7	147.1	96.9	69.5	79.7	22.7		
	Max. (µg kg^{-1})	130.7	357	196	815	407.2	616		
ENB1	Incidence	1	3	3	6	10	6	0.5103	0.0039*
	Frequency	50	50	43	21	50	18		
	Mean[#] (µg kg^{-1})	35.6	114	51.6	98.6	216	51.4		
	Mean[§] (µg kg^{-1})	17.8	57	22.1	21.1	108	9.1		
	Max. (µg kg^{-1})	35.6	196.5	82	251	510	183		
ENs	**Overall incidence**	**2**	**6**	**7**	**12**	**11**	**9**	**-**	**-**
	Overall frequency	**100**	**100**	**100**	**43**	**55**	**26**		

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; n.q.: not quantified; [#]: mean value for samples ≥ limit of quantification (LQ); [§]: mean value for all samples, assuming a zero value for samples ≤ LQ; *: statistically significant different ($p < 0.05$).

4.4.1. Emerging mycotoxin occurrence data

The presence of ENA, ENA1, ENB, ENB1, and BEA in 97 samples of wheat cultivated in three regions of Romania (BV, DB, and TR) in two different harvest years (2014 and 2015) was evaluated. Results showed that 48% of the wheat samples presented detectable levels of ENs or BEA, 47 samples being positive for at least one emerging mycotoxin. ENB was the most often detected (45%), followed by ENB1 (30%), ENA1 (24%), and ENA (9%) (Table XVI). The highest mean (61.9 μg kg^{-1}) and maximum concentration (815 μg kg^{-1}) were also for ENB. Only one wheat sample from BV (2014) was positive for BEA, being contaminated with 9.1 μg kg^{-1}. Simultaneous contamination was observed in 31% of the samples analyzed (64% of the positive samples being contaminated with two to four emerging mycotoxins).

Our results are in agreement with those of other European studies that reported ENB as the most frequent emerging mycotoxin in wheat. Alkadri et al [109], Juan et al in two studies from 2013 [108] and 2016 [66], and Bryła et al [76] found that 42%, 28%, 78%, and 94% of the wheat samples analyzed, respectively, were contaminated with ENB.

4.4.2. Growing region and harvest year

A higher occurrence of emerging mycotoxins was observed in the harvest year 2014 (20 of 29 wheat samples, 69%) than in 2015 (27 of the 68 wheat samples, 40%). Moreover, it was demonstrated that the incidence of emerging mycotoxins in wheat samples varied geographically (8, 19, and 20 positive samples in BV, DB, and TR, respectively). Wheat samples from BV and TR presented the highest mean levels for the detected mycotoxins: samples from BV for ENA (16.1 μg kg^{-1}), and ENB (147.1 μg kg^{-1}) in the harvest year 2015, and samples from TR for ENA1 (55.2 μg kg^{-1}), and ENB1 (108.0 μg kg^{-1}) in 2014 (Table XVI). In 2014, wheat samples from TR presented the highest levels for ENA, ENA1, ENB, and ENB1 (140, 356, 407.2, and 510 μg kg^{-1}, respectively). Interestingly, three of the highest concentrations (ENA, ENA1, and ENB1) were found in the same sample from this county. However, in 2015, the highest levels for the analyzed mycotoxins were found in samples from different regions: ENA and ENA1 (96.6 and 150.4 μg kg^{-1}, respectively) in the same sample from BV, and ENB and ENB1 (815 and 251 μg kg^{-1}, respectively) in the same sample from DB (Table XVI).

When mycotoxins co-occurred, in DB the predominant condition was contamination with two mycotoxins (incidence of 29% and 11% in 2014 and 2015, respectively), whereas in TR the simultaneous presence of three mycotoxins was the frequent situation (incidence of 30% and 9% in 2014 and 2015, respectively). In BV, similar incidences for the co-occurrence of two, three, or four mycotoxins were observed (Fig. 8). The co-occurrence of ENs could be explained by the simultaneous biosynthesis of these mycotoxins produced by the same fungi, particularly *Fusarium graminearum, F. avenaceum* and *F. poae* that develop when unfavourable climatic conditions are present [183].

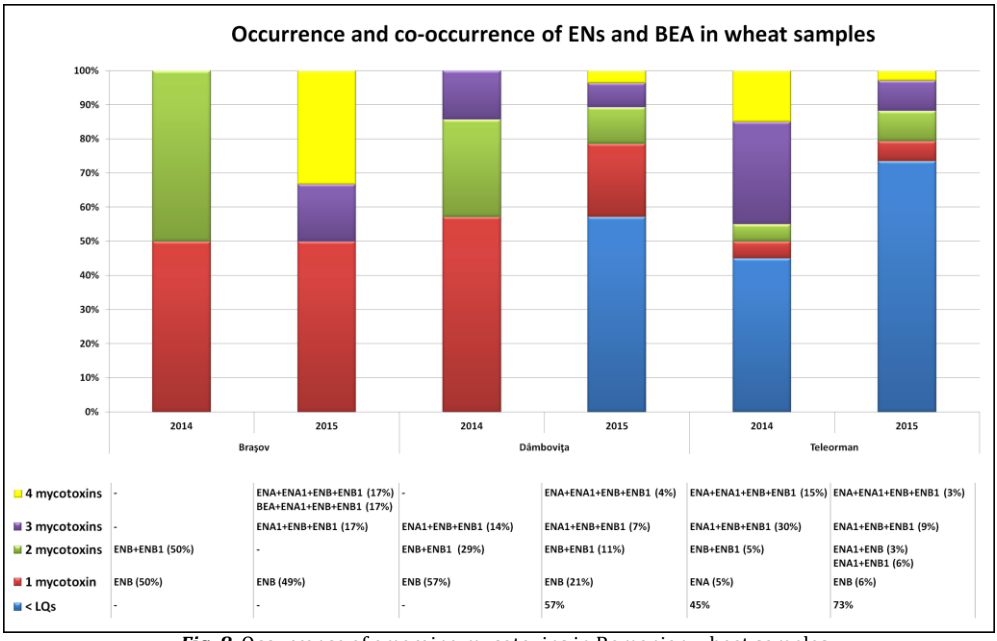


Fig. 8. Occurrence of emerging mycotoxins in Romanian wheat samples harvested in 2014 and 2015.

To interpret observations (samples) in terms of variables (mycotoxin content), the PCA scores and loadings were co-charted in the biplots shown in Fig. 2, where samples with various degrees of mycotoxin infestation analyzed by county and/or harvest years are presented. Samples situated near variables are high in these variables. Regardless of the county (Fig. 9 a, b, c), in all three cases, the first principal component (PC1) describes the load in ENs, whereas the second principal component (PC2) is related to the BEA content. Grouping samples by the year of harvest, in both cases, single-component PCA class models indicate the samples with highest mycotoxin content (Fig. 9 d, e). The color coding shows the magnitude and proportion of samples per county of origin, with the highest mycotoxin contamination (highest in TR in 2014 and DB in 2015).

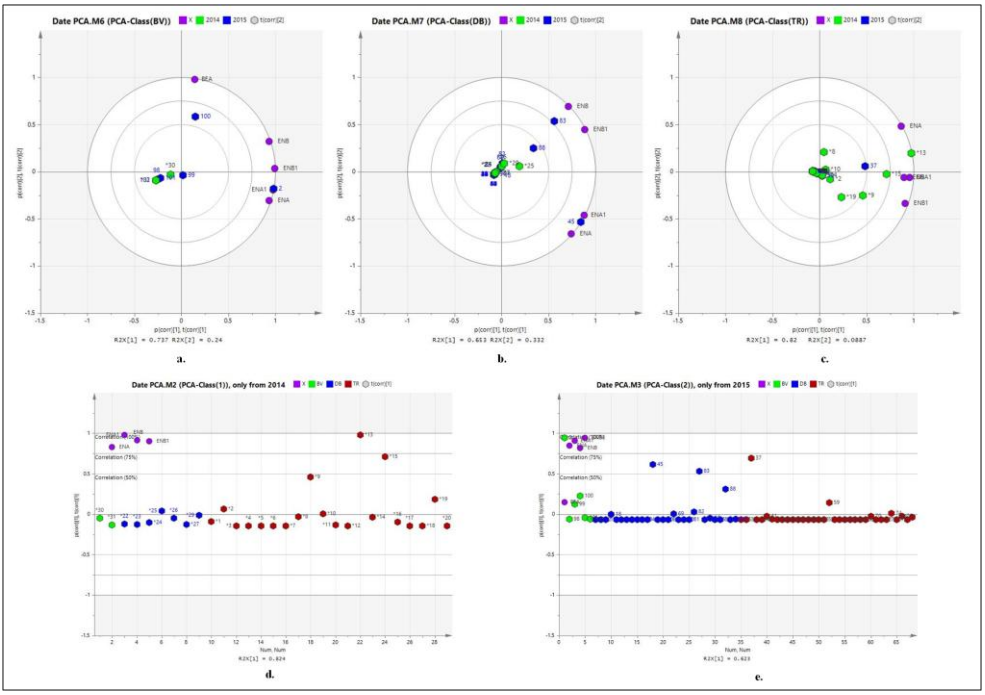


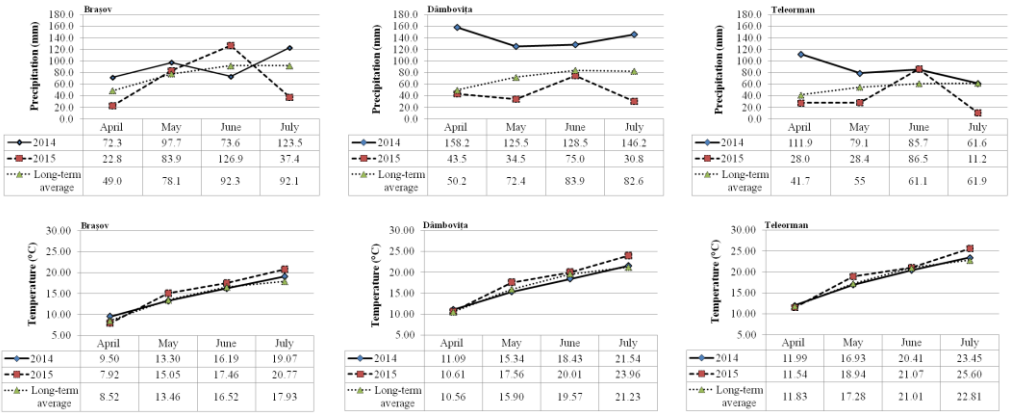
Fig. 9. Biplot loadings and scores for the principal component analysis (PCA) class models obtained in function of the county of origin (**a.** Brașov, **b.** Dâmbovița, **c.** Teleorman) and year of harvest (**d.** 2014, **e.** 2015).

Various studies performed worldwide have described the correlation between mycotoxin contamination in wheat and the growing area or harvest year. One of these studies is a recent Polish study [76] that evaluated 99 wheat samples for the occurrence of 26 mycotoxins and reported that there was no difference for ENB and ENB1 incidence in wheat samples from North, West and South-East of Poland, but a higher occurrence of ENA and ENA1 was observed in the southeastern regions of Poland than in the northern and western regions. Another study performed by Alkadri et al [109] indicated a lower incidence of emerging mycotoxins in Syrian wheat samples (BEA was the most frequent, 21%) than in the Italian ones (ENB was the most common, 49%), although both the countries are in the Mediterranean area. The authors explained this diversity through climatic conditions. Syria has an arid and dry climate, which is very hot in the summer and cold in winter, whereas the climate of Italy is mainly temperate and slightly varies according to the areas; the northern Italian regions have warm humid summers, which makes them more susceptible to mycotoxin contamination.

4.4.3. Climatic conditions correlation

Fusarium mycotoxin development seems in general to be stimulated by a narrow window of climatic factors [53]. To explain the possible differences between the levels of ENs by harvest year or by growing region, climatic conditions in the three

regions were analyzed. The air temperature and precipitation were monitored at each location from April to July in both 2014 and 2015. For each county, the average monthly temperature (°C) and average monthly precipitation (mm) were calculated, including only the locations where wheat samples were collected for the analysis (Fig. 10).



Brașov	April	May	June	July
2014	72.3	97.7	73.6	123.5
2015	22.8	83.9	126.9	37.4
Long-term average	49.0	78.1	92.3	92.1

Dâmbovița	April	May	June	July
2014	158.2	125.5	128.5	146.2
2015	43.5	34.5	75.0	30.8
Long-term average	50.2	72.4	83.9	82.6

Teleorman	April	May	June	July
2014	111.9	79.1	85.7	61.6
2015	28.0	28.4	86.5	11.2
Long-term average	41.7	55	61.1	61.9

Brașov	April	May	June	July
2014	9.50	13.30	16.19	19.07
2015	7.92	15.05	17.46	20.77
Long-term average	8.52	13.46	16.52	17.93

Dâmbovița	April	May	June	July
2014	11.09	15.34	18.43	21.54
2015	10.61	17.56	20.01	23.96
Long-term average	10.56	15.90	19.57	21.23

Teleorman	April	May	June	July
2014	11.99	16.93	20.41	23.45
2015	11.54	18.94	21.07	25.60
Long-term average	11.83	17.28	21.01	22.81

Fig. 10. Monthly precipitation levels (mm) and temperatures (°C) during wheat anthesis and the preharvest period in Brașov, Dâmbovița, and Teleorman, respectively.

In the three regions during anthesis (April and May), higher values of precipitation were registered in 2014 (between 72.3 and 150.5 mm per month) than in 2015 (between 22.8 and 89.3 mm). Moreover, in the late preharvest period (July) higher precipitation was noted in 2014 (between 58.5 and 151.5 mm) than in 2015 for the same period (from 7.7 to 46.5 mm). Furthermore, lower temperatures were registered in 2014 from April to July in all regions studied compared to the corresponding period in 2015 (Fig. 10). It could be considered that high precipitation (at least 20 mm more than long-term monthly precipitation) during wheat anthesis or preharvest period increase the risk of EN occurrence, particularly ENA1, ENB, and ENB1. Thus, it could be stated that wet weather could favor emerging mycotoxin development, as it was demonstrated previously by other authors for *Fusarium* toxins in general [67,200].

The highest mean concentrations for ENA and ENB were observed in the samples from BV in 2015; during this period, high quantities of precipitation were registered at the end of anthesis (83.9 mm in May 2015) and beginning of kernel growth (126.9 mm in June 2015). The highest mean concentrations for ENA1 and ENB1 were found in the wheat samples from TR in 2014; in this region, the average monthly precipitation was 79.1 and 85.7 mm per month during May and June 2014, respectively (Fig. 10).

The sample most contaminated in 2014 was from Islaz (TR) and it presented the highest concentrations of ENA, ENA1, ENB1 and the second highest value of ENB. This area was characterized by high precipitation during anthesis (with a total precipitation of 201 mm in April and May 2014), and by the highest monthly mean temperatures during the four months monitored (12.38, 17.16, 20.80, and 23.57°C from April to July 2014, respectively) from all regions included in the study. In 2015, the highest levels for ENA and ENA1 were found in the same wheat sample from Şona (BV), while the highest levels for ENB and ENB1 were found in the same sample from Dobra (DB). From April to July 2015, Şona region was characterized by high precipitation during final anthesis and beginning of grain growth (with 89.3 mm in May and 122.0 mm in June), followed by a rapid increase of temperature in July (monthly mean of 21.00°C) coupled with low precipitation (monthly mean of 26.7 mm). On the other hand, for the region of Dobra where the highest concentrations of ENB and ENB1 were registered, lower precipitation than normal during the four months monitored (monthly mean of 44.2, 41.8, 63.8, and 30.7 mm from April to July 2015, respectively) and a warm July (mean average of 24.25°C) were reported.

The analysis of variance revealed that no statistically significant difference was present by region for all ENs and by harvest year for ENA and ENB. Statistically significant differences ($p < 0.05$) were identified for ENA1 and ENB1 by harvest year (Table XVI). Notably, calculating the absolute values of Spearman's correlation coefficients (r_s) for ENs and weather parameters (mean temperature and total precipitation during anthesis and preharvest period), a strong ($0.6 < r_s < 0.79$) or very strong ($0.8 < r_s < 1.0$) correlation was registered for ENA1 (r_s=0.8745 for temperature and r_s=0.9326 for precipitation) and ENB1 (r_s=0.7814 and r_s=0.8909, respectively).

Nevertheless, univariate data analysis for correlating climatic conditions and mycotoxin contamination might be regarded a tedious process and prone to a tendentious outcome; therefore, the same study was performed using a multivariate approach. All available climate parameters (annual precipitation, mean annual temperature, and actual mean precipitation, mean air temperature recorded for the months of April, May, June and July in 2014 and 2015), along with the mycotoxin content of each sample, were fed into a PCA model as X-variables. No geographical coordinates were considered as variables to avoid inducing artificial similarities or dissimilarities between the samples merely based on the location of sampling.

The obtained PCA class models (Fig. 11) explain between 88.9% and 99% of the total variation within the X-block, where in all cases, the first component corresponds to the variations in climate parameters and the second component describes the level of mycotoxin contamination in the samples.

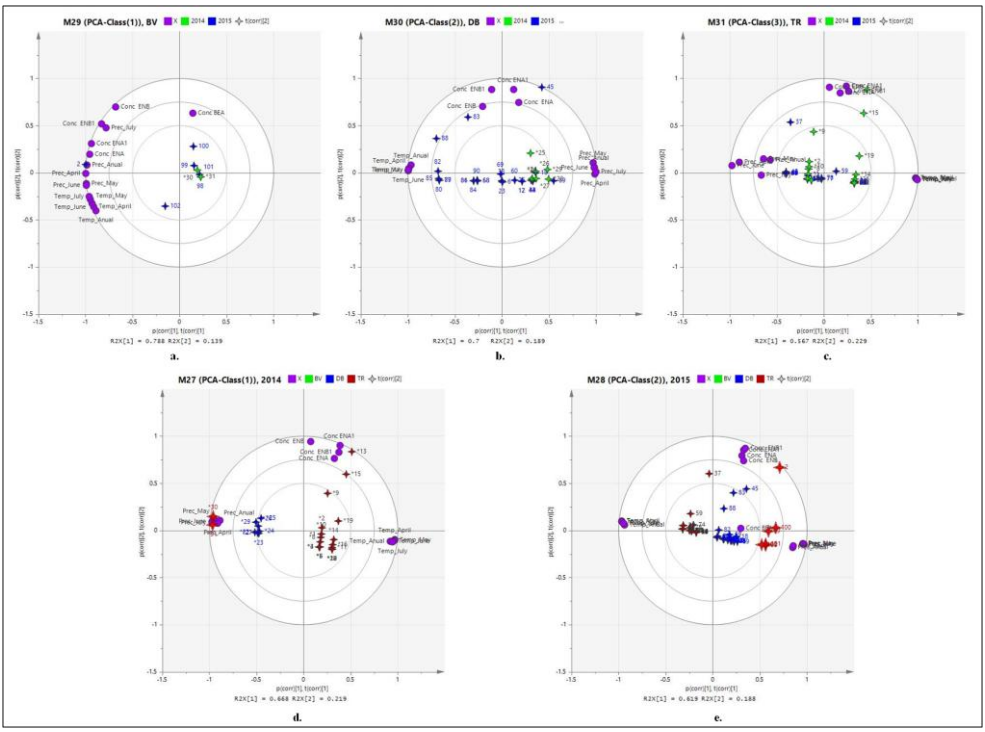


Fig. 11. Biplot loadings and scores for the principal component analysis (PCA) class models considering climate parameters obtained in function of the county of origin (**a.** Braşov, **b.** Dâmboviţa, **c.** Teleorman) and year of harvest (**d.** 2014, **e.** 2015).

In the case of the samples collected in BV (Fig. 11 a), a positive correlation between the levels of precipitation, long-term recorded annual and mean temperatures and the EN levels, and a negative correlation between the climate parameters and BEA levels were observed along PC1 (78.8% of explained variation). The second component (13.9% of explained variation) reveals a positive correlation between the levels of ENs (especially ENB and ENB1) and the recorded levels of precipitation in the month of July and a negative correlation with the recorded temperatures and levels of precipitation in June and May. Therefore, lower average temperatures from anthesis to preharvest period associated with higher levels of precipitation in late preharvest period seem to be related to the increase of ENs, mostly ENB and ENB1.

For DB (Fig. 11 b) and TR (Fig. 11 c), the levels of precipitation and recorded temperatures were inversely correlated along PC1 (70% and 56.7% of the described variation, respectively). For samples originating from DB, the levels of ENA and ENA1 seem to be more favored by higher levels of precipitation and ENB and ENB1 by higher

temperatures (Fig. 11 b). Although the levels of precipitation in May and the long-term annual temperature seem to have the most influence on the levels of ENs (no BEA detected in these samples), none of the studied climatic variables' effects clearly stand out.

