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Determination of mycotoxin contamination in wheat and peanut products using a multi-mycotoxin UPLC-MS/MS analytical method and sorting as a mycotoxin mitigation strategy in wheat grains

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Abbreviations

15Ac-DON	15-acetyl-deoxynivalenol
3Ac-DON	3-acetyl-deoxynivalenol
AF	Aflatoxin
ALT	Altenuene
AME	Alternariol-monomethyl-ether
AOH	Alternariol
ATX	Altertoxin
a_w	Water activity
BEA	Beauvericin
CAP	Cold atmospheric plasma
CIT	Citrinin
CRM	Certified reference material
CV	Variation coefficient
DAD	Diode Array Detector
DAS	Diacetoxyscirpenol
df	Degree of freedom
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3-glycoside
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENN	Enniatin
e-nose	Electronic Nose
FAO	Food and Agriculture Organization
FASFC	Federal Agency for the Safety of the Food Chain
FDK	Fusarium damaged kernel
FHB	Fusarium Head Blight
FLD	Fluorescent Detector
FUM	Fumonisin
GC	Gaz Chromatography
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry

HT2	HT2 toxin
IAC	Immuno Affinity Column
IARC	International Agency for Research on Cancer
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LFIA	Lateral Flow ImmunoAssay
LOD	Limit of detection
LOQ	Limit of quantification
MIP	Molecularly imprinted polymer
ML	Maximum level
MRM	Multiple Reaction Monitoring
MS	Mass spectrometer
ND	Not detected
NIR	Near infrared
NIV	Nivalenol
OTA	Ochratoxin A
OTB	Ochratoxin B
PCA	Principal components analysis
PLS-DA	Partial least squares discriminant analysis
PSA	Primary secondary amines
QuEChERS	Quick Easy Effective Cheap, Effective, Rugged and Safe
RASFF	Rapid Alert System for Food and Feed
RT	Retention time
S/N	Signal over to noise ratio
SPE	Solid Phase Extraction
STE	Sterigmatocystin
T2	T2 toxin
TeA	Tenuazonic acid
UPLC	Ultra Performance Liquid chromatography
VOC	Volatile Organic Compounds
ZEN	Zearalenone

Table of content

Abstract - Résumé 1
Summary *2*
Résumé *4*
General preamble 6
Introduction 8
1. Mycotoxins *9*
1.1 Fusarium produced mycotoxins *9*
1.2 Mycotoxins produced by other fungal genera *13*
1.3 Modified mycotoxins *16*
1.4 Influence of environmental and handling conditions on fungi and mycotoxin contamination *17*
1.5 Impact of other fungal species on mycotoxin contamination *19*
1.6 Health impact of mycotoxins ingestion *21*
1.7 Mycotoxins in wheat *26*
1.8 Mycotoxins in peanuts *28*
1.9 Mycotoxins in other food matrices *30*
1.10 Mycotoxins in feed *31*
2. Mycotoxin issue at the world level *31*
3. Analytical method for mycotoxin determination *34*
3.1. Sampling *35*
3.2. Sample preparation *35*
3.3. Detection and analysis *37*
4. Mycotoxins management *41*
4.1. Mycotoxins contamination prevention practices *41*
4.2. Mycotoxins decontamination processes *43*
4.3. Mycotoxins sorting *46*
Objectives 49
Experimental section 51
Study 1: *Development and validation of a multi-mycotoxin analysis method on wheat by UPLC-MS/MS* *52*
Study 2: *Multi-mycotoxin analyses by UPLC-MS/MS in wheat: The situation in Belgium in 2023 and 2024* *92*
Study 2: *Multi-mycotoxin analyses by UPLC-MS/MS in wheat: The situation in Belgium in 2023 and 2024* *94*
Study 3: *Sorting of mycotoxins contaminated wheat batches: The case of deoxynivalenol and ochratoxin A* *118*
Study 4: *Peanut and peanut-based foods contamination by toxigenic fungi and mycotoxins: Potential risks for Beninese consumer* *147*
Discussion – Perspectives and Conclusions 179
Bibliography 200

Abstract - Résumé

Summary

Mycotoxins are secondary metabolites produced by diverse fungal species such as *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. To date, at least 400 mycotoxins have been identified, and the most studied are aflatoxins, deoxynivalenol, zearalenone, and ochratoxin A. Mycotoxins are found in many plant-based food products (e.g., cereals, apples, tomatoes, spices, and wine).

Mycotoxin contamination may occur during both pre-harvest and post-harvest stages. Before harvest, weather conditions (temperature and humidity), agricultural practices, previous crops, insect infestation, and the use of fertilizers and fungicides may influence fungal infection and mycotoxin production. Inappropriate storage conditions, such as poor drying, may lead to mycotoxin contamination.

Mycotoxin can have acute and chronic adverse effects on animal and human health. The possible effects can be carcinogenic, estrogenic, haemorrhagic, hepatotoxic, immunosuppressive, mutagenic, and neurotoxic. Therefore, some mycotoxins are regulated in the European Union due to their toxicity. Hence, analytical methods to monitor mycotoxin levels in food need to be developed. Ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) allows the detection of multiple mycotoxins in a single analysis.

The first part of this thesis focuses on the development and validation of a multi-mycotoxin analytical method by UPLC-MS/MS on wheat. Initially, the implemented method enabled the detection of 20 mycotoxins and the quantification of 16 of them, including all the regulated mycotoxins in wheat in Belgium and some emerging ones. The validation of the method proved its trueness, reproducibility, and repeatability.

Secondly, this method was applied to wheat samples from the 2023 and 2024 harvests collected from various locations in Belgium, ensuring diverse pedoclimatic contexts and including 11 different varieties. This work provides an updated overview of mycotoxin contamination in Belgian wheat. Mycotoxin presence was higher in 2024 than in 2023, both in terms of the number of contaminated samples and mycotoxin concentrations. In both years, the detected mycotoxins were deoxynivalenol, zearalenone, and enniatins B and B1. Enniatin A1 was only detected in 2024. The most prevalent mycotoxins detected in 2024 were enniatins, with enniatin B present in 68 out of 88 samples ranging from 12 to 488 µg/kg. Differences in fungal infection susceptibility were observed

between the varieties. Moreover, geographic locations also appeared to influence mycotoxin levels, which could be related to previous crops or specific meteorological events.

Thirdly, the effectiveness of different sorting strategies to reduce deoxynivalenol and ochratoxin A levels in contaminated wheat batches was assessed in order to fill existing data gaps regarding sorting efficiency for these mycotoxins. Size, density, and infrared sorting were tested. For infrared-based sorting, a specific *Fusarium*-damaged kernel model was developed and implemented in the sorter. For deoxynivalenol, the *Fusarium*-damaged kernel model proved to be the most effective strategy, whereas size and density sorting showed only limited effectiveness. Regarding ochratoxin A, when the initial concentration was much higher than the legal threshold, sorting processes did not achieve satisfactory performance. Only small quantities of food-compliant grains could be recovered in certain cases.

Finally, the multi-mycotoxin analytical method was successfully extended to peanuts and peanut products. As other mycotoxins were reported to be of interest in peanuts originating from Benin, additional mycotoxins were included in the method, namely aflatoxins (B1, B2, G1, and G2), and fumonisins B2 and B3. Peanut samples collected from different markets in Benin, as well as samples taken from various stages of roasted peanut and *klui-klui (fried pressed peanut cake)* production (to monitor the evolution of mycotoxin concentrations during the process), were analysed. The most prevalent mycotoxins were aflatoxins B1, B2, G1, and G2, as well as ochratoxin A and ochratoxin B. This approach enabled an overview of the contamination levels in Beninese peanuts and peanut-based products, and the identification of critical steps in the processing chain where mycotoxin concentrations may increase.

To conclude, this study led to the development and validation of a reliable analytical method for the detection and quantification of regulated and non-regulated mycotoxins in wheat. This method is flexible and can be extended to other food matrices, and additional mycotoxins could be included according to specific needs. The analysis of the 2023 and 2024 samples provided additional insights into the influence of varieties and meteorological conditions on mycotoxin occurrence in wheat. The prevalence of emerging mycotoxins was also highlighted. In addition, this work provides a basis for evaluating the potential of sorting processes to reduce deoxynivalenol and ochratoxin A concentrations in wheat batches. Finally, the analytical method was successfully extended to peanuts and peanut-based products to generate data on mycotoxin contamination in Beninese products, for which recent information is limited.

Résumé

Les mycotoxines sont des métabolites secondaires produits par diverses espèces fongiques telles que *Fusarium* spp., *Penicillium* spp. et *Aspergillus* spp. À ce jour, au moins 400 mycotoxines ont été identifiées. Les mycotoxines sont présentes dans de nombreux produits alimentaires (céréales, pommes, tomates, épices, vin, etc.). Lors de la culture, les conditions climatiques (température et humidité), les pratiques agricoles, le précédant cultural, la présence d'insectes ainsi que l'utilisation d'engrais et de fongicides peuvent influencer l'infection fongique et la production de mycotoxines. Après récolte, des conditions de stockage inadaptées, telles qu'un séchage insuffisant, peuvent également favoriser leur présence. Les mycotoxines peuvent avoir des effets néfastes aigus et chroniques sur la santé animale et humaine. Des effets cancérogènes, œstrogéniques, hémorragiques, hépatotoxiques, immunosuppressifs, mutagènes et neurotoxiques peuvent être observés. Certaines mycotoxines sont réglementées dans l'Union européenne. Ainsi, le développement de méthodes analytiques permettant de suivre la concentration en mycotoxines dans les denrées alimentaires est nécessaire. La chromatographie liquide d'ultra-haute performance couplée à la spectrométrie de masse en tandem (UPLC-MS/MS) permet la détection de plusieurs mycotoxines en une seule analyse.

La première partie de ce travail est consacrée au développement et à la validation d'une méthode analytique multi-mycotoxines par UPLC-MS/MS appliquée au froment. La méthode développée initialement permet la détection de 20 mycotoxines et la quantification de 16 d'entre elles incluant toutes celles qui sont réglementées dans le froment en Belgique et certaines émergentes. La méthode a été validée pour sa justesse, sa reproductibilité et sa répétabilité.

Dans un second temps, cette méthode a été appliquée à des échantillons de froment issus des récoltes 2023 et 2024, collectés dans diverses régions de Belgique, dans des contextes pédoclimatiques variés et ce pour 11 variétés de froment différentes. Ce travail fournit un état des lieux actualisé concernant l'occurrence de mycotoxines dans le froment belge. La présence de mycotoxines s'est avérée plus élevée en 2024 qu'en 2023, tant en termes de nombre d'échantillons contaminés que de concentrations. Les mycotoxines détectées les deux années étaient le déoxynivalénol, la zéaralénone et les enniatines B et B1. L'enniatine A1 n'a été détectée qu'en 2024. Les mycotoxines les plus fréquemment observées en 2024 étaient les enniatines, l'enniatine B étant présente dans 68 échantillons sur 88, avec des concentrations comprises entre 12 et 488 µg/kg. Des différences de sensibilité à l'infestation fongique ont été observées entre les variétés. De plus, les

localisations géographiques semblaient également influencer les niveaux de mycotoxines, possiblement en lien avec les cultures précédentes ou certains événements météorologiques.

Dans un troisième temps, l'efficacité de différentes stratégies de tri pour réduire les niveaux de déoxynivalénol et d'ochratoxine A dans des lots de froment contaminés a été évaluée afin de combler le manque de données existantes concernant l'efficacité du tri pour ces mycotoxines. Des tris par taille, densité et spectres infrarouges ont été testés. Pour le tri sur base de données obtenues en infrarouge, un modèle spécifique basé sur la détection des grains de froment endommagés par *Fusarium* a été développé et implémenté. Concernant le déoxynivalénol, le modèle infrarouge s'est révélé être la stratégie la plus efficace, tandis que les tris par taille et densité n'ont montré qu'une efficacité limitée. Pour l'ochratoxine A, lorsque la concentration initiale dépassait largement le seuil réglementaire, les procédés de tri n'ont pas permis d'obtenir des résultats satisfaisants, et seule une faible proportion de grains conformes pouvait être récupérée dans certains cas.

Enfin, la méthode multi-mycotoxines a été étendue avec succès aux arachides et produits dérivés. D'autres mycotoxines d'intérêt ayant été rapportées dans les arachides originaires du Bénin, celles-ci ont été ajoutées à la méthode initiale : les aflatoxines (B1, B2, G1 et G2), ainsi que les fumonisines B2 et B3. Des échantillons collectés sur différents marchés du Bénin, ainsi que des échantillons prélevés à diverses étapes de la production d'arachides grillées et de *klui-klui* (galette de pâte d'arachides déshuilée et frite), ont été analysés. Les mycotoxines les plus fréquemment retrouvées étaient les aflatoxines B1, B2, G1 et G2, ainsi que l'ochratoxine A et l'ochratoxine B. Cette approche a permis d'établir un état des lieux des niveaux de contamination des arachides au Bénin, ainsi que d'identifier les étapes critiques de la chaine de transformation.

En conclusion, ce travail a permis le développement et la validation d'une méthode analytique fiable pour la détection et la quantification de mycotoxines réglementées et non réglementées dans le froment. Cette méthode est flexible et peut être étendue à d'autres matrices alimentaires, avec la possibilité d'inclure des mycotoxines supplémentaires selon les besoins. L'analyse des récoltes 2023 et 2024 a apporté de nouvelles connaissances sur l'influence des variétés et des conditions météorologiques sur la présence de mycotoxines dans le froment, tout en soulignant la prévalence de mycotoxines émergentes. Par ailleurs, ce travail fournit des bases sur le potentiel des procédés de tri pour réduire les concentrations de déoxynivalénol et d'ochratoxine A dans les lots de froment. Enfin, la méthode analytique a été transférée avec succès aux arachides et produits dérivés pour générer des données concernant l'occurrence des mycotoxines dans les produits béninois.

General preamble

Mycotoxins, secondary metabolites produced by certain fungal species, represent a major challenge for food safety and security worldwide. Their occurrence in agricultural commodities such as cereals, nuts, and spices is influenced by a wide range of factors, including climatic conditions, agricultural practices and post-harvest storage conditions. The persistence of these compounds, combined with their potential adverse effects on human and animal health, has led to the establishment of regulatory frameworks in many regions, including Europe. Nevertheless, beyond the well-studied regulated mycotoxins, numerous emerging compounds are increasingly being reported in food and feed, raising new concerns for risk assessment and management.

In addition to their health implications, mycotoxins may also have considerable economic consequences. Fungal infection may lead to reduced yields and contaminated batches may need to be discarded or downgraded, resulting in financial losses along the food supply chain. However, in certain cases, strategies that allow the partial recovery of affected batches could provide a more sustainable approach, reducing waste while still ensuring compliance with food safety standards.

The complexity of this issue calls for analytical methods able to simultaneously detect multiple mycotoxins, including both regulated and emerging ones, across different food matrices. Such methods not only support risk assessment but also enable the generation of comprehensive contamination data that reflect real-world scenarios. Moreover, extending the application of these analytical tools to diverse agricultural contexts is essential to capture the variability in mycotoxin occurrence, whether linked to environmental factors, crop varieties, or regional agricultural practices.

This thesis is situated within this broader framework. It seeks to contribute to the development of advanced multi-mycotoxin detection methods, to explore practical approaches for reducing contamination in agricultural products, and to generate reference data that may inform both regulatory bodies and agricultural stakeholders. By focusing on wheat in Belgium, as well as peanuts in Benin, two distinct but relevant case studies, the research addresses both local and global challenges related to mycotoxin contamination.

Introduction

1. Mycotoxins

Mycotoxins are secondary metabolites mainly produced by *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp. and *Penicillium* spp. fungal species (Tola and Kebede, 2016; WHO (World Health Organization), 2023). More than 400 mycotoxins have been reported in the literature. Among them, aflatoxins (AFs), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN) are the most studied compounds due to their frequent occurrence and their impact on human and animal health (Pereira et al., 2014; Smith et al., 2016; Khan et al., 2024).

In wheat, mycotoxin contamination is mainly associated with *Fusarium* species. The most commonly produced *Fusarium* mycotoxins in wheat are trichothecenes, ZEN, and fumonisins (FUMs) (Ji et al., 2019). In addition, several non-regulated or emerging Fusarium mycotoxins such as enniatins (ENNs), beauvericin (BEA) and moniliformin, have been increasingly reported in food and feed matrices (De Felice et al., 2023; Kolawole et al., 2024).

1.1 *Fusarium* produced mycotoxins

1.1.1 Trichothecenes

Trichothecenes constitute a large family of mycotoxins with more than 200 compounds (Ekwomadu et al., 2021). They share a common tricyclic 12,13-epoxytrichothec-9-ene core structure (McCormick et al., 2011), and generally have molecular weights ranging between 200 and 500 Da (Ji et al., 2019). Trichothecenes are mainly produced by *Fusarium* species, and are classified into four groups (A, B, C and D), among which groups A and B are considered the most toxic (Ekwomadu et al., 2021).

Group A trichothecenes include T2 toxin, HT2 toxin and 4,15-diacetoxycirpenol (DAS), whereas group B trichothecenes include nivalenol (NIV), DON and its acetylated derivatives, 3-acetyl-deoxynivalenol (3Ac-DON) and 15-acetyl-deoxynivalenol (15Ac-DON) (McCormick et al., 2011). The toxicity of the trichothecenes may vary depending on the *Fusarium* host-plant (Perincherry et al., 2019).

Fusarium graminearum and *Fusarium culmorum* are among the most economically important trichothecene producers, as they are responsible for Fusarium Head Blight (FHB), a major disease infecting cereal grain crops worldwide (Alshannaq and Yu, 2017). The chemical structures of the above-cited trichothecenes are presented in Figure 1.

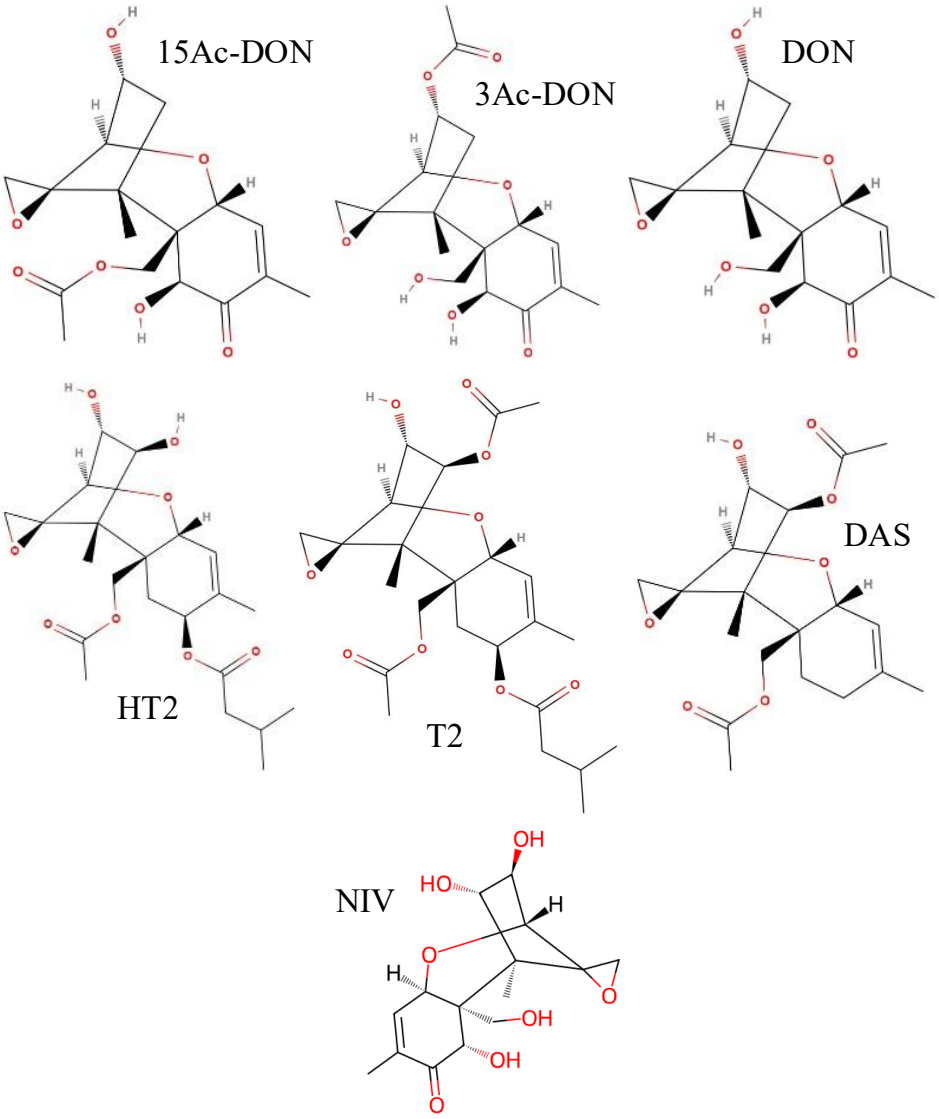


Figure 1: Major trichothecenes chemical structures.

HT2 toxin is a deacylated metabolite of T2 toxin. Due to their structural similarity, both compounds exhibit comparable toxicological profiles and are considered among the most acutely toxic trichothecenes (Ji et al., 2019). The main producer of T2 and HT2 toxins is *Fusarium langsethiae* (Ji et al., 2019).

Deoxynivalenol, also known as vomitoxin, is mainly produced by *Fusarium graminearum*, and is the most widespread and extensively studied trichothecene, although it is considered one of the least toxic within the group (Alshannaq and Yu, 2017). Deoxynivalenol commonly occurs in small grains such as wheat, barley, oats and maize (Ekwomadu et al., 2021). Cereals and cereal-based products represent the primary source of human exposure, with wheat accounting for 56 to 100% (Eskola et al., 2020). Deoxynivalenol frequently co-occurs with ZEN, as both toxins are produced by the same *Fusarium* species (Alshannaq and Yu, 2017; Ekwomadu et al., 2021).

1.1.2 Zearalenone

Zearalenone is mainly produced by *F.graminearum*, *F. crookwellense*, *F. sporotrichioides*, and *F. culmorum* (Ji et al., 2019; Ekwomadu et al., 2021). Zearalenone and its derivatives belong to a subgroup of natural estrogenic compounds called mycoestrogens (Alshannaq and Yu, 2017; Ekwomadu et al., 2021). It is mainly found in cereals, fruits, and cocoa (Khan et al., 2024). The chemical structure of ZEN is presented in Figure 2. The major contributors to ZEN exposure are grains and grains products, as well as bread and fine bakery wares (EFSA, 2011).

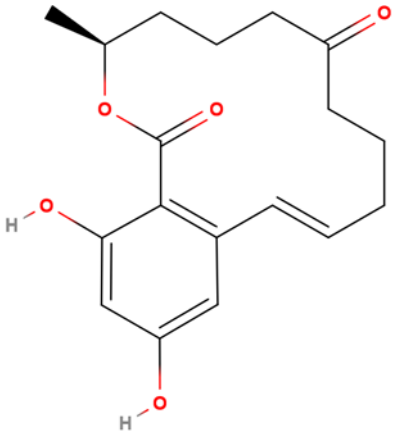


Figure 2: Zearalenone chemical structure

1.1.3 Fumonisins

Discovered in 1988, FUMs are hydrophilic molecules that are soluble in organic solvents (Alshannaq and Yu, 2017). Currently, more than 28 FUMs have been described and classified in four groups (A, B, C, and P) (Alshannaq and Yu, 2017). Fumonisins are primarily produced by *Fusarium verticillioides* (Ekwomadu et al., 2021). They are among the most cytotoxic and carcinogenic mycotoxins, with FUM B1 as the most abundant, representing approximately 70-80% of total FUMs contamination (Alshannaq and Yu, 2017; Ji et al., 2019). Fumonisins mainly occur in maize (Ekwomadu et al., 2021). The chemical structures of FUM B1, B2, and B3 are presented in Figure 3.

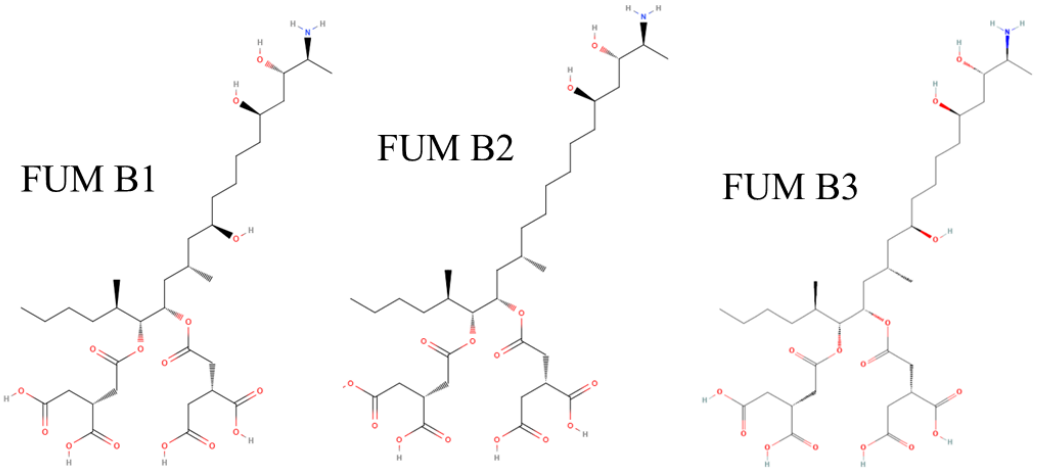


Figure 3: Fumonisins B1, B2 and B3 chemical structures.

1.1.4 Emerging mycotoxins

Emerging mycotoxins are generally defined as mycotoxins that are not regulated or routinely determined in monitoring programs (Kolawole et al., 2024). However, the definition remains heterogeneous, as some authors restrict this category to specific compounds such as fusaproliferin, beauvericin, ENNs, and moniliformin (Gruber-Dorninger et al., 2017).

Although ENNs were discovered in 1947, they have gained increasing attention over the past 15 years as they are considered contaminants of emerging concern (De Felice et al., 2023). To date, 29 species of ENNs have been described, with ENN A, A1, B and B1 being the most widely detected in food and feed (Gruber-Dorninger et al., 2017). ENNs are cyclohexadepsipeptides produced by different *Fusarium* species such as *F. avenaceum*, *F. oxysporum*, *F. poae*, or *F.tricinctum* (Gruber-Dorninger et al., 2017; Behr et al., 2025). A more complex evaluation of their toxicity still needs to be done (De Felice et al., 2023; Behr et al., 2025). Enniatins are mainly present in cereals, such as barley, oats, rice, and wheat, with grains and grain-based products as the major exposure contributors (EFSA, 2014; Gruber-Dorninger et al., 2017; De Felice et al., 2023). Their chemical structures are presented in Figure 5.

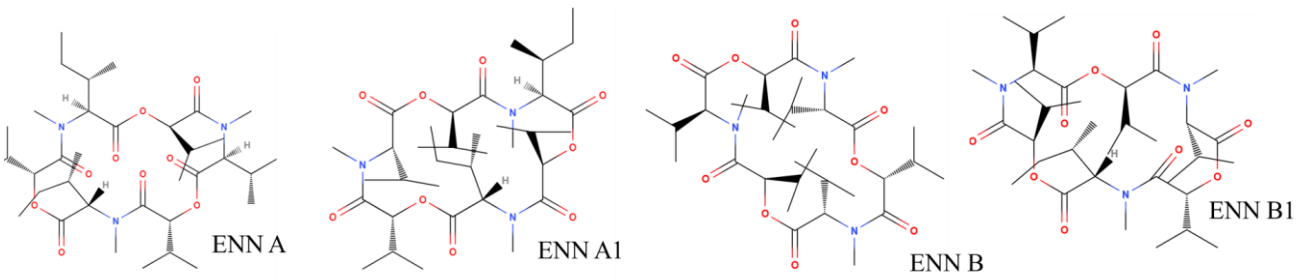


Figure 5: Enniatins A, A1, B and B1 chemical structures

Moniliformin is a small organic acid occurring naturally as potassium or sodium salts (Ekwomadu et al., 2021). It is mainly produced by *Fusarium proliferatum*. Available information about moniliformin is limited (EFSA, 2018). Moniliformin is often detected in cereals such as wheat, oat, barley, rye, maize and rice that are the major contributors to animal exposure (EFSA, 2018; Ekwomadu et al., 2021). Its chemical structure is presented in Figure 4. The co-occurrence of moniliformin with other *Fusarium* mycotoxins, such as ZEN, FUM B1, and DON is frequently observed (Gruber-Dorninger et al., 2017).

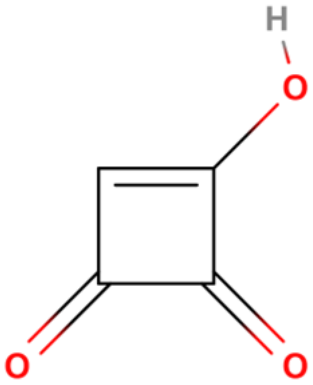


Figure 4: Moniliformin chemical structure.

1.2 Mycotoxins produced by other fungal genera

1.2.1. Regulated mycotoxins

Ochratoxins A (OTA) and B (OTB) are produced by *Aspergillus* and *Penicillium* fungal species, mainly by *A. ochraceus, A. carbonarius and P. verrucosum* (EFSA, 2020). Ochratoxin A is the most abundant and most toxic of the ochratoxins (Kumar et al., 2020). It may occur in cereals, coffee, wine, fresh fruits, spices, etc. (EFSA, 2020). The major contributors to OTA chronic dietary exposure are cheese, preserved meat and cereals (EFSA, 2020). The chemical structures of OTA and OTB are presented in Figure 8.

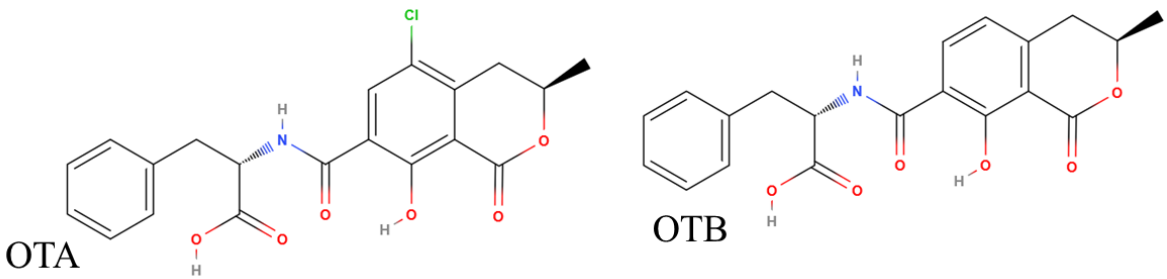


Figure 8: Ochratoxins A and B chemical structures.

Aflatoxins were first identified in 1969 following the "Turkey X disease" incident, during which poultry mortality was linked to contaminated peanuts meal (Alshannaq and Yu, 2017; Khan et al., 2024). Currently, AFs are among the most studied mycotoxins and are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Alshannaq and Yu, 2017). AFB1 is the most prevalent and toxic AF.

In developed countries (e.g. the USA and Europe), AFs mostly represent an economic concern, as excessive concentrations can lead to the rejection of contaminated commodities and subsequent production losses. In contrast, in developing countries in Africa and Asia, AF contamination has more severe consequences. On one hand, mycotoxin contamination compromises product quality, reduces export value, and generates substantial economic losses for producing countries. These losses extend beyond trade, as contaminated products may lead to the waste of natural resources and exacerbate food insecurity, contributing to hunger and malnutrition (Udomkun et al., 2017; Ayelign and De Saeger, 2020). On the other hand, chronic AF exposure is a major public health issue that is responsible for hundreds of cases of hepatocellular carcinoma each year, and can cause growth retardation in children (Lombard, 2014; Alshannaq and Yu, 2017; Abdallah et al., 2018; Ayelign and De Saeger, 2020; Nejad et al., 2023).

AFs are found in various food materials such as cereals, ground nuts, peanuts, walnuts, etc. (Alshannaq and Yu, 2017; Khan et al., 2024). AFM1 is a hydroxylated metabolite of AFB1 formed in the liver of mammals that have ingested AFB1, and excreted in their milk (Alshannaq and Yu, 2017; Iqbal, 2021). AFB1, AFB2, AFG1 and AFG2 chemical structures are presented in Figure 9.

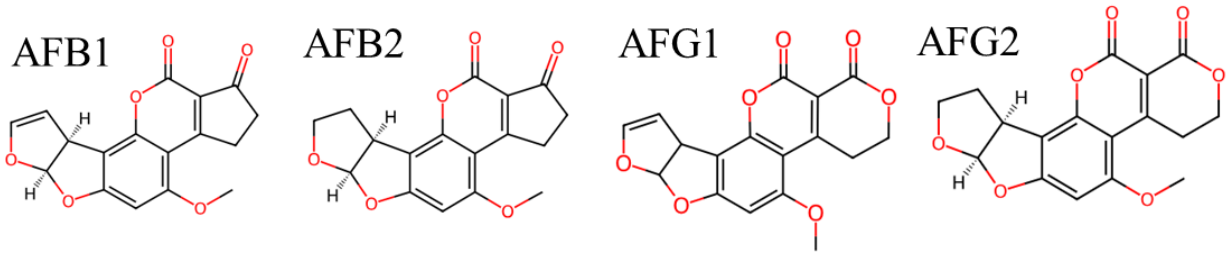


Figure 9: Aflatoxins B1, B2, G1 and G2 chemical structures.

1.2.2. Non-regulated mycotoxins

Sterigmatocystin (STE) is a polyketide mycotoxin mainly produced by *Aspergillus* and acts as a biosynthetic precursor to AFB1, sharing similar biological and structural properties (Zingales et al., 2020). Sterigmatocystin has been reported in grains, nuts, spices, beer and cheese (EFSA, 2013).

Citrinin (CIT) is a polyketide mycotoxin produced by *Aspergillus*, *Penicillium* and *Monascus* species (Gruber-Dorninger et al., 2017). Citrinin is a nephrotoxic mycotoxin that often occurs in grains, beans, fruits, herbs and spices (EFSA, 2012).

Their chemical structures are presented in Figure 7.

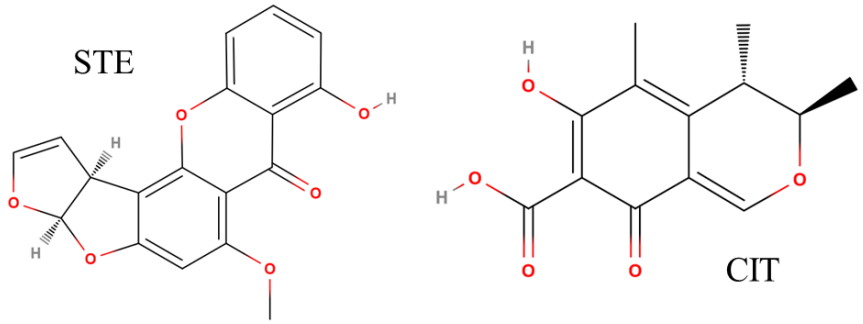


Figure 7: Sterigmatocystin and citrinin chemical structures.

Alternaria species are known to produce more than 70 secondary metabolites, several of which have been identified as mycotoxins. Among these, alternariol (AOH), alternariol-monomethyl-ether (AME), tenuazonic acid (TeA), altenuene (ALT), and altertoxins (ATX) are frequently reported in food commodities (Fraeyman et al., 2017). Alternariol and AME are dibenzopyrone derivatives and are among the most frequently detected *Alternaria* mycotoxins (Ostry, 2008). *Alternaria* mycotoxins have been found in a wide range of products including cereals, fruits and vegetables, nuts and oilseeds, with cereals and cereals-based products, as well as tomatoes, identified as the major contributors to dietary exposure (EFSA, 2016).

Alternaria mycotoxins are not regulated and are generally classified as emerging mycotoxins. Nevertheless, recommended maximum levels are provided by the European Commission (European Commission, 2022). The chemical structures of the above-mentioned *Alternaria* mycotoxins are presented in Figure 10.

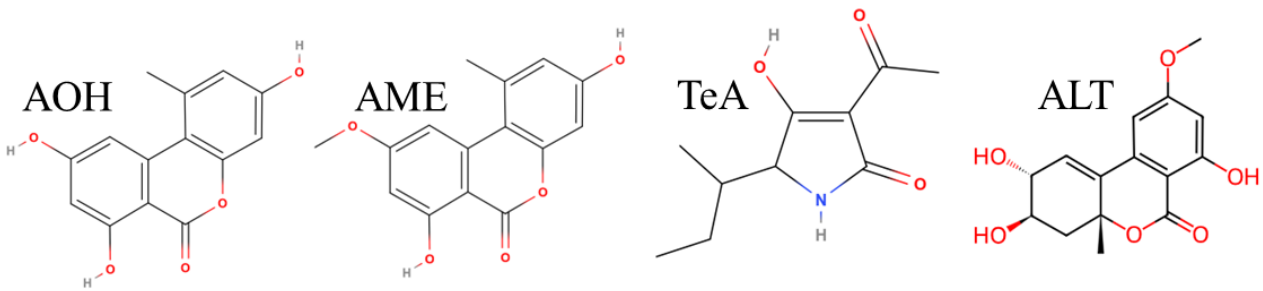


Figure 10: Alternariol, alternariol-monomethyl-ether, tenuazonic acid and altenuene chemical structures.

1.3 Modified mycotoxins

In addition to their native forms, mycotoxins can undergo a wide range of biological and chemical transformations that occur in the plant host, during food and feed processing, or following ingestion by animals and humans. These transformations lead to the formation of so-called modified mycotoxins, which have been reported in food and feed commodities (Ekwomadu et al., 2021; Nešić et al., 2023). As a result, mycotoxin contamination should no longer be considered as limited to parent compounds alone, but rather as a complex and dynamic mixture of native and modified forms.

Among the modified mycotoxins, “masked” mycotoxins are defined as plant metabolites of mycotoxins (Rychlik et al., 2014; Iqbal, 2021). Bound mycotoxins are difficult to detect as they are often attached to proteins or carbohydrates in plants and animals, but they can be separated by chemical processes (Iqbal, 2021; Nešić et al., 2023).

Mycotoxins can be biologically modified through phase I metabolic reactions, including oxidation, reduction or hydrolysis, or through phase II conjugation reactions, where the parent compound is linked to glucose, fatty acids, hemicellulose or glutathione. A well-documented example is deoxynivalenol-3-glucoside (DON-3G), which is formed by glycosylation of DON in plants and is considered one of the most prevalent masked mycotoxins in cereals (Freire and Sant’Ana, 2018; Nešić et al., 2023).

Although mycotoxins are often described as chemically stable compounds, they can also undergo chemical modifications during food and feed processing, including thermal treatments such as roasting, baking, frying or extrusion (Rychlik et al., 2014; Freire and Sant’Ana, 2018). Chemically modified mycotoxins are commonly classified as thermally formed or non-thermally formed derivatives (Rychlik et al., 2014). A representative example is the decarboxylation of ochratoxin A into 14-decarboxy-ochratoxin A during coffee roasting (Rychlik et al., 2014).

From a toxicological standpoint, modified mycotoxins generally exhibit lower acute toxicity than their parent compounds (Iqbal, 2021). However, this assumption cannot be generalized, as some derivatives may display similar or even enhanced toxic effects, depending on their structure and bioavailability. More importantly, modified mycotoxins contribute to the overall mycotoxin burden and may lead to a substantial underestimation of dietary exposure. Indeed, several modified forms can be hydrolysed back into their parent compounds during digestion, either through enzymatic

activity in animal and human metabolism or via the action of intestinal microbiota (Ekwomadu et al., 2021; Iqbal, 2021; Nešić et al., 2023).

To date, data on modified mycotoxins remain limited (Rychlik et al., 2014). The occurrence, stability and conversion processes of modified mycotoxins are currently not well known (Iqbal, 2021). Deoxynivalenol-3-glycoside, a glycosylated derivative of DON, and zearalenone derivatives such as ZEN 4-β-D-glucopyranoside or ZEN 2,4-O-β-diglucoside are relatively well described modified mycotoxins (Nešić et al., 2023).

These knowledge gaps represent a significant challenge for both analytical chemistry and risk assessment. Conventional targeted LC–MS/MS methods may fail to detect modified forms, while the lack of reference standards prevents accurate quantification (Nešić et al., 2023). In this context, advanced analytical approaches such as high-resolution mass spectrometry offer promising perspectives for the detection and characterization of modified mycotoxins (Nešić et al., 2023).

1.4 Influence of environmental and handling conditions on fungi and mycotoxin contamination

Mycotoxins production can occur during pre-harvest, harvest, drying and storage stages if the conditions are appropriate for fungal growth (Marin et al., 2013; Mahato et al., 2021). *Fusarium* species infect crops more often in the field, whereas *Aspergillus* and *Penicillium* species tend to develop during storage (Alshannaq and Yu, 2017).

Mycotoxigenic fungal species contamination does not always lead to mycotoxin presence in food and feed (Tola and Kebede, 2016). Physical, chemical and biological factors may influence mycotoxin production. For example, environmental conditions such as relative humidity, temperature and insect infestation are physical factors that affect mycotoxin synthesis. Chemical factors include the use of fungicides or fertilizers. The interaction between the fungi and their substrate represents a biological factor that influences mycotoxin production (Tola and Kebede, 2016).

The pre-harvest weather conditions, humidity, rainfall and temperature, influence fungal growth and mycotoxin production in the field (Marroquín-Cardona et al., 2014). Oxygen and substrates availability and pH also govern fungal growth and mycotoxin synthesis (Mahato et al., 2021).

Generally, mycotoxin production is enhanced by high water activity (a_w) and temperatures between 20 and 30°C (Mahato et al., 2021). A humidity level of raw material above 15 % will promote mycotoxin production (Mahato et al., 2021).