For the samples collected from TR (Fig. 11 c), increased levels of ENs are supported by higher levels of long-term annual and mean recorded precipitation in May, along with lower levels of annual and mean temperatures. Most of the samples contaminated with ENs were obtained in 2015, where increased levels of mycotoxins appear to be related with higher overall long-term and mean temperatures (especially during July) combined with lower levels of precipitation (mostly during April and June).

PCA class models obtained according to the year of harvest demonstrate the same correlations as described for individual counties, but data patterning in function of county of origin is much more obvious. Thus, the PCA model for samples harvested in 2014 (three component PCA model, Fig. 11 d) indicates a strong grouping of the observations related to the regional and local climate particularities of each county.

The same grouping appears for the five-component PCA model obtained for samples harvested in 2015 (Fig. 11 e), where PC1 (61.9% of explained variability) seems to capture the climate particularities of the three counties and PC2 (18.8% of explained variability) describes the level of EN contamination of the samples. Probably, the sought structured information related to the fine interactions of the weather parameters that can promote higher levels of mycotoxins in the analyzed wheat samples was present in the remaining three components.

The information concerning the presence of emerging mycotoxins in foodstuffs must be taken into account also in the context of the climatic change. Several recent studies have dealt with the climatic changes occurring in Romania, regarding not only the increase of precipitation and air temperature, but also the terrestrial stilling, seasonal or annual modifications in relative humidity, cloud cover, number of sunshine hours, snow-pack decrease, changes in streamflow regime, evapotranspiration, and drought [29]. Moreover, for southern, central, and western Romania, medium to highest negative potential environmental impact of climate change is estimated by the European Environment Agency until the end of the 21st century [201]. Thus, food safety could be affected, including fungi and mycotoxin presence [202].

4.5. Conclusions

In this monitoring study, analyzing 97 Romanian whole wheat samples, it has been observed that the presence of emerging mycotoxins could be correlated with climatic parameters in the cultivation area. Statistically significant differences were identified for ENA1 and ENB1 by harvest year (2014 and 2015), together with a strong

positive correlation with mean temperature (between 15.1 and 18.5°C) and total precipitation (between 240 and 570 mm) from anthesis to preharvest period (from April to July). It has been demonstrated that high precipitation (with at least 20 mm more than long-term normal monthly precipitation) during the months of May and July promote high moisture in wheat kernels, increasing EN occurrence, co-occurrence and mean concentrations, particularly ENA1, ENB, and ENB1. However, PCA class models revealed a different influence of climatic parameters for each region analyzed, possibly because of the additional contribution of geographic, topographic, biologic, agriculture related factors or the preceding crop on mycotoxin contamination.

Our results contribute to the assessment of the global risk posed by emerging mycotoxins and their accumulation in wheat from different regions with distinctive climatic conditions. The frequent occurrence of emerging mycotoxins in Romanian wheat warrants additional studies to investigate their presence in wheat from different Romanian areas, to design a map of mycotoxin incidence according to the weather conditions, and to establish the correct measures decreasing mycotoxin presence in safe food.

5. Study 3. Agricultural practice influence emerging mycotoxin presence in wheat grown in Romania – A 2-year survey

Study included in the article entitled "Presence of Enniatins and Beauvericin in Romanian Wheat Samples: From Raw Material to Products for Direct Human Consumption"

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Abstract

In this study, a total of 244 wheat and wheat-based products collected from Romania were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in order to evaluate the presence of four enniatins (ENs; i.e., ENA, ENA1, ENB, and ENB1) and beauvericin (BEA). For the wheat samples, the influence of agricultural practices was assessed, whereas the results for the wheat-based products were used to calculate the estimated daily intake of emerging mycotoxins through wheat consumption for the Romanian population. ENB presented the highest incidence (41% in wheat and 32% in wheat-based products), with its maximum levels of 815 μg kg^{-1} and 170 μg kg^{-1} in wheat and wheat-based products, respectively. The correlation between the concentrations of ENB and ENB1 in wheat grain samples and farm practices (organic or conventional) was confirmed statistically ($p<0.05$). This is the first study that provides comprehensive information about the influence of agricultural practice on emerging Fusarium mycotoxin presence in Romanian wheat samples and the estimated daily intake of ENs and BEA present in wheat-based products for human consumption commercialized in Romania.

5.1. Introduction

Wheat grains are vulnerable to infections by a wide variety of plant pathogens. Filamentous fungi are a main safety concern due to the production of mycotoxins accumulated in grains as secondary metabolites [52]. The use of different agricultural practices, such as conventional or organic, could play an important role in the growth of various fungi and the biosynthesis of mycotoxins [22]. Production of organic wheat implies a management system that avoids the use of synthetic fertilizers, pesticides, herbicides, or genetically modified organisms. Due to the lack of synthetic fungicides in organic production, speculation has arisen about a possible higher contamination with different mycotoxins in this farming procedure in comparison with conventional practices. Most of the studies performed to clarify this hypothesis were based on trichothecene, fumonisin, or aflatoxin concentrations, and literature presents

heterogeneous statistic results with no general conclusion established. Therefore the issue remains an open area for research [203].

Mycotoxins continue to attract worldwide attention because of their various toxic effects on human health, thus different MLs in foodstuffs and TDIs for some mycotoxins such as aflatoxins (AFLAs), ochratoxin A (OTA), ZEA, DON, FUMOs, and patulin have been set [161,204]. However, the emerging *Fusarium* mycotoxins, including ENs, BEA, FP, and MON [152], are not legislated yet, despite their increasing detection influenced by both increasing frequency and sensitivity of the analysis methods [205].

ENs (ENA, ENA1, ENB, and ENB1) are biosynthesized especially by *Fusarium* sp. (*F. acuminatum*, *F. avenaceum*, *F. equiseti*, *F. langsethiae*, *F. lateritium*, *F. poae*, *F. sambucium*, *F. sporotrichioides*) [183], and BEA is produced mostly by *F. proliferatum*, *F. subglutinans*, *F. verticillioides*, or *F. oxysporum* [71], under the influence of several factors, such as geographic, topographic, climatic, biologic, or management related factors, additional to the preceding crop. Different studies have provided evidence that the incidence of ENB and ENB1 has reached high values in wheat and wheat products like flour, breakfast cereals, pasta or pizza, but the influence of farming systems on emerging mycotoxins presence has been less studied and it is still ambiguous.

The occurrence and toxicity of ENs and BEA are under evaluation, and recently EFSA presented a scientific opinion about these mycotoxins [148]. Results of different studies on the occurrence of ENs and BEA in wheat grains or products from Finland, Norway, Germany, Sweden, The Netherlands, and several Mediterranean countries were presented, but no study from Romania was included in this report. Analyzing the present information about the emerging mycotoxins, EFSA had the following recommendations: the use of liquid chromatography tandem mass spectrometry (LC-MS/MS) methods to analyze ENs and BEA in food and feed, including prepared grain based products; monitoring the co-occurrence with other *Fusarium* toxins and the possible combined effects; and new research on *in vitro* and *in vivo* genotoxicity [148].

5.2. Aims

Due to the scarce information about emerging mycotoxin presence in Romanian wheat and its products, the aims of this work were: (i) to survey the levels of ENs and BEA in Romanian wheat applying a LC-QqQ-MS/MS method; (ii) to evaluate the differences regarding occurrence, co-occurrence, and concentration levels between organic and conventional crops. To the best of our knowledge, this is the first survey concerning the presence of emerging mycotoxins in wheat from Romania by agricultural practice and varieties.

5.3. Materials and methods

5.3.1. Sampling

A total of 133 unprocessed wheat samples were collected during the 2014 and 2015 harvest seasons from different Romanian counties in order to investigate the presence of mycotoxins. The samples were divided by agricultural practice as following: conventional (n = 106) and organic (n = 27). Information about growing area, type of agriculture, and wheat variety was collected. Wheat samples classified as conventional were divided by variety: Alcantara (n = 1), Alex (n = 1), Altigo (n = 1), Arezzo (n = 3), Arieşan (n = 7), Balaton (n = 2), Boema (n = 4), Dropia (n = 1), Exotic (n = 1), Felix (n = 1), Glosa (n = 10), Hyfi (n = 1), ITC-20 (n = 1), Izvor (n = 7), Kontrast (n = 1), Litera (n = 5), Lukulus (n = 1), Miranda (n = 2), Ponomicus (n = 2), Solehio (n = 1), Soxenos (n = 1), Urbanus (n = 1), other types (n = 51). The criterion used to include a wheat sample in the study was that wheat must be produced for human consumption. The number of wheat samples for each type (organic or conventional) and each variety was influenced by the frequency of cultivation.

Sampling was performed according to the European Union guidelines [171]. Three incremental samples of 1 kg unprocessed wheat were collected in the first seven days after harvesting, obtaining an aggregate sample of 3 kg total weight. All samples were milled to a fine powder using a laboratory mill. After homogenization, the samples were packed in plastic bags and stored at −20°C in a dark and dry place until analysis. Three replicates for each sample were weighed for analysis.

5.3.2. Extraction

Extraction was performed according to the procedure presented by Stanciu et al [199], using SLE as it was described in section 3.3.2. All experiments were performed in triplicate.

5.3.3. Mycotoxin analysis

The analysis of the five mycotoxins was performed with a LC-QqQ-MS/MS system, using the method presented in the section 2.2.1. Mobile phases were methanol (0.1% formic acid and 5mM ammonium formate) as phase A, and water (0.1% formic acid and 5mM ammonium formate) as phase B. The multi-mycotoxin method was previously validated by Stanciu et al [199].

Calculations were performed using SPSS software (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA), version 22.0 with a statistical significance set at 95% (p = 0.05). ANOVA single factor test was used to assess the significance of the differences of mycotoxin concentrations determined.

5.4. Results and discussion

5.4.1. Presence of emerging mycotoxins in wheat

A LC-QqQ-MS/MS method was applied in order to evaluate the presence of ENA, ENA1, ENB, ENB1, and BEA in a total of 133 organic and conventional Romanian wheat samples. Sixty-one wheat samples (46%) presented detectable levels of ENs or BEA. Levels between LDs and LQs were found in six situations: two for ENA, one for ENB, one for ENB1, and two for BEA. ENB was the most detected (55/133, 41%), followed by ENB1 (36/133, 27%), ENA1 (27/133, 20%), ENA (11/133, 8%), and BEA (3/133, 2%). Simultaneous contamination was observed in 28% of the samples analyzed (61% of the wheat positive samples were contaminated with two to four emerging mycotoxins). The means of only positive samples (above LQs) were 65.8, 67.6, 135, and 116 μg kg^{-1} for ENA, ENA1, ENB, and ENB1, respectively. Only one wheat sample presented quantifiable levels of BEA (9.1 μg kg^{-1}).

In general, the presence of emerging mycotoxins in Romanian wheat samples found in our study is in agreement with those of other European studies that reported ENB as the most frequent emerging mycotoxin in wheat. Alkadri et al [109] found that 42% of the Italian wheat samples analyzed were contaminated with ENB at levels between 3.1 and 87.2 μg kg^{-1}. On the other hand, recent studies presented higher values for occurrence and concentrations of emerging mycotoxins in wheat. For example, a study carried out by Juan et al [66] reported that 78% of the wheat samples were positive for ENB, in concentrations ranging from 23 to 1826 μg kg^{-1}. Similar results were presented by Bryła et al [76] when evaluating mycotoxin occurrence in wheat samples from Poland; they found that 94% of the samples were contaminated with ENB at concentrations between 1 to 1981 μg kg^{-1}.

5.4.2. Conventional *versus* organic wheat

Concerning the organic wheat samples, 70% of the samples (42 samples) were contaminated with at least one emerging mycotoxin, more than 40% of the conventional positive wheat samples (19 samples). As can be seen in Table XVII, ENB was the most frequent mycotoxin in both types of wheat. The incidences of ENA, ENA1, ENB, and ENB1 were higher for the samples of organic wheat (19%, 30%, 70%, and 41%, respectively) than the corresponding values for conventional wheat (6%, 18%, 34%, and 24%, respectively). Mean values for the four ENs were higher for the organic samples (7.0, 23.7, 102, and 66.0 μg kg^{-1} for ENA, ENA1, ENB, and ENB1, respectively) (Table XVII). Fig. 12 presents the chromatogram of a conventional wheat sample contaminated with four ENs. On the other hand, detectable levels of BEA were found only in three conventional wheat samples at low concentrations, two of them being between the LD and LQ for BEA.

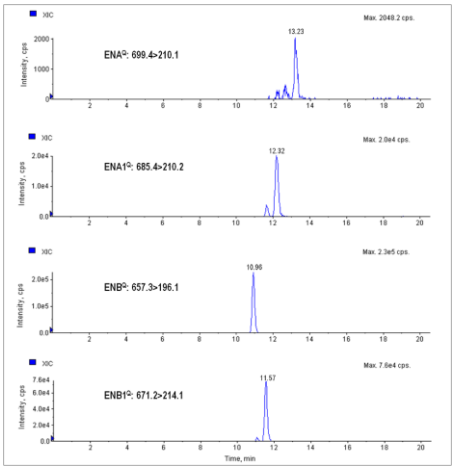


Fig. 12. SRM chromatograms of a conventional wheat sample contaminated simultaneously with ENA (140 µg kg^{-1}), ENA1 (356 µg kg^{-1}), ENB (394 µg kg^{-1}), and ENB1 (510 µg kg^{-1}).

Table XVII. Summary of EN concentrations found in Romanian wheat samples distributed by agricultural practice.

ANALYTE	PARAMETER	CONVENTIONAL (n = 106)	ORGANIC (n = 27)	TOTAL (n = 133)
BEA	Incidence	3	0	3
	LD-LQ	2	0	2
	Frequency	2	0	2
	Mean (µg kg^{-1})	0.07	n.q.	0.07
	Max. (µg kg^{-1})	9.1	n.q.	9.1
ENA	Incidence	6	5	11
	LD-LQ	0	2	2
	Frequency	6	19	8
	Mean (µg kg^{-1})	3.8	7.0	4.5
	Max. (µg kg^{-1})	140	96.6	140
ENA1	Incidence	19	8	27
	LD-LQ	0	0	0
	Frequency	18	30	20
	Mean (µg kg^{-1})	11.2	23.7	13.7
	Max. (µg kg^{-1})	356	272	356
ENB	Incidence	36	19	55
	LD-LQ	0	1	1
	Frequency	34	70	41
	Mean (µg kg^{-1})	42.9	102	54.8
	Max. (µg kg^{-1})	815	487	815
ENB1	Incidence	25	11	36
	LD-LQ	1	0	1
	Frequency	24	41	27
	Mean (µg kg^{-1})	21.5	66.0	30.5
	Max. (µg kg^{-1})	510	510	510

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; LD-LQ: number of samples ≥ LD and ≤ limit of quantification (LQ); Mean: average of total samples, assuming a zero value for samples ≤ LQ; n.q.: not quantified.

Emerging mycotoxin co-occurrence was found in 26 of 42 positive conventional wheat samples (62%), and 11 of 19 positive organic wheat samples (58%). For conventional wheat, the most frequent was the presence of two mycotoxins in the same sample, while for organic wheat the most frequent was the simultaneous contamination with four emerging mycotoxins. Detailed results for co-occurrence are presented in Fig. 13 and Table XVIII. It must be remarked that ENB and ENB1, considered the most toxic ENs, were found together in all positive samples of organic wheat, and in 22 of 26 positive samples of conventional wheat (Table XVIII).

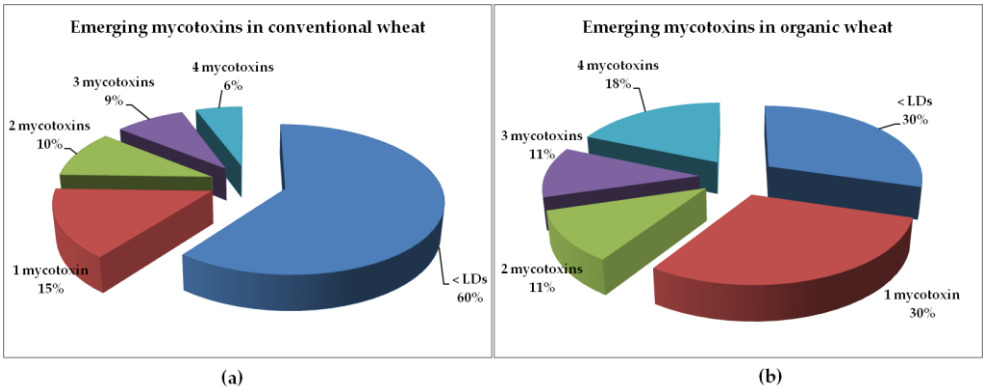


Fig. 13. Emerging mycotoxin frequency for Romanian wheat:
a) conventional; **b)** organic.

Table XVIII. Pairs of emerging mycotoxins found in the positive wheat samples (conventional and organic) from Romania.

CO-OCCURRENCE	NUMBER OF SAMPLES	
	CONVENTIONAL WHEAT (n = 106)	ORGANIC WHEAT (n = 27)
2 Mycotoxins		
ENA1+ENB	1	0
ENA1+ENB1	3	0
ENB+ENB1	7	3
3 Mycotoxins		
ENA1+ENB+ENB1	9	3
BEA+ENB+ENB1	0	0
4 Mycotoxins		
BEA+ENA1+ENB+ENB1	1	0
ENA+ENA1+ENB+ENB1	5	5

The results of one-way analysis of variance (ANOVA) revealed statistically significant differences ($p < 0.05$) between the two agricultural practices for ENB (p = 0.031) and ENB1 (p = 0.018) concentrations. Nevertheless, for ENA and ENA1, no statistically significant difference was observed, while for BEA, the test was not applied due to the low number of positive samples.

Resuming the comparison between conventional and organic wheat from the present study, higher values for incidences, mean levels, and number of mycotoxins found simultaneously were registered for organic wheat samples, but, interestingly, the maximum values found were for conventional samples. This could be explained by the multi-factorial influence in fungal growth and mycotoxin development based on plant substrate, topographic factors, weather parameters, or different management activities [22].

Relating to previous studies about emerging mycotoxin presence in wheat or wheat-based products by farm practice, until now, two published studies compared conventional and organic systems using only wheat-based products. Indeed, Jestoi et al [193], studying the occurrence of sixteen *Fusarium* mycotoxins including six emerging mycotoxins (four ENs, as well as BEA and FP) in different conventional and organic grain-based products, observed that the highest concentrations of ENB (170 μg kg^{-1}) and ENB1 (71 μg kg^{-1}) were in conventional products. On the other hand, Serrano et al [157], comparing organic and conventional pasta, found that 100% of the organic samples and 88% of the conventional samples were contaminated by at least one emerging mycotoxin. Moreover, the incidence percentages of ENA, ENB, ENB1, and BEA were higher in samples of organic pasta, while the concentration levels revealed a heterogeneous distribution: the mean level of ENA (7.3 μg kg^{-1}) was higher for organic wheat pasta, the mean levels of ENB (12.8 μg kg^{-1}) and ENB1 (18.8 μg kg^{-1}) were higher for conventional wheat pasta, and, concerning BEA and ENA1, no significant differences were observed.

The literature presents similar comparisons for wheat or wheat derivatives produced organically or conventionally, using also the levels of other mycotoxins. A study analyzing ten trichothecenes and ZEA in 247 organic and 1377 conventional wheat samples distributed across the whole of the United Kingdom and over five harvest years, identified no significant differences in DON and ZEA concentrations between organic and conventional samples, while the incidence and concentration of positive samples for HT-2 and T-2 were both significantly lower for the organic samples [129]. Bernhoft et al [53] reported significantly lower *Fusarium* infestation and levels of DON, HT-2, and T-2 in samples of organic cereals such as wheat, barley, and oats in comparison with the paired samples of conventional cereals cultivated in Norway, similar with the results of an Italian study [67] that observed a higher contamination by *Fusarium* spp in conventional wheat in comparison to organic wheat. Regarding wheat products, organic and conventional wheat flour samples commercialized in Croatia in 2008 and 2009 were evaluated for OTA and ZEA presence and no statistical differences between organic and conventional products were observed [206]. The same conclusion was also reached by a research group from Slovenia, even if the contamination rate with AFLAs, OTA, FUMOs, DON, ZEA, HT-2, and T-2 was higher for the organic cereal products [124]. In addition, different authors [207]

declared that crop management system is the weakest factor influencing the internal colonization of winter wheat kernels by *Fusarium* fungi.

Due to the high demand for organic foodstuffs and the occurrence of some mycotoxins in wheat, including emerging mycotoxins, research on wheat quality from different farming systems is relevant. Moreover, taking into account the differences in reporting, analytical methods and sensitivities obtained, statistical analyses applied, and agriculture particularities, it is recommended that continuous monitoring studies should be conducted in all types of wheat in an effort to reach final conclusions about best practices in order to inform policies.