The general optimal conditions for *Fusarium* growth are a temperature between 25 and 30°C and a_w higher than 0.88, however, these conditions can vary from one species to another (Marroquín-Cardona et al., 2014; Tola and Kebede, 2016; Mahato et al., 2021; Kos et al., 2023). Nevertheless, the minimal temperature for *Fusarium* growth and DON, ZEN and FUMs production is 5°C (Mannaa and Kim, 2017). Ochratoxin A production by *Aspergillus ochraceus* is enhanced at temperatures of 25 - 30°C, with an a_w of 0.95 (Ben Miri et al., 2024). The optimal temperature for AFs ranges between 24 and 33°C depending on the fungal strain and substrate characteristics (Marroquín-Cardona et al., 2014; Kos et al., 2023), while the optimal a_w is around 0.99 (Kos et al., 2023). A summary of the optimum temperature and a_w is presented in Table 1.

Table 1: Summary of the optimum temperature and water activity (a_w) for fungal growth and mycotoxin production of the different fungal strains

Fungi	Toxins	Optimum temperature (°C)	Optimum a_w
Alternaria spp.	AOH, AME, ATX, …	21-25	> 0.97
Aspergillus spp.	AFs, OTA, STE, …	24-33	0.95-0.99
Fusarium spp.	DON, ZEN, FUMs, …	25-30	> 0.88
Penicillium spp.	OTA, CIT, …	25	0.95-0.99

Optimum temperature and a_w according to Marroquín-Cardona et al., 2014; Tola and Kebede, 2016; Mannaa and Kim, 2017; Mahato et al., 2021; Kos et al., 2023; Ben Miri et al., 2024.

Heavy rainfall during the flowering stage promotes fungal dispersion, leading to higher mycotoxin content in the plants. In particular, intense precipitation during flowering will promote *Fusarium* spread (Marroquín-Cardona et al., 2014). Therefore, significant rainfall in late spring will lead to an increase in mycotoxin contamination such as DON and ZEN (Kos et al., 2023). The risk of ZEN contamination further increases with heavy precipitation combined with long cool periods (Mahato et al., 2021). On the other hand, FUMs production will rather be associated with dry weather and late season rains (Marroquín-Cardona et al., 2014). Low precipitation coupled with high temperature during summer promotes *Aspergillus* spp. dispersion and AFs production (Kos et al., 2023).

Moreover, plant stress due to dry weather and high temperature makes them more vulnerable to pests and creates favourable conditions for fungal growth (Van Der Fels-Klerx et al., 2016). In addition, unseasonable rains may lead to changes in harvest habits, forcing farmers to harvest earlier, when the crops are not completely dry. As mentioned above, the higher humidity of the harvested crops may lead to fungal development (Marroquín-Cardona et al., 2014).

Therefore, global warming seems to have an influence on mycotoxin contamination as the temperature rises and atmospheric humidity increases with greater water evaporation from the Earth (Van Der Fels-Klerx et al., 2016).

Ochratoxin A and AFs are mainly found in tropical and subtropical regions associated with humid and warm climates (Ben Miri et al., 2024; Khan et al., 2024). Enniatins do not appear to have specific preferred climatic conditions, as they are found worldwide (De Felice et al., 2023)

High pH reduces mycotoxin production by repressing the expression of genes involved in the process of mycotoxin synthesis (Marroquín-Cardona et al., 2014). In addition, insect damage can also have an impact on mycotoxin contamination (Mousavi Khaneghah et al., 2019). Finally, the use of nitrogen fertilizers will enhance *Fusarium* growth and thus may increase mycotoxin production (Mahato et al., 2021).

After harvest, the absence of a suitable quality control system, and unsuitable drying, storage and transport conditions that promote fungal development may lead to an increase in mycotoxin contamination (Mahato et al., 2021).

After food processing, the mycotoxin content does not change if the products are stored in conditions that prevent fungal contamination and growth (Marin et al., 2013). However, as mycotoxins are relatively thermally stable, the mycotoxins produced before the food processing will generally remain in the final product (Mousavi Khaneghah et al., 2019). The a_w will determine the food quality and stability, with food having a low a_w being safer regarding fungi contamination. The growth of mycotoxin-producing fungi is inhibited below 0.78 a_w (Mahato et al., 2021).

1.5 Impact of other fungal species on mycotoxin contamination

In addition to the well-known mycotoxigenic genera *Aspergillus*, *Fusarium* and *Penicillium*, a broader diversity of moulds is frequently isolated from food and feed commodities, including species belonging to the genera *Mucor*, *Byssochlamys*, *Geotrichum*, *Monascus*, *Paecilomyces* and *Trichoderma* (Pitt and Hocking, 2022; Khan et al., 2024). These fungi may influence the occurrence, levels, and dissemination of multiple mycotoxins in food matrices, and may also affect the technological and nutritional quality of cereals. Some of these genera include confirmed mycotoxin producers, whereas others exert indirect effects by antagonising or facilitating the growth of recognised toxigenic fungi.

Trichoderma spp. are widespread soil- and plant-associated fungi that colonise plant roots and can provide season-long benefits to crops through the production of antifungal compounds and hydrolytic enzymes that suppress soil-borne phytopathogens, thereby reducing the need for chemical pesticides (Modrzewska et al., 2022; Mitrović et al., 2025). They have been widely applied as biocontrol agents, and several studies have demonstrated the strong antifungal activity of *T. harzianum* against *Aspergillus flavus*, as well as its ability to suppress *Fusarium graminearum* (Mitrović et al., 2025). Beyond growth suppression, recent research has demonstrated that *Trichoderma* spp. can also inhibit DON, 3Ac-DON, 15Ac-DON, NIV and ZEN biosynthesis, acting through competition, antibiosis and mycoparasitism, as well as through metabolite-mediated interference with fungal metabolic pathways (Modrzewska et al., 2022).

Other genera, such as *Paecilomyces*, also play a dual role as both spoilage organisms and potential biocontrol agents. *Paecilomyces niveus* is recognised as an important heat-resistant spoilage fungus in processed fruit products and is capable of surviving pasteurisation and producing patulin in fruit juices (Biango-Daniels et al., 2019). *Paecilomyces* spp. can rapidly colonise plant tissues, and some strains have been successfully used for the biological control of *Fusarium* (Shi et al., 2025). However, the ability of certain *Paecilomyces* strains to produce patulin should be considered before their application in food systems (Shi et al., 2025).

Monascus spp. are traditionally used in the production of fermented foods and are valued for their pigment production (Qin et al., 2023). However, CIT is frequently co-produced by many *Monascus* strains and represents a significant toxicological concern in contaminated food products (Zhang et al., 2021; Qin et al., 2023).

Geotrichum spp. have also been reported to interact with mycotoxigenic fungi, with some strains demonstrating the ability to significantly reduce concentrations of AFs, DON, ZEA and T2, either through toxin adsorption or through inhibition of toxin synthesis (Gastélum-Martínez et al., 2013; Vaičiulienė et al., 2024).

Heat-resistant *Byssochlamys* species also represent an important group of spoilage fungi in pasteurised and canned fruits, as their ascospores can survive heat treatments above 85 °C and grow under low-oxygen conditions (Houbraken et al., 2006). Some *Byssochlamys* species are also capable of producing mycotoxins, including patulin, byssotoxin A, viriditoxin and byssochlamic acid (Houbraken et al., 2006).

In contrast, mycotoxin production has not been reported for *Mucor* spp., although some species can grow and weakly ferment under anaerobic conditions and occasionally spoil beverages (Pitt and Hocking, 2022). In addition, several *Mucor* and related species play beneficial roles in traditional soybean fermentations (Tian et al., 2022).

In general, the presence of a given fungal species can alter the surrounding microenvironment, thereby influencing the growth, metabolism, and mycotoxin production of other co-occurring fungi.

1.6 Health impact of mycotoxins ingestion

The ingestion of mycotoxins can occur through contaminated food or feed. Indirectly, mycotoxin exposure can happen through the consumption of animal by-products from animals that have ingested mycotoxins (Alshannaq and Yu, 2017; Iqbal, 2021; Ben Hassouna et al., 2022). However, thanks to the biotransformation and excretion by animals, the mycotoxin exposure through the consumption of food from animal origin is not very significant, except for AFM1, which is excreted in milk (Mahato et al., 2021).

Mycotoxin ingestion can have acute and chronic effects on animal and human health depending on the dose and the exposure time (Pinton et al., 2025). The possible effects can be carcinogenic, estrogenic, haemorrhagic, hepatotoxic, immunosuppressive, mutagenic and neurotoxic (Ekwomadu et al., 2021; Sarmast et al., 2021; Pinton et al., 2025).

Other mycotoxins can display adverse health effects such as endocrine-disrupting effects, nephrotoxicity, growth retardation and gastrointestinal symptoms (Iqbal, 2021) . More specifically, *Fusarium* mycotoxins can alter intestinal defence mechanisms, including cell proliferation, immunoglobulins and cytokine production, and epithelial integrity (Ekwomadu et al., 2021). In addition, mycotoxin ingestion can alter the proper functioning of the liver, the kidneys, the reproductive system and the immune system (Mousavi Khaneghah et al., 2019).

1.6.1. Aflatoxins

Aflatoxins are among the most toxic mycotoxins. The International Agency for Research on Cancer (IARC) classified AFs in group 1, corresponding to known human carcinogens (International Agency for Research on Cancer, 2012a). Their ingestion can lead to acute toxicity, especially in developing countries, or chronic carcinogenicity, which is a global issue (Alshannaq and Yu, 2017). The most affected organ is the liver, but other organs such as the kidneys, the lungs and the myocardium may

also be affected (Khan et al., 2024). The effects of AFs can be carcinogenic, mutagenic, teratogenic, and immunosuppressive (Alshannaq and Yu, 2017; Pinton et al., 2025).

Chronic dietary exposure to aflatoxins has been increasingly associated with growth retardation in children, particularly in low- and middle-income countries where contamination of staple foods is common (Gong et al., 2002; Lombard, 2014; Tesfamariam et al., 2022; Nejad et al., 2023). Aflatoxins may impair child growth through multiple biological mechanisms, including intestinal damage, immune suppression, and disruption of nutrient absorption and metabolism (Lombard, 2014). Early-life exposure, especially during critical periods of growth and development, including the foetal life, has been linked to stunting and reduced height-for-age indicators (Tesfamariam et al., 2022; Nejad et al., 2023). Consequently, aflatoxin ingestion represents not only a toxicological concern but also a significant contributor to impaired child development and long-term health outcomes (Gong et al., 2002; Lombard, 2014; Tesfamariam et al., 2022; Nejad et al., 2023).

In humans, vomiting, abdominal pain, cerebral or pulmonary oedema, convulsions, coma and death can be the consequences of acute exposure (Alshannaq and Yu, 2017; Pinton et al., 2025). In animals, acute aflatoxicosis may result in reduced reproduction, gastrointestinal dysfunction, decreased of egg and milk production, or anemia (Alshannaq and Yu, 2017). The effects may differ depending on the age, sex and health conditions (Khan et al., 2024).

1.6.2. Ochratoxin A

In humans and in animals, OTA ingestion can have teratogenic, embryotoxic, neurotoxic, genotoxic, and immunotoxic effects (Alshannaq and Yu, 2017). It may inhibit protein synthesis, and cause lipid peroxidation and oxidative damage (Pinton et al., 2025). In animals, it may result in reduced productivity, due to lower feed conversion, and it can also decrease egg production in hens (Alshannaq and Yu, 2017; Khan et al., 2024). Ochratoxin A belongs to group 2B, which includes possibly carcinogenic compounds to humans (International Agency for Research on Cancer, 1993).

1.6.3. Fumonisins

Among the FUMs, FUM B1 is the most prevalent and the most toxic, mainly targeting the liver, the immune system and the kidney (Alshannaq and Yu, 2017; Ekwomadu et al., 2021). FUM B1 is classified in group 2B (possibly carcinogenic compounds) according to IARC (International Agency for Research on Cancer, 1993). Various symptoms are induced by FUM consumption. In humans, it is associated with oesophageal cancer, neural tube defects, weight loss, apoptosis and higher

mortality rates (Ji et al., 2019; Ekwomadu et al., 2021; Pinton et al., 2025). In addition, it enhances cancer development, even if FUMs are not mutagenic (Ji et al., 2019). In animals, FUM ingestion can result in cancer, brain lesions in horses and rabbits, pulmonary oedema in pigs, and can have nephrotoxic effects (Ji et al., 2019; Pinton et al., 2025).

1.6.4. Zearalenone

Zearalenone has an oestrogenic activity and is classified as an endocrine disrupting chemical (Alshannaq and Yu, 2017; Ekwomadu et al., 2021; Pinton et al., 2025). It mainly targets the reproductive system, but can also have hepatotoxic, hematotoxic, immunotoxic and nephrotoxic effects (Ji et al., 2019; Pinton et al., 2025). In humans and animals, ZEN ingestion can cause physical changes in genital organs, reduced fertility in males or cancer development (Mousavi Khaneghah et al., 2019; Ekwomadu et al., 2021). In rats and rabbits, ZEN may be responsible for liver lesions and hepatocarcinoma (Ji et al., 2019). In cattle, ZEN ingestion may be associated with reduced milk production and infertility (Alshannaq and Yu, 2017).

1.6.5. Deoxynivaleol

The ingestion of DON, also called vomitoxin, can lead to digestive disorders (nausea, vomiting, abdominal pain, diarrhea), weight gain suppression, anorexia, headache, dizziness and fever (Alshannaq and Yu, 2017; Ekwomadu et al., 2021; Pinton et al., 2025). Deoxynivalenol is neurotoxic, immunosuppressant, and teratogenic, however, DON is not carcinogenic (Ji et al., 2019). DON may block protein synthesis and inhibit DNA and RNA synthesis (Pinton et al., 2025).

1.6.6. T2 and HT2 toxins

T2 and HT2 have the same effects on health, they are capable of inhibiting protein, DNA and RNA synthesis and have cytotoxic and immunosuppressive effects (Mousavi Khaneghah et al., 2019; Pinton et al., 2025). Acute T2 and HT2 toxicosis is characterized by weight loss, vomiting, abdominal pain, diarrhea, cartilage tissue damage, bloody stools, plasma glucose level reduction and pathological changes in the stomach and liver, in humans and animals (Ji et al., 2019; Ekwomadu et al., 2021; Pinton et al., 2025). T2 and HT2 ingestion may delay cell regeneration of the bone marrow and may result in changes in reproductive organs (Ekwomadu et al., 2021).

1.6.7. Emerging mycotoxins

No genotoxic effects have been observed for ENNs (Gruber-Dorninger et al., 2017). The European Food Safety Authority (EFSA) concluded that acute exposure to ENNS is not a concern, but no conclusions could be drawn for chronic exposure due to a lack of relevant toxicity data (EFSA, 2014).

For moniliformin, the available toxicological data are insufficient to establish acute and chronic tolerance thresholds. Nevertheless, EFSA points out cardiotoxic effects following acute exposure and hematotoxic effects associated with chronic exposure (EFSA, 2018).

Regarding STE, the EFSA report concluded that insufficient contamination data were available to establish a risk assessment of animal and human dietary exposure (EFSA, 2013). Acute toxicity is relatively low, and target organs are the liver and the kidney. In addition, tumorigenicity has been observed, causing angiosarcomas in brown fat, lung adenomas, and hemangiosarcoma (EFSA, 2013).

1.6.8. Mixture toxicity of mycotoxins and other food contaminants

In real dietary exposure scenarios, humans and animals are rarely exposed to a single toxic compound at a time. Instead, mycotoxins frequently co-occur with other food contaminants, including pesticides, plastic-derived chemicals, bacterial toxins, heavy metals, and natural food constituents (Guo et al., 2020). Such combined exposures may lead to additive effects, synergistic effects, or antagonistic interactions (Fu et al., 2022). These mixture effects introduce significant uncertainty into risk assessment, which traditionally evaluates contaminants individually.

Several studies have demonstrated that co-exposure with pesticides can alter the toxicity of major mycotoxins. For example, the cytotoxicity of DON, OTA, ZEN, and AFB1 increases when cells were exposed simultaneously to dichlorodiphenyltrichloroethane, whereas similar potentiation was not observed with dichlorodiphenyldichloroethylene at higher concentrations *in vitro* (Guo et al., 2020; Fu et al., 2022). Likewise, combined exposure to chlorpyrifos, an organophosphorothionate pesticide, and patulin enhanced oxidative stress through increased reactive oxygen species and reduced catalase activity, resulting in synergistic cytotoxicity in liver cells and greater hepatotoxicity in mice (Guo et al., 2020; Fu et al., 2022).

Interactions between mycotoxins and microbial endotoxins have also been reported. Bacterial polysaccharides can trigger immune and inflammatory responses, and the toxicity of DON can be

significantly aggravated by co-exposure, with immune cells acting as a principal target (Guo et al., 2020). Synergistic effects between bacterial polysaccharides and T-2 toxin have also been documented (Guo et al., 2020). In addition, the genotoxic metabolite colibactin produced by *Escherichia coli* showed enhanced DNA-damaging potential when combined with DON, both *in vitro* and *in vivo*, with the magnitude of interaction depending on the dose and exposure time (Guo et al., 2020).

Mycotoxins may also interact with endocrine-active contaminants such as bisphenol A originating from plastics. Mixtures of bisphenol A and ZEN produced additive estrogenic responses, whereas combinations of bisphenol A and AOH produced additive, antagonistic, or synergistic effects depending on mixture ratios (Guo et al., 2020). Similarly, co-exposure to OTA and aristolochic acid I resulted in synergistic genotoxicity *in vivo*, attributed to reduced detoxification of aristolochic acid I and enhanced formation of aristolochic acid I-DNA adducts in liver and kidney tissues (Guo et al., 2020).

Another important group of food contaminants consists of heavy metals, including lead, cadmium, mercury, and arsenic (Guo et al., 2020; Fu et al., 2022). Several studies indicate that co-exposure of mycotoxins and cadmium can intensify toxicity. Simultaneous exposure to patulin and cadmium caused synergistic cytotoxicity in liver and kidney cells and exacerbated organ damage *in vivo* (Guo et al., 2020; Fu et al., 2022). Combined exposure to FUM B1 and cadmium also resulted in synergistic hepatotoxicity, accompanied by body-weight loss and elevated liver enzymes in mice (Guo et al., 2020; Fu et al., 2022). In contrast, the interaction pattern between DON and cadmium appears more complex, ranging from antagonistic to synergistic depending on the dose level, exposure duration, cell type, and mixture ratio (Guo et al., 2020; Fu et al., 2022). Co-exposure to AFB1 and cadmium has also produced additive acute toxicity in animal models (Guo et al., 2020; Fu et al., 2022).

Overall, most reported co-exposure studies involving mycotoxins and other food contaminants describe additive or synergistic effects, although antagonism is also observed in certain contexts. These findings highlight that mixture toxicity must be considered in modern food safety risk assessment, as evaluating single contaminants in isolation may underestimate the true hazard associated with real-world dietary exposure.

1.7 Mycotoxins in wheat

Wheat is one of the most widely cultivated crops in the world (Erenstein et al., 2022). In 2025, the Food and Agriculture Organization (FAO) estimated the global cereal production at 2 971 million tons (Food and Agriculture Organization of the United Nations, 2025). In 2025, in Europe, an average of 26 million hectares of wheat were cultivated, resulting in the production of approximatively 132 million tons of soft wheat (European Commission-Directorate-General for Agriculture and Rural, 2025). This represents more than 50% of cereal production in Europe (European Commission, 2023a).

Wheat stands as a primary source of carbohydrates for human consumption (Erenstein et al., 2022). It provides proteins, essential micronutrients and various non-nutrient bioactive food components such as phenolic acids and flavonoids (Sarmast et al., 2021; Erenstein et al., 2022). The worldwide average wheat consumption per capita is 65.6 kg per year, making it the second most consumed cereal after rice (Erenstein et al., 2022). In Europe, 43.8% of the wheat produced is used for food and 36% for feed, while the other 20.2% is used for seed, subject to losses, or for processing and other uses (Erenstein et al., 2022). In Wallonia, the southern part of Belgium, the situation is different, as only 10% of the wheat is used for food, 45% for feed and 45% for fuel (Delcour et al., 2014). This ratio can reasonably be extrapolated to the whole of Belgium. Wheat intended for human consumption in Belgium is largely imported from other European countries and is predominantly used in breadmaking, biscuit production, and pasta manufacturing.

However, wheat is susceptible to fungal contamination and can be responsible for mycotoxin exposure in consumers. The most prevalent mycotoxins worldwide in wheat are DON, ZEN, AFB1, OTA, HT2, T2, AFs and FUMs (Khodaei et al., 2021). The co-occurrence of mycotoxins in wheat is often observed as fungal species can produce a wide range of different mycotoxins (Alshannaq and Yu, 2017). In addition, ENNs have been more frequently observed in wheat in Europe between 2000 and 2023, with detections in 76% of unprocessed grains (Gruber-Dorninger et al., 2017).

Mycotoxin concentrations higher than the regulatory thresholds have been observed in Europe. According to the Rapid Alert System for Food and Feed (RASFF) portal, 39 notifications of mycotoxins in cereals and bakery products were issued in 2023 including two notifications in wheat, one for DON, originating from Germany, and one for OTA, originating from France (European Commission, 2023b). In 2024, 59 notifications for mycotoxins in cereals and bakery products were issued with

three notifications in wheat, one for ergot alkaloids, originating from France, and two for OTA, both originating from Germany (European Commission, 2024a).

1.7.1 Regulations

Mycotoxins in food are regulated by Commission Regulation EU/2023/915 and its updates (European Commission, 2023c). There is also a European Commission Recommendation (EU) 2022/553 on monitoring the presence of *Alternaria* toxins in food (European Commission, 2022) (section 1.1.2). Although it does not establish maximum levels, it emphasizes the importance of monitoring *Alternaria* mycotoxins. The Commission Regulation is regularly updated according to advances in knowledge regarding the adverse effects and toxicokinetic of mycotoxins.

According to the European Commission, "*the legislation on contaminants is based on scientific advice and the principle that contaminant levels shall be kept as low as can be reasonably achieved following good working practices. Maximum levels have been set for certain contaminants (e.g. mycotoxins, dioxins, heavy metals, nitrates, chloropropanols) in order to protect public health"*[1]. The European Food Safety Authority (EFSA) plays a central role in the risk assessment process by providing to the European Commission independent scientific opinions and risk assessments. EFSA evaluates available data on mycotoxin occurrence, toxicity, and exposure in humans and animals, and where possible may establish health-based guidance values such as tolerable daily intakes. These scientific assessments inform EU policymakers on potential health risks and are a key input for setting regulatory levels which are supposed to protect public health. However, for some chemical contaminants, the maximum levels are established according the "ALARA" (as low as reasonably achievable) principle, based on the background contamination levels of food put on the European market. In some cases (such as aflatoxins), these maximum levels are not protective enough.

All the ML from EU/2023/915 that are relevant in regard to this work are presented in Table 2 and 3.

[1] *https://food.ec.europa.eu/food-safety/chemical-safety_en*

Table 2: Summary of maximal levels (ML) for mycotoxins in unprocessed wheat.

Abbreviation	Products	ML (µg/kg)
AFB1	Cereals and products derived from cereals except for maize and rice subject to sorting and cereals aimed for baby food	2
AFB1 + AFB2 + AFG1 + AFG2	Cereals and products derived from cereals except for maize and rice subject to sorting and cereals aimed for baby food	4
FUM B1 + FUM B2	Wheat, oat and rye	1000 (*)
OTA	Unprocessed cereal grains	5
DON	Unprocessed cereal grains except durum wheat, oat grains and maize grains	1000
ZEN	Unprocessed cereal grains except maize	100
T2 + HT2	Unprocessed cereal grains except barley, maize, durum wheat and oat	50
Ergot sclerotia	Unprocessed cereal grains except rye grains	0.2 (g/kg)
Ergot alkaloids	Milling products of wheat (with an ash content lower than 900 mg/100 g dry matter)	100 (50 as from 1 July 2028)
Ergot alkaloids	Milling products of barley, wheat, spelt and oats (with an ash content equal or higher than 900mg/100g dry matter) Barley, wheat, spelt and oats grains placed on the market for the final consumer	150

The ML are taken from European Commission recommendation EU/2023/915 except for FUM B1 + B2 that are coming from limits of actions established by the Belgian Federal Agency for the Safety of the Food Chain (FASFC) (Belgian Federal Agency for the Safety of the Food Chain (FASFC), 2025).

1.8 Mycotoxins in peanuts

Peanuts, also called groundnuts, are the edible seeds of a legume. Peanuts are mainly grown in warm temperate climate, tropics and subtropics (Melo et al., 2025), and have a rich nutrient profile as they are rich in fibres, proteins and saturated fat (Arya et al., 2016; Melo et al., 2025). In addition, peanuts can also be considered functional food, as they contained bioactive compounds such as phytosterol, phenolic compounds, stilbenes, lignans, and isoflavonoids that may protect against diabetes risk, cancer growth, cardiovascular diseases, etc. (Çiftçi and Suna, 2022). Indeed, peanut consumption provides antioxidants (resveratrol and phenolic acids), minerals (copper, iron,

magnesium, manganese, potassium, selenium and zinc), and vitamins (vitamins B and E) (Arya et al., 2016; Melo et al., 2025).

Peanuts are among the most widely produced nuts worldwide, ranking just after almonds, walnuts, and cashews ("FAOSTAT," 2025). In developing countries, they play a crucial role both as an oilseed and a food crop, destined for local consumption as well as export. Peanuts may be consumed raw, roasted, boiled or processed into fried pressed peanut cake, oil, or peanut paste (Chang et al., 2013; Variath and Janila, 2017). Moreover, peanut consumption has been steadily increasing in Europe, reflecting a growing demand in the market (Melo et al., 2025).

However, mycotoxin contamination of peanuts is a worldwide concern, especially with AF contamination (Arya et al., 2016; Kluczkovski, 2019). Indeed, most peanuts are grown in areas where temperature and relative humidity conditions are favourable for *Aspergillus* spp. development and AF production (Marín and Ramos, 2016). In addition, peanuts are grown below the soil surface, which enhances the contact with soil fungi (Marín and Ramos, 2016). Other mycotoxins have also been detected in peanuts such as FUMs, ZEN, DON, OTA, BEA and ENNs (Marín and Ramos, 2016). In 2023, 48 notifications for mycotoxin contamination in peanuts were recorded on the RASFF portal, mainly forAF B1 in peanuts originating from the United States, and 64 in 2024, mainly in peanuts from the United States, Egypt and India (European Commission, 2025a, 2025b).

From an economic perspective, AFs contamination results in substantial financial losses, driven by export rejections, decreased market value, and the additional expenses linked to testing and mitigation measures. Smallholder farmers are particularly vulnerable, as limited access to adequate storage and monitoring facilities exposes them to greater income instability (Melo et al., 2025).

1.8.1 Regulations

The European regulation EU/2023/915 (European Commission, 2023c) also regulates mycotoxin concentrations in peanuts. The ML for AFB1 and the sum of AFB1, AFB2, AFG1 and AFG2 in raw peanuts that still need to be sorted or physically treated before being available for the final consumer are respectively 8 and 15 µg/kg, while for final products the MLs for AFB1 and the sum of AFs are respectively 2 and 4 µg/kg. The summary of the ML values is presented in Table 3.

Table 3: Summary of mycotoxin maximal levels (ML) in processed and unprocessed peanuts.

Abbreviation	Products	ML (µg/kg)
AFB1	Groundnuts (peanuts) and other oilseeds, to be subjected to sorting or other physical treatment before placing on the market for the final consumer or used as an ingredient in food	8
AFB1 + AFB2 + AFG1 + AFG2	Groundnuts (peanuts) and other oilseeds, to be subjected to sorting or other physical treatment before placing on the market for the final consumer or used as an ingredient in food	15
AFB1	Groundnuts (peanuts) and other oilseeds used as only ingredient or processed products from groundnuts (peanuts) and other oilseeds, placed on the market for the final consumer or use as an ingredient in food	2
AFB1 + AFB2 + AFG1 + AFG2	Groundnuts (peanuts) and other oilseeds used as only ingredient or processed products from groundnuts (peanuts) and other oilseeds, placed on the market for the final consumer or use as an ingredient in food	4

1.9 Mycotoxins in other food matrices

Cereals and dried fruits are among the preferred substrates for toxigenic fungi, and contamination may occur both in the field and during storage (Marin et al., 2013; Pinton et al., 2025). Cereals and cereal-based products constitute a major component of the human diet worldwide and, at the same time, represent highly suitable raw materials for mold growth (Pleadin et al., 2019). In addition to cereals, cereal-based products and oilseeds, other important dietary sources of mycotoxins include nuts and spices. As a result, mycotoxin contamination of food commodities remains a major concern for food safety and public health. In the European Union, mycotoxins are regulated in plant-based food matrices and milk under Commission Regulation (EU) 2023/915.

Aflatoxins B1, B2, G1 and G2 are regulated in dried fruits, groundnuts, cereals and derived products, maize and spices, while aflatoxin M1 is regulated in milk as a consequence of feed-to-food carry-over. In the European diet, grains and grain-based products are the main contributors to aflatoxin exposure, reflecting their high consumption rates (EFSA, 2020b).

Ochratoxin A is regulated in coffee, cocoa, dried fruits, spices, dried herbs, liquorice, cereals and derived products, and wine, with chronic exposure mainly originating from grains and grain-based products, preserved meat and cheese (EFSA, 2020).

Deoxynivalenol and ZEN are regulated in cereals and maize, while FUMs B1 and B2 are regulated exclusively in maize.

In addition, citrinin is regulated in products containing rice fermented with *Monascus purpureus*, patulin in fruit juices and apple-based products, and ergot sclerotia and ergot alkaloids in cereals.

Despite regulatory levels, the widespread occurrence of mycotoxins in food continues to pose challenges due to their persistence, co-occurrence, and resistance to conventional food processing.

1.10 Mycotoxins in feed

Feed has frequently been reported to be contaminated with multiple mycotoxins, particularly cereals and cereal by-products, which form the basis of most compound feeds. Feed supply is a critical component of the animal production industry, as it directly influences animal health, productivity and welfare (Biranjia-Hurdoyal, 2025). When the safety of feed is compromised by mycotoxin contamination, adverse effects on animal performance may occur, leading to economic losses for farmers and potentially increasing the risk of mycotoxin transfer into foods of animal origin, thereby threatening food safety and food security (Biranjia-Hurdoyal, 2025).

Directive 2002/32/EC of the European Parliament and its amendments define a maximum concentration for AFs in feed materials of 20 µg/kg (European Commission, 2002). In addition, recommended values are defined by the European Commission Recommendation 2006/576/EC. The recommended values are 250 µg/kg for OTA, 8000 µg/kg for DON, and 2000 µg/kg for ZEN in cereals and cereals by-products (European Commission, 2006a). A recommended level of 60 000 µg/kg for FUMs B1 and B2 in maize also exists.

These measures aim to reduce animal exposure, limit productivity losses, and prevent the entry of mycotoxins into the food chain through animal-derived products.

2 Mycotoxin issue at the world level

Mycotoxin contamination represents one of the most important issues regarding food safety, as they are widespread in agricultural crops worldwide and can have serious adverse effects on both animal and human health (Zain, 2011; Pleadin et al., 2019). Beyond their direct health impact, fungal contamination of crops can lead to a yield reduction and excessive mycotoxin levels can result in the loss of entire portions of harvests (Kos et al., 2023). Between 2010 and 2015, it was estimated that

around 60 to 80 % of food crops are contaminated with mycotoxins (Eskola et al., 2020), contributing to the wastage of approximately 1.3 billion metric tons of food annually, corresponding to one-third of global food production (Khan et al., 2024).

Huge economic losses can be associated with mycotoxin contamination due to lower food quality, decreases in animal production caused by feed refusal or illness, or higher medical expenses to cover toxicosis treatment (Marroquín-Cardona et al., 2014). Additional expenditures may also arise from the need to find alternative food sources of sufficient quality, to implement relevant management procedures of contaminated supplies, to develop strategies that limit toxin exposure, or to improve detection and quantification methods (Marroquín-Cardona et al., 2014).

Vulnerable populations are particularly at risk, especially rural communities dependent on staple crops prone to fungal infection, and economically disadvantaged groups with limited access to high-quality food (Marroquín-Cardona et al., 2014). By 2050, with the global population expected to surpass nine billion, food supply systems will face unprecedented pressure, compromising both the quantity and the quality of available food (Marroquín-Cardona et al., 2014; Abdallah et al., 2018; “Feeding the future global population,” 2024).

In addition, current climate change is likely to lead to an increase in mycotoxins in crops (Wang et al., 2022). Rising temperatures, water scarcity, land shortages and extreme weather events including wildfires, severe droughts, extreme precipitation are already compromising food safety worldwide (Liu and Van der Fels-Klerx, 2021; Casu et al., 2024). Hence, major consequences on public health are observed (Casu et al., 2024). Extreme weather conditions, elevated carbon dioxide levels and environmental temperature rise are enhancing crop susceptibility to fungal contamination, therefore mycotoxin contamination also tends to increase (Kos et al., 2023). Wheat and maize are more susceptible to *Fusarium* infections with increasing CO_2 concentration (Casu et al., 2024). Extreme wind and rainfall promote fungal spore dispersion (Liu and Van der Fels-Klerx, 2021), while water stress in crops facilitates fungal infection (Casu et al., 2024). Overall, climate change is expected to enhance fungal growth, virulence and geographical spread (Liu and Van der Fels-Klerx, 2021).

Furthermore, climate change is reshaping both crop and fungal geographical distributions. Rising temperatures and altered precipitation regimes are driving thermophilic fungi toward higher latitudes and altitudes, while psychrophilic species retreat to cooler regions (Casu et al., 2024). At the same time, crop production areas are shifting, for instance, maize cultivation is expected to

expand in Northern Europe while declining in the south, exposing crops to unfamiliar fungal communities and environmental conditions (Casu et al., 2024; Garcia-Cela and Gasperini, 2024). Plant physiology will also be affected, as climate change can modify stomatal patterns, transpiration, and photosynthetic capacity, potentially increasing susceptibility to pathogen invasion (Casu et al., 2024).

These environmental changes are already reflected in shifts within fungal populations and associated mycotoxins. In Europe, *Fusarium graminearum*, with an optimal growth temperature of 24–28 °C, is increasingly replacing *F. culmorum* (20–25 °C) in regions such as the Netherlands, Germany, Poland, and the United Kingdom, coinciding with higher *Fusarium* head-blight incidence in southern England (Casu et al., 2024). Warmer and drier conditions have also been linked to a transition from *F. verticillioides* and FUM production to *Aspergillus flavus* and AF production. Aflatoxins, historically most problematic in Sub-Saharan Africa, are now emerging more frequently in Mediterranean regions such as Spain and Italy, where drought risk is increasing (Marroquín-Cardona et al., 2014; Casu et al., 2024). Overall, AF risk in European food is expected to rise from low to medium under future climate scenarios (Kos et al., 2023). Aflatoxins may even overtake OTA as the main mycotoxin in grapes and coffee as conditions become more favourable for thermotolerant *Aspergillus* spp., while OTA occurrence is expected to rise in currently colder regions as *Penicillium verrucosum* will be able to thrive (Medina, 2023; Casu et al., 2024). For FUMs, which peak around 30 °C, a general increase is anticipated in maize, wheat, rice, and soybean production areas, with AF and FUM dominance alternating depending on whether seasons are hotter or wetter (Casu et al., 2024). DON and ZEN contamination are also projected to increase in Northern Europe as it warms, as well as in coastal savannah and humid rainforest regions where high humidity and warm temperatures will favour *Fusarium* and *Penicillium* spp. (Casu et al., 2024). Finally, Alternaria toxins may become more prevalent in currently colder regions such as Poland and less frequent in warmer regions such as Spain (Casu et al., 2024).

In addition to field-level effects, rising temperatures may also enhance fungal development during storage and transport. Increased fungal growth can generate metabolic water and localized humidity, creating favourable micro-environments for AF and OTA formation in stored commodities (Liu and Van der Fels-Klerx, 2021).

On the other hand, climate change is also accelerating crop development, with anthesis and maturity stages projected to occur around two weeks earlier by 2050 (Casu et al., 2024). This

temporal shift may expose crops to weather conditions that heighten the likelihood of fungal infection (Liu and Van der Fels-Klerx, 2021).

Climate change is widely expected to alter plant physiology, and these shifts are likely to modify the interactions between pathogens and their host plants. Under such changing conditions, the complexity of fungus–plant interactions and the resulting patterns of mycotoxin formation, including the occurrence of modified mycotoxins, is likely to increase (Casu et al., 2024). Consequently, there is a clear need for research focused on understanding how climate-induced physiological changes in plants affect pathogen–host interactions and, in turn, influence both the production and transformation of mycotoxins (Casu et al., 2024).

Currently, mycotoxin occurrence in food is almost unavoidable, and the concentration will depend among others on the climatic conditions in the field and the post-harvest storage conditions (Ekwomadu et al., 2021). Some strategies are applied to limit the mycotoxin contamination of food before and post-harvest (section 4).

However, despite the implementation of control strategies, mycotoxins are still present in final food and feed products, posing a risk to animal and human health. Therefore, some mycotoxin concentrations are regulated in Europe and food quality controls are conducted to limit the adverse effects on animal and human health.

Consequently, analytical methods should be developed and implemented for mycotoxin concentration control in food and feed. Furthermore, the analytical method should be validated to ensure reliable and reproducible results.

3 Analytical method for mycotoxin determination

The need for mycotoxins identification and quantification in food has led to the development of various analytical methods. As there is a diversity of mycotoxins, which have various chemical structures, and occur in different complex matrices, method development can be challenging, especially for multi-mycotoxin methods (Alshannaq and Yu, 2017; Mousavi Khaneghah et al., 2019). Additional challenges arise from the need to detect low levels of mycotoxins in complex matrices (Alshannaq and Yu, 2017). Mycotoxin identification and quantification can be performed in different ways, some methods are primarily used for screening (section 3.3.1), while others are used for confirmation (section 3.3.2).

3.1. Sampling

The first step in an analytical method is sampling. It is crucial to collect a representative sample of the whole batch. In addition, this is even more relevant because mycotoxin contamination is not always homogeneous and hotspots can be identified (Iqbal, 2021; Khan et al., 2024). For large batches, a sampling procedure has been defined by the European Union (European Commission, 2006b). An inadequate sampling procedure may lead to mycotoxin concentration determination errors, such as underestimation or overestimation (Sowmya and Ramalingappa, 2023).

3.2. Sample preparation

Sample preparation can be done using different types of extraction procedures. The clean-up and the extraction step of the sample are critical to remove compounds that can interfere with the analyte, and thus mitigate the matrix effects (Alshannaq and Yu, 2017; Janik et al., 2021). These steps improve the specificity and the sensitivity of the detection method, leading to better quantification (Janik et al., 2021).

Liquid-liquid extraction can be used for liquid food samples such as milk, apple juice and wine, for solid materials, such as cereals, peanuts, etc., a liquid-solid extraction can be performed (Turner et al., 2015; Alshannaq and Yu, 2017).

One of the most commonly used extraction methods is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, initially developed for pesticide analysis (Alshannaq and Yu, 2017; Janik et al., 2021; Sowmya and Ramalingappa, 2023). It involves an acidified acetonitrile-water extraction enhanced by the addition of inorganic salts, where the mycotoxins will move to the organic phase. The second step is a dispersive solid phase extraction to remove matrix compounds from the organic phase (Janik et al., 2021; Sowmya and Ramalingappa, 2023). Water presence in the extraction solvent will ease the organic solvent penetration in the food material, while the acidic solvent will help break the strong bonds between the mycotoxins and the food components (Alshannaq and Yu, 2017; Iqbal, 2021). Magnesium sulphate is used to remove water from the organic phase while sodium chloride will reduce the polar interferences, both leading to a better phase separation and mycotoxin recovery in the organic phase (Alshannaq and Yu, 2017). However, some modifications have been made for the extraction of mycotoxins from cereals (Pereira et al., 2014).

The cleanup step is crucial to remove the matrix components that could interfere with the mycotoxins detection, resulting in higher specificity and sensitivity of the analysis (Alshannaq and Yu, 2017; Iqbal, 2021).

The most used clean-up methods for mycotoxins are solid phase extraction (SPE) and immunoaffinity columns (IAC) due to their rapidity and high selectivity (Janik et al., 2021; Sowmya and Ramalingappa, 2023). In SPE, the sample passes through a sorbent-filled cartridge or disk, where mycotoxins are adsorbed and then eluted with an organic solvent. The choice of cartridge depends on the sample nature and the compound of interest. However, SPE does not allow the clean-up of multiple mycotoxins in a single cartridge (Pereira et al., 2014; Alshannaq and Yu, 2017; Sowmya and Ramalingappa, 2023). Regarding IAC, the sample flows through the column and the mycotoxins are bound by specific antibodies, before being released with a miscible solvent or by denaturing the antibodies (Alshannaq and Yu, 2017; Janik et al., 2021). However, the binding ability of the column is limited. Indeed, with very high mycotoxin concentrations, the column can become saturated, leading to unreliable results as some mycotoxins may not be retained by the column (Pereira et al., 2014). In addition, some matrix compounds can interfere with the antibodies (Janik et al., 2021).

In addition, some methods use pressurized liquid extraction that is similar to liquid-solid extraction but accelerated by working under high pressure and temperature (Janik et al., 2021; Sowmya and Ramalingappa, 2023). Furthermore, supercritical fluid extraction is mostly used for the extraction of non-polar organic molecules and leads to the reduction of the use of organic solvents (Xie et al., 2016). Supercritical fluid extraction, microwave-assisted extraction and accelerated solvent extraction allow quicker extraction with smaller volumes of solvents and usually lead to better efficiencies, however these methods can be expensive (Alshannaq and Yu, 2017).

Finally, molecularly imprinted polymers (MIPs) that are synthetic materials engineered to mimic natural recognition systems such as antibodies or biological receptors, can be used for mycotoxin detection (Vasapollo et al., 2011). They are typically obtained through the electrochemical polymerization of monomers and cross-linkers in the presence of a target analyte, such as a mycotoxin (Appell and Mueller, 2016). The MIPs present several advantages, including high selectivity and affinity toward the analyte, excellent chemical stability, easy preparation, and relatively low production costs (Alshannaq and Yu, 2017). They have been successfully developed for the detection of AFs, OTA, DON, and ZEN (Appell and Mueller, 2016). Although their application

to complex food matrices still requires further demonstration and validation (Alshannaq and Yu, 2017).