5.4.3. Cultivar influence on conventional wheat

From the 106 conventional wheat samples analyzed, 55 samples were classified by cultivars, belonging to twenty-two varieties cultivated in Romania, and 51 samples belonged to other unclassified types of wheat. Final results showed that eleven varieties were negative for all emerging mycotoxins evaluated: Alcantara, Alex, Dropia, Felix, Hyfi, ITC-20, Lukulus, Miranda, Ponomicus, Solehio, and Urbanus. BEA was detected in three conventional wheat samples included in the group "Other types", one being quantifiable (9.1 μg kg^{-1}). Concerning the wheat varieties positive for ENs, the lowest levels (mean not exceeding 4 μg kg^{-1}) were obtained for Arezzo, Boema, Glosa, and Kontrast varieties, while the highest means and levels were for Arieşan, Balaton, and Izvor varieties (Table XIX).

The correlation between the presence of emerging mycotoxins in Romanian wheat and the wheat variety is highlighted for the first time in the present study. Detailed analysis for mycotoxin contamination by variety criterion was previously performed in Romania only for DON, when the highest levels of DON were found for Alex, Arieşan, and Exotic wheat varieties, while lower means and concentration levels were observed for wheat varieties such as Balaton, Boema, Glosa, and Ponomicus [208].

Concerning wheat crops in Romania, the most used varieties are: Boema (25% of wheat crops), Glosa (16.5%), and Dropia (16.1%). Boema wheat variety is resistant to Romanian winter conditions, scorching heat and drought; also, it is resistant to yellow rust, medium resistant to brown rust, and it presents better resistance to sprout damages than other varieties. Glosa variety is medium sensitive to brown rust, and medium resistant to yellow rust, *Septoria* contamination, and wheat head fusariosis, while Dropia variety is known to be tolerant to scorching heat and drought, resistant to *Septoria* contamination, medium resistant to yellow and brown rust, and sensitive to wheat head fusariosis. These varieties are recommended for Romanian hill and plain areas [208]. Knowing that Boema, Glosa, and Dropia are the most cultivated wheat varieties, and considering the low levels of emerging mycotoxins in our study, but also the previous study on DON, it can be accepted that safe conventional wheat is produced in Romania.

Table XIX. Summary of enniatin (EN) levels found in Romanian conventional wheat samples distributed by cultivars.

VARIETY	ENA			ENA1			ENB			ENB1			OVERALL INCIDENCE
	Incidence	Mean (μg kg⁻¹)	Max. (μg kg⁻¹)	Incidence	Mean (μg kg⁻¹)	Max. (μg kg⁻¹)	Incidence	Mean (μg kg⁻¹)	Max. (μg kg⁻¹)	Incidence	Mean (μg kg⁻¹)	Max. (μg kg⁻¹)	
Alcantara	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Alex	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Altigo	0/1	n.q.	n.q.	0/1	n.q.	n.q.	1/1	157.4	157	1/1	12.4	12.4	1/1
Arezzo	0/3	n.q.	n.q.	1/3	3.9	11.8	0/3	n.q.	n.q.	1/3	3.8	11.6	1/3
Arieşan	0/7	n.q.	n.q.	1/7	3.6	25.5	6/7	57.7	149	2/7	15.2	71.1	6/7
Balaton	0/2	n.q.	n.q.	2/2	27.0	43.0	2/2	40.5	65.1	1/2	37.0	74.0	2/2
Boema	0/4	n.q.	n.q.	0/4	n.q.	n.q.	1/4	0.5	2.1	0/4	n.q.	n.q.	1/4
Dropia	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Exotic	0/1	n.q.	n.q.	0/1	n.q.	n.q.	1/1	55.7	55.7	1/1	4.5	4.5	1/1
Felix	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Glosa	0/10	n.q.	n.q.	1/10	1.8	17.5	3/10	3.9	33.9	1/10	2.0	20.2	3/10
Hyfi	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
ITC-20	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Izvor	0/7	n.q.	n.q.	1/7	9.4	65.6	1/7	22.7	159	1/7	16.6	116	1/7
Kontrast	0/1	n.q.	n.q.	0/1	n.q.	n.q.	1/1	5.2	5.2	0/1	n.q.	n.q.	1/1
Litera	0/5	n.q.	n.q.	1/5	2.8	14.2	1/5	8.0	40.1	1/5	7.4	37.2	1/5
Lukulus	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Miranda	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2
Ponomicus	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2	n.q.	n.q.	0/2
Solehio	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Soxenos	0/1	n.q.	n.q.	0/1	n.q.	n.q.	1/1	12.6	12.6	0/1	n.q.	n.q.	1/1
Urbanus	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1	n.q.	n.q.	0/1
Other types	6/51	7.9	140	12/51	19.5	356	18/51	70.4	815	16/51	37.1	510	23/51

Incidence: number of samples ≥ limit of detection (LD) / total samples; Mean: average of total samples, assuming a zero value for samples ≤ limit of quantification; n.q.: not quantified.

On the other hand, other varieties (for example Arieşan) were more contaminated. Arieşan wheat variety is resistant to yellow and brown rust, and medium resistant to *Septoria* contamination and wintering. This variety is characteristic for central and northern Romanian regions, where climatic conditions are characterized by variable humidity and higher rainfall [208]. Thus, the higher contamination of Arieşan variety might be the consequence of a complex dependence on several factors, including agricultural practice and weather parameters.

5.5. Conclusions

This study shows that agricultural practices could influence emerging mycotoxin presence in wheat cultivated in Romania. For the four ENs studied, the incidence percentages and mean levels for organic wheat samples were higher than the corresponding values for conventional samples. On a long-time consumption of wheat and wheat-based products, it can be stated that the use of conventional farm practices is safer than organic practices.

Further monitoring studies in different European countries, using sensitive analytical methods, are necessary to confirm the convenience and safety of conventional practices regarding emerging mycotoxin presence in wheat. Also, a multi-variance analysis could be included, as a wide range of compositional, topographic, climatic, and agricultural factors may influence the occurrence and concentrations of mycotoxins in wheat.

6. Study 4. Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions during 2015

Study included in the article entitled "Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions during 2015"

Submitted to Food Control (2017)

Abstract

To observe the influence of climate conditions on mycotoxin presence in wheat, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON), fusarenon-X (FUS-X), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyscirpenol (DAS), neosolaniol (NEO) and zearalenone (ZEA) were evaluated in 102 wheat samples collected from five main wheat growing regions in Romania during 2015. The contamination levels were correlated with the precipitation and temperature values during anthesis and preharvest period. Overall, the contamination frequency decreased as following: DON > ZEA > HT-2 > 3AcDON = 15AcDON > NIV. High incidences for DON (68%) and ZEA (16%) were observed in the North Muntenia, in 2015, this region being characterized by medium to high quantities of rainfall during June and July (41-100 mm/month) and normal temperatures (average of 20.0°C in June and 24.0°C in July), which suggests that prolonged rainy weather during anthesis, dough formation and filling could influence fungi development and mycotoxin production.

6.1. Introduction

Fusarium species are notable among wheat pathogens, being capable to produce trichothecenes and ZEA [57,60]. Trichothecenes are a family of tetracyclic sesquiterpenoid substances, produced by *Fusarium sporotrichioides*, *F. langsethiae*, *F. graminearum*, *F. culmorum*, *F. poae*, and *F. equiseti* [56,83], including DON, NIV, 3AcDON, 15AcDON, FUS-X, HT-2, T-2, DAS, NEO, and also other similar substances [22]. ZEA is a non-steroidal estrogenic mycotoxin, produced mostly by *F. graminearum* and *F. culmorum* [56,209]. According to the available toxicological data concerning carcinogenicity in humans, ZEA, DON, NIV, and FUS-X were included by the IARC in the Group 3 [139]. Considerations on mycotoxin combinatory effects have been initiated over the past years [194], thus, co-occurrence of mycotoxins in foodstuffs became an important topic for mycotoxin presence and risk assessment studies [64].

It is anticipated that climate changes of the planet will produce a warming of the ecosystem, including fungal attacks and mycotoxin production in cereals and their products [210]. Romania has a continental-temperate climate with various influences: oceanic in the central and western regions, continental in the East, and Mediterranean in the South [29]. Evaluation of frequency and tendencies of contamination with mycotoxins on the agrofood chain, particularly for cereals, has a special importance in the context of climate changes predicted for Romania. These changes include not only the increase of temperature by 3-5°C and decrease of rainfall in summer, but also the terrestrial stilling, seasonal changes in relative air humidity linked with changes in streamflow regime, cloud cover and evapotranspiration [29,211].

Fusarium mycotoxin development is dependent of various factors, weather conditions being the most important [212]. The environmental conditions that promote *Fusarium* spp development are moderate temperatures (between 20 and 30°C) in the presence of high relative humidity (90%), frequent rainfall during and after flowering, extended periods of high moisture, and occurrence of air currents. The regional distribution of mycotoxins also depends on endogenous and exogenous factors that can affect mycotoxin production, e.g. agronomic practices, fungicides used, host resistance, preceding crop [60,94,213].

6.2. Aims

Presence of mycotoxins in the agro-food chain is considered a food safety and security issue. Since in the last years Romania is a leader in terms of wheat production in Europe [214] and taking into account its temperate-continental climate, studies on tendencies of mycotoxin contamination are required, particularly in the context of the climate changes predicted for Romania [211]. Therefore, the goals of the present work were: i) to evaluate the presence of nine trichothecenes (DON, NIV, 3AcDON, 15AcDON, FUS-X, HT-2, T-2, NEO, DAS) and ZEA in Romanian wheat harvested in 2015 using a sensitive GC-QqQ-MS/MS analytical method; ii) to assess the influence of the climatic conditions during the grain-growing season on trichothecenes and ZEA presence.

6.3. Materials and methods

6.3.1. Sampling

A total of 102 whole unprocessed wheat samples were collected during 2015 harvest season from five different Romanian regions (Fig. 14) in order to investigate mycotoxin presence. Information about growing area (county and city), cultivation and harvest period was considered. Sampling was performed according to the EU guidelines [171]. After homogenization, samples were packed in plastic bags and kept at -20°C in a dark place. Before the analysis, for all samples, subsamples of 300 g were milled with a blender and divided into three bulks of 100 g each one.

6.3.2. Sample preparation

6.3.2.1. Extraction

Extraction was performed according to the procedure presented by Stanciu et al [199], using SLE as it was described in section 3.3.2. All experiments were performed in triplicate.

6.3.2.2. Derivatisation

A volume of 200 µL of filtrate was placed in a chromatographic vial and was evaporated to dryness at 35°C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, MA, USA)

Over the dry extract 50 µl of derivatisation reagent (BSA+TMCS+TMSI, 3:2:3) were added and the sample was left for 30 minutes. The derivatised sample was diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 seconds. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. Finally, the upper layer was transferred to an autosampler vial for chromatographic analysis.

6.3.3. Mycotoxin analysis

The analysis of the ten mycotoxins was performed using a GC-QqQ-MS/MS system, using the method presented in the section 2.2.2.

6.3.4. Method validation

Validation of the method was performed for linearity, accuracy, repeatability (intraday and interday precision) and sensitivity, following the EU Commission Decision, 2002/657/EC [190]. External standard calibration was used in the validation of the analytical method. The criteria for confirmation of positive findings were: comparison of peak area ratios for quantification (Q) and confirmation (q) transitions with that of the reference standard; peak ratio of the confirmation transition against quantification one; agreement with the retention times.

Matrix-matched calibration curves were constructed at concentration levels between 1 and 1000 µg kg^{-1}. ME was assessed for each analyte by comparing the slope of the standard calibration curve ($a_{standard}$) with that of the matrix-matched calibration curve (a_{matrix}), for the same concentration levels. LD and LQ were estimated for a signal-to-noise ratio (S/N) ≥ 3 and ≥ 10, respectively, from chromatograms of samples spiked at the lowest level validated. Accuracy was evaluated through recovery studies, carried out by spiking blank wheat at three concentration levels: low (LQs), medium (2 times more the LQs), and high (10 times more the LQs). Precision was estimated by calculating the RSD of the results obtained during the same day (intraday), and on three different days (interday) by the repeated analysis three times at the three spiked levels.

6.3.5. Climatic conditions

For the registration of climatic parameters, all time series were extracted from the climatic database of Romanian National Meteorological Administration (Meteo Romania) [196]. Gridded spatially interpolated data was used to register mean precipitation and mean air temperature during anthesis (months of April and May) and preharvest period (months of June and July).

6.3.6. Statistical analyses

Results are reported as the mean ± standard deviation. The correlation between DON and ZEA levels was performed by the Pearson Correlation test. Statistical procedures were performed using SPSS software (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA), version 22.0.

6.4. Results and discussion

6.4.1. Method validation and performance

For identification of compounds using GC-QqQ-MS/MS, two MS/MS transitions were acquired: one for quantitation (Q), and one for confirmation (q). Table XX presents the results obtained for the method validation. ME ranged from 64% (ZEA) to 137% (FUS-X). A good linearity was observed, with corresponding correlation coefficients (r^2) higher than 0.989. The LQs of the mycotoxins analyzed presented a high variability, ranging between 1 μg kg^{-1} (DON) and 20 μg kg^{-1} (NIV and NEO). The accuracy was evaluated for each compound by calculating the recovery values that were between 69 and 127%. Intraday and interday precision values as RSDs were lower than 12% and 19%, respectively. Taking into account our results for the validation of the method, this analysis is sensitive, precise and reproducible. The performance of the method was reflected in the low LQs, and the good recovery rates.

Table XX. Analytical parameters corresponding to the GC-QqQ-MS/MS method used for wheat analysis: retention time (Rt), limits of detection (LD) and quantification (LQ), recovery at three spiked concentration levels, interday relative standard deviation (RSD), matrix effect (ME), and linearity expressed as correlation coefficient (r^2).

ANALYTE	**Rt** (min)	**LD** (μg kg^{-1})	**LQ** (μg kg^{-1})	**RECOVERY (RSD)** (%)						**ME ± RSD** (%)	**LINEARITY** (r^2)
				LQ		2 LQ		10 LQ			
DON	8.45	0.5	1	100	(7)	95	(1)	127	(2)	85 ± 9	0.994
3AcDON	9.45	1.25	2.5	84	(17)	85	(4)	117	(15)	133 ± 7	0.995
15AcDON	9.65	2.5	5	87	(1)	82	(6)	108	(11)	102 ± 6	0.996
FUS-X	9.55	2.5	5	79	(4)	73	(3)	97	(7)	137 ± 4	0.998
DAS	9.56	7.5	15	83	(16)	77	(11)	118	(10)	66 ± 6	0.989
NIV	9.90	10	20	78	(3)	88	(1)	117	(5)	97 ± 9	0.994
NEO	11.30	10	20	75	(4)	80	(1)	86	(9)	129 ± 3	0.999
HT-2	14.40	7.5	15	100	(19)	84	(5)	84	(7)	111 ± 6	0.989
T-2	14.45	2.5	5	103	(1)	107	(1)	113	(5)	101 ± 1	0.989
ZEA	15.46	5	10	69	(8)	106	(8)	97	(3)	64 ± 4	0.999

6.4.2. Mycotoxin occurrence data in Romanian wheat during 2015

The aim of the present study was to monitor the occurrence of nine trichothecenes and ZEA in 102 wheat samples collected during the 2015 growing season from fields located in five different regions of Romania (Fig. 14), with various agroclimatic conditions. Results showed that 67% (68 of the samples) of the samples presented detectable levels of at least one mycotoxin: DON, 3AcDON, 15AcDON, NIV, HT-2, and ZEA (Table XXI). Most of the positive samples were contaminated with one mycotoxin (48%), followed by the presence of two mycotoxins (11%: DON+ZEA or DON+HT-2), three mycotoxins (4%: DON+3AcDON+15AcDON, DON+15AcDON+HT-2 or DON+NIV+HT-2), four mycotoxins (3%: DON+3AcDON+15AcDON+ZEA or DON+ NIV+HT-2+ZEA), and five mycotoxins (1%: DON+3AcDON+NIV+HT-2+ZEA). Three wheat samples exceeded the ML of 100 μg kg^{-1} for ZEA [161] established by the European legislation for unprocessed wheat, with concentrations between 155 and 300 μg kg^{-1}.

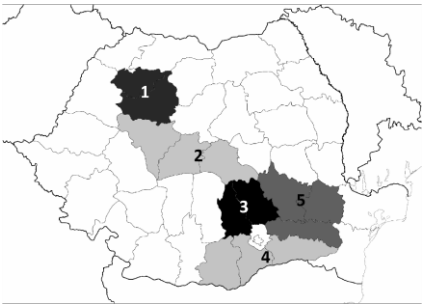


Fig. 14. The five Romanian regions included in the study, belonging to different climatic areas: 1. North-West (*n* = 9), 2. Mideast (*n* = 9), 3. North Muntenia (*n* = 31), 4. South Muntenia (*n* = 37). 5. South-East (*n* = 16).

Recently published data investigating the occurrence of both legislated and non-legislated *Fusarium* mycotoxins in unprocessed wheat from Balkan countries with similar climatic conditions as Romania, e.g. Bulgaria, Croatia, Serbia, Slovenia, is rather scarce. Moreover, depending on the methods applied for the analysis and their sensitivities, a high variability in interpreting the results can appear. Therefore, a brief revision of the literature was conducted with the goal to introduce an insight into the occurrence of *Fusarium* mycotoxins in wheat from Romania during the previous years (Table XXII), observing that there is only sporadic published data in these sense. Most of the studies were focused on DON and ZEA evaluation, only three studies including other trichothecenes such as 3AcDON, 15AcDON, NIV, DAS, NEO, HT-2 or T-2 [119,136,199]. Furthermore, ELISA was the most used method, sometimes having low sensitivities reflected within high LDs, and only two studies used multi-class analysis, one by GC/MS, and another one by LC-MS/MS. Hence, sensitive validated multi-mycotoxin methods are recommended to be carried out with the aim to fill the gap concerning mycotoxin evaluation in wheat and its products from Romania.

Table XXI. Incidence and concentration levels of the mycotoxins detected in the Romanian wheat samples collected during 2015 harvest year.

REGION	PARAMETER	ANALYTE						CO-OCCURRENCE
		DON	3AcDON	15AcDON	NIV	HT-2	ZEA	(%)
North-West	Frequency	100	22	22	11	11	11	33
(*n* = 9)	Mean (μg kg^{-1})	62.4	7.83	12.9	40	41.5	n.q.	
	Range (μg kg^{-1})	2.3 - 323	7.83	8.9 - 16.9	40	41.5	n.q.	
Mideast	Frequency	89	22	11	22	33	33	56
(*n* = 9)	Mean (μg kg^{-1})	43.5	2.7	n.q.	63.8	46.4	42.1	
	Range (μg kg^{-1})	1.3 - 129	2.72	n.q.	63.3 - 64.3	35.2 - 67.4	23.8 - 77.7	
North Muntenia	Frequency	68	3	6	0	10	16	23
(*n* = 31)	Mean (μg kg^{-1})	66.3	12	30	n.q.	60.8	157	
	Range (μg kg^{-1})	1.1 - 955	12	30	n.q.	31.1 - 98.5	12.4 - 300	
South Muntenia	Frequency	43	0	0	0	8	19	11
(*n* = 37)	Mean (μg kg^{-1})	4.7	n.q.	n.q.	n.q.	24.7	60.9	
	Range (μg kg^{-1})	1.2 - 16.4	n.q.	n.q.	n.q.	24.7	11.7 - 155	
South-East	Frequency	38	0	0	0	6	0	0
(*n* = 16)	Mean (μg kg^{-1})	5.5	n.q.	n.q.	n.q.	77.6	n.q.	
	Range (μg kg^{-1})	1.1 - 11	n.q.	n.q.	n.q.	77.6	n.q.	
Overall	Incidence	60	5	5	3	11	16	19
(*n* = 102)	LD - LQ	11	2	2	0	2	5	
	Frequency	59	5	5	3	11	16	
	Mean (μg kg^{-1})	44.3	7.5	18.6	55.9	51.7	90.7	
	Range (μg kg^{-1})	1.1 - 955	2.7 - 12	8.9 - 30	40 - 64.3	24.7 - 98.5	11.7 - 300	
	ML (μg kg^{-1})	1750				100 *	100	

Co-occurrence: the percentage of samples presenting levels ≥ limit of detection (LD) for at least two mycotoxins / total samples; Frequency: the percentage of samples ≥ LD / total samples; Incidence: number of samples ≥ LD; LD-LQ: number of samples ≥ LD and ≤ limit of quantification (LQ); Mean: average of the positive samples; ML: maximum permitted level established by the European regulations for unprocessed wheat [161,162]; *: ML recommended for the sum of HT-2 and T-2 [162]; n.q.: not quantified.