3.3. Detection and analysis

3.3.1. Rapid method

In certain situations, rapid and cost-effective analytical methods are required, even if they are generally more sensitive to matrix interferences than Ultra-Performance Liquid Chromatography (UPLC) or High-Performance Liquid Chromatography (HPLC) methods (section 3.3.2) and are only available for a limited number of mycotoxins. In addition, there is a growing demand for onsite testing that can be performed directly at food production or storage locations.

In this context, immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (LFIA) are commonly used (Janik et al., 2021).

Various formats of ELISA are available: direct assay, competitive direct assay and competitive indirect assay. This technique is based on the interactions between the mycotoxins and antibodies, and relies on enzymatic signal generation, which can be measured using colorimetric, fluorometric or chemiluminescent detection, depending on the assay format (Dib et al., 2023). ELISA can be used as a screening technique as it is simple, accurate and allows the simultaneous testing of multiple samples (Wang et al., 2024). In addition, it requires low sample volume and fewer clean-up procedures (Janik et al., 2021; Dib et al., 2023). However, ELISA must be developed for each mycotoxin of interest and tested for each matrix, as matrix interferences can lead to under- or overestimation of mycotoxin concentration in the sample, before being validated (Singh and Mehta, 2020).

Lateral flow immunoassays are a strip test based on the competition between a labelled antibody and a signal reagent (Janik et al., 2021). The sample is applied on a strip that allows the passage of mycotoxins through a membrane. Qualitative or semi-quantitative data can then be recovered from the test (Khan et al., 2024). The accuracy of the method depends on the signal labels (Wang et al., 2024). Historically, gold nanoparticles were used, but currently, magnetic nanoparticles, carbon nanoparticles or quantum dots are applied (Janik et al., 2021).

In addition, biosensors, such as array biosensors, surface plasmon resonance or fibre-optic probes, can be used for mycotoxins detection (Alshannaq and Yu, 2017; Janik et al., 2021). Biosensors

generally include a biological or biologically derived sensing element to detect specific bio-analytes, combined with a transducer that converts the biological signal into an electrical signal (Janik et al., 2021; Wang et al., 2024). Biosensors can be classified in different groups: optical biosensors, electrochemical biosensors and other sensors (Wang et al., 2024).

An electronic nose (e-nose) can be used for various nonspecific chemical detection to capture and analyse volatile organic compounds (VOCs). By recognizing these patterns, the e-nose provides identification and quantification information on the metabolite. Currently, e-nose are designed to detect fungal presence rather than mycotoxin contamination, allowing the differentiation between toxigenic and non-toxigenic fungi (Alshannaq and Yu, 2017). However, for broader use in detecting mycotoxins, e-noses must be optimized to quantify low levels of these toxins in food (Janik et al., 2021). In addition, most of the mycotoxins are non-volatile, which is problematic for detection (Alshannaq and Yu, 2017).

Infrared spectroscopy can also be used for screening and mycotoxin quantification. By coupling the infrared data to reference values and with the use of principal components analysis (PCA), models could be elaborated to estimate the mycotoxin concentration of samples (Pettersson and Åberg, 2003; Wang et al., 2024). This method is rapid, easy to use, non-destructive and does not require chemicals (Alshannaq and Yu, 2017). In wheat and maize, near-infrared and mid-infrared spectroscopy have been used for the detection of DON (Alshannaq and Yu, 2017; Wang et al., 2024). However, this method still needs to be further improved to reach lower detection limits, and to manage the non-homogeneous distribution of the mycotoxins in the sample (Alshannaq and Yu, 2017). In addition, the specificity for complex samples remains low (Wang et al., 2024). Machine learning techniques and artificial intelligence may be tools to improve the prediction accuracy of the models (Wang et al., 2024). Single kernel near infrared spectroscopy is used to determine grain quality and in some cases can be used to distinguish healthy and infected kernels (Wang et al., 2024).

Capillary electrophoresis could also be a solution for mycotoxin analysis by separating the components based on their electrochemical potential using fluorescence or UV absorbance (Alshannaq and Yu, 2017). This method does not require the consumption of high solvent volume, however, the limits of detection are higher (Alshannaq and Yu, 2017).

To conclude, these rapid analytical methods are easy and quick, and some of them, such as LFIA, are portable, and suitable for onsite use. Nevertheless, the main limitations of these methods include matrix interferences, leading to the necessity of a specific development and validation for each

matrix, the impossibility to reach low-level detection and antibody cross-reactivity. Consequently, these methods are mainly used for screening purposes and sometimes require confirmation by chromatographic techniques (Pereira et al., 2014).

3.3.2. Chromatographic methods

Chromatographic techniques remain the reference methods for mycotoxin analysis due to their high sensitivity, selectivity, and reliability. They can also simultaneously detect multiple mycotoxins at low concentration levels. It exists various types of chromatography that can be combined with many different detection systems.

Gaz chromatography (GC) is often used for the analysis of volatile, thermally stable, with low-polarity compounds. However, mycotoxins have low volatility and a high polarity, therefore, derivatization of the mycotoxins is needed (Dib et al., 2023; Sowmya and Ramalingappa, 2023; Khan et al., 2024). Gaz chromatography can also cause incomplete derivatization and matrix interferences, which can alter reproducibility of the results (Wang et al., 2024). Even if the method is highly sensitive and specific, the main issues of GC are column blockages, cross-contamination from previous samples, response drift, and nonlinearity of calibration with some detectors (Janik et al., 2021).

Liquid chromatography (LC) allows the separation of compounds of interest and, when coupled with an appropriate detector, enables the simultaneous detection and quantification of multiple mycotoxins at low levels without the need for derivatization (Pereira et al., 2014; Dib et al., 2023). The choice and composition of mobile phases are adapted to the physicochemical properties of the analytes and to the detector used (Köppen et al., 2010). Several mycotoxins, including OTA, ZEN and AFs, exhibit natural fluorescence and can therefore be analysed using fluorescence (FLD), UV–visible (UV) or diode array (DAD) detectors (Alshannaq and Yu, 2017; Janik et al., 2021; Dib et al., 2023; Sowmya and Ramalingappa, 2023). However, DAD systems are used less frequently in mycotoxin analysis due to their relatively low sensitivity, which may prevent compliance with regulatory limits in the µg/kg range (Köppen et al., 2010). In some cases, derivatization is applied before or after column separation to introduce a chromophore and improve detectability (Janik et al., 2021; Dib et al., 2023). In addition, LC techniques have also evolved from conventional HPLC to UHPLC systems, where the use of smaller stationary-phase particles increases surface area, column efficiency and theoretical plate numbers, thereby improving resolution, speed and sensitivity while reducing analysis time and cost (Pereira et al., 2014; Turner et al., 2015).

A major development has been the widespread coupling of LC with mass spectrometry (MS), which improves analytical specificity and allows the detection of very low mycotoxin concentrations in complex matrices (Turner et al., 2015; Dib et al., 2023). In particular, LC–MS/MS has become the reference technique for multi-mycotoxin determination in food and feed because of its high selectivity, sensitivity and reliability across chemically diverse toxins and matrices (Janik et al., 2021). Different analysers may be employed, including triple quadrupole, ion trap, time-of-flight and Orbitrap instruments, sometimes combined in hybrid configurations. Among these, the triple quadrupole operating in multiple-reaction-monitoring (MRM) mode is the most widely adopted for routine screening due to its robustness and quantitative performance (Janik et al., 2021). The decreasing cost of MS instrumentation over the last decade has further accelerated the transition from fluorescence-based detection to tandem MS, which offers the advantage of detecting both fluorescent and non-fluorescent toxins within a single run (Turner et al., 2015; Janik et al., 2021).

Despite these advances, LC–MS/MS analysis still faces important challenges, particularly matrix effects in complex commodities. These effects can suppress or enhance ionization and thereby bias quantification. Matrix-matched calibration and the use of isotope-labelled internal standards are commonly applied to compensate, although they cannot fully eliminate matrix interference (Janik et al., 2021; Dib et al., 2023). Furthermore, High-resolution mass spectrometry (HRMS) has gained prominence, particularly for non-targeted screening. Because HRMS records full-scan accurate-mass spectra of precursor and fragment ions, a theoretically unlimited number of compounds from different classes may be detected in a single run at low concentration levels (Righetti et al., 2016). This capability also enables retrospective data analysis, meaning that the presence of newly identified mycotoxins can be investigated in previously analysed samples even in the absence of analytical standards (Righetti et al., 2016).

To conclude, chromatographic methods are often used for mycotoxin analysis as they have a high sensitivity, trueness, reliability and flexibility. However, sample preparation can be complex as matrix effects must be mitigated. In addition, the equipment is quite expensive and cannot be easily transported, in case onsite analysis is needed (Janik et al., 2021).

4. Mycotoxins management

4.1. Mycotoxins contamination prevention practices

Mycotoxin contamination is a global concern. Despite good agricultural and good storage practices, contamination is unavoidable and unpredictable (Alshannaq and Yu, 2017). Mycotoxins are generally considered to be chemically stable compounds, yet food processing can substantially influence their concentration, structure and distribution in the final product. The effects of processing differ according to the toxin involved, the matrix, and the technological conditions applied (Karlovsky et al., 2016; Alshannaq and Yu, 2017; Ekwomadu et al., 2021).

During te pre-harvest stage, good agricultural practices can be implemented. The selection of crop varieties resistant or tolerant to fungal and insect contamination is an effective strategy (Ekwomadu et al., 2021). In addition, crop rotation can be used to avoid the build-up of a local inoculum (Ekwomadu et al., 2021; Mahato et al., 2021; Kos et al., 2023). It has been shown that *Fusarium* contamination tends to decrease when maize – soybean rotation is applied rather than continuous maize cultivation (Ekwomadu et al., 2021). Furthermore, farmers should get rid of weeds, grasses, crop residues or debris that can serve as a reservoir for fungal species (Marroquín-Cardona et al., 2014; Ekwomadu et al., 2021; Mahato et al., 2021). Indeed, fungal spores may persist in the soil for several months and infect crops from one growing season to another (Marroquín-Cardona et al., 2014). Pre-harvest strategies also include soil analysis, seedbed treatment, seed decontamination and irrigation (Mahato et al., 2021; Kos et al., 2023; Casu et al., 2024). Appropriate irrigation can help reduce plant stress, thereby improving crop resistance to fungal infection and limiting mycotoxin production (Casu et al., 2024).

The use of appropriate pesticides, insecticides, herbicides or fungicides can help prevent mycotoxin contamination. However, these products may be harmful to human health and the environment (Ekwomadu et al., 2021; Mahato et al., 2021). In addition, the intensive use of chemicals enhances the development of resistant strains, resulting in a need to increase the frequency of treatment, leading to a greater accumulation of chemicals in the environment and in the food (Ben Miri et al., 2024). Nevertheless, the use of pesticides for toxin reduction is not always effective (Marroquín-Cardona et al., 2014; Casu et al., 2024). On the other hand, nitrogen fertilizer used for promoting crop growth, also enhances *Fusarium* growth and thus mycotoxin production (Mahato et al., 2021).

Currently, new researches are ongoing for the development of biological control agents that can reduce fungal growth and spread (Mahato et al., 2021). Biological control in plant pathology involves

using microorganisms to prevent colonization and suppress the spread of harmful plant pathogens, including mycotoxin-producing fungi (Abdallah et al., 2019; Habschied et al., 2021). Effective biocontrol agents (BCAs) against *Fusarium* Head Blight include *Bacillus*, *Pseudomonas*, and *Streptomyces* species, while some yeasts such as *Cryptococcus* also show strong antagonistic activity (Abdallah et al., 2019; Habschied et al., 2021). Biocontrol agents employ several mechanisms, such as antibiosis, mycoparasitism, competition, and induction of host resistance, and often act through more than one of these mechanisms simultaneously (Abdallah et al., 2019). Antibiosis involves the production of antifungal metabolites, enzymes and lipopeptides that damage fungal membranes or cell walls. For example, *Trichoderma* and *Clonostachys* spp. produce peptaibols that interfere with β-glucan synthesis, while *Bacillus* and *Pseudomonas* spp. synthesize potent lipopeptides (Abdallah et al., 2019). Mycoparasitism has also attracted attention, with *Sphaerodes mycoparasitica* shown to parasitize *Fusarium* spp. and reduce DON production (Abdallah et al., 2019). Competition limits pathogen establishment by occupying ecological niches or competing for essential nutrients needed for growth and toxin biosynthesis (Abdallah et al., 2019). In addition, some BCAs stimulate plant defense pathways, including jasmonic-acid- and salicylic-acid-mediated responses, thereby enhancing resistance. Natural plant-derived compounds such as essential oils are also promising as eco-friendly antifungal and anti-mycotoxin agents (Habschied et al., 2021). For example, Spanish paprika extracts inhibit *Aspergillus* and *Penicillium* species and suppress AFB1, AFG1, and OTA production (Habschied et al., 2021). Although highly promising, biocontrol must be implemented carefully and BCAs themselves must be proven to be non-toxic (Abdallah et al., 2019). However, these biocontrol agents are still relatively little used in practice. Laboratory results are often excellent, but field performance is more variable because application conditions, weather, and the type of formulation strongly influence whether the antagonistic microorganisms are able to successfully establish and develop on the plants.

During harvest, minimizing mechanical damage to crops is crucial, as broken kernels are more prone to mycotoxin contamination (Ekwomadu et al., 2021). Similarly, these damaged seeds and grains should not be in direct contact with the soil to avoid the incorporation of fungal spores into the field (Marroquín-Cardona et al., 2014). In addition, harvesting should be performed at the appropriate time when the crops are mature, early or late harvesting in some crops may lead to higher fungal contamination (Marroquín-Cardona et al., 2014).

Furthermore, some post-harvest strategies to mitigate mycotoxin contamination such as reducing the time between harvest and drying, efficient drying (to reach a moisture content lower than 14 %),

hygiene management, traceability of food, pest control in the storage area, etc. may also be implemented (Ekwomadu et al., 2021; Mahato et al., 2021). Appropriate storage conditions, humidity and temperature are also crucial, the use of modified atmosphere storage with sulphur dioxide may also be used to limit the presence of mycotoxigenic organisms (Mahato et al., 2021).

4.2. Mycotoxins decontamination processes

Despite the risk prevention measures generally taken, mycotoxins are still present in food. Therefore, decontamination procedures have been studied to remove mycotoxins from food materials. Different approaches may be used, including physical, chemical and microbiological processes. The combination of processes is often more effective (Ekwomadu et al., 2021; Khan et al., 2024). However, decontamination should be performed without the creation of toxic residues and the nutritional and technological properties of the food should be preserved (Khan et al., 2024).

Physical treatments include milling, cleaning, sorting and thermal processing, while chemical treatments require the use of compounds containing calcium hydroxide, sulphur and ammonia, or hydrogen peroxide and sodium hypochlorite (Ekwomadu et al., 2021; Khan et al., 2024). However, the use of specific chemicals precludes the utilization of these materials as food or feed (Khan et al., 2024). Finally, biological treatment may be brewing, malting or fermentation (Ekwomadu et al., 2021).

Physical treatments allow the conservation of the food nutritional quality as they do not involve chemicals and are environmental friendly (Ben Miri et al., 2024). Milling, baking, roasting and cooking are the most prevalent food processing procedures in the food industry, which may reduce, redistribute or increase mycotoxin content in the materials (Sarmast et al., 2021). Milling processes cannot reduce the total mycotoxin content but they can redistribute the mycotoxins among the different fractions (Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018). Indeed, the inner part of the grains is less contaminated than the outer part, therefore fractions containing the inner parts could be recovered for food and others may be used for feed if the contamination is not too high (Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018). Sorting, cleaning and milling have shown efficacy to reduce OTA content in the final food product (Ben Miri et al., 2024). By milling maize, mycotoxins are concentrated in the germ and bran fractions (Karlovsky et al., 2016).

Conventional food preparation with temperatures up to 100°C does not greatly affect mycotoxin concentration (Karlovsky et al., 2016). However, thermal treatments, such as boiling, frying,

extrusion, cooking or baking might reduce mycotoxin concentration. A reduction of 50 to 80% of AFs content by extrusion can be achieved depending on grain moisture and temperature (Karlovsky et al., 2016). A reduction in OTA and AFs may be achieved by roasting peanuts, maize or coffee (Karlovsky et al., 2016). Thermal treatment has been reported to be effective for ZEN reduction at temperatures ranging from 120 to 160°C (Ryu et al., 1999; Mahato et al., 2021). Nevertheless, process efficiency depends on pH, temperature and duration (Mahato et al., 2021). Longer physical processing could result in improved mycotoxin removal but in manufacturing situations, process duration is standardized and is not changed from one batch to another. Therefore, maximum efficiency is not always reached (Peng et al., 2018).

Cold atmospheric plasma (CAP) is a physical state obtained when a neutral gas is exposed to an electric field that ionizes some of its atoms or molecules, thereby generating plasma. When the applied voltage exceeds the breakdown threshold, reactive oxygen and nitrogen species are formed, resulting in CAP production. Its application typically consists of subjecting water, buffer solutions, or acids to plasma discharge, which produces sanitizing agents for washing and disinfection. The reactive species generated by plasma can compromise fungal cell walls, causing cytoplasmic leakage and ultimately leading to cell inactivation (Khan et al., 2024). In some cases, CAP may inhibit mycotoxin synthesis by limiting the expression of genes involved in mycotoxin production (de Oliveira et al., 2025). In addition, CAP can induce structural modifications of certain mycotoxins, leading to molecular cleavage and the formation of degradation products with reduced toxicity. CAP has been shown to be effective in the degradation of AFB1 and DON. However, the toxicity and potential health risks associated with the newly formed compounds remain insufficiently characterized and should therefore be carefully assessed (de Oliveira et al., 2025).

In addition, irradiation may be a solution for mycotoxin management. Non-ionizing (UV, microwave, solar) and ionizing (gamma) radiation may eliminate fungi and thus reduce the production of additional mycotoxin in food material. However, changes in the molecular structure of food constituents are observed for some irradiation sources (Karlovsky et al., 2016).

Mycotoxin binders are commonly used as a physical decontamination strategy. These materials act by adsorbing mycotoxins, thereby reducing their bioavailability. For example, activated charcoal has been successfully used to remove patulin from naturally contaminated apple cider, while bentonite has been shown to reduce AFM1 levels in naturally contaminated milk (Karlovsky et al., 2016).

Brewing significantly influences the fate of mycotoxins through steeping, germination, kilning, mashing and fermentation. Because steeping exposes barley to large volumes of water, some water-soluble mycotoxins such as DON and FUMs can be partly leached from the grain. During germination, part of DON may also be converted to its glycosylated form (DON-3-G) (Pascari et al., 2018). However, fungal biomass can increase during this step, and early kilning may still allow some toxin biosynthesis before heat inactivates the fungi (Pascari et al., 2018). A strong redistribution of mycotoxins occurs between wort, spent grains and yeast. Polar toxins tend to pass almost completely into the beer, whereas less-polar compounds are partly retained in spent grains (Inoue et al., 2013; Pascari et al., 2018). Fermentation can also play an important detoxifying role. Mycotoxins such as OTA, ZEN and patulin can undergo enzymatic degradation or be adsorbed to yeast cells, leading to substantial reductions (Inoue et al., 2013; Pascari et al., 2018). Ochratoxin A often drops below ~60% of its initial concentration due mainly to adsorption (Inoue et al., 2013). Adsorption to fermentation residues and spent grains has also been reported for aflatoxins and ZEN (Inoue et al., 2013; Pascari et al., 2018). In contrast, trichothecenes such as DON and NIV are poorly adsorbed and show only limited reduction, meaning they can persist into the finished product and therefore require control at the raw-material stage (Inoue et al., 2013). Overall, the brewing process naturally reduces several mycotoxins through leaching, adsorption to spent grains and yeast, and enzymatic conversion to less-toxic derivatives (Inoue et al., 2013; Karlovsky et al., 2016; Pascari et al., 2018; Pernica et al., 2025).

Concerning chemical treatment, historically, ammonia containing compounds were used for OTA decontamination in various grains such as maize or wheat. However, such processes lead to a decrease in food quality and to the alteration of taste, despite their effectiveness (Ben Miri et al., 2024). Another chemical treatment used was ozonation. With its oxidizing properties, it eradicates fungi and bacteria and thereby helps extend food preservation. However, physicochemical changes in the treated food were also observed (Ben Miri et al., 2024). Currently, chemical treatments of material for food purposes are banned in Europe due to consumer health concerns (European Commission, 2023c).

Biological food treatment may also reduce mycotoxin content. Certain microorganisms are able to transform mycotoxin into less or non-toxic derivatives by changing their chemical and physical properties (Peng et al., 2018). One example is the reductive de-epoxidation of trichothecenes by microorganisms in the hindgut of pigs and poultry. During this process, the epoxide group, responsible for much of the toxicity of trichothecenes, is chemically transformed, resulting in

metabolites with markedly reduced toxicity (Peng et al., 2018). However, in some cases, the degradation products can be equally or even more harmful than the parent toxin. Despite this risk, only a limited number of studies have evaluated the safety of these transformation products, largely because many derivatives are unknown and not detectable with routine analytical techniques (Liu et al., 2022). To ensure safety, both the structure and biological activity of all metabolites must be characterized. This requires a combination of approaches: advanced analytical chemistry (HRMS and non-targeted metabolomics), together with toxicological testing such as *in vitro* cytotoxicity assays, receptor-binding studies, *in vivo* models, and simulated digestion to evaluate bioavailability (Liu et al., 2022).

Food raw materials treatment efficiency is very variable and will depend on the processing type, the matrix and the mycotoxin present, and often results in an incomplete mycotoxin elimination (Ekwomadu et al., 2021; Ben Miri et al., 2024). In addition, the cost of the required specialized equipment can be a limiting factor, especially for smaller producers (Ben Miri et al., 2024). Finally, laboratory experiments do not always reflect the reality of an industrial scale (Schaarschmidt and Fauhl-Hassek, 2018). In addition, in case of large food batches (cereals for example) the heterogeneous distribution of mycotoxins within batches makes it difficult to monitor treatment processes, as contamination may be localized in specific pockets while other areas remain unaffected (Schaarschmidt and Fauhl-Hassek, 2018).

4.3. Mycotoxins sorting

As mentioned above, it has been shown that mycotoxin contamination in batches is not always homogeneous (Nelson, 2016; Schaarschmidt and Fauhl-Hassek, 2018) and, only a few grains with high mycotoxin concentrations may contaminate an entire batch (Ekwomadu et al., 2021; Iqbal, 2021). Hence, sorting procedures have been studied as a tool to reduce the mycotoxin concentration of food materials. Proper cleaning at the early stage of food processing allows the reduction of mycotoxins in raw materials before their introduction into final food products (Schaarschmidt and Fauhl-Hassek, 2018).

Traditionally, hand sorting was often used as a sorting method (Peng et al., 2018). Sorting efficiency depended on the worker's experience but can sometimes reach up to 90% of mycotoxin removal (Peng et al., 2018). However, this method was only feasible in small-scale productions and cannot be transferred to larger-scale due to high costs and low sorting flow (Peng et al., 2018). In large-scale processing, shape, size, density and air resistance of the grains can be used as sorting parameters

for specific devices (Cheli et al., 2013; Schaarschmidt and Fauhl-Hassek, 2018). Multiple cleaning operations can be performed, with scouring, aspiration, sieving, and gravity separation being the most prevalent methods (Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018).

Scouring consists in the removal of the material adhering to the grain surface (Schaarschmidt and Fauhl-Hassek, 2018). In addition, dehulling techniques can be used to remove the outer layers of grains prior to grinding. Mycotoxin concentration reduction is observed when the fungal colonization is not too extensive and thus when mycotoxins are concentrated in surface layers (Karlovsky et al., 2016).

Damaged, broken and smaller kernels often contain a large portion of the mycotoxin content (Karlovsky et al., 2016). Therefore, removing these kernels, and the fine materials and debris that have been collected during the harvest process may lead to a decrease in mycotoxin contamination (Karlovsky et al., 2016).

Fungus-infected kernels have different physical properties than the uninfected kernels. Indeed, grains tend to shrivel and their density decreases when infected with *Fusarium* (Cheli et al., 2013; Karlovsky et al., 2016; Peng et al., 2018). Therefore, grain separation based on their densities can be performed by gravity separators such as gravity tables or by using wet sorting (Cheli et al., 2013; Peng et al., 2018).

Optical sorting was later developed and allows the separation of kernels based on their characteristics such as size, shape, colour and defects. Grains that are infected with *Fusarium* in the field often show a discolouration that can be more specific to *Fusarium* infestation than the density or size of the kernels (Schaarschmidt and Fauhl-Hassek, 2018). Spectral data, from visible and non-visible range, can also be used to detect highly *Fusarium* damaged kernels and therefore be correlated with high mycotoxin levels (Schaarschmidt and Fauhl-Hassek, 2018). However, optical and infrared sorting require more expensive equipment, that cannot always be purchased by the producers or grain storers (Schaarschmidt and Fauhl-Hassek, 2018).

Cleaning and sorting efficiencies are highly variable, depending on the batch quality and the contamination distribution between the kernels, which are influenced by agricultural practices, weather and varieties (Cheli et al., 2013; Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018). The type of sorting procedures and the extent of the contamination will also influence sorting efficiency (Cheli et al., 2013). However, cleaning and sorting procedures may not be sufficient to

obtain mycotoxin levels below the regulatory level if the initial concentrations of mycotoxins are too high (Schaarschmidt and Fauhl-Hassek, 2018). In addition, these sorting methods are not based on mycotoxins themselves but on fungal infection symptoms. Therefore, in the case of asymptomatic fungal contamination, these sorting processes are ineffective (Karlovsky et al., 2016).

Finally, for manufacturers, it is also important to consider both the cost of the devices and their operation, as well as the material loss during cleaning and sorting steps, in order to assess whether the process is worthwhile (Peng et al., 2018).

Objectives

The main goal of this thesis is to contribute to a better understanding and management of mycotoxin contamination in wheat by developing and applying analytical tools as well as practical mitigation strategies. To reach this, multiple complementary goals were highlighted.

Therefore, the first objective is the development and validation of a robust multi-mycotoxin analytical method able to simultaneously detect and quantify regulated and emerging mycotoxins in wheat. Considering the diversity and the co-occurrence of mycotoxins in agricultural products, such a comprehensive approach is essential for complete data collection. The method aims to ensure high sensitivity, specificity, and reproducibility, making it suitable for mycotoxin monitoring and research applications.

Secondly, the aim was to apply the implemented method to wheat samples from two different harvest years. By including samples from different locations in Belgium, the study will also evaluate the impact of specific weather events, pedoclimatic and agricultural conditions on mycotoxin occurrence. In addition, varietal contamination susceptibility will also be assessed by selecting samples from different wheat varieties, providing valuable information for breeding programs.

Thirdly, specific procedures for sorting contaminated batches will be investigated. Size, density, and single kernel infrared-sorting will be studied. The goal behind this approach is to reduce mycotoxin concentration in certain sub-lots, thereby enabling the recovery of at least a part of a batch in cases where overall contamination levels exceed regulatory levels. Developing such practical strategies is particularly important for stakeholders in the agri-food chain, as it may provide an economically viable way to minimize losses while ensuring consumer safety.

Finally, an additional objective is to expand the scope of the developed method to a different matrix and to broaden the panel of detectable mycotoxins. Therefore, the method will be applied to peanuts and peanut based products from Benin, a context where mycotoxin contamination is particularly severe. In addition, aflatoxins and fumonisins will be added to the analytical method. Given the high prevalence of contaminated samples and the often-elevated mycotoxin concentrations observed, peanuts provide a relevant case study to test the flexibility of the developed analytical method. In addition, the generated data will be used to perform a risk assessment related to peanut consumption in Benin. The evolution of mycotoxin concentration during food processing for *klui-klui* and roasted peanuts production will also be evaluated.

Experimental section

Experimental section

Study 1: Development and validation of a multi-mycotoxin analysis method on wheat by UPLC-MS/MS

Preamble

The first study of this work aimed to develop an analytical tool for the detection and the quantification of mycotoxins in wheat. Developing and validating an analytical method is an essential step to ensure the acquisition of reliable data from real samples.

The method included regulated mycotoxins in wheat, namely DON, ZEN, T2, HT2, OTA and OTB. In addition, 3Ac-DON and 15Ac-DON were included as acetylated derivatives of DON, as well as DON-3G, the glycosylated form of DON. Although FUMs are not regulated in wheat but well in maize, they were also incorporated in the method.

Furthermore, a selection of non-regulated mycotoxins was included. Specifically, TeA, AOH and AME, mycotoxins produced by *Alternaria* spp., were added as they are reported in wheat. Diacetoxyscirpenol**,** produced by *Fusarium* spp. and commonly found in cereals, was also included. Citrinin and STE have been previously reported in wheat and were therefore added as well. Finally, due to the increasing interest in ENNs in Europe, these compounds were also incorporated to the method.

After selecting the target mycotoxins and developing the analytical procedure, the method was validated according to European guidelines to ensure the accuracy and reliability of the results.

Abstract

Mycotoxins are secondary metabolites produced by fungal species such as *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. and *Penicillium* spp., that can be found in several foodstuffs such as maize, coffee, spices, wheat. Mycotoxin production can occur during pre-harvest, harvest, drying and storage stages. Wheat is one of the most widely cultivated crops in the world and the primary source of carbohydrates for human consumption. However, wheat is subjected to fungal contamination, and thus to mycotoxin exposure of consumers. Mycotoxins in food and feed are regulated by the Commission Regulation EU/2023/915 and 2002/32/EC and more recent recommended values are available for feed in Recommendation 2006/576/EC, as they can cause adverse effects on health. To prevent this risk, the development of an analytical method to monitor mycotoxin levels in food is essential.

In this work, a multi-mycotoxin analytical method by UPLC-MS/MS was established and validated according to European standards over three days with samples spiked at three concentration levels. The developed and validated method allows the detection and quantification of 16 mycotoxins: deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxinivalenol, zearalenone, T2 and HT2 toxins, ochratoxins A and B, fumonisin B1, alternariol-monomethyl-ether, alternariol, enniatins A, A1, B and B1, sterigmatocystin. The recoveries of the different mycotoxins were ranging between 50.9 to 103.6%. For all mycotoxins, the limits of detection were largely below the regulatory thresholds. The intra-day repeatability variation and the inter-day reproducibility were ranging between 1 and 15 %, and between 8 and 22% respectively. The method validation confirms its accuracy, reproducibility and steadiness.

Key words: Mycotoxins, wheat, UPLC-MS/MS, validation

Introduction

Mycotoxins are toxic secondary metabolites produced by fungi belonging mainly to the genera *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* (Tola and Kebede, 2016; WHO (World Health Organization), 2023). More than 400 different mycotoxins have been identified and described in the literature to date (Pereira et al., 2014; Smith et al., 2016; Khan et al., 2024). Some mycotoxins are regulated in food and feed in Europe due to their impact on human and animal health (European Commission, 2002, 2006a, 2023c). In addition, so called emerging mycotoxins has been increasingly reported. Although no universally accepted definition exists, these compounds are generally described as mycotoxins that are neither routinely monitored nor currently regulated (Kolawole et al., 2024). Regardless of the terminology, the growing recognition of these compounds highlights the need for more comprehensive analytical tools capable of detecting a wider range of contaminants.

Contamination can occur at any stage of the agricultural chain. During pre-harvest, fungal infection of crops is primarily driven by environmental conditions such as temperature, humidity, and rainfall (Marin et al., 2013; Mahato et al., 2021). After harvest, improper drying or poor storage facilities can greatly increase fungal proliferation and toxin production (Mahato et al., 2021).

Despite the implementation of pre- and post-harvest control strategies, mycotoxin contamination of food products is nearly unavoidable (Ekwomadu et al., 2021). Therefore, analytical monitoring is required to ensure food safety. Reliable identification and quantification of mycotoxins in food and feed matrices are essential for regulatory compliance, risk assessment, and consumer protection.

However, multi-mycotoxins analysis method development could be challenging as mycotoxins present diverse chemical structures and physicochemical properties (Alshannaq and Yu, 2017; Mousavi Khaneghah et al., 2019). Moreover, they occur in complex food matrices, often at trace levels close to or below the regulatory thresholds (Alshannaq and Yu, 2017). These challenges necessitate analytical methods with high sensitivity, selectivity, and robustness. A variety of methods have been developed, ranging from immunoassays such as enzyme-linked immunosorbent assay (ELISA) and lateral flow devices, which are useful for rapid screening, to more sophisticated chromatographic methods suitable for confirmation and quantification (Janik et al., 2021).

Liquid chromatography (LC), particularly when coupled with tandem mass spectrometry (LC-MS/MS), is currently one of the most powerful techniques for multi-mycotoxin analysis.

LC-MS/MS combines high sensitivity and selectivity with the ability to detect a broad spectrum of toxins in a single run, without the need for derivatization steps (Pereira et al., 2014; Turner et al., 2015; Janik et al., 2021). Although equipment costs were once prohibitive, technological advances and reduced costs have made LC–MS/MS increasingly accessible to routine laboratories (Turner et al., 2015; Janik et al., 2021). Nonetheless, sample preparation remains a critical step to mitigate matrix effects and ensure accurate quantification. Methods such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), originally developed for pesticide analysis, have been adapted for mycotoxin extraction from solid matrices (Alshannaq and Yu, 2017; Iqbal, 2021; Janik et al., 2021). This approach allows efficient extraction and cleanup, improving recovery and reliability of results.

The present study aims to develop and validate a multi-mycotoxin analytical method in wheat, based on a simplified QuEChERS extraction, without the dispersion step, followed by LC–MS/MS analysis. By covering both regulated and emerging mycotoxins, this method aims to provide a full over-view of mycotoxin contamination. Ultimately, such validated methods contribute to mycotoxin monitoring to limit the inclusion of contaminated raw materials in the food chain, and to inform the sector about the risks.

Material and methods

1. *Chemicals and reagents*

All chemicals and reagents used in this study were of analytical grades: Milli-Q water (Milli-Q® IQ 7010 purification system), acetonitrile AR (Biosolve, France), acetonitrile MS/MS grade (Biosolve, France) acetic acid AR (Fisher Chemical, Belgium), methanol MS/MS grade (Biosolve, France), formic acid (Avantor, VWR, Belgium), ammonium acetate (Fluka, US), anhydrous $MgSO_4$ (Roth, France) and anhydrous NaCl (Roth, France).

A multi-mycotoxin mix solution (named “first standard mix”, including among others the regulated mycotoxins in cereals in the European legislation) was prepared from mycotoxin standard solutions in acetonitrile, which were purchased from Romer-Labs (Romer-Labs, Austria): 15-acetyl-deoxynivalenol (15Ac-DON), 3-acetyl-deoxynivalenol (3Ac-DON), diacetoxyscirpenol (DAS), deoxynivalenol (DON), HT2 and T2 toxins (HT2, T2), tenuazonic acid (TeA), alternariol (AOH), alternariol-monomethyl-ether (AME), zearalenone (ZEN), ochratoxin A (OTA), ochratoxin B (OTB), deoxynivalenol-3-glycoside (DON 3G). The pool was constituted by diluting the individual standard solutions in acetonitrile. Table 1 shows the mycotoxin concentrations in the initial individual standard solution as well as in the pool.

Table 1: Mycotoxin concentrations in the initial individual standard solutions as well as in the mycotoxins first standard mix.

Mycotoxin	Abbreviation	CAS number	Purity	Initial standard concentration (µg/mL)	Volume (µL)	Standard mix concentration (ng/mL)
15-acetyl-deoxynivalenol	15Ac-DON	88337-96-6	99.8 ± 1.0%	100.2	99.8	500
3-acetyl-deoxynivalenol	3Ac-DON	50722-38-8	99.0 ± 1.0%	100.0	100.0	500
Alternariol-monomethyl-ether	AME	26894-49-5	99.2 ± 1.0%	101.4	98.6	500
Alternariol	AOH	641-38-3	99.0 ± 1.0%	100.1	99.9	500
Diacetoxyscirpenol	DAS	2270-40-8	99.2 ± 1.0%	101.1	99.9	500
Deoxynivalenol	DON	51481-10-8	99.38 ± 1.0%	100.0	1250.0	6250
Deoxynivalenol-3-glucoside	DON-3-G	131180-21-7	96.0 ± 1.0%	50.1	199.6	500
HT2 toxin	HT2	26934-87-2	97.3 ± 1.0%	100.0	100.0	500
Ochratoxin A	OTA	303-47-9	99.0 ± 1.0%	10.1	50.0	25
Ochratoxin B	OTB	4825-86-9	97.1 ± 1.0%	10.0	50.0	25
T2 toxin	T2	21259-20-1	99.0 ± 1.0%	100.0	100.0	500
Tenuazonic acid	TeA	610-88-8	98.9 ± 1.0%	100.2	99.8	500
Zearalenone	ZEN	17924-92-4	99.7 ± 1.0%	100.0	100.0	500
Total MYC volume (µL)					2447.6	
ACN volume (µL)					17552.4	
Total volume (µL)					20000.0	

Total MYC volume corresponds to the total volume of mycotoxins standard solutions included into the standard mix. ACN is added to reach a standard mix solution total volume of 20 mL.

In addition, a second mycotoxin standard mix was prepared including the following emerging mycotoxins, enniatins (ENNs) (A, A1, B, B1), moniliformin, citrinin (CIT) and sterigmatocystin (STE), which were purchased from Romer-Labs (Romer-Labs, Austria) except for the ENNs that were purchased from Merck (Merck KGaA, Germany). Fumonisins (FUMs) B1 and B2 were also added to the second standard mix even if they are regulated in food, as they are less common in wheat in Belgium. The second standard mix was prepared using these individual standard solutions in acetonitrile. The volumes of standard solutions are presented in Table 2, as well as the final concentrations of the mycotoxins in the standard mix.

Table 2: Mycotoxin concentrations in the initial individual standard solutions as well as in the second mycotoxin standard mix.

Mycotoxin	Abbreviation	CAS number	Purity	Initial standard concentration (µg/mL)	Volume (µL)	Standard mix concentration (ng/mL)
Citrinin	CIT	518-75-2	99.6 ± 1.0%	100.1	50.0	500
Enniatin A	ENN A	2503-13-1	≥ 95%	100.1	50.0	500
Enniatin A1	ENN A1	4530-21-6	≥ 95%	100.1	50.0	500
Enniatin B	ENN B	917-13-5	≥ 95%	100.1	50.0	500
Enniatin B1	ENN B1	19914-20-6	≥ 95%	100.1	50.0	500
Fumonisin B1	FUM B1	116355-83-0	≥ 99%	50.5	99.0	500
Fumonisin B2	FUM B2	116355-84-1	≥ 99%	50.8	98.4	500
Moniliformin	Moniliformin	31876-38-7	97.0 ± 1.0%	100.2	49.9	500
Sterigmatocystin	STE	10048-13-2	99.0 ± 1.0%	50.1	100.0	500
Total MYC volume					597.3	
ACN volume					9402.7	
Total volume					10000.0	

Total MYC volume corresponds to the total volume of mycotoxins standard solutions included into the standard mix. ACN is added to reach a standard mix solution total volume of 10 mL.

2. Sample preparation and extraction

The extraction procedure described here below was adapted from the QuEChERS method (Zhang et al., 2013; Sun et al., 2016; Annunziata et al., 2017; Solfrizzo et al., 2018; González-Jartín et al., 2021). However, Primary Secondary Amines (PSA) were not used as they reduce the extraction of OTA (Zhang et al., 2013). In addition, no dispersive solid phase extraction (SPE) was used to keep the extraction method less specific, allowing for the recovery of a broader range of different mycotoxins with good efficiency.

The matrix used for the validation of the analytical method was whole wheat flour. The flour was obtained after milling approximatively 1 kg of wheat grains using laboratory mill 3100 (Perten, PerkinElmer Compagny, US). The resulting flour had a particle size below 1 mm. The flour was analysed with the present method before its use for the validation, to ensure that no mycotoxin was detectable in the sample.

Five grams of whole wheat flour were weighted in Falcon tubes (50 mL) and 10 mL of water were added. After one-hour rest, 10 mL of an acetonitrile solution containing 1% of acetic acid were introduced in each tube. The samples were then agitated with a Mixer Mill MM400 (Retsch GmbH) for 10 minutes at 10 Hz. Thereafter, 4 g of anhydrous $MgSO_4$ and 1g of anhydrous NaCl were added to the tubes. The tubes were hand-shaken and then agitated using a Mixer Mill MM400 for 2 minutes at 10 Hz. After stirring, the samples were centrifuged for 5 minutes at 3000 g (Centrifuge 5810 R, Eppendorf).

A volume of 100 µL of the supernatants was collected and diluted with 900 µL of a 50:50 (v/v) solution of water and methanol into injection vials.

3. Calibration curves

The matrix effect was assessed by comparing a seven-points calibration curve prepared in acetonitrile with another one performed by adding mycotoxins in the matrix extract.

Two different calibration curves were prepared, one with the first mycotoxin standard mix and another with the second mycotoxin standard mix. The whole wheat flour (used as a "blank" matrix) was extracted as described above. Specified volumes of blank matrix extract and mycotoxins standard mixes (first and second) were introduced in 1.5 mL Eppendorf tubes to have a total volume of 1 mL. A volume of 100 µL of the obtained solution was diluted with 900 µL of a 50:50 (v/v) solution of water and methanol. Then, the solutions were transferred into injection vials. The obtained mycotoxin concentrations used for the calibration curves are shown in Table 3.

Table 3: Mycotoxins concentrations in µg/kg in the calibration curve.

	1 [µg/kg]	**2 [µg/kg]**	**3 [µg/kg]**
STD01	0.5	10	125
STD02	1.0	20	250
STD03	2.5	50	625
STD04	5.0	100	1250
STD05	7.5	150	1875
STD06	10	200	2500
STD07	15	300	3750

1 = ochratoxins (A and B); 2 = AME, AOH, 3Ac-DON, 15Ac-DON, T2, HT2, ZEN, Enniatins (A, A1, B, B1), FUM B1 and B2, citrinin, moniliformin, sterigmatocystin; 3 = DON.

4. UPLC-MS/MS conditions

The UPLC-MS/MS conditions were based on different papers such as Uhlig and Ivanova, 2004; Jestoi et al., 2009; Soleimany et al., 2012; Stead et al., 2014. However, in these papers, all the selected mycotoxins were not studied, therefore, some modifications were made. Different elution gradients and mobile phase compositions have been tested to optimise the compounds separation in a short time. Finally, the mass spectrometer parameters were adjusted to achieve the highest signal for each mycotoxin.

The samples were injected into a Waters Acquity I-Class (Waters, USA) UPLC system equipped with a BEH C18 column (2.1 × 50 mm, 1.7 µm), coupled to a Xevo TQ-S mass spectrometer (Waters, Milford,

USA). The mobile phase A was a 10 mM ammonium acetate aqueous solution containing 0.1% of formic acid and the mobile phase B was methanol containing 0.1% of formic acid. The elution gradient started with 10% B for 3 min then B increased linearly to 70% in 7 min. The gradient increased again to 90% B in 10 s and kept constant for 2 min. Finally, gradient get back to its original conditions in 10 s and a 3 min equilibration period applied. The column temperature was set at 50°C, the flow rate was 0.3 mL/min, and the injection volume was 2 µL. The samples were kept at 10°C before injection.

The mass spectrometer was used in Multiple Reaction Monitoring (MRM) mode, under positive and negative ionization modes. At least two transitions were selected for each mycotoxin, one for quantification and another for qualification, except for moniliformin which only has one transition as it is a small molecule. Regarding the MS parameters, the capillary voltage was 3.4 kV, the dessolvation temperature was 400°C, the dessolvation gas flow was 800 L/H, the cone gas flow was 150 L/H and the nebuliser pressure was 7 bar. The cone voltage and the collision energy were optimised for each transition.

5. Data processing

For each sample, peaks from the obtained chromatograms were integrated with TargetLynx V4.2 (Waters, Milford, USA).

The matrix-matched calibration curves were determined by plotting the peak area of the quantification transition versus the mycotoxin concentration, and using a linear regression equation $y = ax + b$. Each calibration point was recalculated according to the computed calibration. Then, the percentage of deviation between the back-calculated concentration of calibration solutions and theoretical concentration was calculated for each curve. These percentages of variation were used as validation criteria for the calibration curves. The deviation should not exceed ± 10 % except for the first calibration point, for which the tolerance is ± 20 % (CEN, Comité Européen de Normalisation, 2010). When a calibration point showed a deviation exceeding the acceptance criteria, it was excluded from the calibration curve. Such deviations generally occurred when the volume of standard solution in the vial was insufficient. To have a valid calibration curve, at least five calibration points should be kept (European Commission, 2021).

In each series of analysis, calibration curves were injected twice, once before the samples and once after the samples (bracketed calibration), to consider the potential change in the instrument's

response during the analysis sequence. The average calibration curve was calculated by using the data from the two calibration curves while determining the linear regression. The goodness-of-fit of the data (coefficient of determination R^2) should be reported (European Commission, 2021). For the average calibration curve, the difference should not exceed ± 20 %. Again, to meet these criteria, outliers were removed according to the same principles applied to the individual calibration curves.

The sample concentrations were calculated based on the average calibration curve for each mycotoxin.

6. Identification criteria

Some criteria were used to validate the correct identification of the compound: the transition ratio and the retention time (RT). Also, the signal to noise ratio should be higher than three for identification and higher than 10 for quantification (CEN, Comité Européen de Normalisation, 2022). The transition ratio was calculated as the ratio between the quantification and the qualification transitions and should be within ± 30% of the average transition ratio observed in the calibration solutions (CEN, Comité Européen de Normalisation, 2022). The difference between the RT of the detected compound in the calibration curve and in the sample should not exceed ± 0.05 % of the average RT (CEN, Comité Européen de Normalisation, 2022).

With each series of samples, a control standard solution containing ZEN and DON in acetonitrile at concentrations of 500 µg/L and 6250 µg/L, respectively, was injected. These concentrations correspond to the central point of the calibration curve. The measured response was plotted on a control chart to check the response stability of the UPLC-MS/MS system over time. If the response does not fall within the range of the historical average ± two standard deviations, the issue should be solved before injecting the rest of the samples.

7. Method validation

The validation of the multi-mycotoxin analysis method was performed according to several guidelines including the Commission Decision (UE) 2021/808 (European Commission, 2021), the European analytical norm NBN EN 17641:2022 (CEN, Comité Européen de Normalisation, 2022) and the European technical report CEN/TR 16059:2010 (CEN, Comité Européen de Normalisation, 2010).

The method validation was performed on three non-consecutive days. For the first mycotoxins standard mix containing 15Ac-DON, 3Ac-DON, DAS, DON, HT2, TeA, AOH, AME, T2, ZEN, OTA, OTB,

DON-3G, for each day, the whole wheat flour "blank" matrix was spiked at three different levels of concentration. These concentrations were defined according to the respective maximal levels (ML) of these mycotoxins defined for wheat in the European legislation (European Commission, 2023c). The three levels of concentrations were half of the maximal level (ML/2), ML, and 1.5 times the maximal level (1.5 x ML) (Table 4). The samples at ML were also spiked with the second mycotoxins standard mix at 100 µg/kg including ENNs A, A1, B and B1, FUMs B1 and B2, moniliformin, CIT and STE. The sample preparation and the extraction were performed 6 times per day during the validation.

Table 4: Mycotoxins included into the first and the second mycotoxin standard mix and their associated maximal level (ML) (legal or arbitrary fixed).

Mycotoxins included in the first standard mix	
Abbreviation	**ML (µg/kg)**
15Ac-DON	100 [2]
3Ac-DON	100 [2]
AME	100 [2]
AOH	100 [2]
DAS	100 [2]
DON	1250 [1]
DON-3G	100 [2]
HT2	100 [1]
OTA	5 [1]
OTB	5 [1]
T2	100 [1]
TeA	100 [2]
ZEN	100 [1]
Mycotoxins included in the second standard mix	
Abbreviation	**ML (µg/kg)**
Moniliformin	100 [2]
CIT	100 [2]
ENN A	100 [2]
ENN A1	100 [2]
ENN B	100 [2]
ENN B1	100 [2]
FUM B1	100 [2]
FUM B2	100 [2]
STE	100 [2]

[1] ML defined by the legislation (European Commission, 2023c; Belgian Federal Agency for the Safety of the Food Chain (FASFC), 2025); [2] ML arbitrary fixed for the validation.

For each validation day, 24 samples of 5 g of whole wheat flour were prepared in Falcon tubes (50 mL). From these 24 samples, 6 samples remained unspiked ("blank"), and 6 samples were spiked for each targeted concentration (see above). Hence, respectively 0.5 mL, 1 mL and 1.5 mL of the first mycotoxin standard mix were added to the flour to reach concentrations corresponding to ML/2, ML and 1.5 x ML. One mL of the second mycotoxin standard mix was added to the samples already spiked at ML for the other mycotoxins. The samples were agitated, then left under the fume hood

overnight to allow the acetonitrile to evaporate and were then subjected to the extraction and UPLC-MS/MS analysis as described above.

7.1. Repeatability and reproducibility

For each day, the intra-day reproducibility (also called repeatability) was determined as well as the recovery of measured concentrations. The inter-day deviation (also called reproducibility) was computed using data from the 3 days of validation.

For the repeatability, for each level of concentration, the 6 obtained concentrations were compiled in an Excel file and the coefficient of variation (CV, expressed as percentage) was calculated, along with the relative standard deviation.

If the CV met the criteria of the Horwitz equation (Equation 1), no modifications were made. The intra-day deviation should be lower than 2/3 $CV_{Horwitz}$ and the inter-day deviation should be lower than $CV_{Horwitz}$. The acceptable CVs according to the European guidelines (European Commission, 2021) are presented in Table 5.

$$CV_{Horwitz} = 2^{(1-0.5\log C)}$$

Equation 1
with C: fraction of mass expressed as a power of 10 (for example, 1 mg/g = 10^{-3})

If the variation between samples was too high, the outliers were removed to comply to the CV criteria. These excluded samples were not considered for calculating the average recovery when taking into account the three days of validation.

The reproducibility was calculated in the same way but using the data of the 3 days of validation.

Table 5: Summary of the acceptable CVs (based on Horwitz equation for concentration above 120µg/kg) (adapted from (European Commission, 2021).

Concentration (µg/kg)	Inter-day - Reproductibility $CV_{Horwitz}$	Repeatability 2/3 $CV_{Horwitz}$
2.5	30.00*	20.00*
5	30.00*	20.00*
7.5	30.00*	20.00*
50	25.00*	16.67*
100	22.63*	15.08*
150	21.29	14.19
625	17.17	11.45
1250	15.47	10.31
1875	14.56	9.70

**) For concentration under 120 µg/kg, the CV should be as low as possible.*

7.2. Recovery

For each level of concentration and for each individual sample, the recovery, expressed as a percentage, was calculated as the ratio between the calculated concentration and the theoretical concentration. The recovery should be between 80% and 120% (European Commission, 2021). If the recovery stands between 50% and 80%, the stability of the analytical method needs to be proven by assessing the effect of small modifications in the experimental conditions on the obtained results (CEN, Comité Européen de Normalisation, 2010).

Eight mycotoxins were fully validated, meaning that the repeatability and the reproducibility were determined as well as the transition ratio and the specific RT.

For the other mycotoxins (AME, AOH and mycotoxins from the second standard mix), the recovery was also calculated as well as the average RT and the transition ratio but only for the concentration corresponding to the ML.

All determined concentrations were then corrected with the average recovery.

7.3. Limit of quantification and limit of detection

The limit of quantification (LOQ) was fixed as the lowest calibration point after the zero, after checking that the signal to noise ratio (S/N) calculated in the software Masslynx was higher than 10. The limit of detection (LOD) was also determined by using S/N for the lowest calibration point and the LOD was extrapolated to achieve a S/N of three. Then, a mixture of mycotoxins was prepared with the estimated LOD of each mycotoxin, and 6 “blank” matrices were spiked with this mixture. After extraction and injection, the S/N was calculated again to be sure that this ratio was approximatively three.

7.4. Carry over evaluation

After each calibration curve, a solvent mix containing water and methanol (50:50) was injected to ensure that no remaining mycotoxin signal is observable. In addition, to reduce the risk of carryover contamination during the analysis of unknown samples, different injection sequences were applied for the first and second repetitions, and a solvent mixture was injected after highly contaminated samples.

7.5. Measurement uncertainty

Measurement uncertainty was calculated using two main components, the uncertainty associated with method precision and the uncertainty associated with method trueness, evaluated using an interlaboratory assay sample. The trueness of the method could only be assessed with this sample for 3Ac-DON, 15Ac-DON, DON, T2 + HT2, OTA and ZEA. For the other mycotoxins, measurement uncertainty was only associated with method precision.

The precision component was assessed using an ANOVA with day as a random effect. The repeatability standard deviation ($s_r = \sqrt{\sigma_r^2}$) and intermediate precision standard deviation ($s_{IP} = \sqrt{\sigma_r^2 + \sigma_{day}^2}$) were calculated from the within- and between-day mean squares.

The standard uncertainty of the mean was calculated as: $u_{precision} = \sqrt{\frac{\sigma_r^2}{n \cdot a} + \frac{\sigma_{day}^2}{a}}$

Equation 2

Where: $\sigma_r^2 = variance\ within\ a\ day;$
$\sigma_{day}^2 = variance\ between\ the\ days;$
$n = number\ of\ replicate\ measurments\ per\ day;$
$a = number\ of\ days;$

Method trueness was evaluated through the analysis of a CRM containing the target mycotoxins. The mean measured concentration was compared with the certified value, and the bias was calculated as the difference between the two. The uncertainty associated with the bias, u_{bias}, was obtained by combining the standard uncertainty of the CRM and the uncertainty of the mean CRM measurement:

$$u_{bias} = \sqrt{u_{CRM}^2 + u_{\bar{x}}^2}$$

The combined standard uncertainty of measurement was then obtained by combining the precision and bias components:

$$u_{combined} = \sqrt{u_{precision}^2 + u_{biais}^2}$$

Finally, the expanded uncertainty (U) at 95% confidence was obtained using the Student t-factor corresponding to the effective degrees of freedom. All standard deviations and uncertainties are reported as percent relative standard deviation (%RSD) of the two measurements.

All calculations were conducted using RStudio, version 2025.05.1 (Posit software PBC, USA).

7.6. Stability assessment

After four months of storage at -20°C in the dark in amber vials, the stability of mycotoxins in calibration curves and in sample extracts was assessed by injecting a freshly prepared calibration curve, as well as a calibration curve and spiked sample extracts stored for four months. The new calibration curve was used to back-calculate the concentration of the calibration points of the old calibration curve. Then, the deviation of the determined concentration from the theoretical concentration of the standards was calculated to estimate the stability of the samples. In addition, the concentrations of the old samples were calculated with the new calibration curve and compared to those obtain during the validation.

8. Test of T2 conversion in HT2 during extraction

To test the hypothetical conversion of T2 into HT2 during the extraction procedure, 6 Falcon tubes were filled with 5 g of a "blank" flour. Two samples were spiked with T2, two with HT2 to achieve a concentration of 100 µg/kg and two tubes remained blank. The samples were extracted as described above and injected into the UPLC-MS/MS system together with one T2 calibration curve and one HT2 calibration curve. This experiment was performed on three non-consecutive days and T2 and HT2 were quantified in all the samples.

The aim was to determine if there was formation of HT2 into the samples that were spiked with only T2. To confirm the results, the same experiment was conducted on three non-consecutive days.

9. Application of the method to a certified reference material

Another way to validate the analytical method is to use a certified reference material (CRM). Therefore, one CRM of wheat containing DON (1000 µg/kg), T2 (35.2 µg/kg), HT2 (38 µg/kg), and OTA (6 µg/kg) (Romer-Labs, Austria) was extracted following the previously developed extraction procedure. The sample was extracted twice on two different days.

In addition, a sample from an inter-laboratory assay was extracted and the determined concentrations were compared to the certified one given by the organizing organism, BIPEA (i.e. the average of all the collected data from the different participating laboratories.).

Results

1. *LC-MS/MS parameters for mycotoxins detection and quantification*

The extraction procedure was established based on the literature. In addition, some changes were made during the development of the method to improve the mycotoxins extraction. The UPLC-MS/MS parameters were also tested and optimised to maximize the detection and quantification of as many mycotoxins as possible.

The precursor – product transitions for each mycotoxin were optimised. To do so, standard solutions (100 ng/mL) were injected into the mass spectrometer in infusion mode, meaning that the solutions bypassed the liquid chromatography system and the signal could be observed in real-time. First, the precursor mass over charge ratio (m/z) was selected by looking at the literature. Then, different collision energies were applied in order to identify which one provided the higher response for each transition. Then, the MRM program was implemented. Finally, each mycotoxin RT in liquid chromatography was determined by scanning through the whole injection time and the obtained RT was also added to the acquisition program.

The MRM transitions used for the qualification and the quantification of the mycotoxins are presented in Table 6, as well as RT for each mycotoxin and other LC-MS/MS parameters.

To perform the validation with supplemented samples, a matrix “blank” was identified. Chromatograms of spiked and unspiked “blank” samples are shown in Figure 1.

Examples of chromatograms obtained for both qualification and quantification transitions are shown in Figure 2 for ZEN and DON.

Table 6: Mycotoxins in the validation standard mix with their molecular mass and the mass spectrometer detection parameters.

Mycotoxin	Molecular mass	MODE	M/Z	Cone (V)	Daughter1	Energy1 (eV)	Daughter2	Energy2 (eV)	RT (min)
Moniliformin	98.0	$[M-H]^-$	97.0	15	41.0	10			0.5
3Ac-DON	338.4	$[M+H]^+$	339.1	25	137.0	15	203.1	12	7.0
15Ac-DON	338.4	$[M+NH4]^+$	356.2	5	137.0	16	261.0	14	7.0
DON-3G	458.5	$[M+H]^+$	297.2	20	231.1	11	249.1	10	4.6
Fumonisin B1	721.8	$[M+H]^+$	722.3	15	334.3	35	352.3	35	10.0
AOH	258.2	$[M+H]^+$	259.0	10	185.0	28	213.0	28	9.2
DON	296.3	$[M+H]^+$	297.1	15	231.1	18	249.1	18	3.9
T2	466.5	$[M+NH4]^+$	484.2	7	215.0	18	305.1	12	10.4
OTA	403.8	$[M+H]^+$	404.1	25	239.1	25	357.9	15	10.5
ZEN	318.4	$[M+H]^+$	319.2	20	185.1	25	187.1	19	10.6
OTB	369.4	$[M+H]^+$	370.0	10	187.0	30	204.8	20	9.7
Enniatin B	639.8	$[M+H]^+$	640.7	15	85.9	40	196.0	20	11.5
Citrinine	250.3	$[M+H]^+$	251.1	25	191.0	27	205.1	26	8.8
HT2	424.5	$[M+NH4]^+$	442.2	5	215.0	12	263.1	10	9.8
Enniatin B1	653.8	$[M+H]^+$	655.2	20	85.9	40	196.1	25	11.6
Enniatin A1	667.9	$[M+H]^+$	669.1	30	99.6	40	210.0	25	11.7
Sterigmatocystin	324.28	$[M+H]^+$	325.0	15	253.1	38	281.1	35	10.8
AME	272.3	$[M+H]^+$	273.0	20	128.0	60	228.0	40	10.9
Enniatin A	681.9	$[M+H]^+$	682.6	30	99.6	40	209.8	25	11.8
DAS	366.41	$[M+NH4]^+$	384.2	9	247	12	307.1	10	8.75

In orange: fully validated mycotoxins; in blue: mycotoxins with secondary validation.

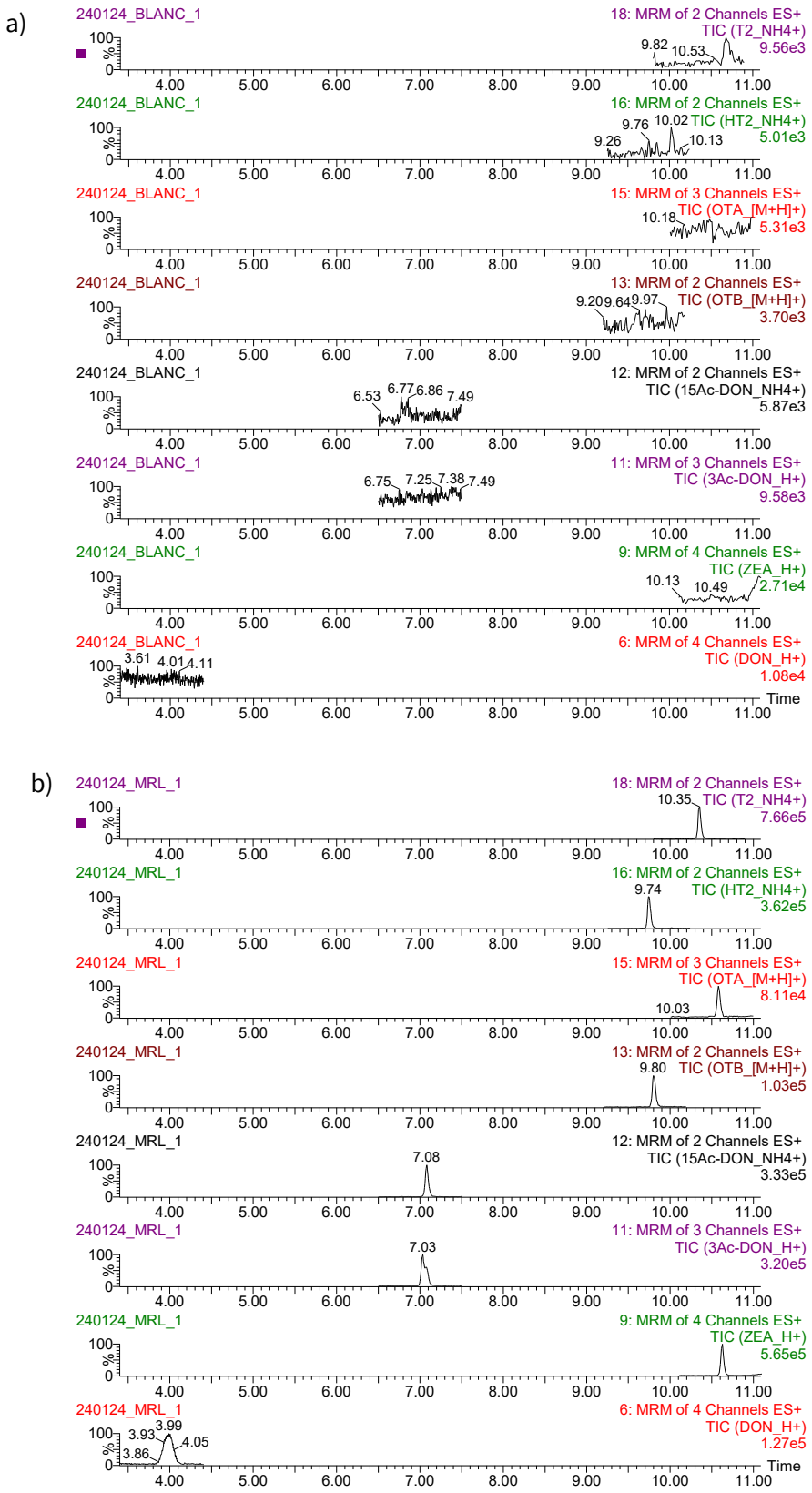


Figure 1: Chromatograms from an unspiked "blank" sample (a) and from a “blank” sample spiked at the ML (b).

Figure 2: Chromatograms for ZEN (left) and DON (right) from the LC-MS/MS analysis of a "blank" sample of wheat flour spiked at the ML of each mycotoxin (100 and 1250 µg/kg, respectively).

2. *Calibration curves*

Matrix matched calibration curves were compared to solvent calibration curves, as illustrated for the eight fully validated mycotoxins in supplementary materials (Figure S1). In some cases, there was almost no matrix effect (for DON for example), but for other mycotoxins, the slope of the matrix match calibration curve was lower than for the solvent calibration curve (for OTA for example). In order to increase the trueness of the quantification, it was decided to use matrix match calibration curves prepared as described in the Material and methods section.

The obtained calibration curves for all eight fully validated mycotoxins on the second day of validation are presented in supplementary material (Figure S2).

3. *Limit of detection and limit of quantification*

The LOD and LOQ for the different mycotoxins are presented in Table 7. The LOD was lower than the ML for all the mycotoxins.

In this study, all mycotoxins were successfully validated at 0.5 × ML, with the exception of 15Ac-DON and 3Ac-DON. Evaluation of the calibration curves showed only limited deviations, even at the lowest calibration level, demonstrating that the method remains stable and reliable at low concentrations. In this work, the LOQ was defined as the lowest non-zero calibration point, provided that the signal-to-noise ratio exceeded 10. However, according to current guidelines, the LOQ should correspond to the lowest analyte concentration that can be measured with acceptable accuracy and statistical certainty (Sloth et al., 2022), and therefore to the lowest successfully validated concentration. In the present work, this concentration was 0.5 × ML, although the analytical

performance observed at this level suggests that lower concentrations could likely be validated if experimentally assessed. Consequently, to fully comply with current guidelines and to achieve lower LOQs, future validation efforts should include concentrations below 0.5 × ML.

Table 7: LOD and LOQ for the different mycotoxins.

Mycotoxins	ML [µg/kg]	LOQ [µg/kg]	LOD [µg/kg]
15Ac-DON	100[2]	10	3.8
3Ac-DON	100[2]	10	3.3
AME	100[2]	10	8
AOH	100[2]	10	8
DON	1000[1]	100	60
ENN A	100[2]	10	8
ENN A1	100[2]	10	5
ENN B	100[2]	10	1
ENN B1	100[2]	10	6
FUM B1	100[2]	10	3
OTA	5[1]	1	0.5
OTB	5[1]	1	0.5
STE	100[2]	10	1.5
T2 + HT2	50[1]	10	0.3
ZEN	100[1]	10	2.4

[1] ML defined by the legislation (European Commission, 2023c; Belgian Federal Agency for the Safety of the Food Chain (FASFC), 2025); [2] ML arbitrary fixed for the validation.

Quality control

One important part of this work was to make sure that the method allows the detection of the molecules of interest in an unknown sample. Criteria about RT and transition ratio are used for that. Data collected during the validation allowed the determination of an average RT and an average transition ratio for each mycotoxin (Supplementary materials, Table S1), that can be used as references by the TargetLynx V4.2 (Waters, Milford, USA) software to check the RT and transition ratio criteria.

Table 8: Summary of the day by day repeatability and the inter-day reproducibility of the eight full validated mycotoxins. a) Samples spiked at the ML/2, b) Samples spiked at the ML, c) Samples spiked at 1.5 x ML.

(a)

		DON Concentration [µg/kg]	3Ac-DON Concentration [µg/kg]	15Ac-DON Concentration [µg/kg]	OTB Concentration [µg/kg]	OTA Concentration [µg/kg]	HT2 Concentration [µg/kg]	T2 Concentration [µg/kg]	ZEN Concentration [µg/kg]
Spiked concentration		625	50	50	2.5	2.5	50	50	50
Day 1	Average	549.8	38.2	29.6	2.8	3.0	79.5	10.0	65.1
	CV [%]	10.1	11.1	5.2	5.8	3.8	2.4	2.9	3.4
Day 2	Average	509.0	29.4	27.1	2.5	2.3	70.4	5.4	44.5
	CV [%]	0.9	6.7	3.8	6.6	8.7	3.8	7.9	3.2
Day 3	Average	478.1	30.2	26.4	2.2	2.7	69.8	5.5	46.4
	CV [%]	8.6	8.6	9.6	12.4	10.1	11.2	15.0	8.2
Global	**Average**	**512.3**	**32.6**	**27.7**	**2.5**	**2.7**	**73.2**	**4.3**	**52.0**
	CV [%]	**9.4**	**15.4**	**8.0**	**13.0**	**12.9**	**8.9**	**41.0**	**19.0**

(b)

		DON Concentration [µg/kg]	3Ac-DON Concentration [µg/kg]	15Ac-DON Concentration [µg/kg]	OTB Concentration [µg/kg]	OTA Concentration [µg/kg]	HT2 Concentration [µg/kg]	T2 Concentration [µg/kg]	ZEN Concentration [µg/kg]
Spiked concentration		1250	100	100	5	5	100	100	100
Day 1	Average	1185.4	58.0	41.7	5.2	5.3	131.1	3.1	106.2
	CV [%]	5.5	4.8	3.4	2.2	6.0	5.0	10.0	3.6
Day 2	Average	1090.6	47.6	44.6	5.0	4.5	146.3	5.4	90.7
	CV [%]	1.1	3.9	1.7	3.2	3.5	2.0	12.7	2.6
Day 3	Average	1003.4	71.3	66.3	4.6	5.8	146.1	14.3	92.1
	CV [%]	2.1	3.3	3.4	6.0	5.6	3.0	14.6	2.7
Global	**Average**	**1093.1**	**59.0**	**50.9**	**4.9**	**5.2**	**141.2**	**7.6**	**96.3**
	CV [%]	**7.8**	**17.3**	**22.4**	**6.8**	**11.4**	**6.1**	**67.1**	**8.0**

		(c)							
		DON **Concentration [µg/kg]**	3Ac-DON **Concentration [µg/kg]**	15Ac-DON **Concentration [µg/kg]**	OTB **Concentration [µg/kg]**	OTA **Concentration [µg/kg]**	HT2 **Concentration [µg/kg]**	T2 **Concentration [µg/kg]**	ZEN **Concentration [µg/kg]**
Spiked concentration		1750	150	150	7.5	7.5	150	150	150
Day 1	Average	1740.6	83.4	65.1	8.7	8.3	247.7	4.0	154.7
	CV [%]	5.7	4.4	7.2	2.8	2.5	2.1	5.5	1.6
Day 2	Average	1610.1	73.2	58.7	7.8	7.4	230.9	8.2	136.7
	CV [%]	1.6	3.0	4.5	3.0	4.4	2.8	10.5	1.8
Day 3	Average	1587.4	91.2	81.1	6.8	9.2	226.0	8.8	137.2
	CV [%]	3.7	7.1	9.4	5.7	4.2	5.4	12.8	4.9
Global	**Average**	**1634.2**	**82.6**	**68.5**	**7.8**	**8.2**	**234.9**	**7.4**	**142.9**
	CV [%]	**5.3**	**10.8**	**16.4**	**11.3**	**9.5**	**5.3**	**29.6**	**6.7**

In red, data that are not compliant with the validation standards.

4. Reproducibility and recovery

The average recovery was calculated at concentration levels, ML/2, ML, and 1.5 x ML and the reproducibility was studied for each mycotoxin. The standard deviation and the coefficient of variation were also computed. Summary of the average and the CV for the concentration and recovery for each day and each mycotoxin is shown in Table 8 8, line "Global".

The recovery of DON, ZEN, HT2 and OTB was between 80% and 120%, that is compliant with the guidelines (CEN, Comité Européen de Normalisation, 2010; European Commission, 2021). In addition, according to the Horwitz equation, the CV needed to be below the $CV_{Horwitz}$ (Table 5). For these mycotoxins, the CV was compliant for the three levels of concentration (Table 8). Hence, the average recovery can be used routinely to correct the concentration of unknown samples.

For T2, 3Ac-DON and 15Ac-DON, the average recoveries were lower than 80%.

4.1. The particular case of T2

Regarding T2, at ML, the average recovery for the first day (43.3%) was higher than the other two, 5.45% for day 2 and 14.3% for day 3. These recoveries were way too low to be compliant with the guidelines (CEN, Comité Européen de Normalisation, 2010). In addition, there is an important variation between the days. Based on these observations and the fact that for inter-laboratory assay no value was given for the concentration of T2 in samples, but only for the sum of T2 and HT2, the hypothesis of a conversion of T2 into HT2 during the extraction was proposed. Furthermore, it is possible that this conversion can vary from one extraction to another depending on the time between the extraction and the injection.

The conversion of T2 into HT2 during the extraction procedure was proven by spiking separately "blank" matrix samples with T2 and HT2. The results obtained are presented in Table 9. Samples spiked with only T2 contained HT2. Indeed, for the T2 spiked samples, the average recovery of T2 is 14.2% and the recovery of HT2 was 60.8%, even if no HT2 was introduced into the samples before the extraction (Table 10). In addition, for samples spiked with HT2, no T2 was detected and the average recovery of HT2 was 72.1%. In comparison with the previously obtained recoveries that were 141.2% for HT2 and 7.6% for T2 in samples spiked with both mycotoxins, the recovery in individually spiked samples were more compliant with the guidelines (CEN, Comité Européen de Normalisation, 2010). These results show that there is a conversion of T2 into HT2 during the

extraction procedure that should be considered while correcting the concentration with the recovery.

Table 9: Concentration of T2 and HT2 in samples spiked with T2 and HT2 individually for 3 days.

			T2 concentration (µg/kg)	HT2 concentration (µg/kg)
Spiked with T2 at 100 µg/kg	Day 1	Sample 1	13.4	60.2
		Sample 2	13.2	61.7
	Day 2	Sample 1	14.3	60.0
		Sample 2	18.6	63.1
	Day 3	Sample 1	12.5	61.6
		Sample 2	13.4	57.6
Spiked with HT2 at 100 µg/kg	Day 1	Sample 1	0.0	81.6
		Sample 2	0.0	81.4
	Day 2	Sample 1	0.0	86.1
		Sample 2	0.0	79.0
	Day 3	Sample 1	0.0	82.5
		Sample 2	0.0	79.7

Table 10: Summary of the results from the assay about T2 conversion into HT2.

	Recovery T2		**Recovery HT2**	
Spiked with T2	14.2%		60.8%	
	St. Dev	CV	St. Dev	CV
	0.02	15.49	0.02	3.06
Spiked with HT2	0.0%		72.1%	
	St. Dev	CV	St. Dev	CV
	0	0	0.02	3.44

By correcting the recovery of T2 used to verify reproducibility with the conversion factor, the average recovery for T2 at ML was 68.4% and the associated CV was 7.5%. Concerning HT2 spiked at the ML, the average recovery was 80.4% and the CV was 10.7%. Therefore, both CV for the recoveries of T2 and HT2 are compliant with the validation criteria previously mentioned while considering the conversion factor.

Therefore, the more accurate approach may be to consider the sum of the two mycotoxins as the conversion of T2 into HT2 can vary slightly from time to time and is also matrix dependent. In addition, this approach is consistent with the European regulation (European Commission, 2023c) which provides a limit for the sum of the two rather than individual regulation. Hence, the average recovery for the sum of T2 and HT2 was calculated using the formula below (Equation 3).

$$Recovery_{T2+HT2}(\%) = \frac{(0.91 \times CC_{T_2}) + CC_{HT_2}}{100 + (0.91 \times 100)} \times 100$$

$$\text{with } \frac{Molar\ mass\ of\ HT2}{Molar\ mass\ of\ T2} = \frac{424.5}{466.5} = 0.91$$

Equation 3

The recoveries of the sum of T2 and HT2 were respectively 84.3%, 83.9% and 86.0% at ML/2, ML and at 1.5 x ML, respectively (Table 11).

4.2. Mycotoxin recoveries and trueness of the method

All the average recoveries obtained for the eight fully validated mycotoxins are summarized in Table 11.

Table 11: Summary of the recoveries of the fully validated mycotoxins at the 3 levels of concentration.

		Recovery (%)						
		DON	**3Ac-DON**	**15Ac-DON**	**OTB**	**OTA**	**T2+HT2**	**ZEN**
ML/2	**Average**	**82.0**	**65.2**	**55.4**	**100.3**	**106.4**	**84.3**	**104.0**
	SD	7.7	10.1	4.4	13.1	13.7	7.6	19.7
	CV	9.4	15.4	8.0	13.0	12.9	9.0	19.0
ML	**Average**	**87.5**	**59.0**	**50.9**	**98.8**	**103.6**	**83.9**	**96.3**
	SD	6.8	10.2	11.4	6.7	11.8	4.7	7.7
	CV	7.8	17.3	22.4	6.8	11.4	5.6	8.0
1.5 x ML	**Average**	**87.2**	**55.0**	**45.7**	**103.7**	**109.4**	**86.0**	**95.3**
	SD	4.6	5.9	7.5	11.7	10.4	6.0	6.4
	CV	5.3	10.8	16.4	11.3	9.5	6.9	6.7

SD: Standard Deviation, CV: Coefficient of variation. Data in yellow are only compliant with the analytical norms if the stability of the method is proved.

The average recoveries, defined in the validation, are used to correct the determined concentrations. Then, the corrected sample concentrations are compared to the theoretical concentrations to assess the trueness of the analytical method. According to European legislation (European Commission, 2021), the trueness acceptance range is from 80% to 120% of the actual concentration for all mycotoxins except for OTA and OTB where the acceptance range is from 70% to 120%. However, if the determined concentration is between 50% to 70%, the steadiness of the method should be proven.

The trueness of the method was studied for each day. The results at ML are presented in Table 12.

Table 12: Summary of the trueness of the analytical method at ML for conventional mycotoxins.

		DON	**3Ac-DON**	**15Ac-DON**	**OTB**	**OTA**	**T2 + HT2**	**ZEN**
Theoretical concentration [µg/kg]		1250	100	100	5	5	200	100
Day 1	**Average [µg/kg]**	1354.8	149.6	161.6	5.3	5.1	203.2	113.8
	Trueness [%]	108.4	149.6	161.6	105.6	101.7	101.6	113.8
Day 2	**Average [µg/kg]**	1246.4	80.7	87.7	5.1	4.4	180.3	97.3
	Trueness [%]	99.7	80.7	87.7	102.0	87.0	90.2	97.3
Day 3	**Average [µg/kg]**	1146.8	120.9	130.2	4.6	/	189.7	98.7
	Trueness [%]	91.7	120.9	130.2	92.6	/	94.8	98.7
Day 4	**Average [µg/kg]**	/	98.3	82.0	/	5.6	/	/
	Trueness [%]	/	98.3	82.0	/	111.1	/	/

Once the concentrations were corrected with the average recoveries, for DON, 3Ac-DON, OTB, OTA, the sum of T2 and HT2, and ZEN, the trueness was compliant with the validation criteria.

Concerning 15Ac-DON, the trueness was not verified for day 3 as it is 130.2% and the maximum allowed value is 120%.

5. Non-regulated mycotoxins

In addition of the fully validated mycotoxins, a more succinct validation was performed for AME, AOH, DON-3G and DAS. These mycotoxins were also included in the first mycotoxins standard mix and were spiked at the three levels of concentration.

For the second standard mix of mycotoxins, only a secondary validation was done. Indeed, instead of spiking the "blank" matrix at three different levels of concentration, only the samples spiked at ML with the first mycotoxin standard mix were also spiked with the second mycotoxin standard mix at 100 µg/kg for three days. The same data treatment as for the first standard mix mycotoxins has been performed.

Table 13 shows the average recoveries for the non-fully validated mycotoxins and Table 14 presents the trueness of the method at ML. For DON-3G and DAS, the recoveries were lower than 50%: around 30% for DON-3-G and less than 10% for DAS. In addition, moniliformin and CIT had too low recoveries. Indeed, the recoveries for these two mycotoxins were approximatively 15% and 40%, respectively. For AME, AOH, ENN A, A1, B and B1, FUM B1 and STE the trueness of the method is between 70% and 120%.

Table 13: Summary of the recoveries (%) of AME and AOH at three levels of concentration and of emerging mycotoxins at one level of concentration.

		Recovery (%)							
		AME	**AOH**	**ENN A**	**ENN A1**	**ENN B**	**ENN B1**	**FUM B1**	**STE**
ML/2	**Average**	**86.7**	**89.7**	ND	ND	ND	ND	ND	ND
	SD	22.6	12.0	ND	ND	ND	ND	ND	ND
	CV	26.0	13.4	ND	ND	ND	ND	ND	ND
ML	**Average**	**90.0**	**89.7**	**90.6**	**92.9**	**93.0**	**92.3**	**58.3**	**90.8**
	SD	7.1	4.3	4.2	6.5	9.5	7.7	6.1	5.2
	CV	7.9	4.8	4.6	7.0	10.2	8.4	10.5	5.7
1.5 x ML	**Average**	**90.3**	**90.8**	ND	ND	ND	ND	ND	ND
	SD	6.6	5.6	ND	ND	ND	ND	ND	ND
	CV	7.3	6.1	ND	ND	ND	ND	ND	ND

ND: Non-determined

Table 14: Summary of the trueness (%) of the analytical method at ML for AME, AOH and emerging mycotoxins.

	Trueness (%)							
	AME	**AOH**	**ENN A**	**ENN A1**	**ENN B**	**ENN B1**	**FUM B1**	**STE**
Day 1	107.2	104.4	104.7	105.7	109.1	103.1	107.1	105.1
Day 2	95.1	97.4	97.5	97.2	97.0	98.7	95.5	96.4
Day 3	97.5	98.3	97.9	97.1	93.9	98.1	97.5	98.3

6. Carry over

Examination of the solvent injection after the highest calibration standard (Figure 3) demonstrated that no carry-over was detected for either mycotoxin (Figure 4).

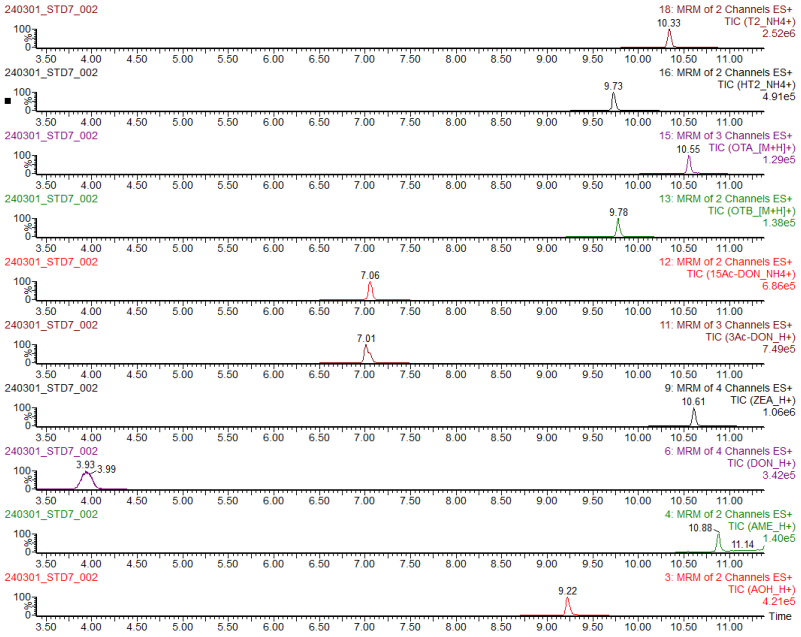


Figure 3: Chromatogram of the highest calibration point injected just before the mobile phase.