Table XXII. *Fusarium* mycotoxin occurrence and levels in wheat in recent surveys in Romania.

YEAR	METHOD	MYCOTOXINS	LD (µg kg^{-1})	N	FREQUENCY (%)	RANGE (µg kg^{-1})	REF.
n.a.	GC/MS	DON	n.a.	42	90	21 - 3395	[119]
		15AcDON			36	6 - 99	
		NIV			2	max. 30	
		DAS			2	max. 19	
		HT-2			50	3 - 18	
		T-2			2	max. 7	
2008	ELISA	DON	n.a.	40	43	max. 95.7	[74]
		ZEA			10	max. 5.5	
2009	ELISA	DON	n.a.	12	83	6.1- 154.3	[75]
		ZEA			50	36.7 - 67.3	
2009	ELISA	T-2	n.a.	2	100	0.8 - 1.0	[136]
2008-2010	ELISA	ZEA	n.a.	20	10	0.88- 3.6	[145]
2010	ELISA	DON	110	26	73	294 - 3390	[73]
		ZEA	22.7		69	37.6 - 1000	
2011	ELISA	DON	110	26	19	254 - 1440	[73]
		ZEA	22.7		77	28 - 105.6	
2012	ELISA	DON	18.5	831	65	<18.5 - 5027	[215]
2013	ELISA	DON	18.5	923	53	<18.5 - 3602	[215]
2014	ELISA	ZEA	n.a.	336	5	17 - 80	[146]
2014	LC-MS/MS	DON	20	31	26	110 - 1787	[199]
		3AcDON	20		0	n.q.	
		15AcDON	150		0	n.q.	
		NIV	150		0	n.q.	
		DAS	30		0	n.q.	
		NEO	7		0	n.q.	
		HT-2	50		0	n.q.	
		T-2	75		0	n.q.	
		ZEA	20		13	327 - 1135	
2015	ELISA	DON	18.5	4	50	<18.5 - 964	[211]

LD: limit of detection reached by the method; Frequency: % of samples ≥ LD / total samples; N: number of samples; n.a.: no data available; n.q.: not quantified.

6.4.3. Geographical distribution and climate influence

The findings obtained in the present study indicated significant differences in mycotoxin frequency and concentration levels throughout the five regions evaluated. On the other hand, similar trends were observed for the mean concentrations of DON, HT-2 and ZEA in wheat samples from Mideast and North Muntenia (Fig. 15). The co-occurrence of mycotoxins was very frequent in the Mideast of Romania (5 wheat samples being contaminated with two to five mycotoxins), the North-West (3 wheat samples being contaminated with three or four mycotoxins), and the North Muntenia (6 wheat samples being contaminated with two to four mycotoxins) (Table XXI). Concerning DON, that was the most frequent mycotoxin, the contamination incidence decreased as following: North-West (100%) > Mideast (89%) > North Muntenia (68%) > South Muntenia (43%) > South-East (38%). The Pearson coefficient indicated a strong positive linear relationship (R^2 = 0.78) between DON and ZEA concentrations. This means that various factors related with the crop or the environment influence DON and ZEA production simultaneously.

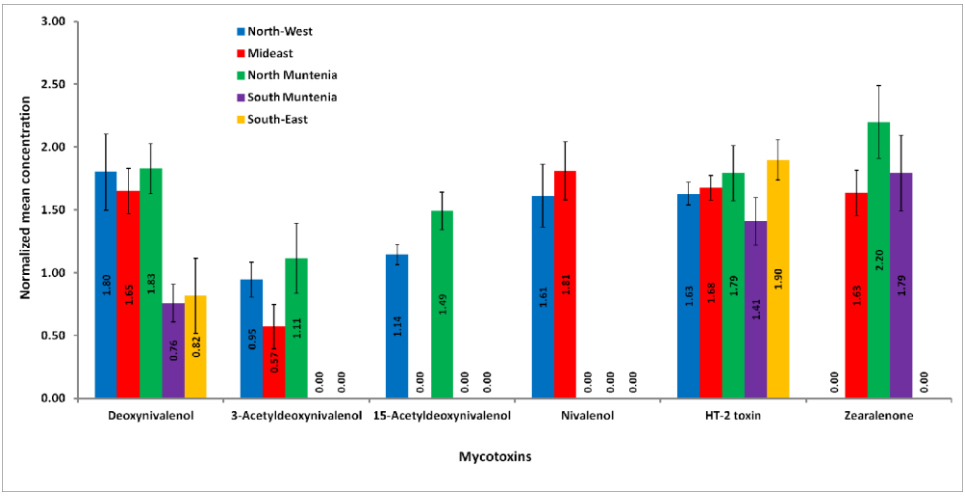
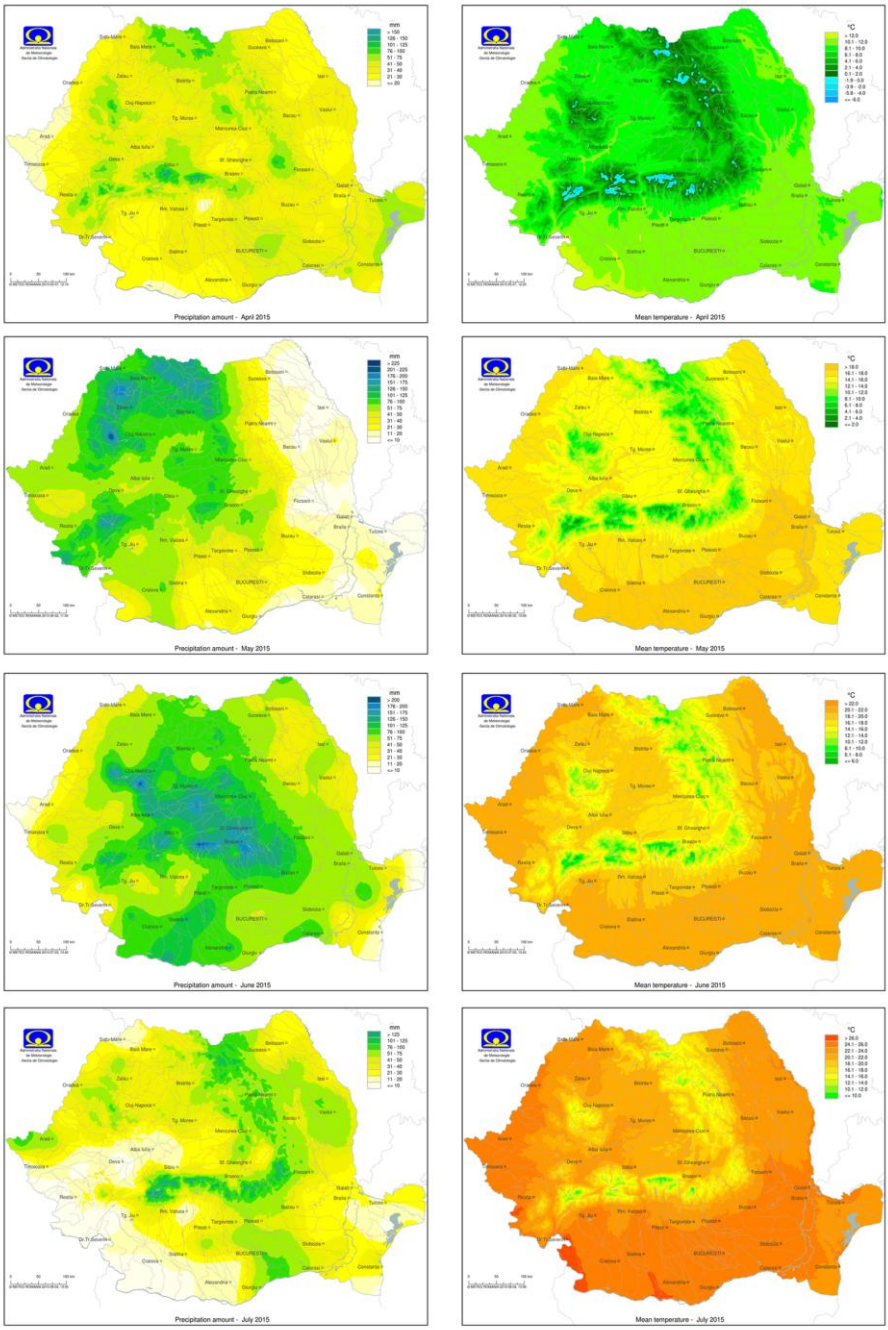


Fig. 15. Normalized mean concentrations of mycotoxins in wheat across five different Romanian regions. Mean concentrations were normalized by Log_{10} (1 + *a*), where *a* is the mean concentration expressed in µg kg^{-1}.

The highest mean concentrations for DON (66.3 µg kg^{-1}), 3AcDON (12 µg kg^{-1}), 15AcDON (30 µg kg^{-1}), HT-2 (60.8 µg kg^{-1}) and ZEA (157 µg kg^{-1}) were found in the same region, North Muntenia. Interestingly, the maximum levels of DON (955 µg kg^{-1}), 3AcDON (12 µg kg^{-1}), 15AcDON (30 µg kg^{-1}), and ZEA (300 µg kg^{-1}) were found in the same sample from this region.

Regarding the weather conditions in Romania during 2015 year (Fig. 16), some particularities have been observed. The region with the highest levels of *Fusarium* mycotoxins in wheat, the North Muntenia, was characterized by moderate quantities of rainfall (31-40 mm) and normal average temperatures (16-18°C) in May (anthesis period), and high quantities of precipitations (51-100 mm and 51-75 mm, respectively) and moderate temperatures (18-20°C and 22-24°C, respectively) in June and July (from late anthesis to yield formation). Furthermore, the second region in terms of mycotoxin presence in wheat samples, the Mideast of Romania, registered high quantities of rainfall (51-75 mm) and lower average temperatures (14-16°C) in May, and abundant to excessive quantities of precipitations (101-175 mm) and moderate temperatures (18-20°C) in June. Also, the North-West Romania, remarked by the highest incidence of DON, was the region that recorded the rainiest month of May during 2015 (126-200 mm) compared with the other regions, coupled with normal temperatures (14-16°C). These remarks suggest that a prolonged rainy weather during earing phase, anthesis, dough formation and filling (01 May to 30 June) could favor high moisture for the wheat crops, consequently influencing fungi development and mycotoxin production, particularly DON, HT-2 and ZEA.



a) Amount of precipitation (mm) in 2015 year (April - July) in Romania.

b) Monthly mean temperatures (°C) in 2015 year (April - July) in Romania.

Fig. 16. **a)** Evolution of amount of precipitation and **b)** monthly mean temperatures in 2015 year (April - July) in Romania [196].

Occurrence of trichothecenes and ZEA in wheat is considered a typical agricultural issue in temperate regions where weather conditions are favorable for *F. graminearum* and *F. culmorum* growth and related mycotoxin production [213]. Environment parameters, particularly temperature, precipitation and relative air humidity, are relevant factors for fungal infection, mycotoxin production and survival. A significant increase in fungal attacks on wheat has been observed worldwide, climate changes being a factor of influence for the occurrence of moulds in cereals, and also the development of mycotoxins [58,59]. Changes in agroclimatic conditions directly affect fungal population and related mycotoxins, and also have indirect impact on mycotoxin contamination, as increased drought stress, insect damage of the plant, and modifications in crop phenology [210].

The results from the present study revealed the correlation between *Fusarium* mycotoxin presence in wheat and weather parameters that are influenced directly by the geographical position. Until now, in Romania studies performed on mycotoxin presence in wheat were focused mostly on DON. For example, Gagiu et al [215] evaluating DON levels in 1754 cereal samples from Romania (common wheat, durum wheat, triticale and wheat) and the meteorological, hydrological and geographical factors, stated that the North-West Romania may present a possible risk for DON contamination. The same conclusion has been reached also by Alexa et al [73], after analyzing 52 wheat samples collected from Western Romania during two consecutive harvest years.

Our results are also in accordance with one of our previous studies regarding the presence of emerging mycotoxins in wheat, that revealed a different influence of the climatic parameters for each region analyzed [216], but it is also in accordance with other studies in countries with similar climate as Romania. A multi-mycotoxin analysis was performed for 54 wheat samples from different regions of Serbia and significant differences have been observed between northern and southern regions within the same year, attributed primarily to the differences in climate conditions and consequently in period of collection. The southern Serbian regions where mycotoxins were not detected in wheat samples were characterized by a specific microclimate with very low amount of precipitation, while in the northern part of Serbia where mycotoxins were detected, particularly DON (ranging from 41 to 309 μg kg^{-1}), precipitation amounts up to seven times higher were recorded [121].

In a recently published study [76], the presence of twenty-six mycotoxins in 99 wheat samples from five regions of Poland was monitored. After analyzing the mycotoxin levels and the trends in the prevalence of temperature and rainfall, it was found that the most contaminated wheat samples belonged to the southeastern regions of Poland, where the highest rainfall and temperature values were recorded during the wheat earing stage and flowering period, compared with the regions located

in the North and West of Poland, where lower temperature and lower air humidity limited mycotoxin biosynthesis.

In addition, a research performed by Alkadri et al [109] indicated a lower incidence of *Fusarium* mycotoxins (DON, 3AcDON, 15AcDON, HT-2, T-2, NIV and ZEA) in Syrian wheat samples than in the Italian ones, although both countries are in the Mediterranean area. The authors explained this diversity through climatic conditions. Syria has an arid climate, very hot in the summer and cold in winter, whereas the climate of Italy is mainly temperate and varies slightly according to the areas.

6.5. Conclusions

A GC-QqQ-MS/MS method was validated for the determination of nine trichothecenes and ZEA in wheat, with good accuracy and sensitivity. The efficiency of the method was proved by evaluating the presence of the ten mycotoxins in 102 Romanian wheat samples collected during 2015 harvest year. Data obtained was correlated with the weather parameters in the growing region.

Based on the present results and available literature on this topic, it can be stated that extreme phenomena – rainy periods at the end of flowering, drought during grain formation or high moisture in the late preharvest period – are favorable particularly for DON, HT-2 and ZEA presence and the simultaneous occurrence of DON and ZEA in wheat. These observations become important in the context of the predicted climate changes that could affect also fungi development and mycotoxin production. The present study can contribute to the effort to reduce fungi and mycotoxin attacks in wheat, and it can represent an important step for the mitigation strategies and the HACCP monitoring process. To protect human health, continuous studies concerning mycotoxin presence in wheat, particularly in the Balkan area, associated with environmental conditions, are required.

7. Study 5. Occurrence of emerging mycotoxins in wheat-based products and exposure assessment for the Romanian population

Study included in the article entitled "Analysis of enniatins and beauvericinby LC-MS/MS in wheat-based products"

CyTA – Journal of Food 15(3) (2017) 433-440

Abstract

Due to the matrix complexity for wheat-based products, a comparative study of different rapid extraction procedures was performed for the extraction of enniatins (ENA, ENA1, ENB, ENB1) and beauvericin in flour, pasta, breakfast cereals, and biscuits. Three different approaches were studied during the extraction and purification steps (shaker, Ultra-Turrax, and QuEChERS) for each matrix. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with electrospray source working in a positive mode was used. For the analysis of the five mycotoxins, the three methods were tested in terms of recovery, matrix effect, and sensitivity, concluding that Ultra-Turrax extraction was the most competent method. The applicability of the validated method was demonstrated by analyzing 16 commercial samples from Romania.

and

Study included in the article entitled "Presence of Enniatins and Beauvericin in Romanian Wheat Samples: From Raw Material to Products for Direct Human Consumption"

Toxins 9 (2017) 189

Abstract

In this study, a total of 244 wheat and wheat-based products collected from Romania were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in order to evaluate the presence of four enniatins (ENs; i.e., ENA, ENA1, ENB, and ENB1) and beauvericin (BEA). For the wheat samples, the influence of agricultural practices was assessed, whereas the results for the wheat-based products were used to calculate the estimated daily intake of emerging mycotoxins through wheat consumption for the Romanian population. ENB presented the highest incidence (41% in wheat and 32% in wheat-based products), with its maximum levels of 815 μg kg^{-1} and 170 μg kg^{-1} in wheat and wheat-based products, respectively. The correlation between the concentrations of ENB and ENB1 in wheat grain samples and farm practices (organic or conventional) was confirmed statistically ($p<0.05$). This is the first study that provides comprehensive information about the

influence of agricultural practice on emerging Fusarium mycotoxin presence in Romanian wheat samples and the estimated daily intake of ENs and BEA present in wheat-based products for human consumption commercialized in Romania.

7.1. Introduction

Wheat is the most consumed cereal worldwide. Mycotoxin contamination in different cereals and foods based on cereals is a major topic due to its remarkable implications for food safety [148]. So, studies on presence of emerging mycotoxins in wheat-based products are useful for the assessment of human exposure to mycotoxins.

The group of ENs (ENA, ENA1, ENB, and ENB1) and BEA has received more and more interest in the past ten years. These mycotoxins are bioactive substances with a cyclic hexadepsipeptide structure. ENs can act as enzyme inhibitors, having antimicrobial, anthelmintic, insecticidal, antifungal, herbicidal, phytotoxic, and cytotoxic potential activity [14] or local central nervous system effects [217]. BEA has shown to be a specific cholesterol acyltransferase inhibitor, with antimicrobial, antiviral, cytotoxic, apoptotic, and immunosuppressive activity [218].

Nowadays, food industry needs rapid, accessible, and preferable multi-mycotoxin analysis [76]. Therefore, it is imperative to create sustainable extraction methods for reliable detection and quantification of ENs and BEA. Approaches on analysis of ENs and BEA commonly use SLE with appropriate solvents, through the application of conventional extractions [66,157,219]. Juan et al [77] tested various solvent mixtures to extract trichothecenes and ZEA from cereal grains, flour, and bread, and the highest recoveries and the lowest matrix influence were reported for the mixture acetonitrile/water (84:16, *v/v*). After this, the method was applied successfully in other studies to extract ENs and BEA too from cereal and cereal products [108]. Moreover, Serrano et al [220] studied acetonitrile, methanol, ethyl acetate, and a mixture of acetonitrile/methanol (50:50, *v/v*) as solvents to extract emerging mycotoxins from pasta using Ultra-Turrax, and the best results were obtained for acetonitrile. QuEChERS methodology has also been employed for multi-mycotoxin analysis [155]. Rodríguez-Carrasco et al [126] presented the optimum steps of QuEChERS procedure for wheat semolina: micro-scale extraction with water/acetonitrile (77:23 or 72:28, *v/v*), $MgSO_4$ and NaCl, and a clean-up based on a d-SPE with $MgSO_4$ and C_{18} used as sorbents to retain co-extracted compounds, such as sugar and fatty acids. So far, only two studies have presented the comparative assessment of different extraction procedures for determination of emerging mycotoxins in pasta and biscuits [220,221].

Different studies have provided evidence that the incidence of emerging mycotoxins has reached high values in wheat [66] and wheat products like flour [199], breakfast cereals [154], pasta [157] or pizza [222]. Even so, until now, no study was published concerning emerging mycotoxin presence in wheat-based products

commercialized in Romania and correspondant exposure to this class of mycotoxins through the consumption of wheat-based products.

7.2. Aims

The aims of this study were: (i) to evaluate three different extraction procedures (Ultra-Turrax homogenizer, rotatory shaker, and QuEChERS) to select the method with the best performance for emerging mycotoxin (ENA, ENA1, ENB, ENB1, and BEA) extraction followed by LC-QqQ-MS/MS analysis on flour, pasta, breakfast wheat-based cereals, and biscuits; (ii) to survey the levels of ENs and BEA in wheat-based products commercialized in Romania; (iii) to estimate the daily intake of emerging mycotoxins through wheat-based product consumption for the Romanian population. This is the first survey concerning the estimated daily intake of emerging mycotoxins through wheat-based product consumption by the Romanian population.

7.3. Materials and methods

7.3.1. Sampling

Mycotoxin-free powdered samples were used as blank material for validation study. Also, a total of 111 samples of wheat-based products were purchased from different markets located in Cluj-Napoca (Romania) during April to June 2016: white wheat flour (n = 41); pasta with minimum of 73% wheat (n = 40); breakfast cereals containing between 54% and 90% wheat (n = 7); integral biscuits containing between 49% and 95% wheat (n = 23). Three wheat-product samples (one biscuit sample and two flour samples) were from organic agriculture. It should be mentioned that in Romania, the organic production and the organic or ecological market offert is still in process of development.

Sampling was performed according to the EU guidelines [171] for the official control of legislated mycotoxins for lots of cereals and cereal products less than 0.5 tons. Consequently, two to six packages for each sample were purchased, obtaining an aggregate sample of at least 1 kg total weight. All samples were milled to a fine powder using a laboratory mill. After homogenization, 500 g samples were packed in plastic bags and kept at -20°C in a dark and dry place until analysis. Three replicates for each sample were weighed for analysis.

7.3.2. Extraction

Two methods using SLE (Ultra-Turrax and rotatory shaker) and the QuEChERS procedure were tested. For extraction using the rotatory shaker, the method of Juan et al [108] was used. Sample extraction with Ultra-Turrax and QuEChERS were performed according to the methods of Serrano et al [157] and Rodríguez-Carrasco et al [126], respectively, with some modifications for each procedure. An Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany) for centrifugation and a multi-sample

Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) for evaporation to dryness at 35°C with a gentle stream of nitrogen were used. On the day of analysis, the residue of each extraction was reconstituted to a final volume of 1 mL with methanol/water (70:30, *v/v*) and filtered through a syringe nylon filter. All experiments were carried out in triplicates.

The efficiency of the three methods was tested to decide the most appropriate extraction procedure for emerging mycotoxins from flour, pasta, breakfast cereals, and biscuits, respectively. Recovery, ME, sensitivity, and extraction time were evaluated for each technique separately. Spiked blanks were analyzed at concentrations of 100, 25, and 12.5 μg kg^{-1} for ENs, and 62.5, 16, and 8 μg kg^{-1} for BEA.

7.3.2.1. SLE with rotatory shaker

Sub-samples were weighed (2 g) and placed into 50 mL PTFE centrifuge tubes, followed by the addition of 10 mL acetonitrile/water (84:16, *v/v*) mixture. The tubes were stirred for 1 hour at 300 shakes min^{-1} using a horizontal shaking device (IKA KS260 basic Stirrer, Staufen, Germany), centrifuged for 5 min at 5°C and 4500 rpm, and then filtered on a Whatman filter paper. Furthermore, 5 mL of supernatant was placed in 15 mL PTFE centrifuge tubes and was evaporated to dryness.

7.3.2.2. SLE with Ultra-Turrax

Sub-samples of 2 g, weighed into 50 mL PTFE centrifuge tubes, were extracted with 20 mL of acetonitrile using IKA T18 basic Ultra-Turrax homogenizer (Staufen, Germany) for 3 min. After this, samples were centrifuged for 5 min at 5°C and 3550 rpm and filtered on a Whatman filter paper. Ten milliliters of supernatant placed in 15 mL PTFE centrifuge tubes was evaporated to dryness.

7.3.2.3. QuEChERS procedure

Sub-samples of 5 g were weighed into 50 mL PTFE centrifuge tubes and 25 mL of distilled water was added prior to sonication for 15 min. The main extraction involved the addition of 10 mL of acetonitrile, 4 g of $MgSO_4$, and 1 g of NaCl. To induce phase separation and mycotoxin partitioning, the tubes were shaken on a vortex for 30 s and centrifuged for 5 min at 5°C and 4500 rpm. Then the upper layer was submitted to a d-SPE clean-up with a mixture of 900 mg of $MgSO_4$ and 300 mg of C_{18}. The tubes were vortexed for 30 s and centrifuged for 1 min at 5°C and 1500 rpm. After purification, the supernatant was transferred into 15 mL PTFE centrifuge tubes and evaporated to dryness.

7.3.3. Mycotoxin analysis

The analysis of the five mycotoxins was performed on a LC-QqQ-MS/MS system, using the method presented in the section 2.2.1. Mobile phases were methanol (0.1% acetic acid and 5mM ammonium acetate) as phase A, and water (0.1% acetic acid and 5mM ammonium acetate) as phase B. Statistical analysis was performed using SPSS software (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA), version 22.0.

7.3.4. Method validation

Validation of the method was performed for linearity, accuracy, repeatability (intraday and interday precision), and sensitivity, following the EU regulation [190] . External standard calibration was used in the validation of the analytical method. The criteria for confirmation of positive findings were: comparison of peak area ratios for quantification (Q) and confirmation (q) transitions with that of the reference standard; peak ratio of the confirmation transition against quantification one; agreement with the retention times.

ME was assessed for each analyte by comparing the slope of the standard calibration curve with that of the matrix-matched calibration curve, for the same concentration levels. For linearity evaluation, matrix-matched calibration curves were constructed at concentration levels between 0.5 and 200 μg kg^{-1}. Sensitivity was evaluated by LD and LQ, that were estimated for S/N ≥3 and ≥10, respectively, from chromatograms of samples spiked at the lowest level validated. Accuracy was evaluated through recovery studies, which were carried out by fortifying blank wheat at three different concentration levels: the same as the LQs, 2 times more than LQs, and 10 times more than LQs. The spiked samples were left to stand for 3 h at room temperature before the extraction to establish equilibration between mycotoxins and the matrix. Intraday precision and interday precision (repeatability) of the method were carried out by spiking wheat at the three levels previously indicated. Method precision was estimated by calculating the RSD using the results obtained during the same day (intraday) and on three different days (interday) by the repeated analysis three times at the three spiked levels.

The validated method was used to quantify emerging myctoxins in real wheat-based samples commercialized in Romania. Matrix-matched calibration curves at concentrations between 1 and 1000 μg kg^{-1} were used for quantification. Samples presenting levels higher than LDs were considered to estimate the incidence (%) for each mycotoxin.

7.3.5. Estimation of daily intake

The dietary exposure to each mycotoxin was evaluated by calculating the EDI. For this, a deterministic method [223] was applied, using the equation:

$$\text{EDI (ng kg}^{-1}\text{ bw day}^{-1}\text{)} = \frac{\sum c * K}{N * Bw}$$

where ∑c is the sum of each mycotoxin in the samples analyzed (μg kg^{-1}), K is the daily average consumption / person for the food commodity included in the study (g $capita^{-1}$ day^{-1}), N is the total number of analyzed samples, and Bw is the body weight used in the population group.

To obtain the sum of each mycotoxin for the samples analyzed, two different scenarios were designed: one underestimating (lower bound - LB) and another one overestimating (upper bound - UB) the exposure. The LB was obtained by setting a zero value for all samples with levels lower than LQ, whereas the UB was achieved by assigning the LD to those samples with undetected levels and the LQ to those samples with levels between the LD and LQ [224]. For the samples with quantifiable levels, the same values were used in both scenarios.

To calculate the EDI, the food supply quantity of wheat and products in Romania was considered 369.5 g capita^{-1} day^{-1}, according to FAOSTAT [33], and a default average body weight of 70 kg was assumed for Romanian population.

As no TDI for emerging mycotoxins was proposed until now, the risk evaluation was performed taking into account the safety guidelines for other *Fusarium* mycotoxins [163,225]. Thus, a hypothetic value of 1000 ng kg^{-1} bw day^{-1} was used for the TDI of the sum of ENs, closer or similar to other PMTDIs established for various mycotoxins (e.g. 1000 ng kg^{-1} bw day^{-1} for the sum of DON and its acetylated derivatives [165]). In addition, the same hypothetic TDI value for ENs (1000 ng kg^{-1} bw day^{-1}) was used previously by other authors [147]. The risk assessment of ENs was carried out by calculating the percentage (%TDI) covered by the EDI from the TDI proposed.

7.4. Results and discussion

7.4.1. Selection of the most suitable extraction procedure

Three of the most used methods to analyze mycotoxins have been studied. Two of them are SLEs and the third one is a QuEChERS extraction procedure. The two methods using SLE differ in the method of shaking and the solvents used. For rotatory shaker, a mixture of acetonitrile/water (84:16, *v/v*) was used and an exterior agitation was applied, while for Ultra-Turrax the solvent was acetonitrile and the agitation was performed into the extract, including homogenization. The extraction with Ultra-Turrax was slightly modified compared to the one presented by Serrano et al [157]. The proportionality between sample and solvent was kept, but we worked with 2 g of sample; to save time and to minimize ME, the step of evaporation with Rotavapor was excluded, and the extract was evaporated to dryness directly under nitrogen. For the third method, a QuEChERS procedure with purification step was applied. The method of Rodríguez-Carrasco et al [126] was used with two modifications. Due to the high absorbability of the wheat products like flour and biscuits, a higher volume of solvent was added (10 mL of acetonitrile, instead of 7.5 mL) and to induce phase separation and mycotoxin partitioning after salts addition, the tubes were centrifuged for more time at low temperature (5 min at 5°C and 4500 rpm, instead of 3 min and 4000 rpm).

Rotatory shaker procedure had very good results (ME and recovery between 72% and 127%) to extract simultaneously ENs and BEA from breakfast cereals, and good results (ME and recovery between 53% and 125%) for the extraction of wheat flour, pasta, and biscuits. Using Ultra-Turrax procedure, which gave the best results to extract ENs and BEA from the four matrices analyzed, the ME and recovery were higher than 70%. QuEChERS procedure has the advantage of giving purified extracts, but, having more steps in the extraction process, recoveries and ME could be negatively influenced. This method offered a wide range of values for these parameters: ME between 32% to 144% and mean recoveries between 50% and 125%. The results from the comparative study of the three methods for EN and BEA extraction from flour, pasta, breakfast cereals, and biscuits are presented in Fig. 17.

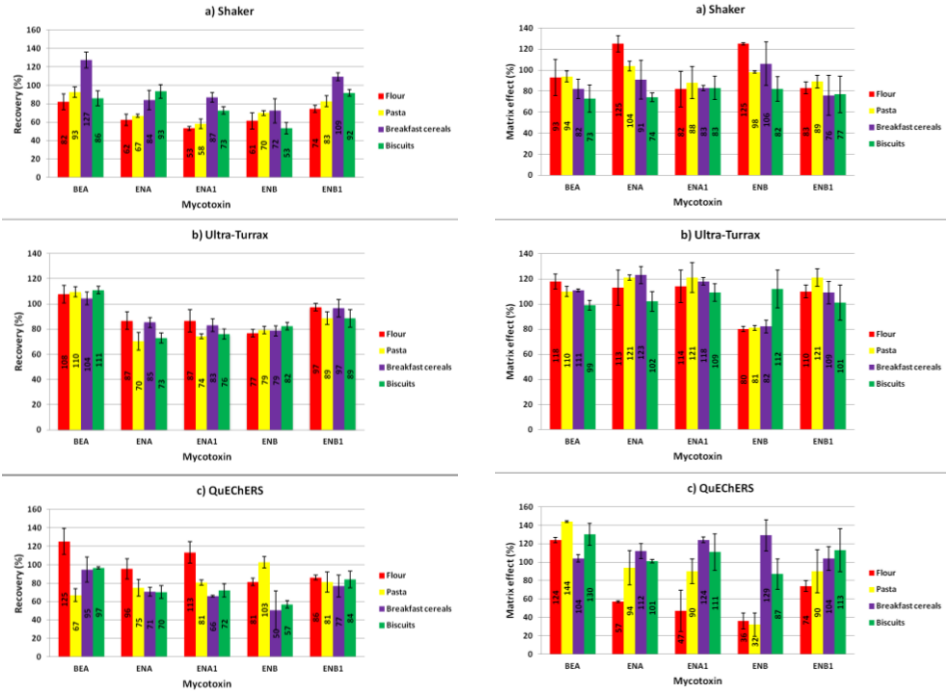


Fig. 17. Mean recoveries and average matrix effect for wheat-based products presented by extraction procedures: **a)** Shaker, **b)** Ultra-Turrax, **c)** QuEChERS.

Based on the comprehensive evaluation of the recovery and ME results [226] and taking into account that the extraction procedure must serve for a simultaneous analysis of ENs and BEA, the most suitable procedure for the extraction of these mycotoxins from flour, pasta, breakfast cereals, and biscuits was the Ultra-Turrax method, giving values higher than 80% and 70% for ME and recovery, respectively. Moreover, the levels for ME and recovery were uniform for this procedure, with a reduced range of values. The use of Ultra-Turrax provided optimum recoveries and

satisfactory values for ME for ENs and BEA, probably because the Ultra-Turrax promotes the contact of the matrix with the solvent. It should be noted that Ultra-Turrax procedure avoids purification of the extract in order to reduce analysis time, but this is possible if a clear extract, suitable for LC-QqQ-MS/MS analysis, is achieved. When purification is needed, QuEChERS procedure can be used successfully, even if this method presents some limitations concerning recovery and ME. Related to shaker method, values closer to those obtained using Ultra-Turrax have been observed, so that rotatory shaker can be an alternative, especially when a high number of samples must be extracted, because it is the fastest of the three procedures.

7.4.2. Method validation of Ultra-Turrax procedure

In order to apply Ultra-Turrax method for analysis of wheat-based products, the method was validated as a quantitative confirmatory method (Table XXIII). The performance of the method is reflected in the LQs, ME, and recoveries rates.

Table XXIII. Analytical performance of Ultra-Turrax method: limit of detection (LD) and quantification (LQ), linearity expressed as correlation coefficient (r^2), matrix effect (ME), recovery, intraday (n=3) and interday (n=3) precision as relative standard deviation.

MATRIX	ANALYTE	LD	LQ	Linearity (r^2)	ME (%)	Recovery (%)			Intraday precision (%)			Interday precision (%)		
		($\mu g\ kg^{-1}$)				LQ	2LQ	10LQ	LQ	2LQ	10LQ	LQ	2LQ	10LQ
Flour	BEA	1	2	0.996	118	113	127	83	11	5	6	16	11	16
	ENA	6	12	0.998	113	96	76	88	6	2	14	9	2	19
	ENA1	2	4	0.993	114	101	69	90	3	11	13	5	12	5
	ENB	0.5	1	0.992	80	61	83	86	7	1	2	7	8	7
	ENB1	1	2	0.989	110	94	102	96	1	4	5	8	6	8
Pasta	BEA	1	2	0.991	110	112	126	91	3	1	9	12	7	12
	ENA	6	12	0.998	121	61	66	84	9	6	7	17	2	21
	ENA1	2	4	0.996	121	73	63	87	5	1	1	12	17	12
	ENB	0.5	1	0.989	81	66	86	86	1	6	1	1	4	1
	ENB1	1	2	0.994	121	91	90	85	6	2	7	6	8	7
Breakfast cereals	BEA	1	2	0.999	111	122	108	83	1	13	1	10	2	21
	ENA	6	12	0.995	123	102	72	82	8	1	4	3	6	2
	ENA1	2	4	0.997	118	84	78	87	14	1	1	11	3	10
	ENB	0.5	1	0.994	82	78	71	87	3	3	7	12	19	16
	ENB1	1	2	0.992	109	111	89	90	8	2	11	11	12	11
Biscuits	BEA	1	2	0.992	99	125	122	86	3	2	3	5	6	8
	ENA	6	12	0.998	102	62	69	88	7	1	3	8	11	3
	ENA1	2	4	0.990	109	69	70	89	3	3	7	3	11	7
	ENB	0.5	1	0.995	112	82	76	89	3	1	5	11	17	5
	ENB1	1	2	0.994	101	91	88	87	9	5	3	6	14	3

Linear regression coefficients of all calibration curves demonstrated a good linearity, with the corresponding correlation coefficients (r^2) higher than 0.990. Ion suppression was observed only for ENB in flour, pasta, and breakfast cereals and for BEA in biscuits. LDs and LQs were between 0.5 and 12 $\mu g\ kg^{-1}$, admitting the same value for each of them in the four matrices. The range of recovery values for the three levels of concentration tested in the four matrices was between 61% (ENB) and 127%

(BEA). Regarding precision values evaluated from the RSDs of intraday precision and interday precision, these were lower than 14% and 21%, respectively. This method is sensitive, of low cost, reproducible, and it presents the advantages of being simple, with no further clean-up, and also environmental friendly due to small amounts of reagents used.

7.4.3. Emerging mycotoxin occurrence in wheat-based products

A total of 111 wheat-based products for direct human consumption (flour, pasta, breakfast cereals, and biscuits) commercialized in Romania were analyzed by LC-QqQ-MS/MS to evaluate the presence of ENA, ENA1, ENB, ENB1, and BEA. Thirty-five samples (32%) presented detectable levels of ENA1, ENB, ENB1, and BEA. ENB was the most detected (35/111, 32%), followed by ENB1 (18/133, 16%), ENA1 (1/111, 1%), and BEA (1/111, 1%). Levels between LDs and LQs were found in eleven situations (Table XXIV).

Table XXIV. Summary of emerging mycotoxin levels in Romanian wheat-based products.

ANALYTE	PARAMETER	Flour (n = 41)	Pasta (n = 40)	Breakfast Cereals (n = 7)	Biscuits (n = 23)	TOTAL (n = 111)
BEA	Incidence	0	0	0	1	1
	LD-LQ	0	0	0	1	1
	Frequency	0	0	0	4	1
	Mean (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
	Max. (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
ENA	Incidence	0	0	0	0	0
	LD-LQ	0	0	0	0	0
	Frequency	0	0	0	0	0
	Mean (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
	Max. (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
ENA1	Incidence	0	1	0	0	1
	LD-LQ	0	1	0	0	1
	Frequency	0	3	0	0	1
	Mean (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
	Max. (μg kg^{-1})	n.q.	n.q.	n.q.	n.q.	n.q.
ENB	Incidence	12	11	2	10	35
	LD-LQ	1	0	0	3	4
	Frequency	29	28	29	43	32
	Mean (μg kg^{-1})	1.8	10.4	1.9	1.7	4.9
	Max. (μg kg^{-1})	38.2	170	7.8	9.7	170
ENB1	Incidence	2	9	1	6	18
	LD-LQ	0	2	0	3	5
	Frequency	5	23	14	26	16
	Mean (μg kg^{-1})	0.5	1.9	0.5	0.7	1.0
	Max. (μg kg^{-1})	16.6	44.8	3.6	6.2	44.8

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; LD-LQ: number of samples ≥ LD and ≤ limit of quantification (LQ); Mean: average of total samples, assuming a zero value for samples ≤ LQ; n.q.: not quantified.

Quantifiable levels were found only for ENB and ENB1, ranging from 1.2 to 170 and from 2.2 to 44.8 μg kg^{-1}, respectively. The averages of the positive samples (above LQs) were 17.5 μg kg^{-1} and 8.7 μg kg^{-1} for ENB and ENB1, respectively. The highest

mean values of ENB and ENB1 (10.4 and 1.9 μg kg^{-1}, respectively) and the highest levels for these mycotoxins (170 and 44.8 μg kg^{-1}, respectively) were found in pasta, followed by flour, biscuits, and breakfast cereals. Fig. 18 shows the chromatograms for standard mix, matched blank flour, and a wheat flour sample contaminated simultaneously with ENB and ENB1.

Simultaneous contamination was observed for 18 (16%) of the samples analyzed (51% of the positive samples were contaminated with two or three emerging mycotoxins). The most frequent situation was the contamination with two mycotoxins simultaneously (ENB+ENB1). Two samples were contaminated with three mycotoxins (one with ENA1+ENB+ENB1 and another one with BEA+ENB+ENB1) [227].

Regarding the organic samples of wheat-based products, the results showed that only one flour sample presented a detectable level of ENB (1.25 μg kg^{-1}), and the other mycotoxins were not detected.

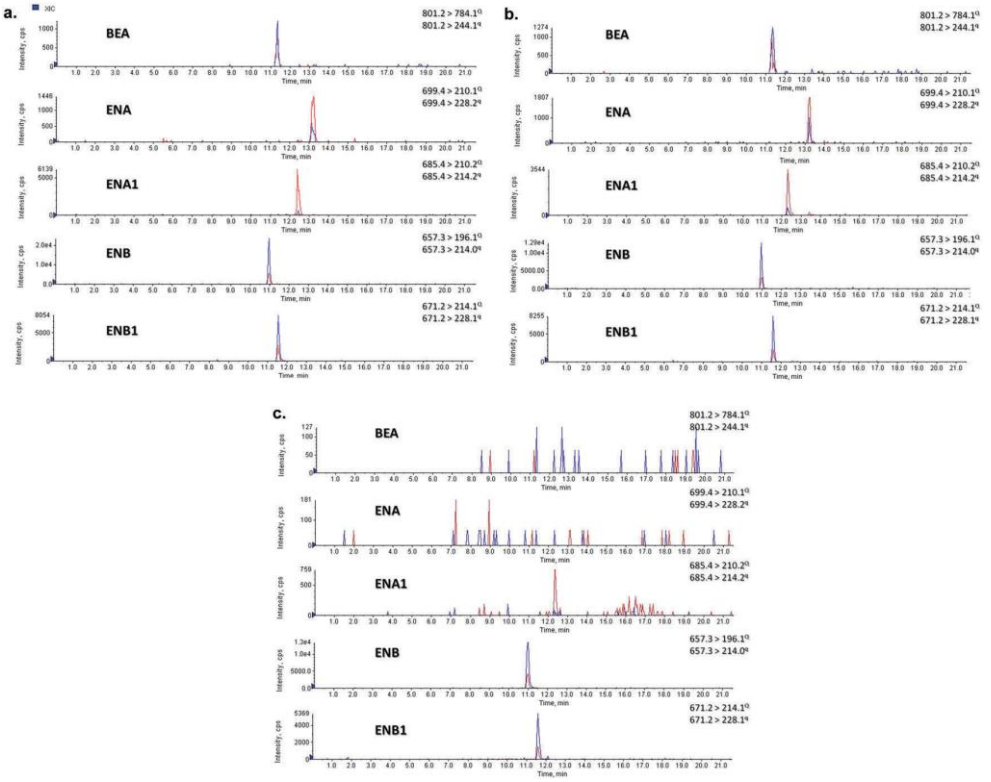


Fig. 18. SRM chromatograms for quantitative (Q) and qualitative (q) ions of: **a)** standard mix at concentrations of 12.5 μg mL^{-1} for ENs and 8 μg mL^{-1} for BEA, **b)** flour blank matched at concentrations of 12.5 μg kg^{-1} for ENs and 8 μg kg^{-1} for BEA, **c)** positive wheat flour sample contaminated with 9.8 μg kg^{-1} of ENB and 2.3 μg kg^{-1} of EB1.