(a)

(b)

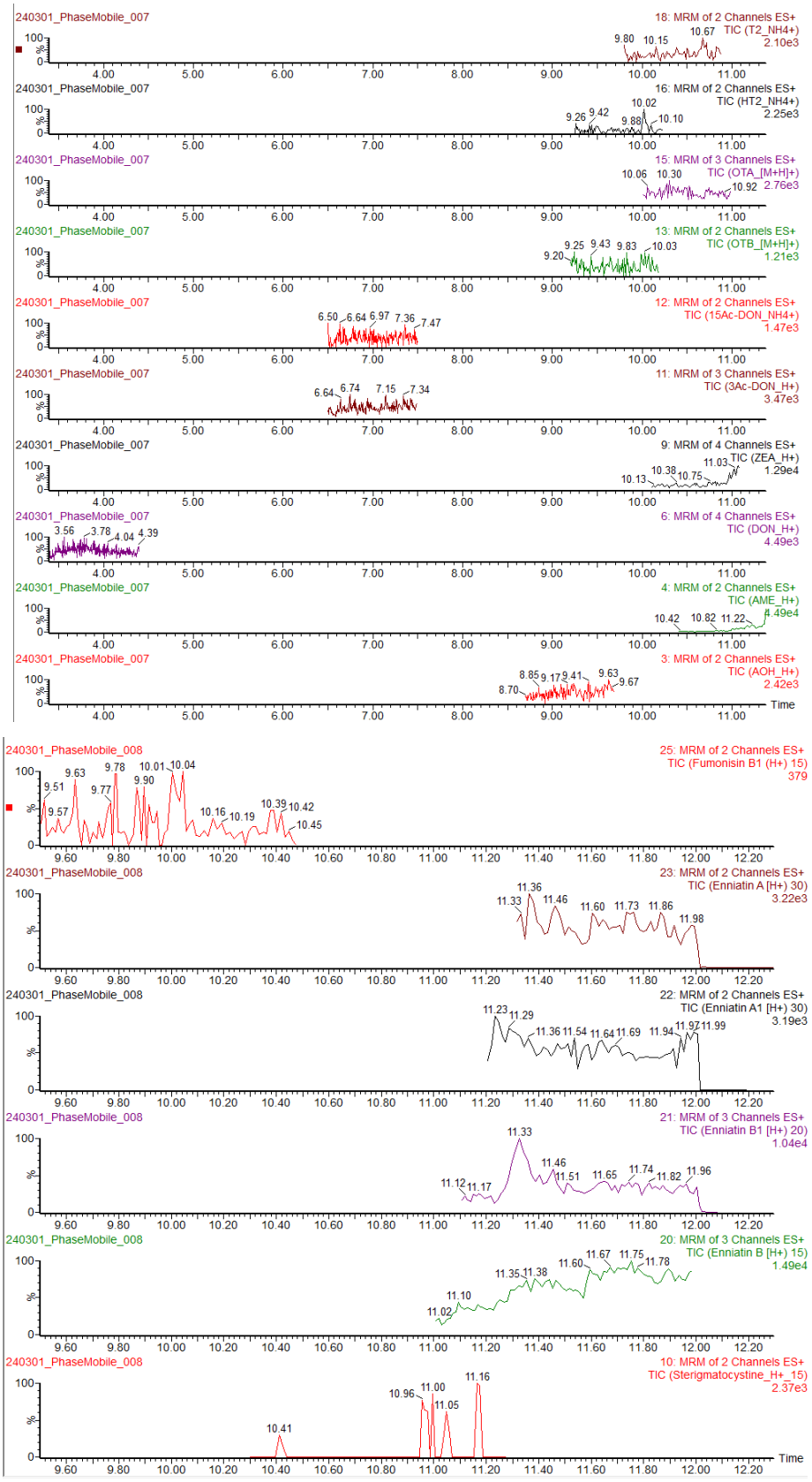


Figure 4: Carry over assessment in a mobile phase injection for (a) mycotoxins from the standard mix 1 and (b) mycotoxins from the standard mix 2.

7. Measurement uncertainty

The uncertainty of the method for each mycotoxin was calculated, and the results are presented in Table 15. For DON, 3Ac-DON, 15Ac-DON, OTA, T2 + HT2, and ZEN, the method uncertainties considered the trueness of the method compared to an interlaboratory assay sample, whereas for OTB, AME, AOH, ENNs, FUM B1 and STE, the method uncertainties were only based on intra- and inter-day variability. Method uncertainties are below 20% for each mycotoxin except for 15Ac-DON and 3Ac-DON that have an uncertainty of respectively 52.2 % and 29.7%.

Table 15: Expended measurement uncertainty for each mycotoxin.

Uncertainties (%)														
DON	**3Ac-DON**	**15Ac-DON**	**OTB**	**OTA**	**T2+HT2**	**ZEN**	**AME**	**AOH**	**ENN A**	**ENN A1**	**ENN B**	**ENN B1**	**FUM B1**	**STE**
10.3	29.7	52.2	14.6	13.1	18.8	10.4	7.3	12.5	8.2	8.4	15.1	4.3	9.1	8.7

8. Stability

The stability test showed that the extracted and diluted samples are stable when stored at -20°C in the dark, in amber injection vial. Indeed, by looking at Table 16, the relative difference obtained for samples injected directly after extraction and the same samples injected after four months of conservation as described above, is lower than 15% (European Commission, 2021) for DON, 3Ac-DON, 15Ac-DON, and ZEN meaning that there was no significant degradation of these mycotoxins in the samples. For OTA and OTB, the deviation was slightly over 15% since the concentration of OTA and OTB increased with time. Regarding T2 and HT2, by looking at the sum of the two mycotoxins, the deviation between the two determined concentrations is not significative at 0.5 x ML and ML, and low for 1.5 x ML.

In addition, about the stability of the calibration curves, after four months of storage at -20°C in the dark in amber injection vial, the deviations between the back-calculated concentrations of the calibration points and the theoretical ones were lower than 20% for DON and ZEN (Figure S3 and Table S2). For the other mycotoxins, the deviation was below 30%. In general, the lower points of calibration were more affected by the storage.

Table 16: Summary of the calculated concentration for the same samples injected directly after extraction and after four months of storage in the dark at -20°C

		DON		3Ac-DON		15Ac-DON		OTB		OTA		T2		HT2		T2 + HT2		ZEN	
		23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24	23-02-24	27-06-24
0.5 x ML	IND. AVG (µg/kg)	509.0	538.3	29.4	32.5	27.1	31.8	2.5	3.1	2.3	3.2	5.4	2.3	70.4	79.0	75.3	81.2	44.5	44.9
	RD (%)	5.5		9.8		14.8		19.1		28.6		133.8		18.9		92.817.2		99.30.7	
ML	IND. AVG (µg/kg)	1090.6	1133.1	47.6	49.2	39.3	43.4	5.0	6.0	4.5	5.9	5.4	2.8	146.3	166.8	151.3	169.4	90.7	86.0
	RD (%)	3.8		3.3		9.5		16.1		23.1		92.8		12.3		10.7		5.5	
1.5 x ML	IND. AVG (µg/kg)	1610.1	1652.4	73.2	77.9	58.7	65.9	7.8	9.4	7.4	9.6	8.2	5.9	230.9	280.0	238.4	285.4	136.7	131.5
	RD (%)	2.6		6.0		11.0		16.2		22.2		37.6		17.5		16.5		3.9	

IND.AVG: individual average including the 6 samples of the same level of concentration injected directly after extraction and after four months at -20°C.

*RD: Relative difference = 100 - ((IND. AVG 23-02-24/IND. AVG 27-06-24)*100).*

In red values with a RD difference above 15%.

9. Certified reference material

Certified reference material for wheat naturally contaminated with mycotoxins are quite difficult to find. Hence, even though it's a good way to validate an analytical method, it was not possible to base the validation entirely on this kind of samples.

The CRM found was a naturally contaminated wheat flour with DON (1000 µg/kg), T2 (35.2 µg/kg), HT2 (38 µg/kg) and OTA (6 µg/kg) (Romer-Labs, Austria).

The CRM was extracted twice on two different days, and the calculated concentrations were corrected with recoveries previously determined. The results, presented in Table 17, show that the measurement concentrations are well in acceptable range for each analysed mycotoxin, demonstrating an acceptable accuracy of the method.

Table 17: Results of the analysis of the certified reference material on two different days (D1 and D2).

	Xpt (µg/kg)	VT (µg/kg)	D1 (µg/kg)	D2 (µg/kg)
OTA	6.0	± 3.0	8.8	5.7
DON	1000.0	± 300.0	820.5	795.8
HT2	38.0	± 12.7	48.7	47.6
T2	35.2	± 16.3	24.9	39.4
T2 + HT2	70.0	± 14.5	71.4	83.4

Xpt: Expected value; VT: Tolerance value (given by the supplier).

In addition, a sample from inter-laboratory assay was also extracted and analysed twice and the results were compared to those given by the organization (BIPEA, France), that are an average of the concentration determined by the participating laboratories. Table 18 shows the results.

Table 18: Results of the analysis of the interlaboratory assay on two different days (D1 and D2).

	Xpt (µg/kg)	VT (µg/kg)	D1 (µg/kg)	D2 (µg/kg)
OTA	2.5	± 1.5	2.2	1.8
DON	1061.0	± 477.0	1045.0	1023.3
3Ac-DONG	60.0	± 46.0	/	/
3Ac-DON	74.0	± 40.0	33.5	40.9
15Ac-DON	92.0	± 49.0	35.6	53.5
NIV	131.0	± 69.0	/	/
HT2	29.0	± 18.0	29.9	39.3
T2HT2	60.0	± 33.0	29.2	53.6
ZEN	15.0	± 11.0	11.2	10.1

Xpt: Expected value; VT: Tolerance value (given by the organization).

Discussion

1. *Validation procedure*

The validation criteria used came from three different reference documents (CEN, Comité Européen de Normalisation, 2010, 2022; European Commission, 2021). However, there is consistency between these three norms, but among these documents some were more precise than others for some criteria and that is the reason why these are more specifically cited.

The validation process was not based on CRM as the availability of such samples is really limited on the market. Indeed, it was not possible to find wheat samples naturally contaminated by as many mycotoxins, even only including the eight fully validated mycotoxins. However, one CRM was analysed to confirm the trueness of the method, for OTA, DON, T2 and HT2. In addition, the method was tested as a part of an inter-laboratory assay with samples analysed in different laboratories. The results obtained and corrected by the average recoveries were in the acceptable range meaning that the trueness of our analytical method is verified.

In this case and for further analysis, no internal standard was used as the purchase of these compounds is very expensive. In addition, 20 different internal standards should be used as the extraction dynamic can vary from one mycotoxin to another, meaning that many internal standards should be purchased. The extraction of samples spiked with a known concentration allows estimating the recovery and thus correcting the concentration based on the mycotoxins that are really extracted from the naturally contaminated samples. As internal standards are not routinely used, several measures were implemented to ensure the reliability and accuracy of the measured concentrations. During method validation, average recoveries were determined for each mycotoxin, allowing the correction of calculated concentrations by taking into account the extraction efficiency. In addition, all samples were analyzed in duplicate, and a maximum deviation of 20% between the two measurements was accepted. The results reported correspond to the mean value of the two measurements. Furthermore, for each analytical batch, multiple quality control steps were applied to minimize the risk of erroneous quantification. These included the injection of a standard solution prepared in solvent to verify the instrument response, the analysis of an internal reference sample linked to a control chart, and the extraction of a spiked sample alongside the batch. In addition, bracketed calibration was systematically applied to compensate for potential signal drift throughout the analytical sequence.

2. Limit of detection

Concerning the LOD, even if the greatest care is taken in the proper maintenance of the system, the sensitivity of the mass spectrometer can change over time with clogging of the device, modifications of the parameters due to device servicing and so on. Thus, this variation of sensibility can lead to changes of LOD. However, when looking at regulated feed and food contaminants, the LOD is not a critical information, as what matters is to see if the contaminant concentration is above or below the legal limit, and not to detect a prohibited compound in a given matrix.

None of the papers shown in Table 19 has the exact same procedure as the one used in this study. However, the calculated LOD can be compared to the ones found in the literature. Leite et al. (2023) performed multi-mycotoxin analysis using QuEChERS extraction followed by dispersive solid phase extraction with a C18 tube and detection and quantification by UPLC-MS/MS. Sun et al. (2016) employed additional salts, sodium citrate and sodium hydrogen citrate sesquihydrate, to perform the salting out effect, the mobile phases and the column were the same. Finally, Zhang et al. (2013) performed the extraction with methanol and water using no acetonitrile or salts. The obtained LOD for DON was much higher than those found in literature. However, it is not a big concern as the LOD determined in the laboratory is much lower than the ML of DON in wheat. For 3Ac-DON, 15Ac-DON, OTA, T2, HT2 and ZEN, the determined LOD was in the same range than the ones found in the papers. No data were found for OTB.

Table 19: Comparison of the LOD obtained during the validation and the ones from the literature (Zhang et al., 2013; Sun et al., 2016; Leite et al., 2023).

	This study	**Leite et al., 2023**	**Sun et al., 2016**	**Zhang et al., 2013**
	Wheat [µg/kg]	Maize [µg/kg]	Cereals [µg/kg]	Wheat [µg/kg]
DON	60	3.2	5	1.5
3Ac-DON	3.3	/	2	/
15Ac-DON	3.8	/	2	/
OTB	0.5	/	/	/
OTA	0.5	3.4	0.1	1.4
T2	0.1	5.8	0.1	0.6
HT2	0.3	4.8	0.1	0.8
ZEN	2.4	2.5	1.0	/

3. Recoveries and reproducibility

Obtained recoveries can be compared to the ones found in the literature (Table 20). De Santis et al. (2017) did not use salt for the extraction. The calculated recoveries for DON and ZEN were like those reported in the papers. For OTA, the recovery was higher and for 3Ac-DON and 15Ac-DON, the

recoveries were lower. For T2 and HT2, the recovery is similar but, in our case, we consider the sum of the two toxins. Even if the recovery of 3Ac-DON and 15Ac-DON is lower than that in the literature, no change will be made to the method as it allows for the extraction of more mycotoxins, and the trueness is still compliant.

Table 20: Comparison of the recoveries obtained during the validation and the ones from the literature (Zhang et al., 2013; Sun et al., 2016; De Santis et al., 2017; Solfrizzo et al., 2018).

	Recovery (%)				
	This study	De Santis et al., 2017	Solfrizzo et al., 2018	Sun et al., 2016	Zhang et al., 2013
	Wheat	**Wheat**	**Feed**	**Cereals**	**Wheat**
DON	87.5 ± 6.8	92.0	79.0	97.9	96.0
3Ac-DON	59.0 ± 10.2	/	/	104.7	/
15Ac-DON	50.9 ± 12.6	/	/	100.8	/
OTB	98.8 ± 6.7	/	/	/	/
OTA	103.6 ± 11.8	83.0	82.0	88.7	92.0
T2	83.9 ± 4.7	95.0	76.0	69.7	94.0
HT2		90.0	106.0	73.0	84.0
ZEN	93.3 ± 7.7	101.0	63.0	73.5	/

The reproducibility can also be compared to that from the literature (Table 21). The variation between the days was like the variations from papers for DON, OTA and ZEN. Based on the validation, the CV for the sum of T2 and HT2 was considered and not the individual CVs, contrary to what was found in literature. Regarding 3Ac-DON and 15Ac-DON, the variation was more significant in the developed method compared to that in the papers. Finally, no information was found for OTB.

Table 21: Comparison of the CV (%) obtained during the validation and the ones from the literature (Zhang et al., 2013; Sun et al., 2016; De Santis et al., 2017; González-Jartín et al., 2021)

	CV (%)				
	This study	De Santis et al., 2017	González-Jartín, 2021	Sun et al., 2016	Zhang et al., 2013
	Wheat	**Wheat**	**Milk**	**Cereals**	**Wheat**
DON	7.8	15.0	6.8	7.0	16.0
3Ac-DON	17.3	/	6.3	7.3	/
15Ac-DON	22.4	/	5.7	7.1	/
OTB	6.8	/	/	/	/
OTA	11.4	10.0	6.6	9.3	16.0
T2	5.6	15.0	5.4	7.2	12.0
HT2		15.0	6.9	7.9	17.0
ZEN	8.0	15.0	3.5	6.5	/

4. Calibration

The central point of calibration corresponds to the ML for the regulated mycotoxins (European Commission, 2023c) and to the arbitrary value of 100 µg/kg for the non-regulated ones. This value was chosen as it was the ML of ZEN and the recommended value for T2 and HT2 (before July 2024). In case of regulatory changes, a secondary validation should be done. For example, due to an update

in the regulation for DON that applies from the 1st of July 2024, the ML switches from 1250 µg/kg to 1000 µg/kg. Therefore, an additional day of validation with a calibration range centred around 1000 µg/kg was done to verify that no subsequent changes occurred. About emerging mycotoxins, as there are not many data about the situation in Belgium, it might be possible that the calibration range should be adapted to the actual situation. The aim should be to include most of the contaminated samples within the calibration range.

5. The specific case of T2 and HT2

The experiment conducted on separately spiked "blank" samples with T2 and HT2 revealed a conversion of T2 into HT2 during sample extraction and analysis. This transformation can not be neglected when determining the individual concentrations of the two mycotoxins. Therefore, since European regulations are based on the sum of T2 and HT2, considering their combined concentration is more relevant than assessing each compound separately.

6. Validation conclusions

About the different aspects that were studied within the validation, the recovery for most mycotoxins was between 70% and 120%. For 3Ac-DON and 15Ac-DON, the recovery was between 50% and 70%. The steadiness of the method was verified.

During this validation process, the reproducibility was investigated with the inter-day variation, as the operator was different for each day of validation, and the repeatability was assessed via the intra-day variation. Considering the inter-day variation, it can be said that the method was reproducible.

The calibration was done using a linear model as specified in the analytical norms. The matrix effect was investigated before the beginning of the validation process. For some mycotoxins, the matrix effect of wheat is almost inexistent (DON, ZEN, T2, HT2, 15Ac-DON and 3Ac-DON) but for others it can increase the response such as OTA and OTB.

6.1. Additional mycotoxins

In addition to the eight fully validated mycotoxins, a secondary validation was done for other mycotoxins such as AME, AOH, DON-3G, DAS, TeA, ENNs A, A1, B and B1, FUMs B1 and B2, moniliformin, CIT and STE. These mycotoxins were not fully validated as they are not regulated or frequently found in wheat in Belgium.

Tenuazonic acid was not detected with the current liquid chromatography conditions. By changing some parameters such as mobile phases composition while adding emerging mycotoxins into the analytical method, some mycotoxins were not detected anymore, namely NIV and TeA. The decision was made to drop these two mycotoxins from the multi-mycotoxins method as they are known for a long time and no specific interest or regulation has been shown by the authorities. However, it is possible to change the UPLC-MS/MS conditions and create another method specifically for these mycotoxins. The extraction procedure should remain the same.

Among the non-fully validated mycotoxins, the recoveries of the determined concentration of DON-3G, DAS, moniliformin and CIT were too low to be accepted. To improve the recovery of these molecules, the extraction procedure should be adapted. However, modifications to the extraction procedure can lead to the loss of other mycotoxins. Nevertheless, an additional extraction procedure can be developed specifically for these mycotoxins if needed.

7. Stability

The stability of the samples was shown during the validation process. Hence, in case of a sudden device issue, it is known that the extracted samples can be conserved at -20°C in the dark pending injection.

In addition, the stability of the calibration curves and the intermediate solutions prepared to do the calibration curves was proven, meaning that they can be reused for later analysis. Therefore, the standard solutions consumption can be decreased, and it can save time as there is no need to do a new calibration curve for every batch of analysis.

Conclusions

An analytical method is a dynamic process. Initially, the method needs to be developed and then validated. However, as the method is used routinely, it may evolve, or the needs may change. This evolution can lead to method improvements and new development phases, which must be validated again to ensure continued accuracy and reliability. Quality controls will be maintained to ensure the accuracy of results, and the calculated concentrations of real samples will be corrected based on assessed extraction recoveries. The validation process, including multiple days, underscores the steadiness and reliability of the multi-mycotoxin analysis method by UPLC-MS/MS. Despite challenges with some mycotoxins, the method is effective for most mycotoxins of interest, allowing its application in routine mycotoxin monitoring.

Supplementary materials

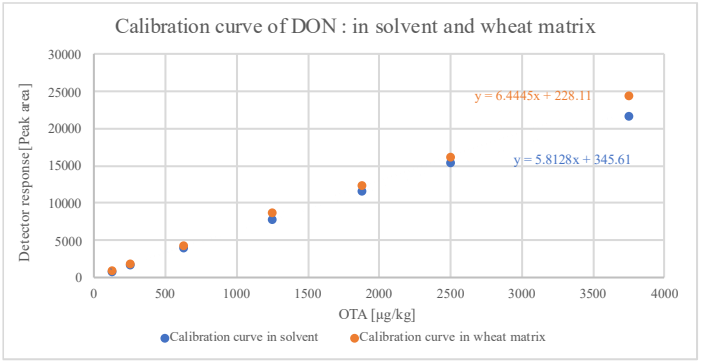
Table S1: Summary of the qualitative parameters for the identification of the mycotoxins.

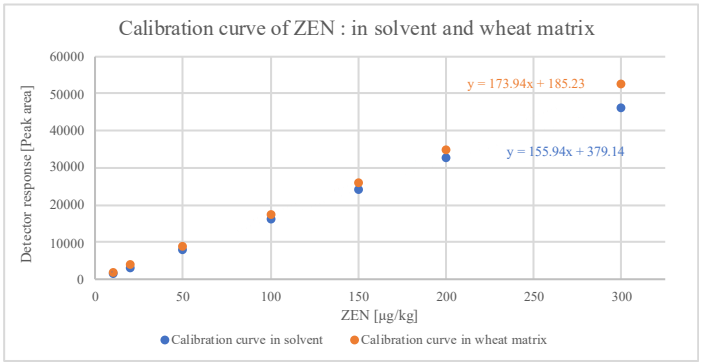
	Retention time			Transition ratio		
Mycotoxin	**Average**	**Max**	**Min**	**Average**	**Max**	**Min**
15Ac-DON	7.07	7.42	6.72	0.510	0.663	0.357
3Ac-DON	7.01	7.36	6.66	0.618	0.803	0.432
DAS	8.75	9.19	8.31	0.563	0.732	0.394
DON	3.95	4.15	3.75	0.853	1.109	0.597
HT2	9.74	10.23	9.25	0.955	1.242	0.669
TeA	/	/	/	/	/	/
AOH	9.22	9.68	8.76	0.648	0.842	0.454
AME	10.88	11.42	10.34	0.064	0.083	0.045
T2	10.34	10.86	9.82	0.900	1.170	0.630
ZEN	10.62	11.15	10.09	0.830	1.079	0.581
OTA	10.57	11.10	10.04	0.490	0.637	0.343
OTB	9.79	10.28	9.30	0.417	0.542	0.292
DON-3-G	4.55	4.78	4.32	0.496	0.644	0.347
STE	10.84	11.38	10.30	0.130	0.169	0.091
CIT	8.85	9.29	8.41	0.679	0.882	0.475
Moniliformin	0.82	0.86	0.78	/	/	/
FUM B1	9.99	10.49	9.49	0.802	1.043	0.561
FUM B2	/	/	/	/	/	/
ENN A	11.83	12.42	11.24	0.251	0.327	0.176
ENN A1	11.72	12.31	11.13	0.220	0.287	0.154
ENN B	11.53	12.11	10.95	0.944	1.227	0.661
ENN B1	11.63	12.21	11.05	0.943	1.225	0.660

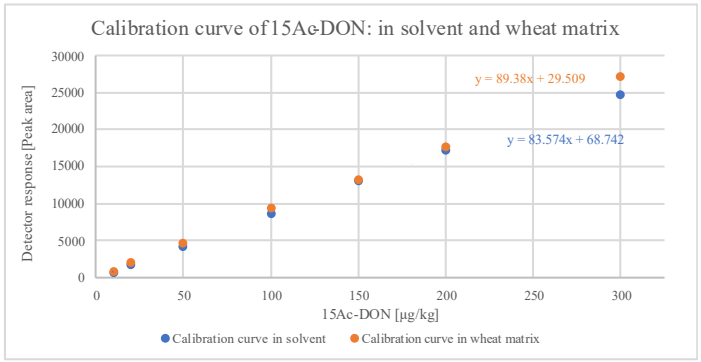
Max and min RT are defined as average RT ± 0.05%. Max and min transition ratio are defined as the average transition ratio ± 30% (n = 54)

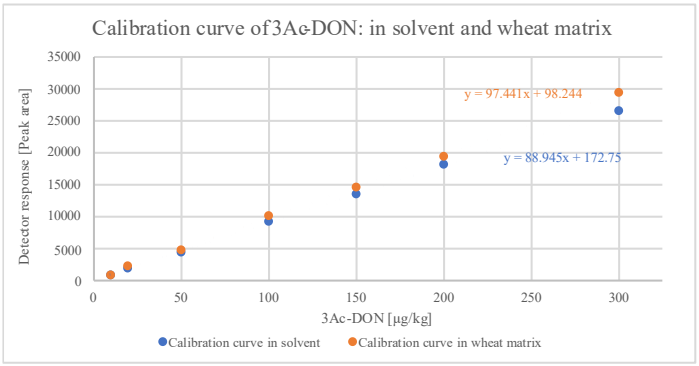
Table S2: Deviation of the calibration curve injected directly after preparation and another injected after four months in the dark at – 20°C for DON and ZEN.

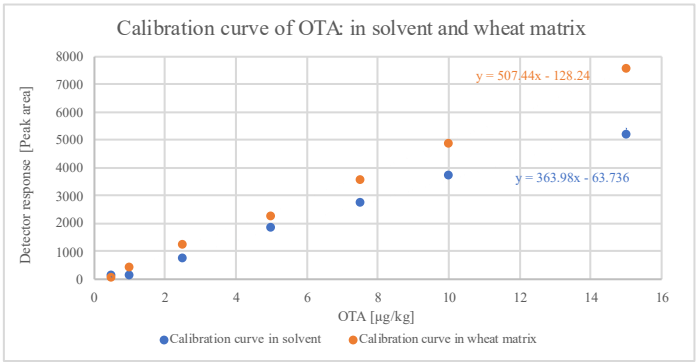
DON			**ZEN**		
Theoretical concentration (µg/kg)	**Back-calculated concentration (µg/kg)**	**Deviation (%)**	**Theoretical concentration (µg/kg)**	**Back-calculated concentration (µg/kg)**	**Deviation (%)**
125.0	100.0	20.0	10.0	11.4	13.7
250.0	204.0	18.4	20.0	16.9	15.4
625.0	598.4	4.3	50.0	45.3	9.4
1250.0	1208.0	3.4	100.0	92.6	7.4
1875.0	1724.9	8.0	150.0	127.4	15.1
2500.0	2258.5	9.7	200.0	165.5	17.3
3750.0	3506.6	6.5	300.0	265.7	11.4

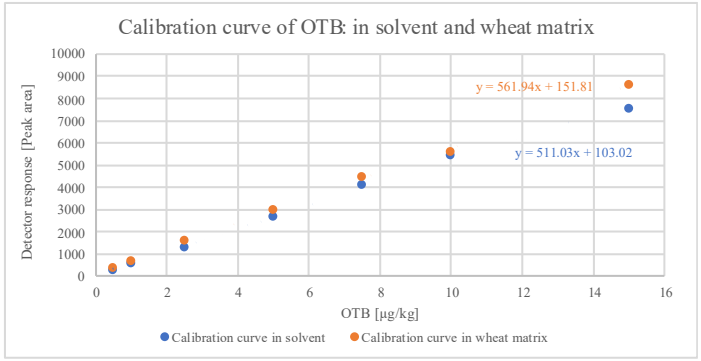


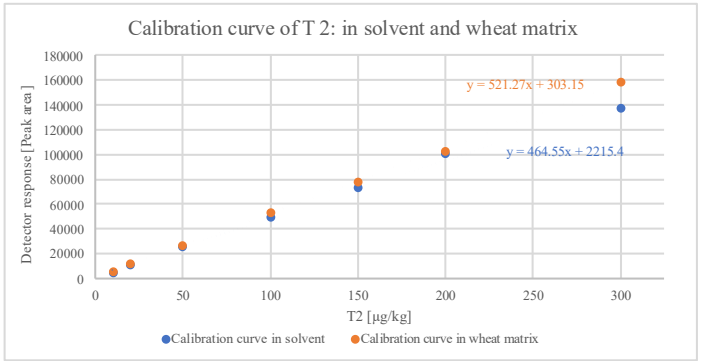












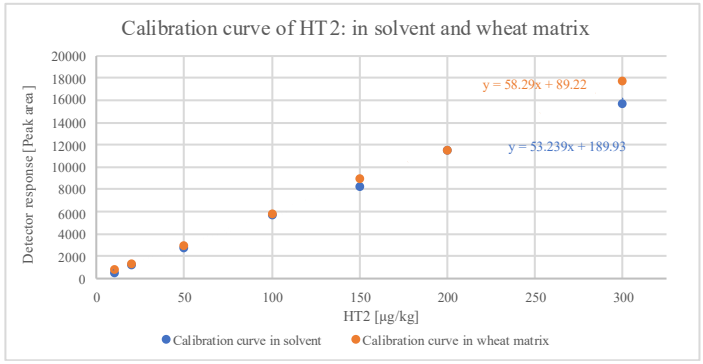


Figure S1: Calibration curves of the eight fully validated mycotoxins in solvent and in wheat matrix

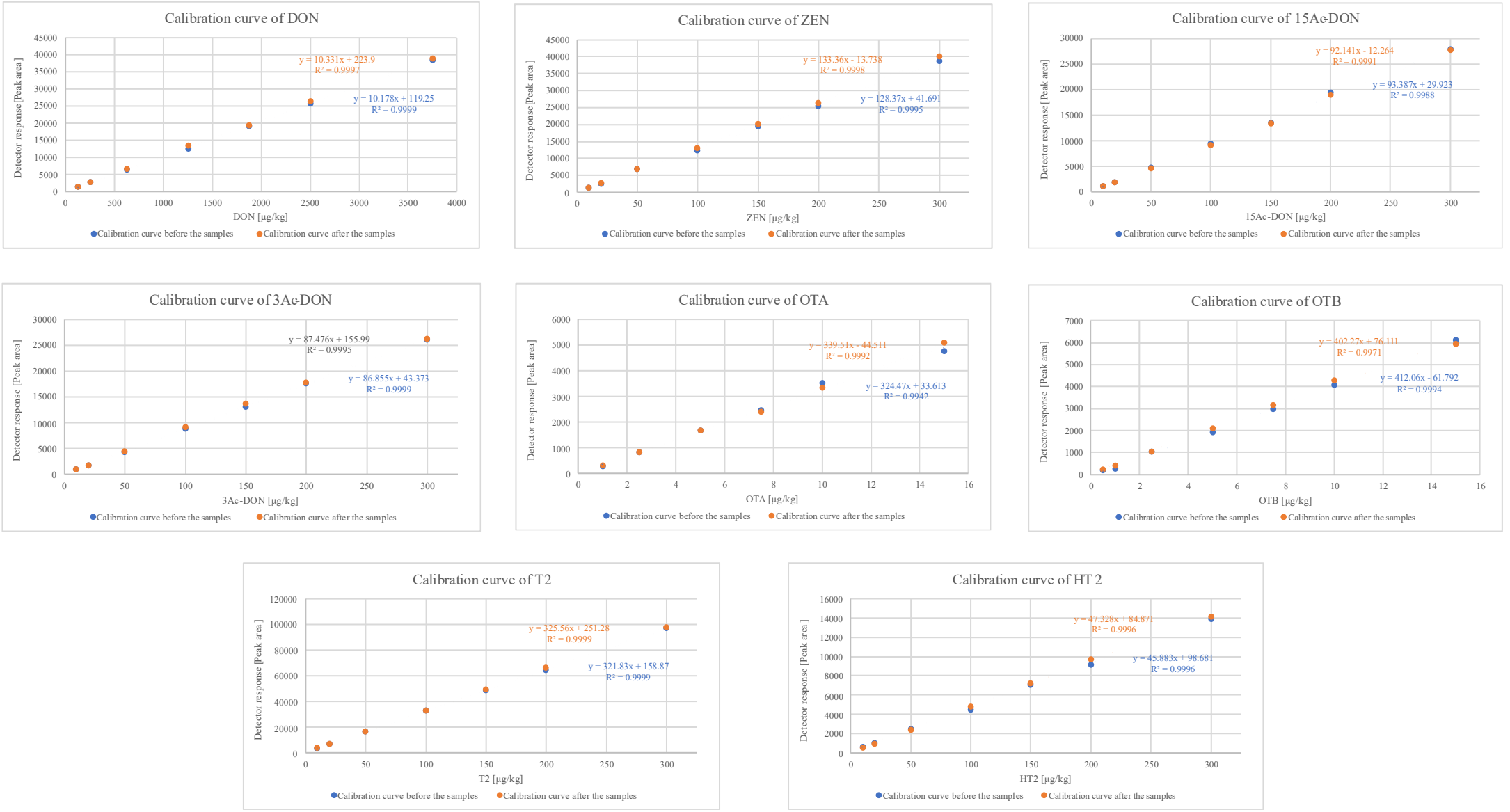


Figure S2: Calibration curves of the eight fully validated mycotoxins: calibration curve injected before and after the samples (second day of validation)

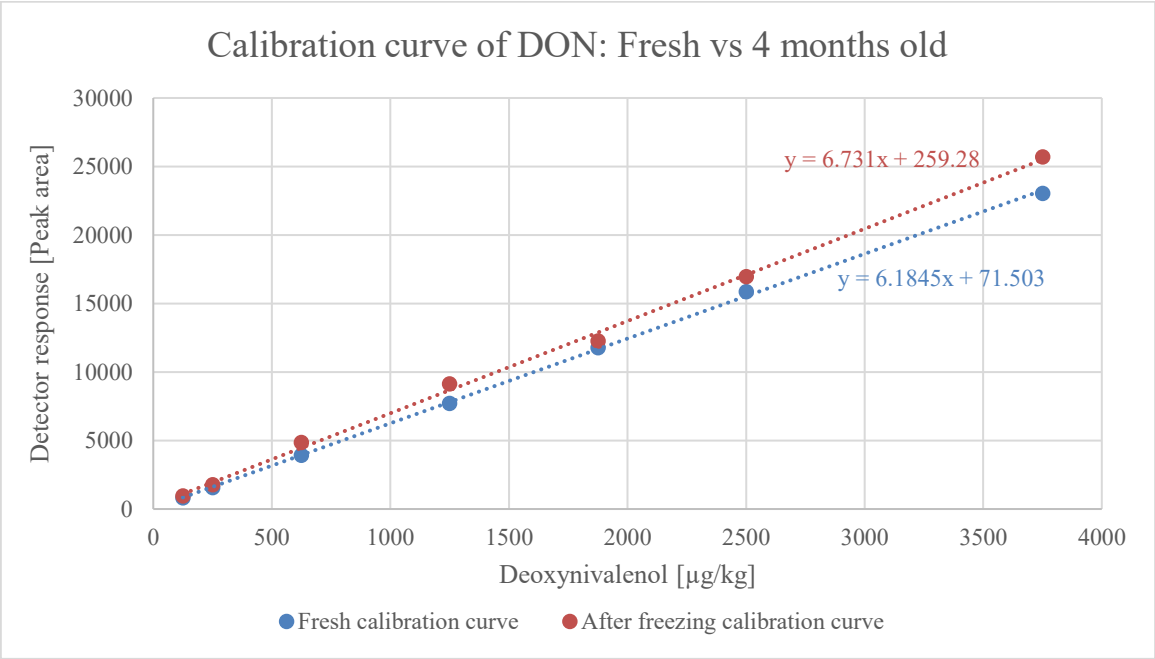


Figure S3: Comparison of a calibration curve injected directly after preparation and another injected after four months in the dark at – 20°C for DON.

Experimental section

Study 2: Multi-mycotoxin analyses by UPLC-MS/MS in wheat: The situation in Belgium in 2023 and 2024

Adapted from Foods 2025, 14(13), 2300; https://doi.org/10.3390/foods14132300

Camille Jonard [1], Anne Chandelier [1], Damien Eylenbosch [1], Joke Pannecoucque [2], Bruno Godin [1], Caroline Douny [3,*], Marie-Louise Scippo [3] and Sébastien Gofflot [1]

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Preamble

The analytical method developed and validated in Study 1 was subsequently applied to wheat samples of interest. To this end, different wheat varieties harvested in 2023 and 2024 from various locations across Belgium were collected in order to gain an overview of mycotoxin contamination during these two years. The main objective was to monitor the occurrence of mycotoxins in Belgian wheat, thereby enabling early risk anticipation, sector warnings, and reducing the entry of contaminated batches into the processing chains.

In addition, the analysis of different varieties may help identify these that are more susceptible to mycotoxin contamination. Such information is highly valuable for both breeders and farmers, as it can support the development and the cultivation of more resistant varieties.

Moreover, since the collected samples originated from different geographical regions, the influence of pedoclimatic conditions and agricultural practices on mycotoxin occurrence could also be investigated. Understanding these relationships may contribute to better management strategies for mycotoxin prevention and mitigation in future growing seasons.

Experimental section

Study 2: Multi-mycotoxin analyses by UPLC-MS/MS in wheat: The situation in Belgium in 2023 and 2024

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Abstract

This work proposes an insight into the mycotoxins detected in wheat from the 2023 and 2024 harvests in Belgium and highlights the link between agronomic conditions and mycotoxin contamination. The study utilized samples from a Belgian trial network, covering nine locations in 2023 and eight in 2024, ensuring diverse pedoclimatic contexts and including 11 different varieties. Sowing and harvest dates, previous crops and meteorological data were collected for these locations. A validated UPLC-MS/MS multi-mycotoxin method able to detect 20 mycotoxins, regulated or not, was used. Deoxynivalenol, zearalenone, and enniatins B and B1 were detected in the 2023 and 2024 samples. Enniatin A1 was only detected in the 2024 samples. Mycotoxin contamination was higher in 2024 compared to 2023, in terms of both the number of contaminated samples and the contamination levels. Enniatins B and B1, non-regulated mycotoxins, were widely detected in the 2024 wheat samples, with enniatin B detected in 68 out 88 samples ranging from 12 to 488 µg/kg. Differences between the wheat varieties were observed, with some varieties showing significantly higher contamination. Additionally, geographic location appeared to influence contamination levels, which could be related to previous crops or meteorological events. In conclusion, this research provides a comprehensive analysis of mycotoxin co-contamination in wheat samples from diverse pedoclimatic contexts in Belgium based over 2 years. It shows the importance of weather conditions on mycotoxin contamination. It also emphasizes the importance of variety selection to manage mycotoxin contamination.

Keywords: mycotoxins; UPLC-MS/MS; winter wheat; Belgium.

Introduction

Mycotoxins are secondary metabolites produced by fungal genera such as *Aspergillus* spp., *Alternaria* spp., *Fusarium* ssp. and *Penicillium* spp. (Tola and Kebede, 2016; WHO (World Health Organization), 2023). More than 300 mycotoxins have been reported in the literature. Among mycotoxins, the most studied compounds are aflatoxins (AFs), deoxynivalenol (DON), ochratoxin A (OTA) and zearalenone (ZEN), because of their toxicity to both animals and humans, their occurrence and because they are regulated (Pereira et al., 2014; Smith et al., 2016). In addition, some emerging mycotoxins, such as enniatins (ENNs), beauvericin (BEA) or sterigmatocystin (STE), defined as not regulated or not routinely monitored mycotoxins, are currently more frequently observed in raw agricultural products, used as food or feed (De Felice et al., 2023; Kolawole et al., 2024).

Deoxynivalenol, also called vomitoxin, is mainly produced by *Fusarium graminearum*. The main *Fusarium* species that produce ZEN are *F. graminearum*, *F. crookwellense*, *F. sporotrichioides* and *F. culmorum*. Deoxynivalenol and ZEN can co-occur as they are produced by the same Fusarium species (Ekwomadu et al., 2021).

Ochratoxins A and B (OTB) are produced by species of genera *Aspergillus* and *Penicillium* (EFSA, 2020). The main producers of AFs are *Aspergillus flavus* and *Aspergillus parasiticus*. Among the AFs, the most widespread and the most dangerous molecule is aflatoxin B1 (AFB1)(International Agency for Research on Cancer, 1993; Zain, 2011).

Enniatins are produced by different Fusarium species such as *F. avenaceum*, *F. oxysporum*, *F. poae* or *F. tricinctum* (Gruber-Dorninger et al., 2017). Currently, ENNs are not regulated. The last European Food Safety Authority (EFSA) report concluded that there was a lack of acute toxicity and genotoxicity data (EFSA, 2014).

Mycotoxins can be found in various foodstuffs such as coffee, beans, nuts, spices, maize, wheat, etc. (Marin et al., 2013). Mycotoxin contamination is a worldwide issue for human and animal health, crop cultivation and meat production (Zain, 2011; Pleadin et al., 2019). Mycotoxins are thermally stable, meaning that they are not destroyed during food processing (Bullerman and Bianchini, 2007; Ji et al., 2019; Mousavi Khaneghah et al., 2019). Consumption of contaminated products may lead to bioaccumulation of the toxins in the organs, leading to acute or chronic effects on humans and animals depending on the dose and duration of exposure (Zain, 2011; Sun et al., 2014; Mousavi Khaneghah et al., 2019). The possible effects can be mutagenic, teratogenic, carcinogenic,

hepatotoxic, cytotoxic or immunosuppressive (EFSA, 2020; Ekwomadu et al., 2021; De Felice et al., 2023). Therefore, some mycotoxin concentrations are regulated worldwide and controlled in food (Tola and Kebede, 2016). In the European Union, the Commission Regulation 2023/915 (European Commission, 2023c) and its amendments (European Commission, 2024b, 2024c) define the maximum levels (MLs) of mycotoxins in food. For unprocessed cereals, the ML for AFB1 is set at 2 µg/kg, while the total concentration of AFs must not exceed 4 µg/kg. The combined concentration of fumonisins B1 and B2 (FUM) is limited to 1000 µg/kg. Additionally, the MLs are 5 µg/kg for OTA, 1000 µg/kg for DON, 100 µg/kg for ZEN and 50 µg/kg for the sum of T-2 and HT-2 toxins. In feed, Directive 2002/32/EC of the European Parliament and its amendments define maximum contents for certain mycotoxins (European Commission, 2002). The MLs are 250 µg/kg for OTA, 8000 µg/kg for DON, and 2000 µg/kg for ZEN. These regulations are updated in line with advances in knowledge about mycotoxins' toxic effects.

Mycotoxin contamination can occur during pre-harvest, harvest and storage stages if the conditions are appropriate (Marin et al., 2013; Mahato et al., 2021). Infections by toxigenic fungi are influenced by weather conditions, especially humidity and temperature, agricultural practices, insect damage and storage conditions (Khodaei et al., 2021; Mahato et al., 2021; Kolawole et al., 2024). In cereals, heavy rains, leading to high air humidity, during flowering can induce Fusarium infection of ears, which can lead to higher mycotoxin content in the plants (Marroquín-Cardona et al., 2014). Fusarium growth increases at temperatures between 20 °C and 30 °C and in a matrix with water activity above 0.88 (Marroquín-Cardona et al., 2014). The probability of contamination with ZEN increases with heavy precipitation and long cool periods (Mahato et al., 2021). In addition, late harvest coupled with favorable weather conditions may enhance ZEN production (Moraes et al., 2023). On the other hand, *Aspergillus* growth is enhanced under warm temperatures, between 25 °C and 35 °C, and dry weather (Erenstein et al., 2022). *Aspergillus* mycotoxin and FUM productions are higher above 22 °C (Erenstein et al., 2022). Dry weather and late season rains may lead to FUM production (Marroquín-Cardona et al., 2014).

Wheat is one of the most widely cultivated crops in the world (Erenstein et al., 2022). It is a major source of carbohydrates and essential micro-nutrients in the human diet (Sarmast et al., 2021; Erenstein et al., 2022). In 2023, the Food and Agriculture Organization (FAO) estimated the global cereal production at 2847 million tons (Food and Agriculture Organization of the United Nations, 2025). In Europe, an average of 26 million hectares of wheat is cultivated, leading to the production of approximatively 254.6 million tons for food and feed ingredients (Food and Agriculture

Organization of the United Nations, 2025). The worldwide average amount of wheat consumption per capita is 65.6 kg annually, and it is the second most consumed cereal after rice (Erenstein et al., 2022). In Europe, 43.8% of the wheat produced is used for food and 36% for feed, while the other 20.2% is used for seed, processing and other uses, or subjected to losses (Erenstein et al., 2022). In Wallonia, the southern part of Belgium, the situation is different, as only 10% of the wheat is used for food, 45% for feed and 45% for fuel (Delcour et al., 2014).

The most prevalent mycotoxins in wheat are DON, ZEN, AFB1, OTA, HT2, T2, AFs and FUMs (Khodaei et al., 2021). In addition, some emerging mycotoxins have also been detected in wheat, such as ENNs, BEA and moniliformin (Ekwomadu et al., 2021). Co-occurrence of mycotoxins is a common phenomenon, as fungal species can produce multiple mycotoxins (Smith et al., 2016).

Mycotoxins are one of the most important issues regarding food safety, as they are observed worldwide in crops and can cause adverse effects on human health (Alshannaq and Yu, 2017). In addition, fungal infection and mycotoxin contamination have a huge economic impact on agriculture and the food industry, as they can lead to a decrease in production yield or destruction of contaminated products (Pitt and Miller, 2017; Khodaei et al., 2021; Mahato et al., 2021). Up to 60 to 80% of food crops are contaminated with mycotoxins, depending on the mycotoxin (Eskola et al., 2020). Furthermore, due to climate change, mycotoxin contamination patterns tend to evolve, and mycotoxin contamination is expected to increase (Khodaei et al., 2021; Wang et al., 2022; Casu et al., 2024). Therefore, continuous monitoring and analysis of conventional and emerging mycotoxins is of paramount importance.

Different methods allow mycotoxin detection in food matrices. Many chromatographic technics can be used for this purpose (Vargas Medina et al., 2021). More specifically, liquid chromatography (LC) coupled with tandem mass spectrometry (UPLC-MS/MS) is one of the most widely used analytical methods for multi-targeted mycotoxin monitoring, thanks to its accuracy, even in complex matrices (Turner et al., 2015). Modern chromatographic technics can also be used to analyze mycotoxins such as nano-LC to increase sensibility and decrease sample volume. However, such technics require a higher clean-up of the sample to avoid system clogging (Vargas Medina et al., 2021). For untargeted analysis, high-resolution liquid chromatography is generally used.

Currently, mycotoxin contamination patterns in Belgium remain poorly understood. As these patterns are likely to evolve with climate change, it is important to investigate the current situation. This study provides an overview of the occurrence of mycotoxins in wheat harvested in Belgium in

2023 and 2024, two agronomic years characterized by markedly different rainfall patterns during anthesis and harvest, which are critical periods for fungal contamination and mycotoxin production. Various wheat samples were collected at diverse locations and analyzed using a validated multi-mycotoxin analysis method by UPLC-MS/MS.

Material and methods

1. Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade: Milli-Q water (Milli-Q® IQ 7010 purification system) (MilliporeSigma, Burlington, MA, USA), acetonitrile AR (Biosolve, Dieuze, France), acetonitrile MS/MS grade (Biosolve, Dieuze, France), acetic acid AR (Fisher Chemical, Merelbeke, Belgium), methanol MS/MS grade (Biosolve, Dieuze, France), formic acid (Avantor, VWR, Leuven, Belgium), ammonium acetate (Fluka, Mint Street, NC, USA), MgSO4 (Roth, Karlsruhe, Germany) and NaCl (Roth, Karlsruhe, Germany).

Mycotoxin standard solutions of known concentration for 15-acetyl-deoxynivalenol (15Ac-DON), 3-acetyl-deoxynivalenol (3Ac-DON), DON, HT2, alternariol (AOH), alternariol-monomethyl-ether (AME), T2, ZEN, OTA, OTB, FUM B1 and STE were purchased from Romer-Labs (Romer-Labs, Getzersdorf, Austria). Enniatins (A, A1, B and B1) standards were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2. Sample collection

A total of 45 samples of wheat grains from the 2023 harvest and 88 samples from the 2024 harvest were analyzed.

In 2023, the samples came from a trial network having nine different locations distributed across Belgian territory (Thorembais, Hannut, Gesves, Terwagne, Enghien, Leffinge, Poperinge, Merelbeke and Bassevelde). This trial network was set up by CRA-W and ILVO for variety registration in Belgium. These trials aim to compare varieties in contrasting pedoclimatic growth conditions and are conducted without fungicide protection or growth regulator. In addition, samples were collected only from regions where wheat is commonly cultivated. Consequently, no samples originated from the Ardennes (Southern Belgium), as fields in this region are predominantly used for pasture or for the cultivation of spelt and oats.

Varieties are cultivated in microplots (11 to 15 m2, depending on the trials) and are harvested using a special combine harvester, which allows the separate harvesting of each plot and avoids mixing the samples taken from each plot. From each location, five varieties were analyzed.

In 2024, the samples were collected from the same trial network from only eight different locations, as the Bassevelde field could not be sown due to poor climatic conditions in winter 2023. For each location, 11 varieties were collected. The different locations of sample collection are presented in Figure 1.

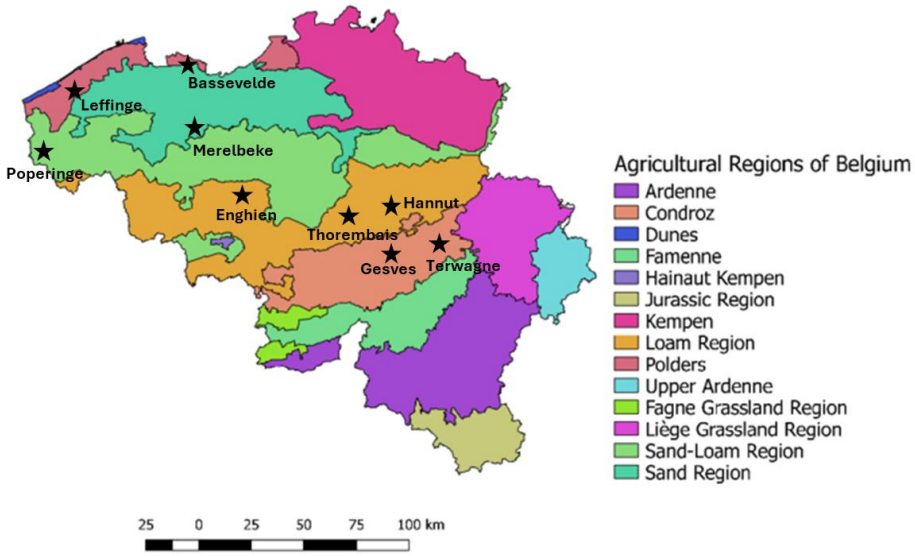


Figure 1. Pedologic map of Belgium with the location of the cultivation sites. Adapted from reference (De Frutos Cachorro et al., 2018).

The trials were sown between October and December and the samples were harvested in July and August, depending on the location. The sowing and harvest dates, the presence or absence of ploughing, and previous crops cultivated on the field are presented in Table 1.

Table 1. Agricultural data for the trial network in 2023 (a) and 2024 (b).

		(a)		
		Harvest 2023		
	Sowing Date	**Harvest Date**	**Previous Crop**	**Ploughing**
Gesves	5 November 2022	10 August 2023	Potatoes	No
Terwagne	31 October 2022	14 August 2023	Sugar beet	No
Hannut	25 October 2022	13 August 2023	Flax	No
Thorembais	26 October 2022	11 August 2023	Flax	Yes
Enghien	16 October 2022	20 July 2023	Flax	No
Merelbeke	18 October 2022	18 July 2023	Potatoes	Yes
Poperinge	19 October 2022	18 July 2023	Corn silage	Yes
Leffinge	19 October 2022	26 July 2023	Potatoes	Yes
Bassevelde	19 October 2022	19 July 2023	Grain corn	Yes
		(b)		
		Harvest 2024		
	Sowing Date	**Harvest Date**	**Previous Crop**	**Ploughing**
Gesves	18 December 2023	12 August 2024	Potatoes	Yes
Terwagne	17 October 2023	06 August 2024	Rapeseed	No
Hannut	18 December 2023	06 August 2024	Sugar beet	No
Thorembais	22 November 2023	30 July 2024	Flax	Yes
Enghien	1 December 2023	30 July 2024	Canned peas	Yes
Merelbeke	18 October 2023	29 July 2024	Potatoes	Yes
Poperinge	18 December 2023	30 July 2024	Sugar beet	Yes
Leffinge	18 October 2023	30 July 2024	Potatoes	Yes

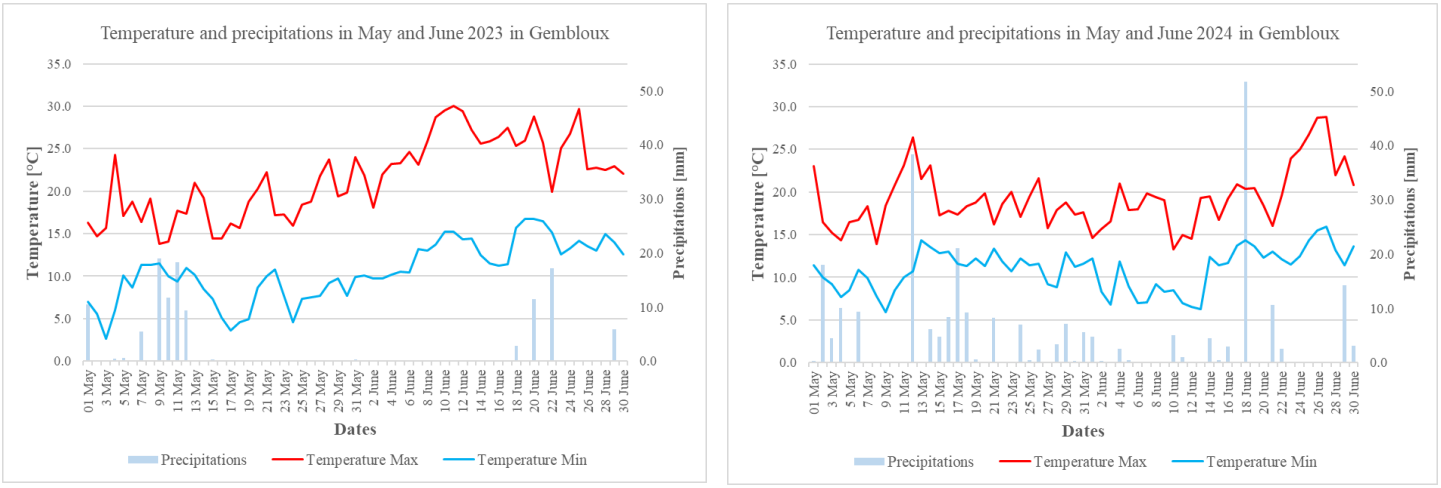
Flowering and maturity dates have been estimated based on the Phénoblé tool (CRA-W, Gembloux, Belgium). In 2023, wheat flowering occurred between the 7th and the 10th of June, and the wheat reached maturity around the 20th of July. In 2024, flowering occurred between the 2nd and the 4th of June, and maturity was reached around the 22nd of July.

3. Meteorological considerations

During the first season of sampling, October and November 2022 were dry and warm in Belgium, while December was marked by several days of frost. Precipitation remained within normal levels. On average, January 2023 was mild and wet, whereas February was particularly dry. The spring of 2023 was extremely rainy, with a colder-than-average April. From mid-May to the end of June, at the time of ear emergence and flowering of wheat crops, the weather was very dry and sunny. Late July and early August had significant rainfall, delaying much of the harvest.

During the second season of sampling, rain returned from mid-October 2023 onward, delaying most sowing activities. The winter was very wet and generally mild, except for a period of frost and snow in mid-January. Spring of 2024 was extremely rainy, as was June. However, precipitation decreased in July and August, allowing for a successful wheat harvest under good conditions.

Daily temperatures and precipitations in May and June 2023 and 2024 are presented in Figure 2.



(**a**) (**b**)

Figure 2. Temperature (°C) and precipitation (mm) in May and June 2023 (a) and 2024 (b) in Gembloux. Data from IRM station in Gembloux.

4. Sample preparation

The mycotoxin analysis was performed on whole-wheat flour, obtained after milling approximately 50 g of wheat grains using a laboratory mill (Cyclotec, FOSS, Nanterre, France). The resulting flour had a particle size under 1 mm.

The extraction procedure was adapted from the QuEChERS method (Zhang et al., 2013; Sun et al., 2016; Annunziata et al., 2017; Solfrizzo et al., 2018; González-Jartín et al., 2021).

Five grams of whole-wheat flour was weighed in Falcon tubes (50 mL) and 10 mL of water was added. After a one-hour rest, 10 mL of acetonitrile solution containing 1% acetic acid was introduced in each tube. The samples were then agitated using a Mixer Mill MM400 (Retsch GmbH, Aartselaar, Belgium) for 10 min at 10 Hz. Thereafter, 4 g of MgSO4 and 1 g of NaCl were added to the tubes. The tubes were hand shaken and then agitated using a Mixer Mill MM400 for 2 min at 10 Hz. After stirring, the samples were centrifuged for 5 min at 3000 g (Centrifuge 5810 R, Eppendorf, Leipzig, Germany).

A volume of 100 µL of the supernatants was collected and diluted with 900 µL of a 50:50 (v/v) solution of water and methanol into injection vials.

5. Standard solutions and calibration

Two mycotoxin standard mixes were constituted by diluting the initial individual standard solution in acetonitrile. The first standard mix was composed of 15Ac-DON, 3Ac-DON, DON, HT2, AOH, AME, T2, ZEN, OTA and OTB, with 15Ac-DON, 3Ac-DON, DON, HT2, AOH, AME, T2, ZEN at 100 µg/kg, DON at

1000 µg/kg and OTA and OTB at 5 µg/kg. The second standard mix was prepared including the following emerging mycotoxins: ENNs (A, A1, B, B1), FUM B1 and STE, all at 100 µg/kg.

To address potential matrix effects, matrix-matched calibration curves were established by spiking mycotoxins into a sample extract free of the targeted mycotoxins.

Two different calibration curves were produced, one with each mycotoxin standard mix. The whole-wheat flour (used as a "blank" matrix) was extracted as described above. Specified volumes of blank matrix extract were introduced in 1.5 mL Eppendorf tubes. Then, specific volumes of the mycotoxin standard mix solution were added to the Eppendorf tubes to make a total volume of 1 mL. A volume of 100 µL of the obtained solution was sampled and 900 µL of a 50:50 (v/v) solution of water and methanol was added. Then, the solutions were transferred into injection vials. The mycotoxin concentrations used for the calibration curves are shown in Table 2.

Table 2. Mycotoxin concentrations in µg/kg in the calibration curve.

	1 [µg/kg]	**2 [µg/kg]**	**3 [µg/kg]**
Standard 01	0.5	10	100
Standard 02	1.0	20	200
Standard 03	2.5	50	500
Standard 04	5.0	100	1000
Standard 05	7.5	150	1500
Standard 06	10	200	2000
Standard 07	15	300	3000

1 = ochratoxins (A and B); 2 = AME, AOH, 3Ac-DON, 15Ac-DON, T2, HT2, ZEN, Enniatins (A, A1, B, B1), FUM B1 and B2, citrinin, moniliformin, sterigmatocystin; 3 = DON.

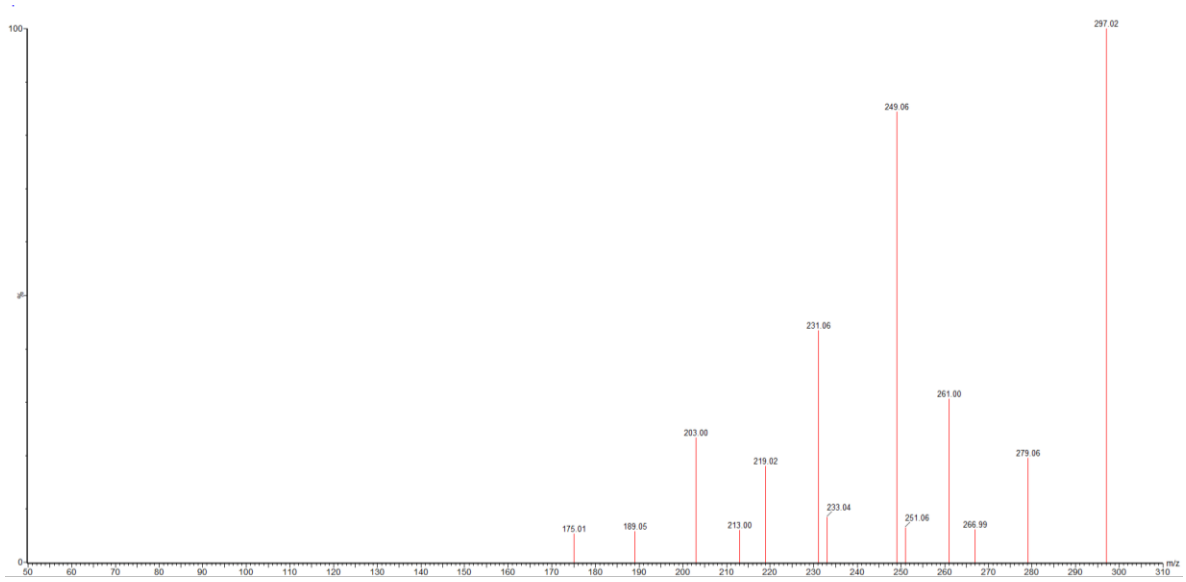
6. UPLC-MS/MS conditions

The UPLC-MS/MS conditions were optimized based on different studies such as (Zhang et al., 2013; Sun et al., 2016; Annunziata et al., 2017; Solfrizzo et al., 2018).

The samples were injected into a Waters Acquity I-Class (Waters, Milford, USA) UPLC system equipped with a BEH C18 column (2.1mm × 50 mm, 1.7 µm), coupled to a Xevo TQ-xS mass spectrometer (Waters, Milford, USA). Mobile phase A was a 10 mM ammonium acetate–water solution containing 0.1% formic acid, and mobile phase B was methanol with 0.1% formic acid. The column temperature was set at 50 °C, the flow rate was 0.3 mL/min and the injection volume was 2 µL. The elution gradient started at 10% B, held for 3 min and increased linearly to 70% in 7 min; then increased again of 10% in 10 s and kept constant for 2 min. Finally, B decreased to 10% in 10 s and equilibrated for 3 min.

The mass spectrometer was used in Multiple Reaction Monitoring (MRM) under positive ionization modes. Two transitions were selected for each mycotoxin, one for quantification and another for qualification. Regarding the mass spectrometer parameters, the capillary voltage was 3.4 kV, the desolvation temperature was 400 °C, the desolvation gas flow was 800 L/Hr, the cone gas flow was 150 L/Hr and the nebuliser pressure was 7 bar. The cone voltage and the collision energy were optimized for each transition by direct infusion. The transitions used for each mycotoxin and the associated energies and cone voltages are presented in Table 3. Examples of mass spectra for the DON and ENN B are shown in Figure 3.

(a)



(b)

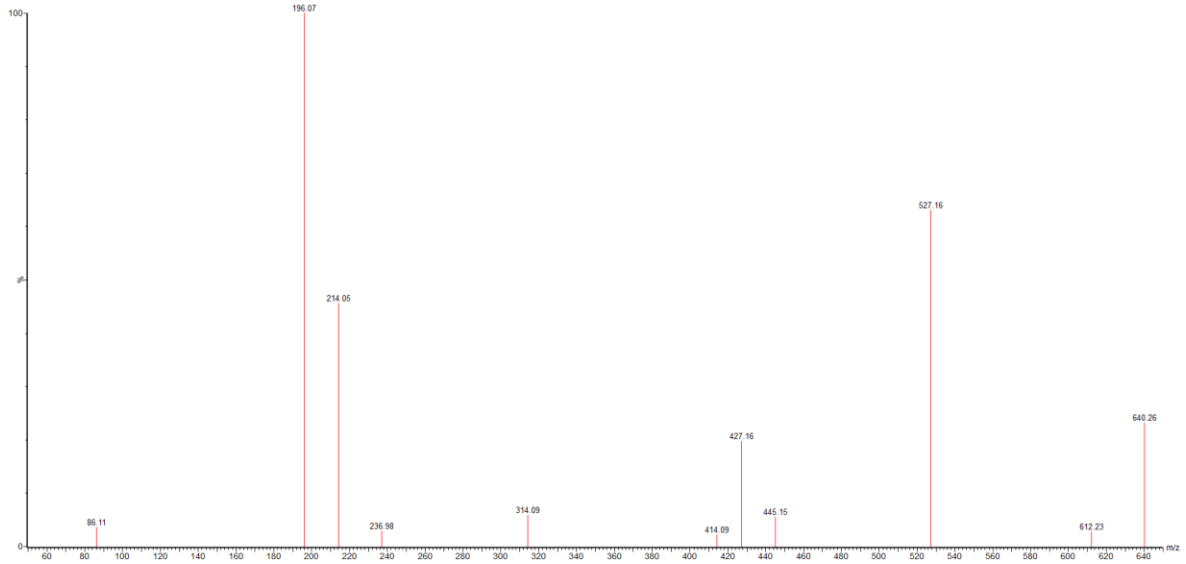


Figure 3. Mass spectra for DON (a) and ENN B (b).

Table 3. Optimized MS/MS parameters for the multi-mycotoxin detection method in MRM mode.

Mycotoxin	Molecular Mass	MODE	*m/z*	Cone (V)	Daughter 1	Energy1 (eV)	Daughter 2	Energy2 (eV)	RT (min)
AME	272.3	$[M+H]^+$	273.0	20	128.0	60	228.0	40	10.9
AOH	258.2	$[M+H]^+$	259.0	10	185.0	28	213.0	28	9.2
DON	296.3	$[M+H]^+$	297.1	15	231.1	18	249.1	18	3.9
3Ac-DON	338.4	$[M+H]^+$	339.1	25	137.0	15	203.1	12	7.0
15Ac-DON	338.4	$[M+NH_4]^+$	356.2	5	137.0	16	261.0	14	7.0
ZEN	318.4	$[M+H]^+$	319.2	20	185.1	25	187.1	19	10.6
T2	466.5	$[M+NH_4]^+$	484.2	7	215.0	18	305.1	12	10.4
HT2	424.5	$[M+NH_4]^+$	442.2	5	215.0	12	263.1	10	9.8
OTA	403.8	$[M+H]^+$	404.1	25	239.1	25	357.9	15	10.5
OTB	369.4	$[M+H]^+$	370.0	10	187.0	30	204.8	20	9.7
Enniatin A	681.9	$[M+H]^+$	682.6	30	99.6	40	209.8	25	11.8
Enniatin A1	667.9	$[M+H]^+$	669.1	30	99.6	40	210.0	25	11.7
Enniatin B	639.8	$[M+H]^+$	640.7	15	85.9	40	196.0	20	11.5
Enniatin B1	653.8	$[M+H]^+$	655.2	20	85.9	40	196.1	25	11.6
Fumonisin B1	721.8	$[M+H]^+$	722.3	15	334.3	35	352.3	35	10.0
Sterigmatocystin	324.28	$[M+H]^+$	325.0	15	253.1	38	281.1	35	10.8

7. Data treatment

For each sample, the peaks from the obtained chromatograms were integrated using TargetLynx V4.2 (Waters, Milford, USA). The calibration curves were determined by plotting the peak area of the quantification transition versus the mycotoxin concentration and using the linear regression equation y = ax + b. An average calibration curve, calculated from the calibration curves injected before and after the samples (bracketed calibration), was used for quantification.

The sample concentrations were calculated based on the average calibration curve for each mycotoxin and corrected using the exact mass of the sample intake and the recovery of each mycotoxin determined during method validation.

Recoveries were determined by spiking samples with known amounts of each mycotoxin. Samples were spiked to obtain three levels of concentration according to the MLs for the regulated mycotoxins, ML/2, ML and 1.5× ML. For non-regulated mycotoxins, the central value was 100 µg/kg. The spiked samples were then extracted as described above. For each level of concentration and for each individual sample, the recovery, expressed as a percentage, was calculated as the ratio between the calculated concentration and theoretical concentration. These recoveries are presented in Table 4. The lowest points of calibration used as the limit of quantification (LOQ) and the limit of detection (LOD) determined during method validation are also presented in Table 4. For OTA and OTB, the second-lowest point of calibration was used as the LOQ, because signal-to-noise (S/N) ratio was too low for the lowest point of calibration. The LOD was determined by extrapolating

a concentration corresponding to a S/N of 3, then experimentally verified by spiking standard solutions in blank matrices to adjusted concentrations until this ratio was achieved.

Table 4. Average recovery, limit of quantification (LOQ) and limit of detection (LOD) determined during method validation for the different mycotoxins.

Mycotoxins	Recovery [%]	LOQ [µg/kg]	LOD [µg/kg]
15Ac-DON	49	10	3.8
3Ac-DON	59	10	3.3
DON	88	100	60
AOH	90	10	8
AME	90	10	8
T2 + HT2	84	10	0.3
ZEN	96	10	2.4
OTA	104	1	0.5
OTB	99	1	0.5
ENN A	91	10	8
ENN A1	93	10	5
ENN B	93	10	1
ENN B1	92	10	6
FUM B1	58	10	3
STE	91	10	1.5

Each sample was extracted and analyzed twice. The difference between the two calculated concentrations was required not to exceed 20%. Otherwise, the sample was re-extracted in duplicate.

All the statistical analyses were conducted using RStudio, version 2024.12.0 (Posit software PBC, Boston, MA, USA). The differences between the wheat varieties and the wheat cultivation sites were assessed through a Kruskal–Wallis test followed by a Dunn test.

Results

1. *Mycotoxin occurrence in samples collected in 2023 and 2024*

A total of 133 samples were analyzed using the UPLC-MS/MS multi-mycotoxins analytical method. In 2023 (Table 5), nine out of 45 samples, corresponding to one-fifth of the total, were contaminated with mycotoxins at concentrations exceeding the LOQ. The mycotoxins detected were DON, ZEN and ENN B; the other mycotoxins included in the method were not detected in these samples. Only two samples showed co-contamination by at least two mycotoxins.

Table 5. Mycotoxin contamination of the 2023 wheat samples (n = 45).

Mycotoxin	ND (n)	<LOQ (n)	>LOQ (n)	Mean ± SD (µg/kg)	Min–Max of Samples > LOQ (µg/kg)	Median of Samples > LOQ (µg/kg)
DON	39	1	5	63 ± 104	171–567	317
ZEN	40	0	5	2.7 ± 4.4	12–18	15
ENN B	38	5	2	4.5 ± 19.7	28–131	79
ENN B1	44	1	0	3.0 ± 0.3	All < LOQ	All < LOQ

n: number of samples (n total = 45); ND: non-detected; for the calculation of the mean, the middle-bound approach was used, meaning that for ND samples, the values were LOD/2 and for samples < LOQ, the values were LOQ/2; the min, max and median included samples above the LOQ only.

In 2024 (Table 6), 74 out of 88 samples displayed higher concentrations than the LOQ for at least one mycotoxin, meaning that 84% of the samples contained quantifiable amounts of mycotoxins. Deoxynivalenol, ZEN, ENN A1, ENN B and ENN B1 were the mycotoxins detected by UPLC-MS/MS. Co-contamination occurred in 59 samples. ENN B was the most prevalent mycotoxin in the 2024 samples. It was detected in 68 samples with concentrations ranging from 12 to 488 µg/kg. Deoxynivalenol was detected in 52 samples, ENN B1 in 49, ENN A1 in 17 and ZEN in 10 samples. One sample was contaminated with DON at 1479 µg/kg, exceeding the level of 1000 µg/kg fixed by the European regulation for grains intended to be used for food [20]. No sample was over the limit for ZEN as the maximum level detected was 55 µg/kg, while the ML set in the European legislation is 100 µg/kg [19]. The maximum levels detected for ENN A1, B and B1 were 49, 488 and 185 µg/kg, respectively. Currently, ENNs are not regulated in Europe. Mycotoxin contamination was higher in 2024 than in 2023, in terms of both the number of contaminated samples and the contamination levels.

Table 6. Mycotoxin contamination of the 2024 wheat samples (n = 88).

Mycotoxin	ND (n)	<LOQ (n)	>LOQ (n)	Mean ± SD (µg/kg)	Min–Max of Samples > LOQ (µg/kg)	Median of Samples > LOQ (µg/kg)
DON	11	25	52	255 ± 268	131–1478	370
ZEN	53	25	10	5.4 ± 9.5	16–55	28
ENN A1	35	36	17	6.5 ± 7.0	10–49	14
ENN B	3	17	68	89 ± 109	12–488	66
ENN B1	21	18	49	29 ± 37	7–185	38

n: number of samples (n total = 88); ND: non-detected; for the calculation of the mean, the mid-dle-bound approach was used, meaning that for ND samples, the values were LOD/2 and for samples < LOQ, the values were LOQ/2; the min, max and median included samples above the LOQ only.

As few samples from the 2023 harvest were positive or had mycotoxin concentrations exceeding the LOQ, no further data analysis was performed. In 2024, most of the samples were contaminated by at least one mycotoxin, allowing for an assessment of the differences in contamination between varieties and cultivation sites. All the data are in the Supplementary Materials (Tables S1 and S2).

2. Differences in concentration between the varieties in 2024 wheat samples

For each mycotoxin, the differences in mycotoxin concentration between the varieties were assessed, as 11 different varieties of wheat were cultivated in each location. Hence, eight samples of each variety were available to be analyzed by UPLC-MS/MS. The results are shown in Figure 4 and the Supplementary Material (Table S2). The significance of the differences between the varieties was evaluated by performing a Kruskal–Wallis test (Table 7).

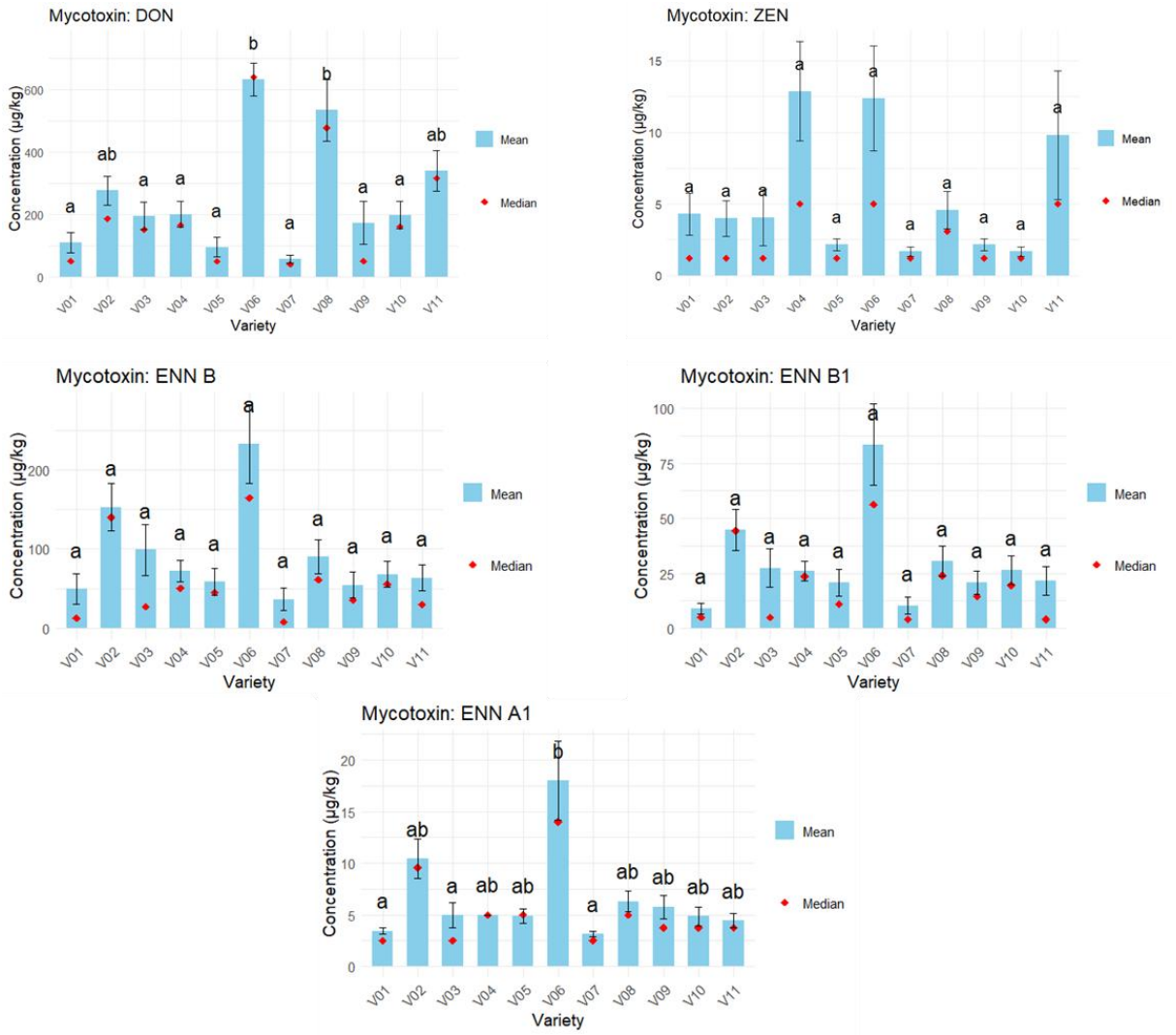


Figure 4. Mycotoxin contamination depending on the variety (n = 8 per variety) for 2024 wheat samples. Significance letters were assigned using a Dunn test performed after a Kruskal–Wallis test. Varieties sharing the same letter (a, ab or b) are not significantly different (p > 0.05). For the calculation of the mean, the middle-bound approach was used, meaning that for samples where the mycotoxin was not detected, the values were LOD/2 and for samples < LOQ, the values were LOQ/2.

Table 7. Significance of the differences of mycotoxin contamination between the varieties in 2024 (Kruskal–Wallis test).

	Mean Concentration ± SD (µg/kg)	**Kruskal–Wallis X^2**	**df**	***p*-Value**	**Significance**
DON	255 ± 268	42.239	10	6.80×10^{-6}	***
ZEN	5.4 ± 9.5	23.644	10	0.008604	**
ENN A1	6.5 ± 7.0	24.693	10	0.005958	**
ENN B	89 ± 109	16.969	10	0.07506	n.s.
ENN B1	29 ± 37	19.086	10	0.03918	*

df: degree of freedom. The differences between the varieties are considered significant (*) for p-value < 0.05, (**) for p-value < 0.01, (***) for p-value < 0.001, n.s. = non- significant.

For ENN B, there was no significant difference in concentration between the varieties ($p < 0.05$). For the other mycotoxins (DON, ZEN, ENN B1 and ENN A1), a significant difference between the varieties was found.

Statistically, the DON concentration was higher in varieties 06 and 08. In addition, variety 06 was more contaminated with ENN A1 than the other varieties. For ENN B, ENN B1 and ZEN, the mycotoxin concentrations in the different varieties could not be statistically differentiated. However, variety 06 had the higher means among the analyzed varieties for ENN B and ENN B1. Therefore, within the scope of this work, considering the specific conditions of the 2024 study, variety 06 appears to be at a higher risk for mycotoxin contamination. On the other hand, variety 07 is among the least contaminated varieties for each mycotoxin; this variety seems to be more resistant to mycotoxin contamination. In addition, field observations are consistent with these results, as almost no wheat ears showed symptoms from Fusarium infection. However, these results should be confirmed with additional trials during subsequent years.

3. Differences in mycotoxin concentration between the cultivation sites in 2024 wheat samples

The location effect was studied by comparing the contamination level for each mycotoxin among the 11 varieties harvested in each location (Figure 5). The significance of the differences between the locations was evaluated by a Kruskal–Wallis test (Table 8).

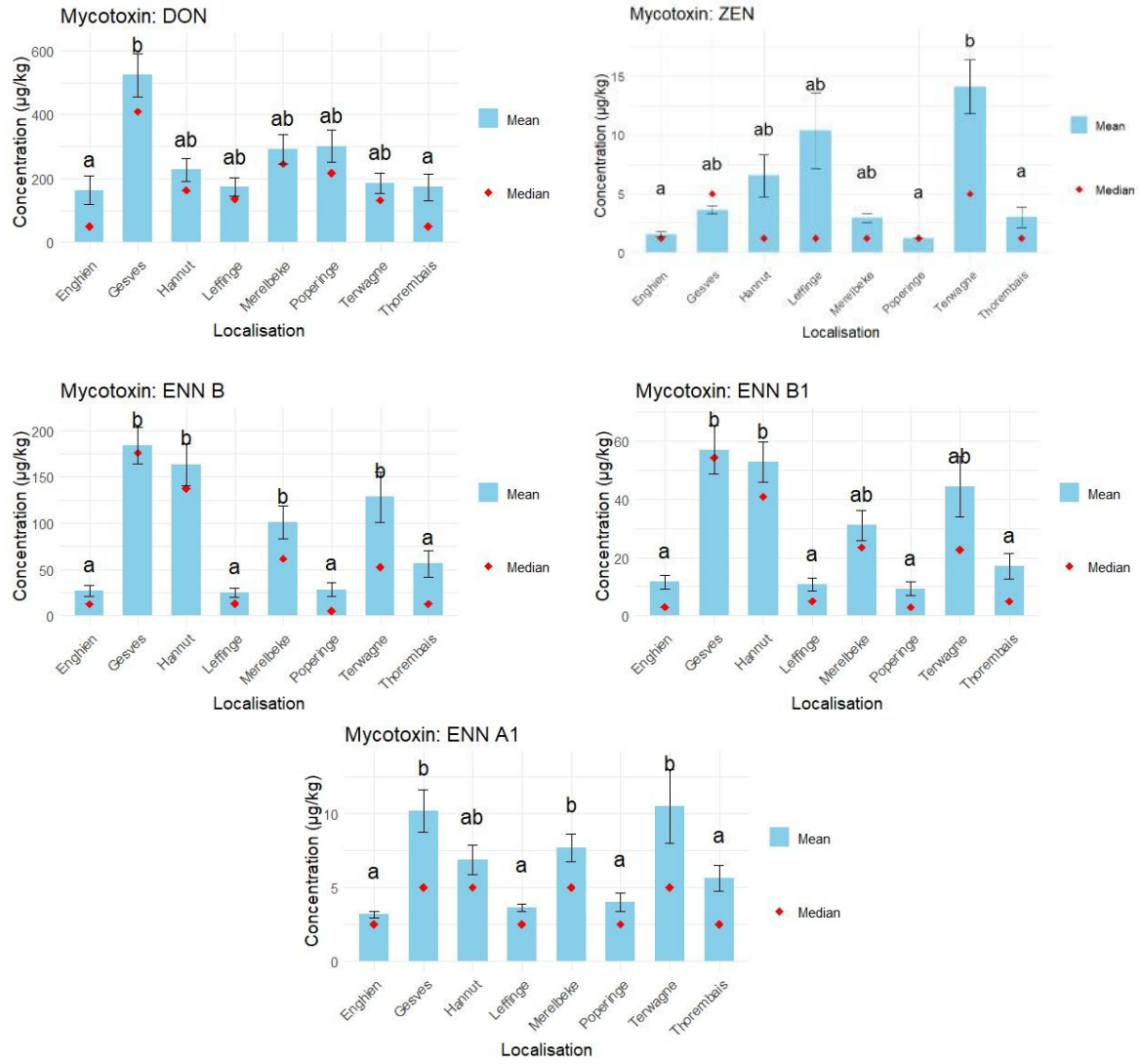


Figure 5. Mycotoxin contamination depending on the location for 2024 wheat samples (n = 11 per location). Significance letters were assigned using a Dunn test performed after a Kruskal–Wallis test. Varieties sharing the same letter (a, ab or b) are not significantly different (p > 0.05). For the calculation of the mean, the middle-bound approach was used, meaning that for samples where the mycotoxin was not detected, the values were LOD/2 and for samples < LOQ, the values were LOQ/2.

Table 8. Significance of the differences of mycotoxin contamination of wheat between the cultivation sites in 2024 (Kruskal–Wallis test).

	Mean Concentration ± SD (µg/kg)	Kruskal–Wallis X^2	df	*p*-Value	Significance
DON	255 ± 268	15.108	7	0.03464	*
ZEN	5.4 ± 9.5	29.384	7	0.0001231	***
ENN A1	6.5 ± 7.0	25.715	7	0.0005662	***
ENN B	89 ± 109	40.970	7	8.21×10^{-7}	***
ENN B1	29 ± 37	34.123	7	1.63×10^{-5}	***

df: degree of freedom. The differences between the varieties are considered significant (*) for p-value < 0.05, (***) for p-value < 0.001.

For each mycotoxin, the cultivation site had a significant effect on the concentration. Within the scope of this work, for DON contamination, the most critical cultivation site was Gesves, and for ZEN it was Terwagne. For ENNs, mycotoxin contamination was significantly higher in Gesves, Hannut,

Terwagne and Merelbeke. In 2024, Gesves seemed to be the most affected growing site for all mycotoxins. On the other hand, for ENNs, Enghien, Leffinge, Poperinge and Thorembais were less affected by mycotoxin contamination. For DON and ZEN, the sites the least affected by mycotoxin contamination were Enghien and Thorembais. Poperinge was not subjected to ZEN contamination at all.