As was also noticed previously by other authors [228], type B ENs were more detected than type A ENs and BEA. The incidences varied from 28% (pasta) to 43% (biscuits) for ENB and from 5% (flour) to 26% (biscuits) for ENB1. The fact that no ENA was detected in the wheat-based products analyzed is interesting, since low levels of ENA were detected in 8% of the wheat samples evaluated. This could be explained by the influence of the cleaning and debranning procedures used during the technological process of wheat that could reduce mycotoxin content [229]. Like in our study, unquantifiable levels of BEA were observed in pasta and biscuits from Italy [154,221]. Concerning ENs, similar incidences have been reported for Italian pasta (4% for ENA, ENA1, and ENB1 and 44% for ENB) [154], and Romanian flour (9%, 11%, 80%, and 17% for ENA, ENA1, ENB, and ENB1, respectively) [199]. Additionally, higher occurrences have been reported in other studies which analyzed Italian pasta (33%, 94%, and 90% for ENA, ENA1, and ENB, respectively) [230], or Spanish refrigerated pizza dough (8% for ENA and 100% for ENA1, ENB, and EB1) [222].

Regarding the concentrations of ENs found in the wheat products, the levels from the present study were higher than those obtained by Quiles et al. [222] in refrigerated pizza dough (maximum of 14.96 μg kg^{-1} for ENA), but were similar to those noted in a study of Juan et al. [154] which reported a maximum concentration of 106 μg kg^{-1} for ENs in pasta. On the other hand, higher levels for ENs were reported by other authors in pasta (max. of 710 μg kg^{-1} for ENB [230] or 979 μg kg^{-1} for ENB1 [157]), multicereal baby food (max. of 1100 μg kg^{-1} for ENB) [154], and wheat semolina couscous (max. of 651 μg kg^{-1} for ENA) [231]. It must be mentioned that, generally, other ingredients than wheat (e.g., oat, maize, rye, rice and some fruits such as nuts, peanuts, raisins) included in wheat-based product recipes could be a source of mycotoxins; in this study, the contribution of these ingredients to mycotoxin content was minimized, as the products analyzed were selected on account of being composed mostly from wheat or white wheat flour.

7.4.4. Estimation of daily intake

To assess the risk for the Romanian population related to the exposure to emerging mycotoxins through the wheat product consumption, the EDIs were calculated at two different levels. The present study has the advantage of including a wide variety of products for direct human consumption commercialized in Romania, such as pasta, flour, breakfast cereals, and biscuits, the estimation being more accurate. At a LB scenario, the EDIs of the emerging mycotoxins analyzed ranged from 0 (BEA, ENA, and ENA1) to 25.8 ng kg^{-1} bw day^{-1} (ENB), whereas the EDIs at the UB scenario ranged between 5.3 (BEA) and 31.7 ng kg^{-1} bw day^{-1} (ENA). All EDI values calculated for ENs were lower than the hypothetic TDI proposed for the sum of ENs (1000 ng kg^{-1} bw day^{-1}) [163,225]. The total contribution of ENs to the hypothetic TDI for the Romanian population was 3.12% and 8.05% for LB and UB scenario, respectively (Table XXV).

Table XXV. Emerging mycotoxin exposure and risk assessment for the Romanian population through the consumption of wheat-based products.

MYCOTOXIN	EDI (ng kg^{-1} bw day^{-1})		%TDI	
	LB	UB	LB	UB
BEA	0	5.3	n.c.	n.c.
ENA	0	31.7	0	3.17
ENA1	0	10.7	0	1.07
ENB	25.8	27.8	2.58	2.78
ENB1	5.4	10.3	0.54	1.03
Sum of ENs	31.2	80.5	3.12	8.05

EDI: Estimated daily intake; LB: low bound scenario, calculated assuming a zero value for the samples ≤ limit of quantification (LQ); UB: upper bound scenario, calculated assuming the limit of detection (LD) value for the samples ≤ LD and the LQ value for the samples LD-LQ; %TDI: the percentage covered by the EDI from a proposed hypothetic tolerable daily intake (TDI) for the sum of ENs (1000 ng kg^{-1} bw day^{-1}); n.c.: not calculated because no TDI was proposed for BEA.

Due to a lack of *in vivo* toxicity data on BEA and ENs, a TDI or an acute reference dose for BEA or the sum of ENs for humans was not set until now. To obtain some insight into the possible risks of dietary exposure to BEA and the sum of ENs at the estimated levels of exposure, the CONTAM Panel proposed to compare the estimated chronic exposure levels with the doses reported to cause adverse effects upon therapeutic use of the drug fusafungine via nasal/oromucosal spraying taking a worst case approximation for converting the nasal/oromucosal dose levels to oral dose levels. An oral dose of 90 to 170 μg kg^{-1} bw day^{-1} was used as a rough estimate for a LOAEL for a mixture of ENs. In the absence of toxicity data on repeated exposure for BEA, the CONTAM Panel also used this range for BEA. On the other hand, using the Threshold of Toxicological Concern approach for human risk assessment (probability of adverse health effects and possible human health risks), the CONTAM Panel found a value of 0.025 μg kg^{-1} bw day^{-1} for BEA and 1.5 μg kg^{-1} bw day^{-1} for the sum of ENs [148]. Considering this data and other PMTDIs established for various mycotoxins [165], a hypothetic value of 1000 ng kg^{-1} bw day^{-1} was used for the TDI of the sum of ENs.

Comparing the results obtained in the present study (5.3 and 80.5 ng kg^{-1} bw day^{-1} for BEA and the sum of ENs, respectively, for high consumers) with the scenarios proposed by the CONTAM Panel and the hypothetic value proposed in this study, it is easy to observe that the EDIs calculated were lower than all these values. However, the EDI values from the present study were higher than the EDIs of emerging mycotoxins calculated for the Spanish population through the consumption of different wheat-based products. For example, 4.69, 1.08, or 13.19 ng kg^{-1} bw day^{-1} of ENs could be ingested by the Spanish population as a potential result of high consumption of refrigerated pizza dough [222], bread loaf [147], or pasta [157], respectively. Also, the EDIs of BEA were 0.5 ng kg^{-1} bw day^{-1} consuming pasta [157] and 4.94 ng kg^{-1} bw day^{-1} consuming refrigerated pizza dough [222], with both of these values representing an overestimation.

7.5. Conclusions

After a comparative study of three different extraction methods, the SLE using Ultra-Turrax procedure coupled to LC-QqQ-MS/MS method provided optimum recoveries and satisfactory values of ME for simultaneous extraction of ENs and BEA in flour, pasta, breakfast cereals, and biscuits. The main advantages of the proposed technique were the accessibility and rapidity. Moreover, good recoveries, low influence of the matrix, and good precision were observed, indicating the suitability of the method. The method was successfully applied to analyze for the first time in Romania the occurrence of emerging mycotoxins in 111 wheat-based products for direct human consumption (pasta, flour, breakfast cereals, and biscuits) commercialized in this country. Results showed that 32% of them presented detectable levels of BEA, ENA1, ENB, or ENB1. A low EDI of emerging mycotoxins was calculated for the Romanian population (with the maximum being a total of 86 ng kg^{-1} bw day^{-1}). The approximate risk assessment showed that the total contribution of ENs to the hypothetic TDI (1000 ng kg^{-1} bw day^{-1}) did not exceed 10%.

8. Study 6. Occurrence of trichothecenes and zearalenone in wheat-based products and exposure assessment for the Romanian population

Study included in the article entitled "Evaluation of trichothecenes and zearalenone by GC-QqQ-MS/MS in flour, pasta, bread, biscuits, and breakfast cereals from Romania"

in process of submitting (2017)

Abstract

In this study, a dietary exposure assessment of mycotoxins was conducted for the Romanian population using the contamination data of a various categories of wheat-based products for direct human consumption. Wheat-based foods (n=181) commercialized in Romania, including flour, bread, biscuits, breakfast cereals and pasta, were evaluated for the occurrence of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON), fusarenon-X, nivalenol, HT-2 and T-2 toxins, diacetoxyscirpenol, neosolaniol and zearalenone (ZEA). Exposure of Romanian adult population was assessed by joining national consumption data on wheat products in 2016 (365 g per capita per day) with the analytical results, supposing two scenarios, one underestimating (lower bound – LB) and another one overestimating (upper bound – UB) the exposure. Estimated daily intake (EDI) values calculated were compared with the available tolerable daily intake (TDI) values established. The highest EDIs were observed for the sum of DON+3AcDON+15AcDON (669 ng kg^{-1} bw day^{-1} at LB, and 689 ng kg^{-1} bw day^{-1} at UB), being lower than the TDI set (1000 ng kg^{-1} bw day^{-1}). For ZEA, a maximum EDI of 26 ng kg^{-1} bw day^{-1} was calculated, below the TDI (200 ng kg^{-1} bw day^{-1}).

8.1. Introduction

Wheat is a highly-consumed cereal, particularly in Europe. Romania presents a high consumption of wheat and wheat-based products (365 g per capita per day), being the the third highest consumer of wheat and products, after Italy and Malta [33]. The flour milling industry is the main consumer of wheat, this cereal being the key substrate for bread production [211,232]. Wheat-based products are very important and highly consumed in the human diet, and their quality and safety could affect directly human health status. Therefore, quality control during wheat processing throughout the entire food chain is a determinant strategy in the public health area [84].

Trichothecenes and ZEA are important *Fusarium* mycotoxins with high occurrence in wheat and wheat-based products [22,138,233]. Due to the high occurrence and the possible adverse effects for human health, EFSA and JECFA encouraged

continuous research on mycotoxin presence and risk assessment linked with these compounds, including also the masked forms [165,234]. Based on toxicological studies, MLs in various classes of wheat products [161,162], and TDI or PMTDI values for certain mycotoxins (e.g. DON, NIV, ZEA, HT-2 and T-2) were set by the international organizations [163–166].

Monitoring exposure to various mycotoxins has become a key part of ensuring food safety. Dietary exposure is defined as the amount of a certain substance that is consumed and is usually estimated by combining food consumption data with data on the concentration of chemicals in food [223]. For this, deterministic or probabilistic assessements can be performed. Deterministic estimations of exposure assume that all individuals consume certain group of foods at the same period of time, at a same level, and the parent mycotoxins and their masked forms are present continuously at an average level. On the other hand, in probabilistic analysis every possible value that each variable can have and the weight of each possible scenario for the probability of its occurrence are taken into consideration [234]. The surveillance and exposure studies are indispensable for human health concern and these investigations gain higher importance for vulnerable groups in the population, such as babies and young children [235].

8.2. Aims

Taking into account the Romanian background in the field of mycotoxin research in products for direct human consumption, the aims of this study were: (i) to validate a selective multi-mycotoxin GC-QqQ-MS/MS method for the analysis of nine trichothecenes and ZEA in flour, pasta, breakfast wheat-based cereals, bread and biscuits; (ii) to survey the levels of these mycotoxins in wheat-based products commercialized in Romania; (iii) to estimate the daily intake of mycotoxins through wheat-based product consumption for the Romanian population. This is the first survey concerning the estimated daily intake of both legislated and non-legislated mycotoxins through wheat-based product consumption by the Romanian population.

8.3. Materials and methods

8.3.1. Sampling

Mycotoxin-free powdered samples were used as blank material for validation study. Also, a total of 181 samples of wheat-based products were purchased from different markets located in Cluj-Napoca (Romania) during April to October 2016: white wheat flour (n = 41); pasta with minimum of 73% wheat (n = 40); breakfast cereals containing between 54% and 90% wheat (n = 7); integral biscuits containing between 49% and 95% wheat (n = 23); wheat flour-based bread (n = 70). Two to six packages for each sample were purchased, obtaining an aggregate sample of at least 1

kg total weight. All samples were milled to a fine powder using a laboratory mill. After homogenization, 500 g samples were packed in plastic bags and kept at -20°C in a dark and dry place until analysis. Three replicates for each sample were weighed for analysis.

8.3.2. Extraction

Extraction was performed according to the procedure described in section 3.3.2, using SLE. All experiments were performed in triplicate. After residue reconstitution and filtration, a volume of 200 µL of filtrate was placed in a chromatographic vial and was evaporated to dryness at 35°C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, MA, USA). Over the dry extract, the derivatization was performed as it was described in section 6.3.3.2. Finally, the upper layer was transferred to an autosampler vial for chromatographic analysis.

8.3.3. Mycotoxin analysis

The analysis of the ten mycotoxins was performed with a GC-QqQ-MS/MS system, using the method presented in the section 2.2.2. Statistical analysis was performed using SPSS software (IMB Corp, IBM SPSS Statistics, Armonk, NY, USA), version 22.0.

8.3.4. Method validation

Validation of the method was performed for linearity, accuracy, repeatability (intraday and interday precision) and sensitivity, following the EU Commission Decision, 2002/657/EC [190], as it was presented in the section 7.3.4. External standard calibration was used in the validation of the analytical method. The criteria for confirmation of positive findings were: comparison of peak area ratios for quantification (Q) and confirmation (q) transitions with that of the reference standard; peak ratio of the confirmation transition against quantification one; agreement with the retention times.

The validated method was used to quantify emerging myctoxins in real wheat-based samples commercialized in Romania. Matrix-matched calibration curves at concentrations between 1 and 2000 µg kg^{-1} were used for quantification. Samples presenting levels higher than LDs were considered to estimate the incidence (%) for each mycotoxin.

8.3.5. Estimation of daily intake

The dietary exposure to each mycotoxin was evaluated by calculating the EDI. For this, a deterministic method [223] was applied, using the equation:

$$\text{EDI (ng kg}^{-1}\text{ bw day}^{-1}) = \frac{\sum c * K}{N * Bw}$$

where ∑c is the sum of each mycotoxin in the samples analyzed (μg kg^{-1}), K is the daily average consumption/person for the food commodity included in the study (g $capita^{-1}$ day^{-1}), N is the total number of analyzed samples, and Bw is the body weight used in the population group.

To obtain the sum of each mycotoxin for the samples analyzed, two different scenarios were designed: underestimating (LB) and overestimating (UB) the exposure. The LB was obtained by setting a zero value for all samples with levels lower than LQ, whereas the UB was achieved by assigning the LD to those samples with undetected levels and the LQ to those samples with levels between the LD and LQ [224].

To calculate the EDI, the food supply quantity of wheat and products in Romania was considered 365 g $capita^{-1}$ day^{-1}, according to FAOSTAT [33], and a default average body weight of 70 kg was assumed for Romanian population.

8.4. Results and discussion

8.4.1. Method validation

All mycotoxins included in the study exhibited a good linearity over the working range in both standard solution and matrix-matched calibration curves, with corresponding correlation coefficients (r^2) higher than 0.989. LDs of the mycotoxins analyzed were: 0.5 μg kg^{-1} (DON), 1.25 μg kg^{-1} (3AcDON), 2.5 μg kg^{-1} (15AcDON, FUS-X, and T-2), 5 μg kg^{-1} (ZEA), 7.5 μg kg^{-1} (DAS and HT-2), and 10 μg kg^{-1} (NEO and NIV), whereas LQs were: 1 μg kg^{-1} (DON), 2.5 μg kg^{-1} (3AcDON), 5 μg kg^{-1} (15AcDON, FUS-X, and T-2), 10 μg kg^{-1} (ZEA), 15 μg kg^{-1} (DAS and HT-2), and 20 μg kg^{-1} (NEO and NIV). ME ranged between 64% and 133% for flour, between 59% and 141% for pasta, between 61% and 157% for breakfast cereals, between 69% and 142% for biscuits, and between 59% and 134% for bread (Fig. 19). Concerning recovery studies, it was remarked that values lower than 100% were predominant for the low spiked levels (LQ and 2LQ), whereas at high spiked level (10LQ), recoveries higher than 100% were calculated (Fig. 20). Regarding precision values evaluated from the RSD of intraday and interday precision, these were lower than 15% and 21%, respectively. This analysis is sensitive, precise and reproducible, presenting the advantages of being a multi-mycotoxin method, user-friendly and possessing a high performance.

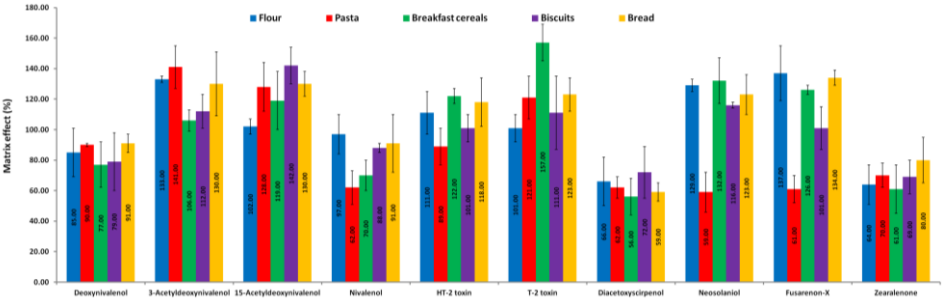


Fig. 19. Matrix effect (%) of the GC-QqQ-MS/MS method for wheat-based products analysis.

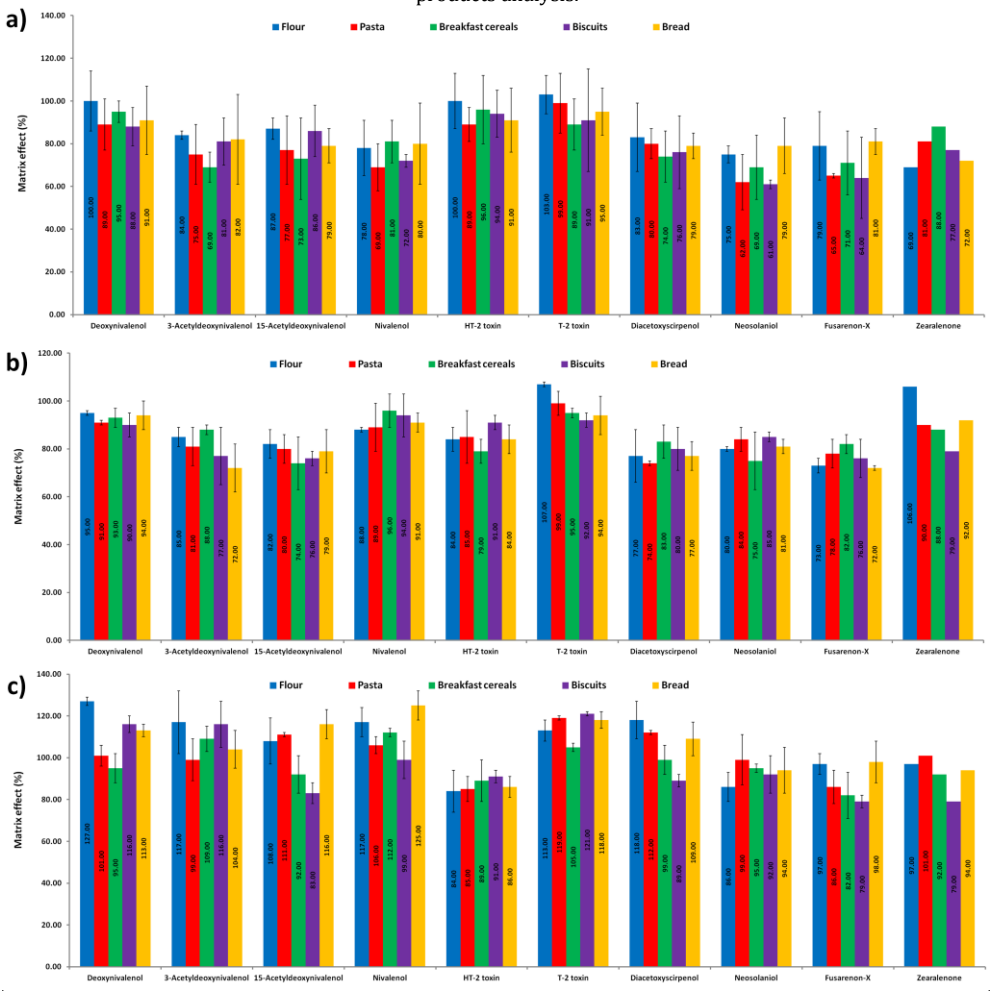


Fig. 20. Recovery values (%) of the GC-QqQ-MS/MS method for wheat-based products analysis at three spiked levels: **a)** LQ, **b)** 2LQ, **c)** 10LQ.