Discussion

1. *Effect of the year of harvest of wheat on the mycotoxin contamination*

Samples collected from the 2024 harvest were more contaminated by mycotoxins in terms of number of contaminated samples and level of contamination. However, only one sample was contaminated at a level exceeding the European regulation for food. As reported in the literature (Marroquín-Cardona et al., 2014; Perrone et al., 2020; Platzer et al., 2025), rainy conditions and high relative humidity during the flowering of the wheat may enhance fungal contamination and mycotoxin production. Hence, the higher contamination of the 2024 samples (approximatively 86%) compared to those from 2023 (20% of positive samples) may be explained by weather conditions, as the period from mid-May to June 2023 was dry in Belgium, whereas the spring of 2024 was rainy. However, European data on mycotoxin contamination in cereals are available and indicate no significant difference in contamination levels between 2023 and 2024. For example, for DON, Cargill reported that 88% of samples were contaminated in both 2023 and 2024, while dsm-Firmenich reported 77% in 2023 and 76% in 2024 (Cargill, 2024; dsm-firmenich, 2024). In contrast, the present study, conducted within a more geographically restricted area, reveals greater interannual variation, likely due to the stronger influence of local weather conditions on fungal development and mycotoxin production.

Other factors may explain the difference in mycotoxin contamination of wheat between these two years. Climate change is often cited as a factor influencing mycotoxin contamination patterns, as shifts in temperature and seasonal variations can alter the wheat growth cycle, potentially making it more susceptible to contamination (Medina et al., 2015; Liu and Van der Fels-Klerx, 2021; Medina, 2023). However, to have a better understanding of the impact of climate changes on mycotoxin contamination of wheat in Belgium, this study should be continued for several years.

Both in 2023 and 2024, the same mycotoxins were detected except for ENN A1, which was detected only in 2024. DON and ZEN are commonly present in wheat (Perrone et al., 2020), and this study

confirms this observation. Enniatins were the most prevalent mycotoxins in the samples harvested in 2024, whereas they were only detected in seven samples in 2023. Enniatins are considered emerging mycotoxins as they are not regulated or routinely analyzed. However, there is a growing concern about these mycotoxins as they are more often detected and at a higher concentration (Medina, 2023). dsm-Firmenich highlights a notable global presence of ENN B, supporting the observation made in this study of increased ENN B levels in Belgian wheat samples from 2024 (dsm-firmenich, 2024). The EFSA report from 2014 indicates that acute exposure to ENNs is not a concern for human health (EFSA, 2014). However, the data are not sufficient to draw firm conclusions about the chronic exposure for these mycotoxins. Therefore, it is relevant to gather contamination data if a new risk assessment is performed.

2. *Differences in mycotoxin concentration between the wheat varieties*

Significant differences in concentration for DON, ZEN, ENN A1 and ENN B1 were observed between the wheat varieties ($p < 0.05$). For each mycotoxin, the differences in mycotoxin concentration between the varieties were assessed, as 11 different varieties of wheat were cultivated in each location. Hence, eight samples of each variety were available to be analyzed by UPLC-MS/MS. The results are shown in Figure 4 and the Supplementary Material (Table S2). The significance of the differences between the varieties was evaluated by performing a Kruskal–Wallis test (Table 7). This highlights that certain varieties may be more susceptible to mycotoxin contamination than others. For example, studies showed that lower anther retention is linked with high resistance to fungal infection (Michel et al., 2024). These trials should be repeated over several years to confirm that the observations are due to varietal effect and not only an annual effect. Such differences in susceptibility could be attributed to genetic factors influencing fungal infection and toxin production. These findings emphasize the importance of selecting and breeding varieties with enhanced fungal resistance, which is one of the criteria for the acceptance and commercialization of new varieties. Incorporating resistance traits into breeding programs contributes to reducing mycotoxin contamination and improving the overall safety and quality of harvested grains. The selection of mycotoxin-resistant varieties represents a more effective strategy than fungicide application for reducing the risk of contamination.

3. *Differences in mycotoxin concentration between the wheat cultivation sites*

There is a significant effect of location on the mycotoxin presence in wheat. The location effect was studied by comparing the contamination level for each mycotoxin among the 11 varieties harvested

in each location (Figure 5). The significance of the differences between the locations was evaluated by a Kruskal–Wallis test (Table 8).

The most impacted site in 2024 was Gesves, which had been subjected to heavy rainfall and mudflows that had damaged the cultivation site. Other significantly affected sites included Hannut, Terwagne and Merelbeke. Each site belongs to a different pedological region, except for Gesves and Terwagne, which are situated in Condroz. From these data, no clear relationship can be established between the pedological region and mycotoxin contamination. Based on crop history data from the past 7 years, the Terwagne site has frequently been cultivated with wheat and silage maize. This cropping pattern may have contributed to the buildup of a local inoculum. In contrast, silage maize was not present at the other Walloon sites.

At this stage, no obvious link can be established with the previous crop, sowing, ploughing or harvest dates. It could be interesting to investigate whether the same sites are the most affected by fungal contamination and mycotoxin presence each year, while others appear to be better preserved. Therefore, more data should be collected in the coming years to draw broader conclusions about the link between mycotoxin contamination and agricultural factors, helping to mitigate specific effects.

Conclusions

This study provides updated data on mycotoxin contamination in wheat harvested in Belgium during the 2023 and 2024 growing seasons. A total of 113 samples were analyzed, revealing a higher occurrence of mycotoxins in 2024, particularly ENNs. Notably, one sample exceeded the European ML for DON in 2024, while all the samples from 2023 remained below the regulatory thresholds. These findings underscore the strong influence of weather conditions, especially the rainy period during wheat flowering in 2024, which likely promoted fungal growth and subsequent mycotoxin production. Although climate change is frequently cited as a driver of such contamination patterns, long-term monitoring will be essential to better evaluate its specific impact in Belgium.

The results also highlight the importance of wheat variety selection and cultivation site conditions in managing mycotoxin risk. Certain varieties showed different levels of susceptibility to contamination, reinforcing the need for breeding programs focused on improving resistance to fungal pathogens. Additionally, annual variations in climate can significantly influence mycotoxin

development, making some locations appear more or less favorable depending on specific weather events rather than inherent site characteristics.

By providing a two-year comparative dataset, this study provides an overview of mycotoxin occurrence in wheat in Belgium. Multiple factors may influence the mycotoxin presence in crops, such as weather (temperature and humidity), agricultural practices, previous crops, pests, etc., making it difficult to fully understand the underlying mechanisms. To better understand these trends and develop effective mitigation strategies, further data collection over multiple years is essential. This will provide a clearer picture of potential contamination patterns and help refine agricultural practices to minimize mycotoxin risks and ensure safer wheat production in the future.

Supplementary materials

Table S1: Average mycotoxin concentrations (µg/kg) of 2023 wheat samples (n=2).

DON	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke	Watervliet
V01	ND	351 ± 7	214 ± 3	ND	ND	ND	ND	ND	ND
V02	317 ± 11	ND	ND	ND	ND	171 ± 15	ND	ND	ND
V03	ND	ND	ND	ND	ND	ND	ND	ND	ND
V04	ND	ND	ND	ND	ND	567 ± 22	ND	ND	ND
V05	ND	ND	ND	ND	ND	<LOQ	ND	ND	ND

ZEN	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke	Watervliet
V01	ND	18	ND	ND	ND	ND	ND	ND	ND
V02	15 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
V03	ND	ND	12 ± 1	ND	ND	ND	ND	ND	ND
V04	16 ± 1	ND	13 ± 3	ND	ND	ND	ND	ND	ND
V05	ND	ND	ND	ND	ND	ND	ND	ND	ND

ENN B	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke	Watervliet
V01	ND	131 ± 7	ND	ND	ND	ND	ND	<LOQ	ND
V02	ND	28 ± 4	< LOQ	ND	ND	ND	ND	<LOQ	ND
V03	ND	ND	ND	ND	ND	ND	ND	ND	ND
V04	ND	ND	<LOQ	< LOQ	ND	ND	ND	ND	ND
V05	ND	ND	ND	ND	ND	ND	ND	ND	ND

ENN B1	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke	Watervliet
V01	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND
V02	ND	ND	ND	ND	ND	ND	ND	ND	ND
V03	ND	ND	ND	ND	ND	ND	ND	ND	ND
V04	ND	ND	ND	ND	ND	ND	ND	ND	ND
V05	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table S2: Average mycotoxin concentrations (µg/kg) of 2024 wheat samples (n=2).

DON	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke
V01	< LOQ	ND	361 ± 28	< LOQ	ND	ND	< LOQ	277 ± 26
V02	165 ± 3	161 ± 56	649 ± 38	131 ± 1	200 ± 41	172 ± 1	217 ± 5	508 ± 70
V03	< LOQ	533 ± 65	150 ± 9	379 ± 66	< LOQ	217 ± 2	ND	150 ± 29
V04	< LOQ	245 ± 20	382 ± 27	495 ± 3	131 ± 6	< LOQ	198 ± 1	< LOQ
V05	< LOQ	< LOQ	409 ± 102	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
V06	749 ± 87	568 ± 16	869 ± 42	488 ± 14	887 ± 43	264 ± 61	710 ± 50	519 ± 40
V07	ND	< LOQ	177 ± 1	< LOQ	ND	ND	ND	< LOQ
V08	485 ± 52	447 ± 30	1479 ± 49	161 ± 1	257 ± 2	477 ± 10	485 ± 85	476 ± 52
V09	ND	ND	199 ± 36	ND	< LOQ	136 ± 9	850 ± 68	< LOQ
V10	< LOQ	163 ± 12	569 ± 63	158 ± 4	< LOQ	< LOQ	290 ± 13	247 ± 25
V11	196 ± 2	226 ± 1	520 ± 44	< LOQ	< LOQ	426 ± 24	405 ± 54	834 ± 39

ZEN	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke
V01	ND	ND	< LOQ	18 ± 1	ND	ND	ND	< LOQ
V02	ND	ND	< LOQ	16 ± 12	ND	< LOQ	ND	ND
V03	ND	ND	ND	24 ± 4	ND	ND	ND	ND
V04	17 ± 2	< LOQ	< LOQ	31 ± 2	ND	37 ± 3	ND	< LOQ
V05	ND	< LOQ	ND	< LOQ	ND	ND	ND	ND
V06	< LOQ	33 ± 1	< LOQ	40 ± 3	< LOQ	< LOQ	ND	< LOQ
V07	ND	ND	ND	< LOQ	ND	ND	ND	ND
V08	ND	17 ± 1	< LOQ	ND	ND	< LOQ	ND	< LOQ
V09	ND	ND	< LOQ	< LOQ	ND	ND	ND	ND
V10	ND	ND	ND	< LOQ	ND	ND	ND	ND
V11	ND	< LOQ	< LOQ	< LOQ	ND	55 ± 10	ND	< LOQ

ENN A1	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke
V01	ND	< LOQ	< LOQ	< LOQ	ND	ND	ND	ND
V02	ND	< LOQ	14 ± 1	24 ± 6	ND	< LOQ	14 ± 1	16 ± 1
V03	ND	< LOQ	ND	< LOQ	ND	ND	ND	17 ± 1
V04	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
V05	ND	< LOQ	12 ± 3	< LOQ	ND	< LOQ	ND	< LOQ
V06	14 ± 2	21 ± 2	31 ± 4	49 ± 4	< LOQ	< LOQ	< LOQ	14 ± 1
V07	ND	ND	< LOQ	ND	ND	ND	ND	< LOQ
V08	ND	11 ± 1	14 ± 1	< LOQ	< LOQ	< LOQ	ND	< LOQ
V09	12 ± 2	ND	14 ± 3	< LOQ	ND	ND	ND	< LOQ
V10	14 ± 1	ND	< LOQ	< LOQ	ND	ND	ND	< LOQ
V11	ND	10 ± 1	< LOQ	< LOQ	ND	ND	ND	< LOQ

ENN B	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke
V01	< LOQ	92 ± 2	224 ± 50	32 ± 1	13 ± 3	13 ± 3	< LOQ	12 ± 1
V02	165 ± 1	137 ± 28	137 ± 4	422 ± 8	17 ± 3	39 ± 6	142	163 ± 47
V03	24 ± 5	207 ± 21	31 ± 4	138 ± 44	< LOQ	13 ± 1	< LOQ	368 ± 27
V04	< LOQ	57 ± 5	173 ± 21	124 ± 18	41 ± 7	99 ± 11	44 ± 1	34 ± 5
V05	< LOQ	101 ± 1	209 ± 11	52 ± 1	< LOQ	52 ± 2	< LOQ	38 ± 1
V06	228 ± 27	488 ± 15	474 ± 9	436 ± 55	70 ± 9	20 ± 1	46 ± 1	101 ± 1
V07	ND	43 ± 8	176 ± 2	12 ± 1	< LOQ	< LOQ	< LOQ	46 ± 4
V08	< LOQ	258 ± 22	180 ± 10	61 ± 12	103 ± 5	25 ± 8	29 ± 2	62 ± 6
V09	32 ± 4	63 ± 9	195 ± 35	38 ± 7	< LOQ	ND	< LOQ	98 ± 26
V10	134 ± 10	194 ± 21	73 ± 9	53 ± 1	27 ± 3	ND	< LOQ	59 ± 5
V11	13 ± 1	154 ± 4	148	41 ± 13	< LOQ	< LOQ	19 ± 2	126 ± 4

ENN B1	Thorembais	Hannut	Gesves	Terwagne	Enghien	Leffinge	Poperinge	Merelbeke
V01	ND	32 ± 5	67 ± 10	14 ± 1	ND	< LOQ	ND	< LOQ
V02	< LOQ	41 ± 1	50 ± 1	124 ± 2	< LOQ	22 ± 5	48 ± 12	63 ± 8
V03	< LOQ	56 ± 3	< LOQ	44 ± 10	ND	< LOQ	ND	99 ± 3
V04	< LOQ	22 ± 1	57 ± 8	44 ± 5	25 ± 1	36 ± 1	14 ± 1	< LOQ
V05	ND	35 ± 1	73 ± 15	17 ± 2	ND	28 ± 3	ND	< LOQ
V06	75 ± 8	144 ± 2	179 ± 4	185 ± 1	30 ± 2	< LOQ	12 ± 1	38 ± 3
V07	ND	< LOQ	50 ± 6	< LOQ	ND	ND	ND	11 ± 1
V08	< LOQ	80 ± 4	65 ± 1	24 ± 4	36 ± 2	< LOQ	< LOQ	24 ± 3
V09	26 ± 6	24 ± 3	64 ± 10	< LOQ	ND	ND	ND	37 ± 3
V10	53 ± 1	79 ± 2	22 ± 2	23 ± 1	13 ± 1	ND	ND	17 ± 2
V11	ND	63 ± 1	54 ± 2	ND	ND	ND	< LOQ	38 ± 2

Experimental section

Study 3: Sorting of mycotoxins contaminated wheat batches: The case of deoxynivalenol and ochratoxin A

Preamble

For this third study, the impact of different sorting processes on mycotoxin reduction was investigated for two specific mycotoxins. The first was deoxynivalenol, a field mycotoxin frequently detected in Belgian wheat. Based on the results from Study 2, deoxynivalenol was found in 60 % of the wheat samples collected in 2024, with one sample exceeding the maximum limit established by European legislation. Deoxynivalenol is produced by *Fusarium* species before harvest if environment conditions are favourable.

The second mycotoxin selected was ochratoxin A, produced by *Aspergillus* species. In contrast to deoxynivalenol, ochratoxin A is primarily produced when storage conditions are not suitable. Elevated ochratoxin A concentrations can occasionally be observed, making the development of efficient sorting strategies particularly relevant to prevent downgrading or rejection of entire grain batches.

Since sorting procedures are generally based on physical symptoms of fungal infection rather than on the direct quantification of mycotoxins, it is both relevant and scientifically interesting to evaluate their effectiveness for two mycotoxins produced by different fungal genera and occurring at distinct stages of the production chain.

Regarding the sorting technologies investigated, both low-cost options, such as size and density-based sorters, and more advanced systems, such as near-infrared spectroscopy sorter, were assessed to compare their efficiency and potential applicability at the industrial scale.

Experimental section

Study 3: Sorting of mycotoxins contaminated wheat batches: The case of deoxynivalenol and ochratoxin A

Abstract

Deoxynivalenol is a mycotoxin mainly produced by *Fusarium graminearum* mostly during pre-harvest and harvest stages. Deoxynivalenol concentration in wheat is regulated by the European Commission with a maximum level of 1000 µg/kg. Ochratoxin A is a mycotoxin that is mainly produced by *Aspergillus* spp. when storage conditions are not appropriate. Ochratoxin A is classified by the IARC in the group 2b which are possibly carcinogenic compounds to humans and is regulated at 5 µg/kg in wheat in Europe.

Different strategies are used to limit the presence of mycotoxins in food and feed ingredients, such as the selection of resistant varieties, the application of fungicides, and good agricultural and storage practices. However, mycotoxins presence in food is unavoidable. Mycotoxin contamination in batches is not homogeneous and, only a few grains with high mycotoxin concentration can contaminate a whole batch. Therefore, sorting strategies have been investigated as a tool to reduce the contamination of batches.

In this study, an evaluation of the efficiency of three different sorting methods for the reduction of deoxynivalenol and ochratoxin A concentrations in wheat grain batches with different initial levels of concentration was performed. Kernels were sorted according to their size, their density and their infra-red spectra. Mycotoxins concentrations of the different fractions were assessed by UPLC-MS/MS.

For deoxynivalenol discrimination, near infrared single-kernel sorting with the developed *Fusarium*-damaged kernel model emerged as the most promising strategy, as it maximized the recoverable fraction while effectively excluding the most contaminated kernels. By excluding 20% of the most damaged kernels, the deoxynivalenol concentrations were nearly tripling in the rejected fraction, while the remaining fraction concentration was below the maximum level. In contrast, the cleaner separator and the gravity table could serve as cost-effective pre-cleaning steps, although their stand-alone efficiency remains partial. Regarding ochratoxin A, all tested sorting devices showed only limited effectiveness. The development of ochratoxin A-specific spectral models, or alternative discrimination criteria, should therefore be further investigated to improve removal efficiency.

Key words: Mycotoxins, wheat, sorting, infrared single-kernel sorting, UPLC-MS/MS.

Introduction

Mycotoxins are secondary metabolites produced by several fungal species such as *Aspergillus* spp., *Alternaria* spp., *Fusarium* spp., and *Penicillium* spp. (Marin et al., 2013; Tola and Kebede, 2016). Currently, more than 400 mycotoxins have been reported in the literature, and the most studied are deoxynivalenol (DON), zearalenone (ZEN), aflatoxins (AFs) and ochratoxin A (OTA) because of their occurrence, toxicity and as they are regulated in Europe (Pereira et al., 2014; Smith et al., 2016).

Mycotoxins represent a huge risk for food safety worldwide as they are found in many food stuffs, raw ingredients and processed food, such as coffee, cereals, spices, etc., (Marin et al., 2013; Alshannaq and Yu, 2017; Iqbal, 2021; Khan et al., 2024) and as they can have adverse effects on animal and human health (Ji et al., 2019; Khan et al., 2024). Mycotoxins can lead to acute or chronic diseases and can have mutagenic, hepatotoxic, neurotoxic and carcinogenic effects (Ji et al., 2019; Ekwomadu et al., 2021; De Felice et al., 2023). In addition, fungal contamination of crops can lead to a decrease of the production yield or to the loss of the production due to high mycotoxins concentration (Pitt and Miller, 2017; Khodaei et al., 2021). Hence, it can have a huge economic impact on agriculture and food industries.

Mycotoxins contamination may occur during pre-harvest, harvest and storage stages. In the field, fungal contamination is influenced by weather conditions, more specifically, the humidity and the temperature, and agricultural practices (Mousavi Khaneghah et al., 2019; Mahato et al., 2021).

Deoxynivalenol is mainly produced by *Fusarium graminearum* and mostly occur during pre-harvest and harvest stages. International Agency for Research on Cancer (IARC) classified DON in the group 3, which are those that can not be classified in regard to their carcinogenicity to humans, because of insufficient data (International Agency for Research on Cancer, 1993). Chronic ingestion of DON may lead to reduced weight gain, and growth hormone production fluctuations (Marin et al., 2013; Ekwomadu et al., 2021). On the other hand, acute exposure may lead to gastroenteritis and emesis (Marin et al., 2013; Ekwomadu et al., 2021). *Fusarium* infection is enhanced by heavy rains during flowering, as air humidity increases. In addition, *Fusarium* growth increases in temperature range between 20°C and 30°C (Marroquín-Cardona et al., 2014). In Europe, DON concentration is regulated in food. In wheat, the maximum level (ML) of DON is 1000 µg/kg (European Commission, 2024c).

Ochratoxin A is a mycotoxin that is mainly produced by *Aspergillus* spp. (EFSA, 2020; Kumar et al., 2020). It is classified by the IARC in the group 2b which are possibly carcinogenic compounds to

humans (International Agency for Research on Cancer, 1993). Ochratoxin A contamination may occur in the field, especially in hot-tropical region dehydrated (Alshannaq and Yu, 2017; Kumar et al., 2020). However, OTA contamination mainly occurs during post-harvest stage, when storage conditions are inadequate, for example when the crops are not sufficiently dried (Schaarschmidt and Fauhl-Hassek, 2018). Water activity, moisture content and temperature will influence the presence of the toxigenic fungus producing OTA. Optimal temperature for OTA production is between 25 and 30°C for *Aspergillus ochraceus* and *Aspergillus niger* (Ben Miri et al., 2024). A water activity above 0.95 will also enhance fungal development and OTA production (Ben Miri et al., 2024). European regulation fixed the ML of OTA in wheat at 5 µg/kg (European Commission, 2023c).

Wheat is one of the most widely cultivated crops worldwide. In the world, the average yearly consumption per capita of wheat is 65.6 kg (Erenstein et al., 2022). In Europe, wheat production stands for an average of 26 million of cultivated hectares, with 254.6 million tons for food and feed ingredients (Food and Agriculture Organization of the United Nations, 2025). In Europe, 43.8% of wheat production is intended for food and 36% for feed. The remaining 20.2% are used for seed, other uses or subjected to losses (Erenstein et al., 2022). In Wallonia, the southern part of Belgium, only 10% of the wheat is intended for food, 45% is used for feed and 45% for fuel production (Delcour et al., 2014).

Mycotoxins are widely found in wheat. The most prevalent are DON, ZEN, T2, HT2, AFs and OTA (Khodaei et al., 2021). Mycotoxin contamination is one of the most important issues regarding food safety. Indeed, 60 to 80% of the agricultural crops are contaminated by one or more mycotoxins (Eskola et al., 2020). In addition, due to climate changes, contamination patterns are expected to evolve, and mycotoxin contamination to increase in Europe (Khodaei et al., 2021; Wang et al., 2022; Casu et al., 2024).

Different strategies are used to limit the presence of mycotoxins in food and feed ingredients, such as the selection of resistant varieties, the application of fungicides, and good agricultural and storage practices (Kumar et al., 2020; Khan et al., 2024). However, it is still not possible to guarantee that the concentration of mycotoxins in raw ingredients remains below legal levels. On the other hand, mycotoxins are thermally stable and their elimination is difficult or impossible (Ben Miri et al., 2024). Decontamination methods have been studied but they rarely lead to an elimination of the mycotoxins (Khan et al., 2024). Furthermore, dilution of contaminated batches into less contaminated ones and chemical detoxification are prohibited by European regulation (European Commission, 2023c).

Mycotoxin contamination in batches is not homogeneous (Nelson, 2016; Schaarschmidt and Fauhl-Hassek, 2018) and, only a few grains with high mycotoxin concentration can contaminate a whole batch. Therefore, sorting strategies have been investigated as a tool to reduce the contamination of batches. Traditionally, hand-sorting was used and proved effective in removing up to 90% of highly contaminated kernels (Peng et al., 2018). However, this method is not feasible on an industrial scale. In larger scale, mechanical sorting can be performed according to the shape, size, density and air resistance of the grains (Cheli et al., 2013; Schaarschmidt and Fauhl-Hassek, 2018).

Removing the materials adhering to the grain surface may lead to a decrease of mycotoxin contamination (Schaarschmidt and Fauhl-Hassek, 2018). Density sorting is based on the hypothesis that fungal infection of the kernel may lead to the decrease of its density (Peng et al., 2018). Optical sorting is based on the grains characteristics such as size, shape, colour, defects. Spectral data can also be used to identify *Fusarium* damaged kernel (Schaarschmidt and Fauhl-Hassek, 2018).

However, pre-milling sorting efficiency is very variable depending on the contamination and its extend, the initial grain conditions and the type of cleaning processes used (Cheli et al., 2013; Peng et al., 2018). In addition, if the initial mycotoxin concentrations are too high, sorting may not be sufficient to lower the concentration below the European levels.

In this study, an evaluation of the efficiency of three different sorting methods for the reduction of DON and OTA concentrations in wheat grain batches with different initial levels of concentration was performed. Kernels were sorted according to their size, their density and their infra-red spectra. Mycotoxins concentrations of the different fractions were assessed by UPLC-MS/MS.

Materials and methods

1. Chemicals and reagents

For this study, chemicals and reagents were all of analytical grade. Milli-Q water was obtained through Milli-Q® IQ 7010 purification system from MilliporeSigma (Burlington, USA). Acetonitrile AR, acetonitrile MS/MS grade and methanol MS/MS grade were purchased from Biosolve (Dieuze, France). Acetic acid AR was obtained from Fisher Chemical (Merelbeke, Belgium), formic acid from Avantor (Leuven, Belgium) and ammonium acetate from Fluka (Mintstreet, USA). Anhydrous $MgSO_4$ and anhydrous NaCl were purchased from Roth (Karlsruhe, Germany).

Mycotoxin standard solutions of known concentration for DON and OTA were purchased from Romer Labs (Getzersdorf, Austria).

2. Single kernel analysis

Four wheat samples originating from *Fusarium* spp. inoculation trials in Gembloux (Wallonia, Belgium) were collected. Field inoculation of wheat was carried out using a spray boom. Inoculations took place early in the morning to take advantage of the presence of dew and the natural opening of the wheat spikes, both of which favour infection. The inoculum consisted of an aqueous suspension of a wild *Fusarium graminearum* strain isolated from wheat collected during previous year's harvest. From each sample, 40 kernels were randomly selected, weighted and placed in a 2 mL Eppendorf tube. A single-kernel extraction procedure was applied to the whole grain. For this purpose, 1 mL of an acidified acetonitrile-water solution (80:20, v/v, 2% acetic acid) was added to the tube. The kernels were left in the solvent overnight to improve solvent penetration into the grain and thereby enhance mycotoxin recovery. The Eppendorf tubes were then centrifugated at 3000 g for 10 minutes. 100 µL of the supernatant was collected and diluted with 900 µL of a water-methanol solution (50:50, v/v). Mycotoxin detection and quantification were subsequently performed using UPLC-MS/MS.

3. Batch samples preparation

Sorting was done according to two mycotoxins, DON, a field mycotoxin and OTA, a storage mycotoxin. For each mycotoxin, three samples with different levels of concentration were constituted as described here below.

3.1. DON contaminated samples

Naturally contaminated wheat grains were collected during the 2024 harvest in Wallonia (Belgium), with DON concentration of approximatively 2000 µg/kg. The contaminated batch was mixed with another batch from the same variety than the naturally contaminated one but with DON concentration lower than the limit of detection (LOD) (60 µg/kg). Three samples of 3 kg (named N1, N2 and N3) with three different levels of concentration were obtained, as presented in Table 1.

Table 1: DON contaminated samples preparation: Mass of the constituents and final concentration in the three samples (n=3).

	Mass of naturally contaminated grains (g)	Mass of the batches with DON < LOD (g)	Concentration (µg/kg)
N1	750	2250	737 ± 144
N2	1500	1500	1008 ± 197
N3	2250	750	997 ± 194

3.2. Ochratoxin A contaminated samples

Samples contaminated with OTA were obtained by inoculation with *Aspergillus ochraceus*. 3 kg of wheat grains that did not contain DON or OTA at concentration higher than the LOD, 60 µk/kg and 0.5 µg/kg respectively, were moisturized with 300 mL of water to reach 22% of humidity. The sample and water were mixed with a mixer (Chopin, KPM Analytics, Westborough, USA) for 30 minutes.

An inoculation solution was prepared by suspending *Aspergillus ochraceus (MUCL 39534 from the (agro)industrial fungi & yeasts collection of the UCLouvain, Belgium)* spores in a water solution. The concentration of the solution of 8.8 x 10^6 spores/mL was determined by contrast phase microscopy with a Nageotte cell. Wheat sample was inoculated with one mL of this solution. After agitation, the sample was kept in a heating chamber for 3 weeks à 28°C.

The inoculated sample reached a concentration of approximatively 130 µg/kg. The contaminated sample was mixed with a non-inoculated sample of the same wheat batch to have three levels of concentration (Table 2).

Table 2: Ochratoxin A contaminated sample preparation: Mass of the constituents and final concentration (n=3).

	Mass of naturally contaminated grains (g)	Mass of the batches with OTA < LOD (g)	Concentration (µg/kg)
N1	150	2850	16 ± 3
N2	300	2700	34 ± 7
N3	450	2550	51 ± 10

4. Samples sorting

Samples contaminated with DON and OTA were sorted according to three different sorting devices: a cleaner-separator (SLN sample cleaner, Pfeuffer, Kitzingen, Germany), a small-scale laboratory gravity separator (LA-K, Westrup, Slagelse, Denmark) and a high-speed single kernel near infra-red (NIR) sorter (QSorter Explorer 2.0, QualySense, Schafisheim, Switzerland).

4.1. Cleaner separator

The cleaner separator is used to sort grains based on their sizes by combining different grids with elongated or circular holes. The cleaning process begins with the removal of light impurities, which are aspirated by an air suction system and separated in the cyclone before being collected in the dust bag. The sample then passes through a first sieve that retains coarse impurities. The cleaner separator used is presented in Figure 1a. The first sieve used is perforated with round holes of 6 mm diameter, followed by a second sieve perforated with 3.5 mm round holes that removes medium-sized impurities, the third sieve, pierced with 2.5 mm oblong holes, and the fourth sieve, pierced with 3 mm oblong holes, separate smaller material (Figure 1b and c). Sorting sieves were selected to have sufficient grains in each obtained fraction.

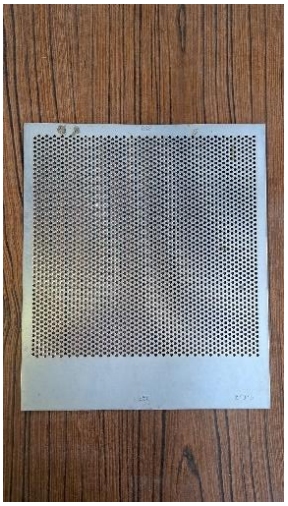
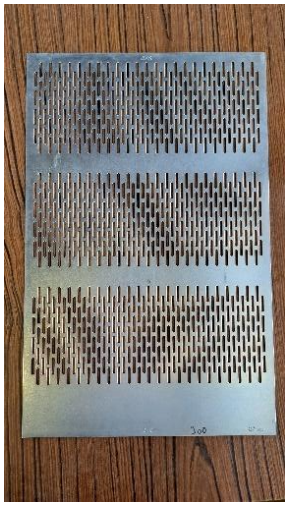
(a) (b)  (c) 

Figure 1: (a) Cleaner separator device used for the study, (b) sieve of 3.5 mm round holes, (c) sieve of 3 mm oblong holes.

500 g of each of the samples were introduced in the cleaner-separator. At the end of the process four fractions were collected. The first one contained the smallest grains and the fourth one consisted of the biggest grains.

4.2. Gravity separator

The gravity separator operates by exploiting small differences in the specific weight of kernels, thereby allowing the separation of heavy and light kernels. Precise separation is achieved through the combination of components, oscillation of triangular grading deck, and uniform airflow across the entire deck. Used gravity separator is showed in Figure 2.

Figure 2: Gravity separator device used for the study.

2 kg of grains of each sample were introduced on the gravity separator. The separated grains are collected into five distinct fractions with the first one constituted of the lighter grains and the denser grains were in the fifth fraction.

4.3. NIR-sorter

A high-speed single kernel infra-red sorter was used (Figure 3). It separates the kernel following visible or spectral data.

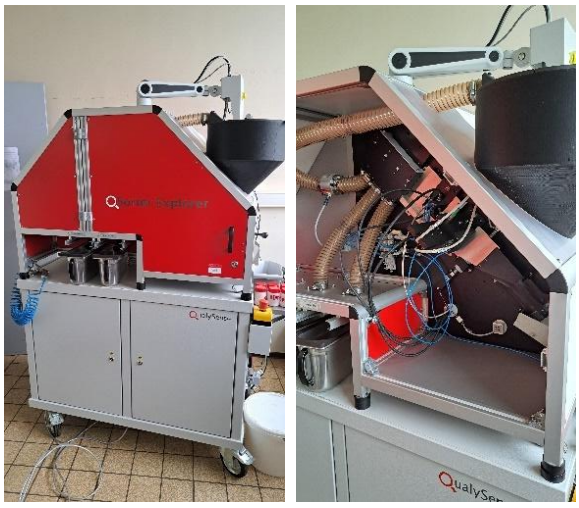


Figure 3: High-speed single kernel infra-red sorting device used for the study.

For this study, a *Fusarium*-damaged kernel model was developed and implemented in the sorter. This model was developed as follow, a total of 30 grains samples originating from *Fusarium* spp. inoculation trials were selected to develop a discrimination model (15 samples from 2023 and 15 from 2024). Kernels used for model development came from two different harvest year to avoid the introduction of an annual effect on the model. In addition, two naturally *Fusarium*-infected samples

were reserved for external validation of the calibration. Each sample was manually sorted to obtain approximately 150 symptomatic kernels, characterized by a discoloured, pinkish, chalky, shrivelled, and/or shrunk appearance. A total of 4,867 kernels from the inoculation trial was used for calibration and 339 naturally contaminated kernels for validation. In addition, between 150 and 200 healthy or asymptomatic kernels per sample were collected, corresponding to 5,641 kernels for calibration and 315 kernels for validation. Asymptomatic kernels were exhibited a plump morphology and a brownish to golden coloration. Symptomatic and asymptomatic kernels are presented in Figure 4.

Figure 4: Kernels with symptoms of Fusarium infection (left) vs kernels with no visible symptoms of Fusarium infection (right).

All kernels used for calibration and validation were analysed with the high-speed single kernel infra-red sorter, generating spectral datasets for each individual kernel (Figure 5). A two-class partial least squares discriminant analysis (PLS-DA) model (symptomatic vs. asymptomatic kernels) was subsequently developed and optimized with respect to spectral preprocessing and the selection of the optimal number of latent variables. The model was then implemented in the sorter.

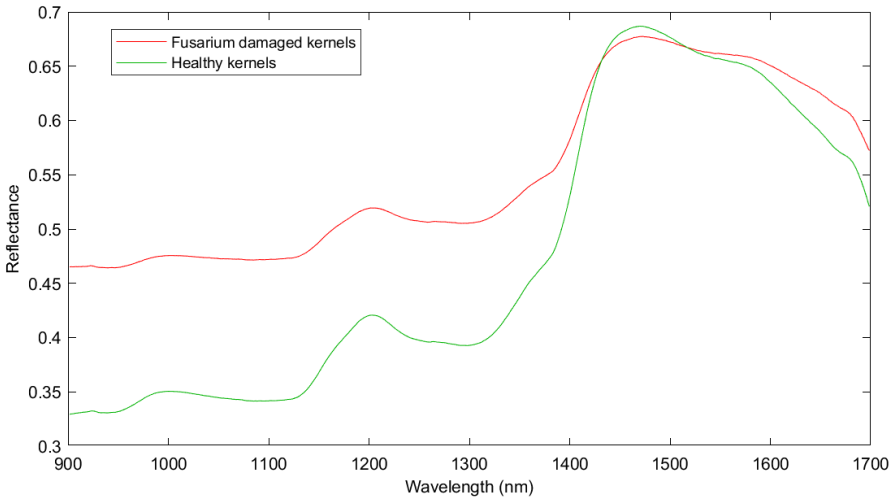


Figure 5: Average spectra for symptomatic(green) and asymptomatic (red) kernels from the calibration dataset.

Then, 200 g of grains from each sample were introduced into the NIR sorter. The sorting properties were adjusted to allow the separation of the 20% most *Fusarium*-damaged kernels from the remaining 80% of the grains.

5. Mycotoxin analysis by UPLC-MS/MS

Mycotoxin analysis by UPLC-MS/MS was performed according to Jonard et al. (2025) and Sogbossi Gbétokpanou et al. (2025).

5.1. Samples preparation

For extraction, 5 g of whole-wheat flour were weighed into 50 mL Falcon tubes and mixed with 10 mL of water. After standing for 1 h, 10 mL of acetonitrile containing 1% acetic acid were added. The samples were homogenized using a Mixer Mill MM400 (Retsch GmbH, Aartselaar, Belgium) at 10 Hz for 10 min.

Subsequently, 4 g of magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl) were added. The tubes were shaken manually and then agitated again in the Mixer Mill MM400 (Retsch GmbH, Aartselaar, Belgium) for 2 min at 10 Hz. After mixing, the samples were centrifuged at 3000 × g for 5 min (Centrifuge 5810 R, Eppendorf, Leipzig, Germany).

Finally, a 100 µL aliquot of the supernatant was diluted with 900 µL of water/methanol (50:50, v/v) and transferred into vials for UPLC-MS/MS injection.

5.2. UPLC-MS/MS conditions

Sample analysis was carried out using a Waters Acquity I-Class UPLC system (Waters, Milford, USA) coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation was performed on a BEH C18 column (2.1 mm × 50 mm, 1.7 µm) maintained at 50 °C. The mobile phases consisted of (A) 10 mM ammonium acetate in water with 0.1 % formic acid and (B) methanol with 0.1 % formic acid. The injection volume was 2 µL.

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with positive electrospray ionization. Source parameters were as follows: capillary voltage 3.4 kV, desolvation temperature 400 °C, desolvation gas flow 800 L/h, cone gas flow 150 L/h, and nebulizer pressure 7 bar. The specific transitions together with their optimized parameters for DON and OTA are provided in Table 3.

Table 3: Optimized MS:MS parameters for DON and OTA detection in MRM mode.

Mycotoxin	Molecular Mass	MODE	*m/z*	Cone (V)	Daughter 1	Energy1 (eV)	Daughter 2	Energy2 (eV)	RT (min)
DON	296.3	$[M+H]^+$	297.1	15	231.1	18	249.1	18	3.9
OTA	403.8	$[M+H]^+$	404.1	25	239.1	25	357.9	15	10.5

6. Data treatment

Peak integration of chromatograms was performed using TargetLynx V 4.2 (Waters, Milford, USA). Mycotoxin quantification was carried out using bracketed calibration, in which an average calibration curve was generated from calibration standards injected before and after the sample set. Calibration curves were constructed by linear regression (y = ax + b).

Calculated concentrations were corrected according to the exact sample intake and the recovery rates determined for each mycotoxin during method validation. The LOD, limits of quantification (LOQ), and recovery values are summarized in Table 4.

Table 4: Average recovery, limit of quantification (LOQ) and limit of detection (LOD) determined during method validation for DON and OTA.

Mycotoxins	Recovery [%]	LOQ [µg/kg]	LOD [µg/kg]
DON	88	100	60
OTA	104	1	0.5

Results

1. Single kernel mycotoxin analysis

The single kernel analysis of mycotoxins in four wheat samples originating from *Fusarium* spp. inoculation trials revealed distinct DON contamination profiles across the samples. Zearalenone was the only other mycotoxin also detected in the samples. In samples 1 and 2, the majority of kernels were contaminated above the regulatory threshold of 1000 µg/kg (Figure 6). In contrast, samples 3 and 4 contained only a limited number of kernels with very high DON concentration (Figure 6). Therefore, selectively removing these highly contaminated kernels could significantly reduce the average DON concentration in the sample. However, for sample 1 and 2, since for most of the kernels DON concentrations were above the threshold, many kernels should be eliminated, making sorting less relevant.