8.4.2. Trichothecene and zearalenone occurrence in wheat products

A total of 181 wheat-based products for direct human consumption (flour, pasta, bread, breakfast cereals, and biscuits) commercialized in Romania were analyzed by GC-QqQ-MS/MS to evaluate the presence of DON, 3AcDON, 15AcDON, NIV, FUS-X, NEO, DAS, HT-2, T-2, and ZEA. DON was the most detected (114/181, 63%), followed by 15AcDON (10/181, 5.5%), while the other mycotoxins were not detected. Levels between LDs and LQs were found in four bread samples. Quantifiable levels were found for DON and 15AcDON, ranging from 1.9 to 1947 and from 14.2 to 32.6 µg

kg^{-1}, respectively. The averages of the positive samples (above LQs) were 202 μg kg^{-1} and 25.2 μg kg^{-1} for DON and 15AcDON, respectively (Table XXVI).

For DON, the highest frequency was observed for pasta (34/40, 85%), followed by bread (46/70, 66%) and biscuits (14/23, 61%), whereas for 15AcDON the highest frequency was found in flour (4/41, 10%). The highest mean value of DON (353 μg kg^{-1}) was calculated for flour, and concerning 15AcDON, very close mean values were calculated for flour (24.6 μg kg^{-1}) and pasta (26.4 μg kg^{-1}). The highest levels of DON and 15AcDON (1947, and 32.6 μg kg^{-1}, respectively) were found in flour. Three flour samples (817, 977, and 1947 μg kg^{-1}, respectively), and two samples of pasta (752, and 899 μg kg^{-1}, respectively) exceeded the ML of 750 μg kg^{-1} established by the EC [161]. The same situation was registered also for three samples of bread (542, 553, and 1594 μg kg^{-1}, respectively) and one biscuit sample (1010 μg kg^{-1}), exceeding the ML of 500 μg kg^{-1} set by the EC [161].

Simultaneous contamination was observed in 10 (5.5%) of the samples analyzed, 8.8% of the positive samples being contaminated with DON and 15AcDON at the same time. 15AcDON was found in all situations together with DON.

Table XXVI. Summary of trichothecene levels in Romanian wheat-based products.

ANALYTE	PARAMETER	Flour (n = 41)	Pasta (n = 40)	Bread (n = 70)	Breakfast Cereals (n = 7)	Biscuits (n = 23)	TOTAL (n = 181)
DON	Incidence	17	34	46	3	14	114
	LD-LQ	0	0	0	0	0	0
	Frequency	42	85	66	43	61	63
	Mean$^{\#}$ (μg kg^{-1})	353	181	190	17.4	148	202
	Mean§ (μg kg^{-1})	147	154	125	7.5	90	127
	Max. (μg kg^{-1})	1947	899	1594	24.2	1010	1947
15AcDON	Incidence	4	2	4	0	0	10
	LD-LQ	0	0	4	0	0	4
	Frequency	10	5	5.7	0	0	5.5
	Mean$^{\#}$ (μg kg^{-1})	24.6	26.4	n.q.	n.q.	n.q.	25.2
	Mean§ (μg kg^{-1})	2.4	1.3	n.q.	n.q.	n.q.	0.83
	Max. (μg kg^{-1})	32.6	29.4	n.q.	n.q.	n.q.	32.6

Frequency: % of samples ≥ limit of detection (LD) / total samples; Incidence: number of samples ≥ LD; LD-LQ: number of samples ≥ LD and ≤ limit of quantification (LQ); n.q.: not quantified; $^{\#}$: mean value for samples ≥ LQ; §: mean value for all samples, assuming a zero value for samples ≤ LQ.

The presence of different trichothecenes and ZEA in various wheat products was also observed during previous studies. De Boevre et al [234], evaluating the presence of trichothecenes, ZEA and their masked forms in cereal-based foods from Belgium found that DON is a major contaminant in the wheat based matrices, a total of 85% and 44% of the fibre and bran-enriched bread samples being contaminated with DON at low levels (max. of 138 μg kg^{-1}) considering the ML set by the EC of 500 μg kg^{-1}. On the other hand, breakfast cereals presented concentrations of DON higher than the ML of 500 μg kg^{-1} (max. of 718 μg kg^{-1}). On the contrary of the results from our study, De Boevre et al [234] observed that 14%, 22%, and 39% of the bran-enriched bread

samples were contaminated with T-2, HT-2, and ZEA, respectively, and Tolosa et al [230] reported high incidence percentages for T-2 (77%), HT-2 (90%), and ZEA (93%), while in the present study none of these mycotoxins was detected in wheat-based products. Similar to our study, the absence of T-2 and HT-2 was confirmed also by Pleadin et al [236], analyzing pasta and wheat flour from Croatia and Bosnia and Herzegovina. Another recent study from Croatia, comparing organic and conventional cereal products, found that 60% and 33% of the wheat flour samples analyzed were contaminated with DON and ZEA, respectively, DON ranging from 27.1 to 126 μg kg^{-1}, and ZEA from 4.1 to 10.1 μg kg^{-1} [101].

A study from Italy [108], applying a multi-mycotoxin analysis on different commercialized organic cereals, revealed that 28% of the wheat samples analyzed were contaminated with DON (9.6-99.6 μg kg^{-1}), 9% with ZEA (2.35-27.15 μg kg^{-1}), 5% with HT-2 (6.78-60.10 μg kg^{-1}), 4% with T-2 (7.14-17.8 μg kg^{-1}), 19% with NIV (12-106 μg kg^{-1}), while other mycotoxins such as NEO, NIV, or 3AcDON were not detected. Rodríguez-Carrasco et al [237] also found a high incidence for DON (95/119, 80%) in wheat-based cereals, presenting a maximum level of 83.2 μg kg^{-1}. In the same study, 17% and 13% of wheat-based cereal samples were contaminated with HT-2 and NIV, respectively, while 3AcDON, FUS-X, NEO, and T-2 were detected in less than 4% of the samples, and DAS and ZEA were not detected.

Regarding other studies on wheat flour, heterogeneous results are publised. For example, in Serbia, DON was found in 87% of wheat flour samples (max. of 976 μg kg^{-1}) [122], and similar results were calculated in Danish [103] and Portuguese [116] flour samples, with an incidence of 89% (max. 2591 μg kg^{-1}), and 80% (max.77 μg kg^{-1}), respectively. On the other hand, research on wheat flour samples reported modest values for maximum concentration of ZEA, as 21.1 μg kg^{-1}, 37.2 μg kg^{-1}, and 39.4 μg kg^{-1} in Serbian, Dutch, and Portuguese wheat flour samples, respectively [122,144]. ZEA was also detected in bread loaf from Spain, 65% of the 80 samples analyzed being positive for ZEA, with a maximum level of 905 μg kg^{-1} [147].

In the last years, literature presents also studies concerning mycotoxin presence and risk assessment on wheat-based products less studied, including semolina, couscous semolina, or refrigerated pizza dough. For example, Zinedine et al [231] found that ZEA is one of the most prevalent (29/84, 35%) mycotoxin in wheat semolina couscous, presenting levels between 22 and 132 μg kg^{-1}, DON was less present (18/84, 21%) at levels from 20.6 to 106.6 μg kg^{-1}, and NIV was present in 15% of the samples (13/84), at higher levels (from 52.4 to 462.2 μg kg^{-1}). In the same study [231], the low occurrence of 3AcDON (1/84, 1%), 15AcDON (6/84, 7%), and T-2 (2/84, 2%), and the absence of DAS and HT-2 in wheat-based products were confirmed. On the other hand, after analyzing the occurrence of various *Fusarium* mycotoxins in fresh refrigerated pizza dough samples, Quiles et al [222] remarked that 100% of the samples presented

detectable levels of ZEA, ranging from 28.64 to 176.28 μg kg^{-1}, 12% being higher than de maximum legislated level.

8.4.3. Estimation of daily intake

Results from the present study allowed assessing the risk for the Romanian population related to the exposure to trichothecenes and ZEA through wheat product consumption. Therefore, EDIs were calculated at two different levels, under- and overestimating. The present study has the advantage of including a wide variety of products for direct human consumption commercialized in Romania, such as pasta, flour, breakfast cereals, bread and biscuits, the estimation being more accurate. At a LB scenario, the EDIs ranged from 0 ng kg^{-1} bw day^{-1} (NIV, NEO, DAS, FUS-X, ZEA, sum of HT-2 and T-2,) to 668.9 ng kg^{-1} bw day^{-1} (the sum of DON, 3AcDON and 15 AcDON), whereas the EDIs at the UB scenario ranged between 13 ng kg^{-1} bw day^{-1} (FUS-X) and 689.6 ng kg^{-1} bw day^{-1} (the sum of DON, 3AcDON and 15 AcDON). All EDI values calculated for the mycotoxins evaluated were lower than the TDI set by the EC (Table XXVII).

Table XXVII. Trichothecene and zearalenone exposure and risk assessment for the Romanian population through the consumption of wheat-based products.

MYCOTOXIN	TDI or PMTDI (ng kg^{-1} bw day^{-1})	EDI (ng kg^{-1} bw day^{-1})		%TDI	
		LB	UB	LB	UB
DON	1000 [163]	664.6	665.5	66	67
3AcDON		0	6.5	n.c.	n.c.
15AcDON		4.35	17.5	n.c.	n.c.
Sum of DON+3AcDON+15AcDON	1000 [165]	668.9	689.6	67	69
HT-2		0	13	n.c.	n.c.
T-2		0	52.1	n.c.	n.c.
Sum of HT-2+T-2	100 [166]	0	65.2	0	65
NIV	700 [163]	0	39.1	0	6
NEO		0	52.1	n.c.	n.c.
DAS		0	39.1	n.c.	n.c.
FUS-X		0	13	n.c.	n.c.
ZEA	200 [164]	0	26.1	0	13

EDI: Estimated daily intake; LB: low bound scenario, calculated assuming a zero value for the samples ≤ limit of quantification (LQ); UB: upper bound scenario, calculated assuming the limit of detection (LD) value for the samples ≤ LD and the LQ value for the samples LD-LQ; %TDI: the percentage covered by the EDI from the tolerable daily intake (TDI) or provisional maximum TDI (PMTDI); n.c.: not calculated because no TDI was proposed.

Human exposure to mycotoxins mainly occur via the food chain [2,79,84]. Up until now, no data is available in the literature on the presence of *Fusarium* mycotoxins in wheat-based products for direct human consumption commercialized in Romania. Comparing the results obtained in the present study with the TDI set by the international organizations, it is easy to observe that the EDIs calculated were lower than all these values. Even so, special attention should be paid to the values for DON, the EDI of DON through the wheat-based product consumption by the Romanian population representing more than 50% of the TDI (Table XXVII). Thus, further

monitoring surveys concerning *Fusarium* mycotoxin presence in foodstuffs from Romania are required.

However, the EDI values of ZEA from the present study were higher than the EDIs calculated for the Spanish population through the consumption of different wheat-based products. For example, 15.8, 2.9, or 0 ng kg^{-1} bw day^{-1} of ZEA could be ingested by the Spanish population as a potential result of high consumption of refrigerated pizza dough [222], bread loaf [147], or wheat-based products [237], respectively. Also, lower EDI values of DON than those from the present study were obtained by Rodríguez-Carrasco et al [237] or Pleadin et al [101], the contribution of these values to the TDI not exceeding 11% at a high consumption of wheat-based products from Spain and Croatia, respectively. Closer EDI values to the results from our study for DON+3AcDON+15AcDON, ZEA, and HT-2+T-2 were observed in the survey published by Sirot et al [204] in the second French total diet study, while Zinedine et al [231] reported higher EDI levels than our results for these groups of mycotoxins.

8.5. Conclusions

This investigation provides the first data on multi-mycotoxin occurrence in wheat-based products for humans consumed in Romania. Moreover, the risk associated with the exposure to both regulated and unregulated *Fusarium* mycotoxins through intake of wheat-based products has never been studied for the Romanian population. Even if same samples exceeded the ML of DON (500 or 750 μg kg^{-1}, depending on the food commodity [161]), this study concluded that there is no risk linked with the DON intake through the consumption of wheat-based products in Romania, even at an overestimated exposure. Furthermore, for HT-2, T-2, NIV, and ZEA too, the EDI values calculated did not exceed the TDI allowed. As for some mycotoxins like NEO, DAS, and FUS-X there are no official data regarding the TDI, the intakes estimated in this study are for orientation purposes. To have an overview of the presence of mycotoxins in wheat based products from Romania and the contribution of these products to mycotoxin intake by the Romanian population, a further attention to this type of studies should be given.

9. General discussion

To achieve the objectives of the thesis, a total of 349 samples from Romania were analyzed, distributed as following:

- **133 wheat samples**:
  - 31 samples harvested in 2014,
  - 102 samples harvested in 2015.
- **216 wheat-based products**:
  - 35 wheat flour samples purchased in 2014,
  - 41 wheat flour samples purchased in 2016,
  - 40 samples of pasta purchased in 2016,
  - 70 samples of bread purchased in 2016,
  - 23 samples of biscuits purchased in 2016,
  - 7 samples of breakfast cereals purchased in 2016.

The methods used to evaluate *Fusarium* mycotoxin presence in these samples include accessible and rapid techniques of extraction, without clean-up step, followed by LC-QqQ-MS/MS or GC-QqQ-MS/MS analysis. The choice of the analysis method (LC-QqQ-MS/MS or GC-QqQ-MS/MS) for each mycotoxin was influenced by the limit of detection, the method with the lowest values being selected. Good recoveries, low influence of the matrix, good precision, linearity and sensitivity were observed. The methods proposed were validated in order to evaluate *Fusarium* mycotoxin presence in six types of matrices (whole wheat, flour, pasta, bread, breakfast cereals, and biscuits), and the suitability of the methods was confirmed.

Once validated, the methods were applied in order to evaluate fourteen *Fusarium* mycotoxins in wheat and wheat-based products from Romania, including legislated (DON, ZEA, HT-2, T-2), and non-legislated (NIV, 3AcDON, 15AcDON, NEO, DAS, FUS-X, ENs, BEA) mycotoxins. DON and ENs showed the highest frequencies. With respect to the legislated mycotoxins, DON was the most detected in Romanian wheat (60/102, 59%), and wheat-based products (114/181, 63%). The highest frequency of DON was observed for pasta (34/40, 85%), whereas the highest levels of DON (mean value of 353 μg kg^{-1}) were found for wheat flour. From the emerging mycotoxins, ENB was the most prevalent in both wheat samples (55/133, 41%), and wheat-based products (35/111, 32%).

It was confirmed that the incidences and concentrations of mycotoxins are correlated with the geographic area and weather paramaters, particularly for ENs, DON, ZEA, and HT-2. Multivariate study for emerging mycotoxins underlined that the influence of climatic parameters on mycotoxin levels is specific for each region. For Braşov county, low temperatures and precipitation in May and June or high levels of precipitation in preharvest period (July) favor ENB and ENB1 presence, while in Dâmboviţa county, high total precipitation seem to increase ENA and ENA1 levels, and higher temperatures favor ENB and ENB1 development. For wheat from Teleorman county, increased levels of ENs are linked with high precipitation and lower mean temperatures in May, or lower levels of precipitation (mostly during April and June) combined with higher mean temperatures (particularly during July).

Nowadays, many consumers prefer organic rather than non-organic food, considering that organic production makes no use of synthetic fungicides and fertilizers. These practices can improve the nutritional qualities of food, but, at the same time, there exists the awareness that poorer use of fungicides may go in favour of mycotoxin presence in natural chemical-free products. Due to the high interest in organic food in the last years and the occurrence of various mycotoxins (including emerging mycotoxins) in wheat and its products, studies on wheat quality from different farming systems are justified and required. Analyzing the results from our studies related to organic and conventional Romanian wheat, higher incidences were registered for organic wheat, but, interestingly, the maximum levels were found for conventional samples. This could be explained by the multi-factorial influence in fungal growth and mycotoxin development based on plant substrate, topographic factors, weather parameters, or different management activities.

Concerning the dietary exposure to mycotoxins and health risk assessment for the Romanian population through the consumption of wheat-based products, a deterministic approach was performed. The EDIs of *Fusarium* mycotoxins were calculated for all mycotoxins (with or without a TDI established by the European regulations), trying to complete the toxicological data in field. The results confirm that there is no significant risk for the Romanian population correlated with the mycotoxin presence, but, continuous studies are required because certain samples for direct human consumption presented high levels of *Fusarium* mycotoxins and this can increase the risk.

10. General conclusions

The studies included in the present thesis allowed concluding the following:

1. The methods proposed, based on solid-liquid extractions and LC-QqQ-MS/MS or GC-QqQ-MS/MS analysis, have been satisfactory validated in order to analyze nine trichothecenes, ZEA and five emerging mycotoxins in six types of matrices: whole wheat, flour, pasta, bread, breakfast cereals, and biscuits.

2. DON and emerging mycotoxins are the most frequent *Fusarium* mycotoxins in wheat and wheat-based products for direct human consumption collected in Romania.

3. Closer frequencies and levels of DON are present in both wheat and wheat-based products. The highest frequency of DON corresponds to pasta, whereas the highest concentrations of DON are found in wheat flour. The fact that similar levels of DON are observed for wheat and wheat products such as flour, pasta, bread, biscuits and breakfast cereals too can be explained by the high transfer of DON through food chain from unprocessed wheat to its derivatives, possible contribution to mycotoxin content of other ingredients in wheat products for direct human consumption, or fungi development in packing bags.

4. Reduction of EN content during industrial processes such as baking or pasta elaboration is confirmed, as significant lower levels of emerging *Fusarium* mycotoxins are detected in wheat-based products in comparison with unprocessed wheat levels.

5. When mycotoxins co-occurr in wheat and its derivatives, two to five mycotoxins are present in the same sample, DON and its acetylated derivatives, or ENB and ENB1, being frequent combinations found.

6. The correlation between mycotoxin levels in wheat and climatic and geographic parameters in the grain-growing region is confirmed. Generally, extreme phenomena – rainy periods at the end of flowering, drought during grain formation or high moisture in the late preharvest period – are favorable for fungi development. Particularly, high precipitation during the months of May and July increase ENA1, ENB, and ENB1 occurrence and co-occurrence, while prolonged rainy weather during the months of May and June favors DON, HT-2 and ZEA occurrence.

7. Agricultural practices influence emerging mycotoxin presence in wheat cultivated in Romania. On a long-time consumption of wheat and wheat-based

products, it can be stated that the use of conventional farm practices is safer than organic practices. The most resistant cultivars to emerging mycotoxin contamination include Alcantara, Alex, Dropia, Felix, Hyfi, ITC-20, Lukulus, Miranda, Ponomicus, Solehio, and Urbanus cultivars.

8. Estimates of daily intakes of *Fusarium* mycotoxins through wheat-based product consumption by the Romanian adult population indicate that there is no remarkable risk for human health. However, monitoring studies are permanently required in order to mitigate mycotoxin contents.

10. Concluzii generale

Studiile incluse în prezenta teză au condus la următoarele concluzii:

1. Metodele propuse, bazate pe extracţii solid-lichid şi analize LC-QqQ-MS/MS sau GC- QqQ -MS/MS, au fost validate satisfăcător pentru a evalua nouă tricotecene, ZEA şi cinci micotoxine emergente în şase tipuri de matrici: grâu integral, făină, paste, pâine, cereale pentru micul dejun şi biscuiţi.

2. DON şi micotoxinele emergente sunt cele mai frecvente micotoxine ale genului *Fusarium* în grâu şi produse pe bază de grâu pentru consumul uman provenite din România.

3. Niveluri şi incidenţe asemănătoare ale DON sunt prezente în grâu şi produse pe bază de grâu. Cea mai mare incidenţă a DON corespunde pastelor, iar cele mai mari concentraţii de DON se găsesc în făina de grâu. Faptul că nivele similare de DON sunt observate pentru grâu şi produsele pe bază de grâu, cum ar fi făină, paste, pâine, biscuiţi şi cereale pentru mic-dejun, poate fi explicat de transferul ridicat de DON prin intermediul lanţului alimentar din grâu neprelucrat la derivatele sale, posibila contribuţie la conţinutul de micotoxine al altor ingrediente din produsele pe bază de grâu pentru consum uman sau dezvoltarea mucegaiurilor în pungile de ambalare.

4. Reducerea conţinutului de ENs în timpul proceselor industriale, cum ar fi panificaţia sau elaborarea pastelor, este confirmată, întrucât nivelele de micotoxine emergente detectate în produsele pe bază de grâu sunt cu mult mai scăzute decât cele din grâul neprocesat.

5. Când diferite micotoxine sunt înregistrate simultan în grâu sau produse derivate, două până la cinci micotoxine sunt prezente în aceeaşi probă, DON şi derivaţii săi acetilaţi şi, respectiv, ENB şi ENB1 fiind combinaţii frecvent regăsite.

6. Corelaţia dintre nivelele de micotoxine din grâu şi parametri climatici şi geografici din regiunea de cultură este confirmată. În general, fenomenele extreme - perioadele ploioase la sfârşitul înfloririi, seceta în timpul formării bobului sau umiditatea ridicată în perioada anterioară recoltării - sunt favorabile pentru dezvoltarea mucegaiurilor. În mod particular, precipitaţiile ridicate în lunile mai şi iulie favorizează apariţia şi co-apariţia de ENA1, ENB, şi ENB1, iar o vreme ploioasă prelungită în cursul lunilor mai şi iunie favorizează apariţia DON, HT-2 şi ZEA.

7. Practicile agricole influenţează prezenţa micotoxinelor în grâul cultivat în România. Pe termen lung, la consumul de grâu şi produse pe bază de grâu, se poate afirma că utilizarea practicilor agricole convenţionale este mai sigură decât practicile agriculturii organice. Soiurile cele mai rezistente la contaminarea cu micotoxine emergente includ soiurile Alcantara, Alex, Dropia, Felix, Hyfi, ITC-20, Lukulus, Miranda, Ponomicus, Solehio şi Urbanus.

8. Estimările aportului zilnic de micotoxine ale genului *Fusarium* prin intermediul consumului de produse pe bază de grâu de către populaţia adultă din România indică faptul că nu există un risc remarcabil pentru sănătatea umană. Cu toate acestea, studii de monitorizare sunt necesare în permanenţă, cu scopul de a preveni şi reduce conţinutul de micotoxine.

10. Conclusiones generales

Los estudios realizados en la presente tesis, han permitido concluir:

1. Los métodos propuestos, basados en las extracciones sólido-líquido, seguido del análisis multimicotoxina por LC-QqQ-MS/MS o GC-QqQ-MS/MS, han sido validados satisfactoriamente con el fin de evaluar la presencia de nueve tricotecenos, ZEA y cinco micotoxinas emergentes en seis tipos de matrices: trigo integral, harina, pasta, pan, cereales para el desayuno y galletas.

2. El DON y las micotoxinas emergentes son las micotoxinas de *Fusarium* más prevalentes en las muestras analizadas de trigo y productos a base de trigo destinados al consumo humano procedentes de Rumanía.

3. Las concentraciones y frecuencias de DON en trigo y productos a base de trigo fueron similares. El DON se detectó con más frecuencia en pasta, en cambio las concentraciones más elevadas de esta micotoxina se detectaron en harina de trigo. El hecho de que se observen niveles similares de DON en trigo y sus productos como harina, pasta, pan, galletas y cereales para el desayuno se puede explicar por la alta transferencia de DON a través de la cadena alimenticia, del trigo entero a sus derivados, la posible contribución al contenido de micotoxinas de otros ingredientes en productos de trigo para consumo humano directo, o el desarrollo de hongos en las bolsas de embalaje.

4. La reducción del contenido de ENs en los procesos industriales frecuentemente utilizados, tales como la panificación o la elaboración de pasta, se ha confirmado, al detectarse cotenidos inferiores de micotoxinas emergentes de *Fusarium* en los productos a base de trigo en comparación con los obtenidos en trigo sin procesar.

5. Se ha detectado coexistencia de micotoxinas en trigo y sus derivados, siendo las combinaciones de dos hasta cinco micotoxinas en la misma muestra. Las combinaciones más frecuentes encontradas fueron DON y sus derivados acetilados, o ENB y ENB1.

6. Se confirma la correlación entre los niveles de micotoxinas en trigo y los parámetros climáticos y geográficos en la región de cultivo de cereales. En general, fenómenos extremos - períodos de lluvia al final de la floración, la sequía en la

formación de grano o la humedad elevada en el periodo antes de la cosecha - son favorables para el desarrollo de hongos. En particular, las precipitaciones elevadas en los meses de mayo y julio aumentan la existencia y coexistencia de ENA1, ENB, y ENB1, mientras que épocas de lluvia prolongada en los meses de mayo y junio favorecen la presencia de DON, HT-2 y ZEA.

7. Las prácticas agrícolas influyen en la presencia de micotoxinas emergentes en el trigo cultivado en Rumania. Con respecto al consumo de trigo y productos a base de trigo a largo plazo, se puede afirmar que utilizar prácticas agrícolas convencionales es más seguro que utilizar las prácticas ecológicas. Las variedades de trigo más resistentes a la contaminación por micotoxinas emergentes son Alcantara, Alex, Dropia, Felix, Hyfi, ITC-20, Lukulus, Miranda, Ponomicus, Solehio, y Urbanus.

8. Las estimaciones de las ingestas diarias de micotoxinas de *Fusarium* a través del consumo de productos a base de trigo por la población adulta rumana indican que no existe un riesgo para la salud humana. Sin embargo, se requieren estudios de monitorización de forma permanente con el fin de vigilar el contenido de micotoxinas.

11. Originality and innovative contributions of the thesis

The original contribution of the thesis consists in the simultaneous analysis of both regulated (DON, ZEA, HT-2, T-2) and unregulated (other trichothecenes or emerging mycotoxins) *Fusarium* mycotoxins, in various types of wheat or wheat-based products produced or commercialized in Romania, from raw material (unprocessed wheat) to products for direct human consumption (wheat flour, bread, pasta, breakfast cereals, biscuits). In addition, using sensitive multi-component methods such as LC-QqQ-MS/MS and GC-QqQ-MS/MS, a complex and comprehensive investigation was performed.

The present work brings an important contribution in the field of food safety, evaluating fiftheen *Fusarium* mycotoxins, and being the first research in Romania including in mycotoxin study the newest discovered class of *Fusarium* mycotoxins, the emerging mycotoxins. Moreover, the studies in the thesis provide for the first time information about the possible influence of the climatic parameters (such as average temperature and total precipitation), agricultural practices (organic or conventional), and cultivar in conventional agriculture on emerging mycotoxin occurrence and concentrations in wheat harvested in Romania. Also, this research complements other studies in DON and ZEA presence in wheat crops in Romania correlated with the weather conditions. Thus, this part of the investigation was particularly conducted in the context of climate changes predicted for Romania during the next century that could affect also fungi development and mycotoxin production.

Furthermore, the thesis presents the results of an exhaustive study concerning the occurrence and co-occurrence of *Fusarium* mycotoxins in wheat-based foodstuffs commercialized in Romania. Data from this part of the research was used to estimate for the first time the dietary exposure to mycotoxins of the Romanian population through wheat-based food. This study has the advantage of including a wide variety of products for direct human consumption, the estimation being more accurate.

Finally, this thesis contributes to the effort to reduce fungi and mycotoxin attacks in wheat, and it can represent an important step for the mitigation strategies and the HACCP monitoring process.

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ANNEXES

Annex 1. List of tables

Annex 2. List of figures

Annex 3. Articles published *in extenso*

DE GRUYTER OPEN

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OCCURENCE OF FUSARIUM MYCOTOXINS IN WHEAT FROM EUROPE – A REVIEW

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Abstract: The quality of cereals is very important for both human and animal nutrition. Fusarium mycotoxins include a great number of compounds. Trichothecenes, zearalenone (ZEN) and fumonisins are the major Fusarium mycotoxins occurring in cereal grains, animal feeds and forages. Conditions that predispose to mycotoxin production by *Fusarium* species include humidity, temperature, aeration and substrate type. Even if a great number of fungal metabolites have been designated as mycotoxins, a small number are known to have significant animal/human health and economic significance. For this, the world-wide impact of mycotoxins on human and animal health is likely underestimated and the future in this area is to identify additional specific biomarkers and group of biomarkers that can be used to establish the exposition of human and animals to individual mycotoxins.

Keywords: *Fusarium* mycotoxins, trichothecenes, fumonisins, zearalenone, wheat, Europe.

INTRODUCTION

Nowadays, industrialization, globalization and liberalization make it possible to have greater varieties of foods worldwide. But globalization and technological development lead also to increased risks in food chain (Smyth

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Occurrence and co-occurrence of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania

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Deoxynivalenol (PubChem CID: 40024)
Enniatin A (PubChem CID: 16727691)
Enniatin A1 (PubChem CID: 16727692)
Enniatin B (PubChem CID: 164754)
Enniatin B1 (PubChem CID: 11262300)
Neosolaniol (PubChem CID: 13818797)
Zearalenone (PubChem CID: 5281576)

ABSTRACT

In this study, the presence of fourteen *Fusarium* mycotoxins, legislated by the European Union – deoxynivalenol, zearalenone, HT-2 and T-2 toxins (EC/1881/2006; 2013/165/EU), or non-legislated (five trichothecens and five "emerging" mycotoxins), was evaluated in 31 whole unprocessed wheat samples and 35 white wheat flour samples from different areas of Romania. For this purpose, a validated multi-mycotoxins liquid chromatography tandem mass spectrometry method was applied. Seventy three percent of the analyzed samples contained at least one mycotoxin. The highest occurrence was for enniatin B, 71% of the analyzed samples being positive (21–407 μg kg^{-1}). Regarding the legislated mycotoxins, deoxynivalenol was detected in 14% (111–1787 μg kg^{-1}) of the samples, while zearalenone was detected in 9% (51–1135 μg kg^{-1}). Only one sample was positive for neosolaniol. Concerning the co-occurrence, 42% of the samples were contaminated with two to five mycotoxins, the most frequent being the binary or tertiary combinations of enniatins. This is the first study applied to Romanian wheat grains and flour samples using a high sensitive multi-mycotoxins method, and which included also "emerging" mycotoxins.

1. Introduction

Mycotoxins can be present in vegetable foods which can serve as a substrate for the growth of filamentous fungi of the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*.

There are many factors that predispose to mycotoxin production by fungi including substrate type and availability, climate conditions, storage and processing conditions (Covarelli, Beccari, Antonio et al., 2015, Covarelli, Beccari, Prodi et al., 2015; Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003). Cereals contamination is the most important for mycotoxins occurrence, particularly for wheat, maize and rice which are of major importance (Pereira, Fernandes, & Cunha, 2014). The Rapid Alert System for Food and Feed of the European Union reports mycotoxins on the third position according to the total number of hazard notifications (RASFF, 2015).

Wheat is considered to be the main strategic crop in the world with a global production of 729 million tonnes in 2014. The European Union (EU) is the world's largest wheat producer. Romania is one of the five biggest wheat producers in the EU with a harvested area of 2,107,813 hectares and an annual production of 7,584,814 tonnes in 2014 (RINS, 2015). More than half of Romanian annual wheat production is exported in different countries around the world (USDA, 2015). On the other hand, for Romanian population a high wheat and wheat products consumption is registered (364.6 g/capita/day), more than the European average (295.4 g/capita/day), and the double of the world average (178.8 g/capita/day) (FAOSTAT, 2015).

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Climatic conditions influence emerging mycotoxin presence in wheat grown in Romania – A 2-year survey

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ABSTRACT

The correlation between the occurrence of four enniatins (ENA, ENA1, ENB, and ENB1) and beauvericin (BEA) and the weather parameters during anthesis and preharvest period was studied in 97 wheat samples collected in 2014 and 2015 across three counties from central and south Romania (Braşov, Dâmboviţa, and Teleorman). The highest mean values of ENA (16.1 μg kg^{-1}) and ENB (147.1 μg kg^{-1}) were measured in the samples from Braşov county in the harvest year 2015, whereas for ENA1 and ENB1 the highest means (55.2 μg kg^{-1}, and 108.0 μg kg^{-1}, respectively) were noted in samples from Teleorman county in 2014. Statistically significant differences ($P < 0.05$) were identified between ENA1 and ENB1 and the harvest year, coupled with a strong correlation with the weather parameters (ENA1: $r_s = 0.8745$ and $r_s = 0.9326$; ENB1: $r_s = 0.7814$ and $r_s = 0.8909$, for temperature and precipitation, respectively). Principal component analysis revealed that the influence of weather parameters on emerging mycotoxin concentrations in wheat samples varied by region. This study showed that the presence and levels of emerging mycotoxins are related to weather parameters from the respective Romanian region.

1. Introduction

Mycotoxins, the secondary metabolites of various fungi, enter into the food chain through naturally contaminated food and feed, mainly cereals. These toxins can cause biochemical, physiological, and/or pathological changes in both human and animal species (Stanciu et al., 2015). *Fusarium* spp. produce mycotoxins that are legislated in the European Union (EU) as trichothecenes, zearalenone, and fumonisins (EC, 2013, 2006a) in addition to a group of "emerging" mycotoxins such as fusaproliferin, beauvericin (BEA), enniatins (ENs: ENA, ENA1, ENB, and ENB1) and moniliformin. In the past years, ENs and BEA gained a high interest and recently toxicity and risk assessment have been performed (EFSA, 2014; Escrivá et al., 2015). The toxic effects of BEA and ENs cause a negative effect on food commodities including cereals such as wheat (Covarelli et al., 2015).

There are many factors that influence the occurrence of mycotoxins in cereals, e.g., plant substrate (composition, pH, and water activity), management factors (tillage, harvesting, storage and processing conditions), topographic factors (relief position and topographic wetness index), but weather parameters (rainfall, humidity and temperature) represent the key determinants for fungal colonization and mycotoxin production (Milani, 2013; Müller et al., 2010). In this context, climate changes might influence crop yield and the degree to which the crops are contaminated with mycotoxins or could increase the development of fungi not identified previously within a given area (EC, 2007; Magan et al., 2011).

Wheat (*Triticum aestivum* L.) is the main strategic crop worldwide (USDA, 2016). The EU is the world's largest wheat producer. Romania is the fifth biggest producer of wheat in the EU, after

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Article

Presence of Enniatins and Beauvericin in Romanian Wheat Samples: From Raw Material to Products for Direct Human Consumption

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Abstract: In this study, a total of 244 wheat and wheat-based products collected from Romania were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in order to evaluate the presence of four enniatins (ENs; i.e., ENA, ENA1, ENB, and ENB1) and beauvericin (BEA). For the wheat samples, the influence of agricultural practices was assessed, whereas the results for the wheat-based products were used to calculate the estimated daily intake of emerging mycotoxins through wheat consumption for the Romanian population. ENB presented the highest incidence (41% in wheat and 32% in wheat-based products), with its maximum levels of 815 μg kg^{-1} and 170 μg kg^{-1} in wheat and wheat-based products, respectively. The correlation between the concentrations of ENB and ENB1 in wheat grain samples and farm practices (organic or conventional) was confirmed statistically ($p < 0.05$). This is the first study that provides comprehensive information about the influence of agricultural practice on emerging *Fusarium* mycotoxin presence in Romanian wheat samples and the estimated daily intake of ENs and BEA present in wheat-based products for human consumption commercialized in Romania.

Keywords: emerging mycotoxins; LC-MS/MS; cereals; organic; conventional; wheat products; estimated daily intake

1. Introduction

Wheat (*Triticum aestivum* L.) is the main strategic crop worldwide, with recent data reporting a total area harvested of 224.7 million hectares, and an annual global production around 734 million metric tones [1]. In Romania, wheat has a special contribution in traditional agriculture, with 2.04 million hectares being used for wheat cultivation, with an annual production of about 7.85 million tons [2]. Moreover, the Romanian population registers a high wheat and wheat product consumption (133.09 kg/capita/year) [1].

Cereal grains are vulnerable to infections by a wide variety of plant pathogens. Filamentous fungi are a main safety concern due to the production of mycotoxins accumulated in grains as secondary metabolites [3]. The use of different agricultural practices, such as conventional or organic, could play an important role in the growth of various fungi and the biosynthesis of mycotoxins [4]. Production of organic wheat implies a management system that avoids the use of synthetic fertilizers, pesticides, herbicides, or genetically modified organisms. Due to the lack of synthetic fungicides in

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Analysis of enniatins and beauvericin by LC-MS/MS in wheat-based products

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ABSTRACT

Due to the matrix complexity for wheat-based products, a comparative study of different rapid extraction procedures was performed for the extraction of enniatins (ENA, ENA1, ENB, ENB1) and beauvericin in flour, pasta, breakfast cereals, and biscuits. Three different approaches were studied during the extraction and purification steps (shaker, Ultra-Turrax, and QuEChERS) for each matrix. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with electrospray source working in a positive mode was used. For the analysis of the five mycotoxins, the three methods were tested in terms of recovery, matrix effect, and sensibility, concluding that Ultra-Turrax extraction was the most competent method. The applicability of the validated method was demonstrated by analyzing 16 commercial samples from Romania.

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Emerging mycotoxins; LC-MS/MS; method validation; cereal-based products

Análisis de eniatinas y beuvericina en productos elaborados con trigo por LC-MS/MS

RESUMEN

Debido a la complejidad de los productos elaborados con trigo, se ha realizado un estudio comparativo de diferentes procesos de extracción rápida de eniatinas (ENA, ENA1, ENB, ENB1) y beauvericina en harina, pasta, cereales para el desayuno y galletas. Se estudiaron tres procedimientos (agitación, Ultra-Turrax, QuEChERS) en cada matriz con distintos pasos de extracción y purificación. Se utilizó la cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS) con fuente de electroespray en modo positivo. Los tres métodos fueron estudiados para el análisis de las cinco micotoxinas, en términos de recuperación, efecto matriz y sensibilidad, concluyendo que el método de extracción con Ultra-Turrax fue el que mejores resultados proporcionó. La aplicabilidad del método validado se demostró con el análisis de 16 muestras comercializadas en Rumania.

PALABRAS CLAVE
Micotoxinas emergentes; LC-MS/MS; validación; productos elaborados con trigo.

Introduction

Some fungi can produce a wide variety of mycotoxins as a result of their secondary metabolism. Many important food commodities can be contaminated by *Fusarium* mycotoxins which include trichothecenes, zearalenone, and fumonisins, with their derivatives, and also the newest group of emerging mycotoxins composed mainly of enniatins (ENs), beauvericin (BEA), fusaproliferin (FUS), and moniliformin (MON) (Stanciu et al., 2015).

The occurrence and toxicity of emerging mycotoxins are currently under evaluation by the European Food Safety Authority (EFSA) Panel on Contaminants in Food Chain (CONTAM), and according to scientific opinion, there is insufficient information on the risk characterization of mycotoxins. In this regard, one EFSA recommendation was to develop and validate sensitive methods for the analysis of emerging mycotoxins in food, especially in products for direct human consumption (EFSA, 2014).

Regarding emerging mycotoxins, the class of ENs (ENA, ENA1, ENB, and ENB1) and BEA has received more interest in the past decade. These mycotoxins are bioactive substances with a cyclic hexadepsipeptide structure. ENs can act as enzyme inhibitors, having antimicrobial, anthelmintic, insecticidal, antifungal, herbicidal, phytotoxic, and cytotoxic potential activity (Escrivá, Font, & Manyes, 2015) or local central nervous system effects (Taevernier et al., 2016). BEA has shown to be a specific cholesterol acyltransferase inhibitor, with antimicrobial, antiviral, cytotoxic, apoptotic, and immunosuppressive activity (Ruiz, Franzova, Juan-Garcia, & Font, 2011). Furthermore, due to their similar chemical structures, it has been demonstrated by *in vitro* studies that ENs and BEA can present additive or synergistic cytotoxic effects in several cell lines (Juan-Garcia, Ruiz, Font, & Manyes, 2015).

Mycotoxin contamination in different grains and food based on grains is of major importance due to its remarkable implications for food safety (EFSA, 2014). Wheat is the most consumed cereal worldwide. So, studies on presence of emerging mycotoxins in wheat-based products are useful for the assessment of human exposure to mycotoxins. Several authors reported high occurrence of ENs and BEA in grains and wheat-based products like pasta, infant formulas, breakfast cereals, and biscuits, with incidences between 40% and 90% (Blesa, Marín, Lino, & Mañes, 2012; Jestoi, 2008; Juan, Mañes, Raiola, & Ritieni, 2013a; Juan, Ritieni, & Mañes, 2013b; Mahnine et al., 2011; Malachova et al., 2011; Oueslati, Meca, Mliki, Ghorbel, & Mañes, 2011). Regarding the simultaneous presence of ENs and BEA or co-occurrence with other

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