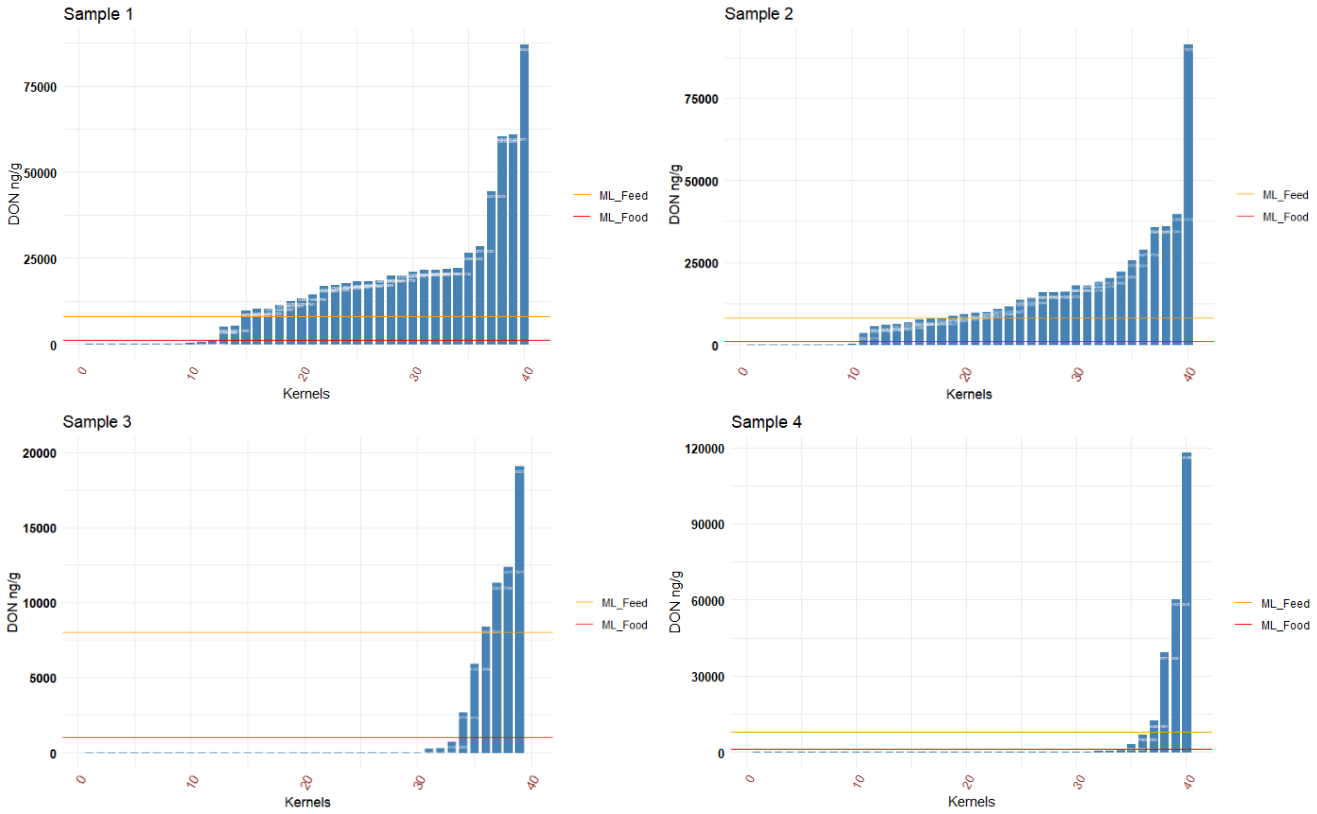


Figure 6: Single kernels DON concentration in 40 kernels from four samples of Fusarium *spp. inoculated wheat.*

2. Cleaner separator

Figure 7 shows the variation in DON concentrations for each sample throughout the sorting process by the cleaner separator. All determined concentrations and the masses of each fraction are presented in Table 5. For all samples, the initial concentrations in the raw materials were not substantially higher than the ML, with values of 737, 1008, and 997 µg/kg for N1, N2, and N3, respectively. The first sorting fraction (S1) showed a reduction in DON concentration for all samples. The subsequent fractions (S2 and S3) showed a slight decrease of the concentrations. The maximum concentration is observed in S4, indicating a possible accumulation of more contaminated grains in this fraction during the sorting process, with the S4 of N3 exceeding the ML.

Overall, these results suggested that the first sorting fraction, containing the smaller grains, was the most effective in reducing DON contamination, while fractions S2 and S3 had a more limited effect. Fraction 4, constituted of the biggest grains, concentrated the most contaminated grains.

Most of the grains were collected in S3 that represented around 55% of the whole batch for the three levels of concentration (Figure 8). The only fraction with an important concentration decrease was

S1 but this fraction corresponded only to 10 to 15% of the samples. Contaminated grains concentrated in S4 represented between 20 and 26% of the baches.

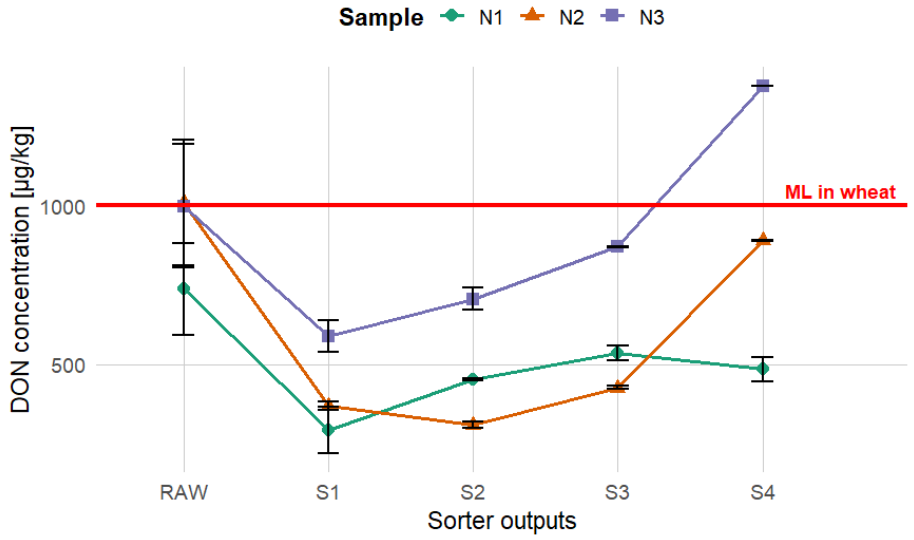


Figure 7: Effect of the cleaner separator on DON concentration for the three levels of contamination.

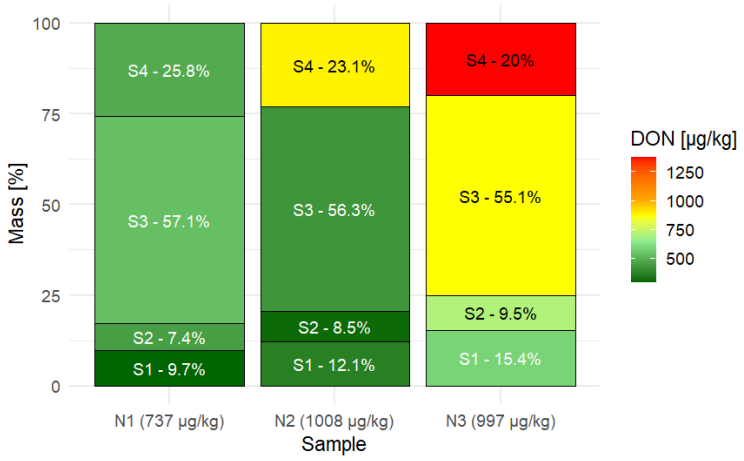


Figure 8: Mass distribution and DON concentration after sorting with the cleaner separator. Red fraction concentrations are above the ML in wheat of 1000 µg/kg.

Table 5: Average DON concentrations and masses of the different fractions resulting from size sorting (n=3).

	Initial concentration (µg/kg)	S1 concentration (µg/kg)	S1 mass fraction (%)	S2 concentration (µg/kg)	S2 mass fraction (%)	S3 concentration (µg/kg)	S3 mass fraction (%)	S4 concentration (µg/kg)	S4 mass fraction (%)
N1	737 ± 144	293 ± 73	9.7	452 ± 3	7.4	536 ± 23	57.1	485 ± 38	25.8
N2	1008 ± 197	370 ± 13	12.1	310 ± 9	8.5	427 ± 5	56.3	889 ± 2	23.1
N3	997 ± 194	588 ± 49	15.4	706 ± 34	9.5	870 ± 2	55.1	1375 ± 1	20.0

For OTA, variations in concentration were also observed among the different sorter outputs (Figure 9). In all samples, the initial concentrations in the raw materials were well above the ML, with values of 16, 42, and 59 µg/kg for N1, N2, and N3, respectively. The first and third fractions (S1 and

S3) showed an increase in OTA concentration across all samples, while the concentration in S2 was approximately similar to the one of the unsorted samples. A reduction in OTA concentration was observed in S4, which contained the largest grains. However, only S4 from N1 reached a concentration below the ML.

Overall, these results indicated that only S4 exhibited a reduction in concentration, making it the only fraction potentially recoverable after sorting. Nevertheless, when the initial contamination level was too high, sorting alone was insufficient to reduce OTA concentrations below regulatory limits. This fraction (S4) represented approximately 45% of the initial batch (Figure 10).

All determined concentrations and the masses of each fraction are presented in Table 6.

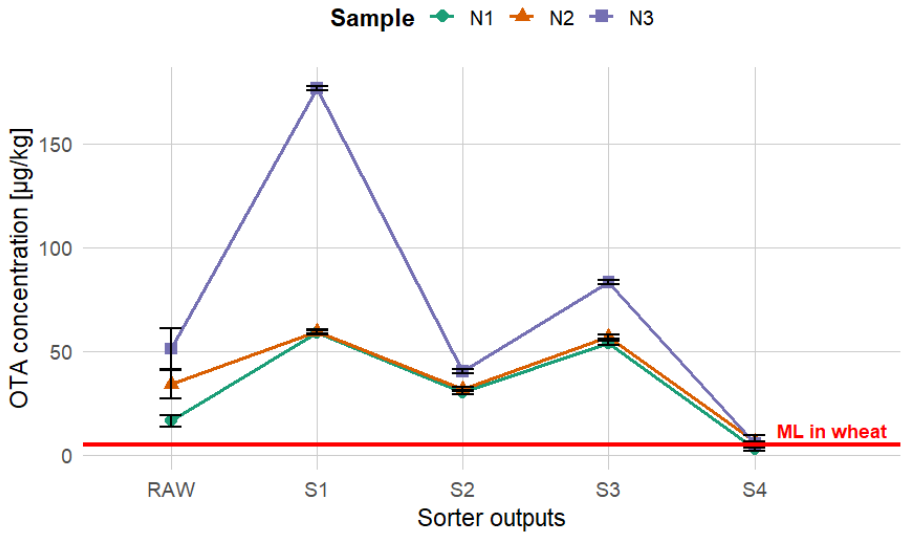


Figure 9: Effect of the cleaner separator on OTA concentration for the three levels of contamination.

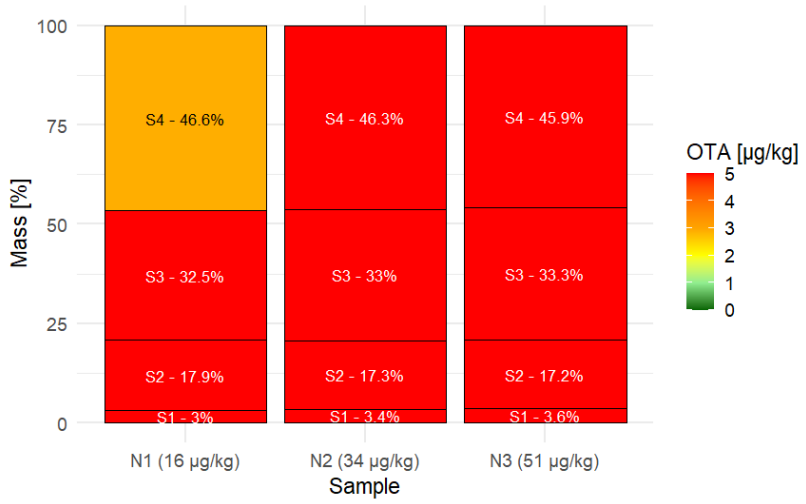


Figure 10: Mass distribution and OTA concentration after sorting with the cleaner separator.

Red fraction concentrations are above the ML in wheat of 5 µg/kg.

Table 6: Average OTA concentrations and masses of the different fractions resulting from size sorting (n=3).

	Initial concentration (µg/kg)	S1 concentration (µg/kg)	S1 mass fraction (%)	S2 concentration (µg/kg)	S2 mass fraction (%)	S3 concentration (µg/kg)	S3 mass fraction (%)	S4 concentration (µg/kg)	S4 mass fraction (%)
N1	16 ± 3	59 ± 1	3.0	30 ± 1	17.9	54 ± 1	32.5	3 ± 1	46.6
N2	34 ± 7	59 ± 1	3.4	32 ± 1	17.3	57 ± 1	33.0	7 ± 3	46.3
N3	51 ± 10	177 ± 1	3.6	40 ± 1	17.2	83 ± 1	33.3	6 ± 1	45.9

3. *Gravity sorter*

Sorting with gravity table resulted in different concentrations among the five fractions, with a similar trend observed for all three samples. The highest DON concentrations were consistently found in the first fraction (S1), corresponding to the least dense kernels. Fractions S3 to S5 generally showed lower DON concentrations than the raw materials, except for S3 in sample N3. For N1 and N2, the concentrations in S2 were approximately equal to those of the unsorted sample, whereas for N3, the S2 concentration was still about twice the one of the unsorted batches (Figure 11).

S1, which concentrated the most contaminated grains, accounted for about 15% of the total batch mass. Together, S4 and S5, which showed reduced concentrations compared to the raw materials, represented approximately 25% of the batch (Figure 12).

Complete results are presented in Table 7.

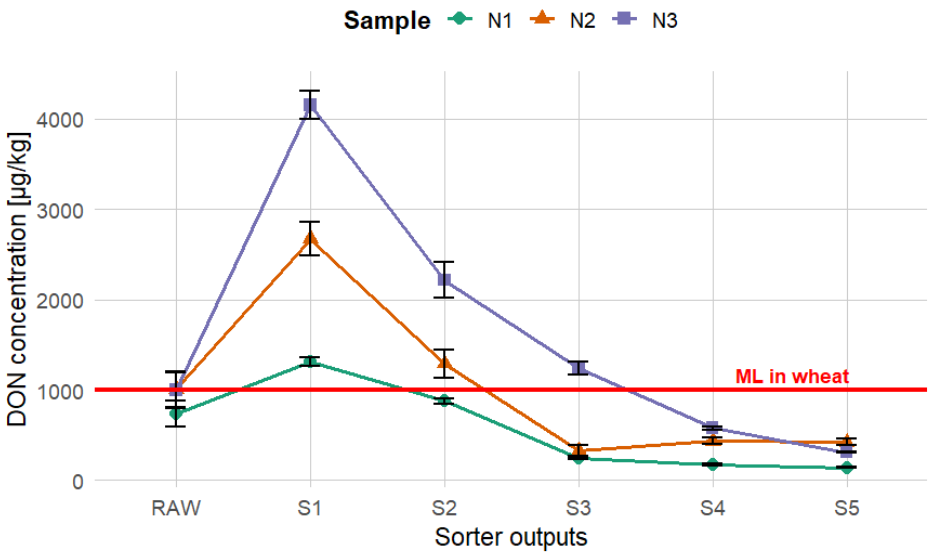


Figure 11: Effect of the gravity table on DON concentration for the three levels of contamination.

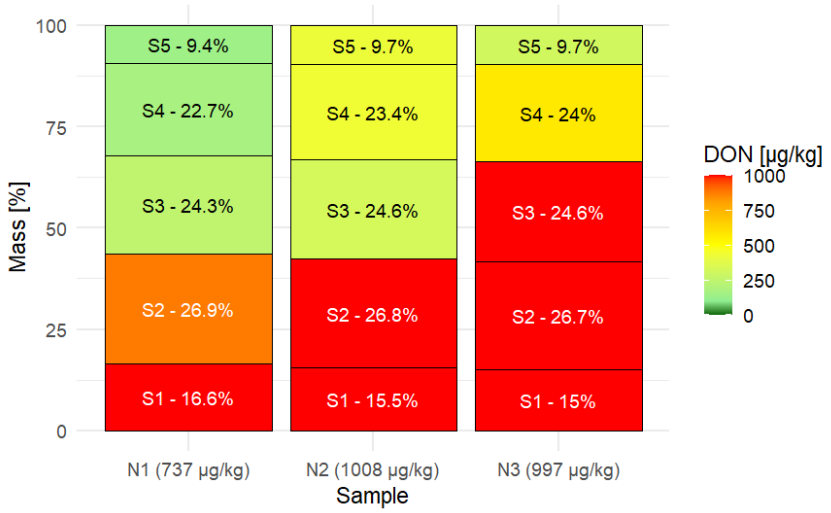


Figure 12: Mass distribution and DON concentration after sorting with the gravity table. Red fraction concentrations are above the ML in wheat of 1000 µg/kg.

Table 7: Average DON concentrations and masses of the different fractions resulting from density sorting (n=3).

	Initial concentration (µg/kg)	S1 CC (µg/kg)	S1 mass fraction (%)	S2 CC (µg/kg)	S2 mass fraction (%)	S3 CC (µg/kg)	S3 mass fraction (%)	S4 CC (µg/kg)	S4 mass fraction (%)	S5 CC (µg/kg)	S5 mass fraction (%)
N1	737 ± 144	1311 ± 47	16.6	880 ± 30	26.9	248 ± 16	24.3	172 ± 9	22.7	140 ± 5	9.4
N2	1008 ± 197	2672 ± 189	15.5	1291 ± 152	26.8	325 ± 63	24.6	436 ± 35	23.4	424 ± 31	9.7
N3	997 ± 194	4154 ± 159	15.0	2218 ± 201	26.7	1241 ± 71	24.6	576 ± 15	24.0	311 ± 9	9.7

For OTA, a consistent trend in concentration repartition across the three samples was observed among the different sorting fractions obtained with the gravity table (Figure 13). Fractions S1 and S2 showed an accumulation of contaminated grains, resulting in higher OTA concentrations than in the unsorted materials. In contrast, the fifth fraction (S5), corresponding to the densest kernels, exhibited a reduction in OTA concentration. This fraction represented approximately 10% of the total batch (Figure 14). However, only for the least contaminated initial sample, the concentration in S5 did fall below the ML. Fractions 3 and 4 (S3 and S4) showed concentrations similar to those of the raw materials.

Given the high initial concentrations, only about 10% of the least contaminated sample could be recovered for food use.

All the data are presented in Table 8.

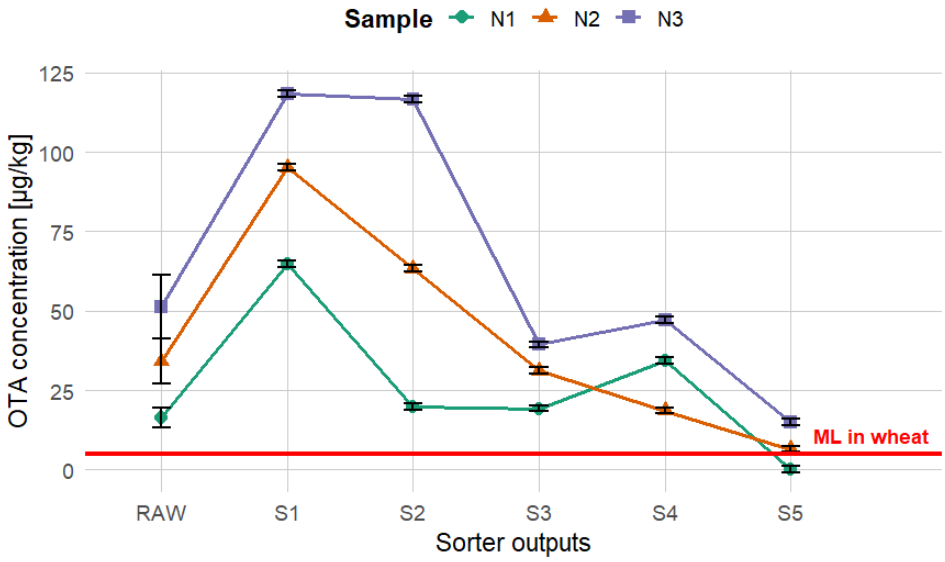


Figure 13: Effect of the gravity table on OTA concentration for the three levels of contamination.

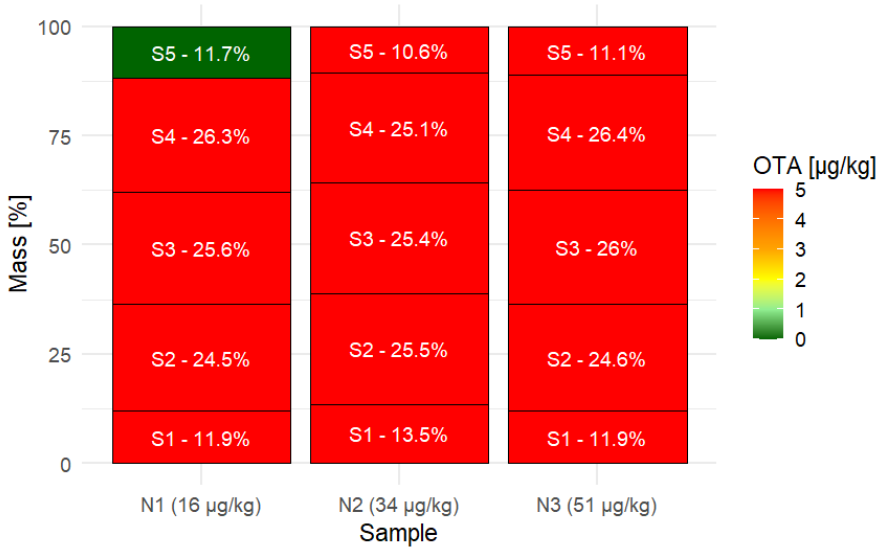


Figure 14: Mass distribution and OTA concentration after sorting with the gravity table. Red fraction concentrations are above the ML in wheat of 5 µg/kg.

Table 8: Average OT A concentrations and masses of the different fractions resulting from density sorting (n=3).

	Initial concentration (µg/kg)	S1 CC (µg/kg)	S1 mass fraction (%)	S2 CC (µg/kg)	S2 mass fraction (%)	S3 CC (µg/kg)	S3 mass fraction (%)	S4 CC (µg/kg)	S4 mass fraction (%)	S5 CC (µg/kg)	S5 mass fraction (%)
N1	16 ± 3	65 ± 1	11.9	20 ± 1	24.5	19 ± 1	25.6	34 ± 1	26.3	ND	11.7
N2	34 ± 7	95 ± 1	13.5	63 ± 1	25.5	31 ± 1	25.4	20 ± 1	19	6 ± 1	10.6
N3	51 ± 10	118 ± 1	11.9	117 ± 1	24.6	39 ± 1	26.0	47 ± 1	26.4	15 ± 1	11.1

4. Near-infrared single kernel sorter

Finally, the removal of the 20% of the most *Fusarium*-damaged kernels by the NIR sorter resulted in a strong concentration of DON in the excluded fraction (S2), with levels nearly tripling across all three

samples. This indicates that the implemented model was effective in separating the most contaminated grains. The remaining fraction (S1), corresponding to 80% of the batch, was suitable for food use except for sample N2 (Figure 15, Figure 16 and Table 9).

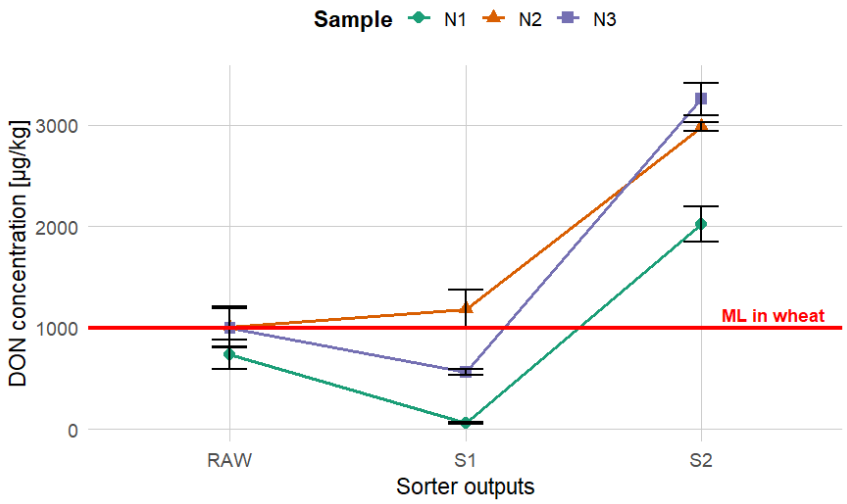


Figure 15: Effect of NIR sorting with the Fusarium-damaged kernel model on DON concentration for the three levels of contamination.

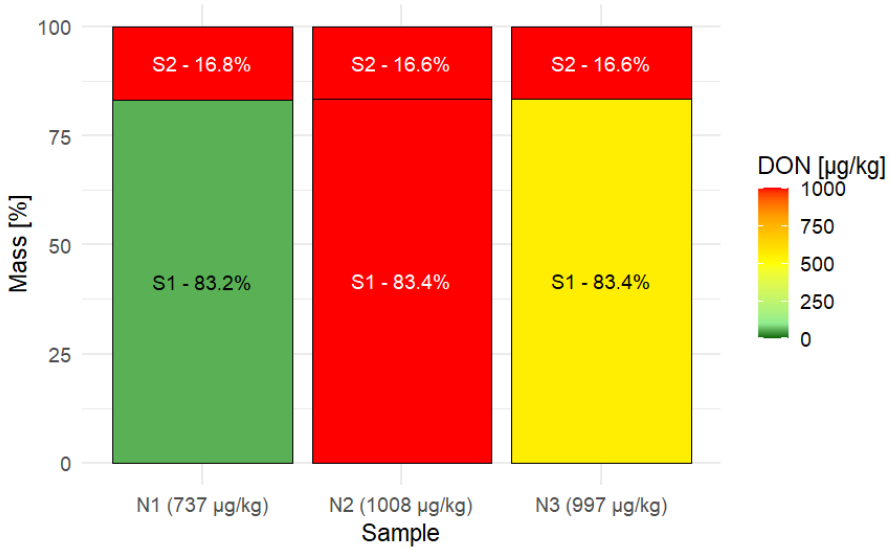


Figure 16: Mass distribution and DON concentration after the NIR sorting.
Red fraction concentrations are above the ML in wheat of 1000 µg/kg.

Table 9: Average DON concentrations and masses of the different fractions resulting from NIR sorting (n=3).

	Initial concentration (µg/kg)	S1 concentration (µg/kg)	S1 mass fraction (%)	S2 concentration (µg/kg)	S2 mass fraction (%)
N1	737 ± 144	58 ± 11	83.2	2021 ± 174	16.8
N2	1008 ± 197	1184 ± 187	83.4	2982 ± 40	16.6
N3	997 ± 194	558 ± 29	83.4	3257 ± 158	16.6

For OTA, the application of the *Fusarium*-damaged kernel sorting model did not result in a consistent concentration pattern between the two fractions (Figure 17, Figure 18 and Table 10). In addition, the sorting was not effective as none fractions reached concentrations below the ML. This outcome was expected, since OTA is not produced by *Fusarium* spp., and the kernel damaged associated with OTA contamination differs from that caused by *Fusarium* infection.

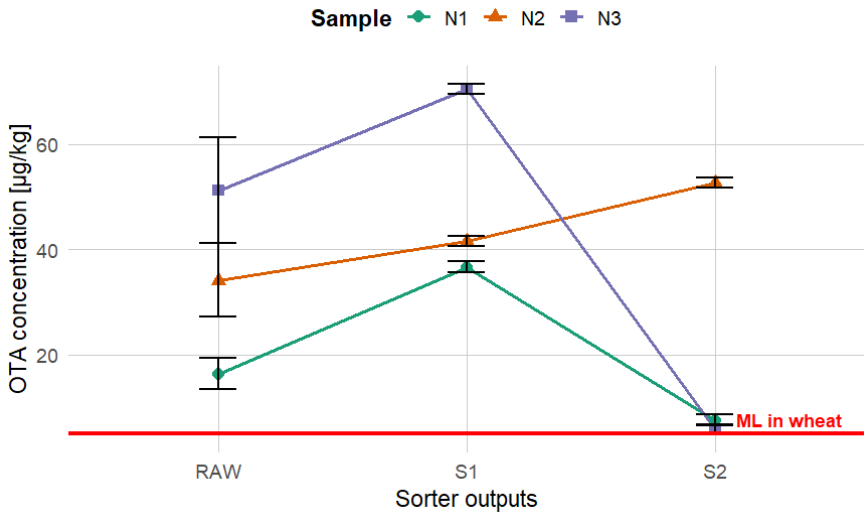


Figure 17: Effect of NIR sorting with the Fusarium-damaged kernel model on OTA concentration for the three levels of contamination.

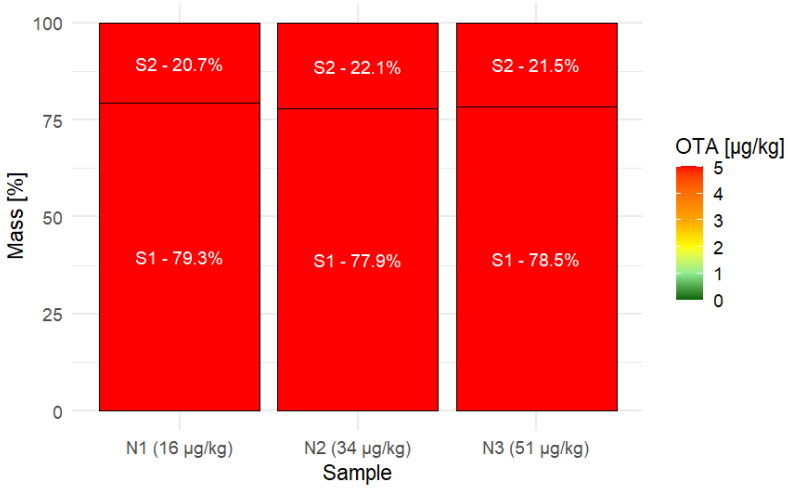


Figure 18: Mass distribution and OTA concentration after the NIR sorting. Red fraction concentrations are above the ML in wheat of 5 µg/kg.

Table 10: Average OTA concentrations and masses of the different fractions resulting from NIR sorting (n=3).

	Initial concentration (µg/kg)	S1 concentration (µg/kg)	S1 mass fraction (%)	S2 concentration (µg/kg)	S2 mass fraction (%)
N1	16 ± 3	37 ± 1	79.3	8 ± 1	20.7
N2	34 ± 7	42 ± 1	77.9	53 ± 1	22.1
N3	51 ± 10	70 ± 1	78.5	6 ± 1	21.5

All results are summarized in Table S1 and S2.

Discussion

1. Sorter efficiency

The present study compared the efficiency of three sorting devices, a cleaner separator, a gravity table, and a NIR single kernel sorter, in reducing contamination levels of DON and OTA in wheat.

Sorting efficiency depended not only on the sorter used but also varied from sample to sample due to the different contamination profile of the kernels. In most cases, the concentration patterns were consistent across samples.

1.1. DON

Sorting of DON contaminated samples by the cleaner separator resulted in a reduction of concentrations in 75 % of the material, while the most contaminated grains were concentrated in the remaining 25 %. The lowest concentrations were observed in fraction S1, where DON levels decreased by 37 to 56 %, with a decrease of concentration for the three initial levels of concentration included between 400 and 600 µg/kg. Size sorting can thus be a solution to recover a part of a contaminated batch (Figure 7 and Figure 8).

With the gravity separator, about 55 % of the sample could be recovered with equal or lower DON concentrations compared to the initial material. However, a huge increase of DON concentrations was observed in fraction S1, which represented 15 % of the sample, with DON concentrations rising by 178 to 417 %, corresponding to an increase of 574, 1664 and 3157 µg/kg for the three levels of concentration. Density sorting is efficient to sort DON contaminated samples (Figure 11 and Figure 12).

The *Fusarium*-damaged kernel model implemented in the NIR single kernel sorter proved to be the most effective strategy. By excluding 20% of the most damaged kernels, the DON concentrations were nearly tripled in the rejected fraction, while the remaining fraction concentration (80%) went bellow the ML (Figure 15 and Figure 16).

In contrast, the cleaner separator and the gravity table showed only partial effectiveness. Although these devices achieved some redistribution of DON among fractions, only limited portions of the batch showed a reduction in DON concentrations, and the reduction is not always substantial.

A huge variability of the sorting efficiency was observed between the samples that may come from the diversity of batch quality depending on the cultivar, weather conditions and agricultural practices (Schaarschmidt and Fauhl-Hassek, 2018). This variability is also observed in the literature (Cheli et al., 2013; Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018). For example, Tibola et al. (2016) reported between 74.7 % and 88.9 % of DON concentrations reduction by gravity separator with only 28% to 32.9 % of mass loss. On the other hand, Cheli et al. (2013) reported a pre-milling processes efficiency for DON removal from 7 to 63 %. In this study, size-sorting analysis indicated that the larger kernels were the more contaminated. This is in opposition with reports in the literature which generally state that smaller kernels tend to be more contaminated as *Fusarium* infection tends to decrease the seeds growth (Bakker and McCormick, 2019). However, as the study was performed on several mixtures constituted from a single contaminated sample combined with a non-contaminated one, the results may not be fully representative of real-world conditions.

In this study, the analysed samples were not contaminated with DON with level highly exceeding the ML of 1000 µg/kg, due to challenging collection of naturally contaminated material in huge amount. A collection of naturally contaminated sample is required, as the analysis of artificially contaminated or artificially constituted samples may introduce a bias into the results. Hence, in practical situations, sorting should not be applied for this kind of samples. However, this study showed valuable information about the concentration patterns, increase and decrease between the fractions, that are expected during sorting.

1.2. Ochratoxin A

First, with the cleaner separator, OTA concentrations increased substantially in fractions S1 and S3 (162 to 457 %), representing ~3% and ~30% of the batch, respectively. Indeed, OTA concentration in S1 increased from 43, 16 and 126 µg/kg for the three levels of concentrations. In addition, OTA concentration increase was between 23 and 38 µg/kg in S3. No significant changes of concentrations were observed in S2. While S4, corresponding to 45 % of the batch, showed an important reduction in OTA concentration (80 to 90 %), respectively 13, 27 and 45 µg/kg for each sample (Figure 9 and 10).

The gravity table enable to concentrate OTA in S1 and S2, which accounted together for 35 to 40% of the batch, with an increase of 49 to 67 µg/kg in S1 and 4 to 66 µg/kg in S2. Only fraction S5, corresponding to the densest kernels, showed a meaningful decrease of OTA concentrations, with a

decrease between 68 and 100 %, corresponding to 16, 28 and 36 µg/kg for the three levels of concentration. However, this fraction represented only 10 % of the batch (Figure 13 and 14).

Finally, NIR sorting with the *Fusarium*-damaged kernel model was not relevant for OTA, which was expected since OTA is not primarily associated with *Fusarium* infection but with *Aspergillus* and *Penicillium* species (EFSA, 2020) (Figure 17 and 18). Specific models based on fungal infection symptoms or moisture content should be developed and tested.

Overall, for OTA, none of the tested sorting processes achieved satisfactory sorting when initial concentrations largely exceeded the ML, at least three times higher. The cleaner separator and gravity table showed reductions in some fractions, but the recoverable safe material remained very small. The NIR sorter as used, being *Fusarium*-specific, was not relevant for OTA mitigation. These findings highlight the need for OTA-targeted sorting models or complementary mitigation strategies.

Variability between samples was also observed due to the diversity of kernel concentration profile.

Currently, developed methods for OTA concentration reduction by cleaning and sorting are not really effective (Duarte et al., 2010; Karlovsky et al., 2016).

2. *Mycotoxins concentration heterogeneity in the batches*

Mycotoxin contamination is heterogeneous within a batch as showed in the first point of the results and in the literature (Nelson, 2016; Schaarschmidt and Fauhl-Hassek, 2018). This is the key feature of sorting as the aim is to remove the most contaminated grains. This heterogeneity explains two of the observations in this study.

First, low-mass fractions can show very high concentrations, for example, S1 from the cleaner separator for OTA or S1 from the gravity separator for DON.

Second, the mass × concentration balance is not always consistent: when recalculating the “theoretical initial concentration”, the value does not always match the measured concentration in the raw materials. Besides heterogeneity, other factors may contribute to these observations, sub-sampling errors before and after sorting as well as the measurement uncertainty of the analytical method.

3. Limitations and perspectives

An important limitation of this study is that DON levels in the naturally contaminated raw samples only slightly exceeded the ML of 1000 µg/kg. Starting from levels lower or close to the ML facilitates obtaining fractions below the ML after sorting. Data for DON should therefore be interpreted mainly as a proof of concept of contamination distribution patterns, rather than as evidence that sorting will consistently bring highly contaminated lots into compliance.

Hence, extrapolation to highly contaminated batches must be cautious. Further trials with higher DON concentrations should be performed to establish robust dose–response curves for each sorting technology.

Within this study, the operational parameters of the sorting devices were not further optimized. For example, alternative sieve combinations could be tested with the cleaner separator, followed by mycotoxin analysis of the resulting fractions to assess potential improvements in efficiency. For the gravity table, adjustments to the inclination of the grading deck and the air flow rate may enhance sorting efficiency. Regarding the NIR sorter, additional models could be developed for OTA discrimination, such as model based on kernels moisture or other specific symptoms of fungal infection. For DON, the proportion of the excluded fraction could be optimized depending on the initial contamination level of the batch.

All used sorting devices were designed for laboratory scale. Even though similar devices are available at the industrial scale, it is necessary to evaluate whether their performances are comparable.

Furthermore, combinations of different sorting devices could be investigated to enhance overall decontamination efficiency. It would also be valuable to assess sorting effects on other regulated mycotoxins. However, a major limitation remains the availability of sufficient quantities of naturally contaminated samples, ideally covering a wide range of contamination levels for each target mycotoxin.

Conclusion

For DON discrimination, NIR single-kernel sorting with the developed *Fusarium*-damaged kernel model emerged as the most promising strategy, as it maximized the recoverable fraction while effectively excluding the most contaminated kernels. However, this type of device may be expensive for supply chain stakeholders, which could limit its adoption at large scale. In contrast, the cleaner separator and the gravity table could serve as cost-effective pre-cleaning steps, although their stand-alone efficiency remains partial. Wheat is a food product with a relatively low added value. Therefore, this type of procedure may be economically more relevant for higher-value food matrices such as coffee or peanuts.

Regarding OTA, all tested sorting devices showed only limited effectiveness. The development of OTA-specific spectral models, or alternative discrimination criteria, should therefore be further investigated to improve removal efficiency.

For both mycotoxins, the initial contamination level plays a decisive role. While sorting can reduce concentrations within certain fractions, it does not guarantee compliance with ML. Testing a wider range of samples, including those with higher DON levels and other regulated mycotoxins, would help establish feasibility thresholds for each device and contamination scenario.

With the expected increase in mycotoxin occurrence under climate change, sorting technologies may become a necessity to secure a sufficient and safe food supply. Extending this research to various and higher contamination levels and to additional mycotoxins would therefore be highly relevant.

Finally, beyond technical efficiency, the economic dimension must be considered. Assessing the cost–benefit balance of each sorting device under industrial conditions is essential to determine the actual interest of implementing sorting technologies at scale. Developing a screening or decision tool to determine whether sorting a batch is relevant should be considered.

Supplementary materials

Table S1: Average DON concentration and batch percentage for the three different sorting processes (n=3).

		N1		N2		N3	
		Concentration (µg/kg)	**Batch percentage (%)**	**Concentration (µg/kg)**	**Batch percentage (%)**	**Concentration (µg/kg)**	**Batch percentage (%)**
Cleaner-separator	RAW	737 ± 144	100.0	1008 ± 197	100.0	997 ± 194	100.0
	S1	293 ± 73	9.7	370 ± 13	12.1	588 ± 49	15.4
	S2	452 ± 3	7.4	310 ± 9	8.5	706 ± 34	9.5
	S3	536 ± 23	57.1	427 ± 5	56.3	870 ± 2	55.1
	S4	485 ±38	25.8	889 ± 2	23.1	1375 ± 1	20.0
Gravity table	RAW	737 ± 144	100.0	1008 ± 197	100.0	997 ± 194	100.0
	S1	1311 ±47	16.6	2672 ± 189	15.5	4154 ± 159	15.0
	S2	880 ± 30	26.9	1291 ± 152	26.8	2218 ± 201	26.7
	S3	248 ± 16	24.3	325 ± 63	24.6	1241 ± 71	24.6
	S4	172 ± 9	22.7	436 ± 35	23.4	576 ± 15	24.0
	S5	140 ± 5	9.4	424 ± 31	9.7	311 ± 9	9.7
Single kernel NIR sorter	RAW	737 ± 144	100.0	1008 ±b197	100.0	997 ± 194	100.0
	S1	58 ± 11	83.2	1184 ± 187	83.4	558 ± 29	83.4
	S2	2021 ± 174	16.8	2982 ± 29	16.6	3257 ± 158	16.6

Table S2: Average OTA concentration and batch percentage for the three different sorting processes (n=3).

		N1		N2		N3	
		Concentration (µg/kg)	Batch percentage	Concentration (µg/kg)	Batch percentage	Concentration (µg/kg)	Batch percentage
Cleaner-separator	RAW	16 ± 3	100.0	42 ± 7	100.0	59 ± 10	100.0
	S1	59 ± 1	3.0	60 ± 1	3.4	177 ± 1	3.6
	S2	30 ± 1	17.9	32 ± 1	17.3	41 ± 1	17.2
	S3	54 ± 1	32.5	57 ± 1	33.0	83 ± 1	33.3
	S4	3 ± 1	46.6	7 ± 3	46.3	6 ± 1	45.9
Gravity table	RAW	16 ± 3	100.0	42 ± 7	100.0	59 ± 10	100.0
	S1	65 ± 1	11.9	95 ± 1	13.5	119 ± 1	11.9
	S2	20 ± 1	24.5	63 ± 1	25.5	117 ± 1	24.6
	S3	19 ± 1	25.6	31 ± 1	25.4	39 ± 1	26.0
	S4	34 ± 1	26.3	19 ± 1	25.1	47 ± 1	26.4
	S5	ND	11.7	7 ± 1	10.6	15 ± 1	11.1
Single kernel NIR sorter	RAW	16 ± 3	100.	42 ± 7	100.0	59 ± 10	100.0
	S1	37 ± 1	79.3	42 ± 1	77.9	71 ± 1	78.5
	S2	8 ± 1	20.7	53 ± 1	22.1	6 ± 1	21.5

Experimental section

Study 4: Peanut and peanut-based foods contamination by toxigenic fungi and mycotoxins: Potential risks for Beninese consumer

Adapted from *Toxins 2025, 17, 532.* https://doi.org/10.3390/toxins17110532

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Preamble

The fourth part of this work aimed to evaluate the transferability of the developed analytical method to other food matrices. In this context, additional mycotoxins were incorporated into the method to meet specific requirements related to the analysis of peanut and peanut-based products. A brief validation procedure was carried out to ensure the reliability and steadiness of the method when applied to this new food matrix.

In addition, the analysis of peanuts and peanut-derived products originating from Benin enable both the assessment of mycotoxin occurrence and the evaluation of associated consumer exposure risks. A few studies reported the presence of mycotoxins in peanuts. However, there remains a significant lack of data on how processing practices influence contamination levels, as well as on the overall dietary exposure of Beninese consumers. This study therefore aims to fill these knowledge gaps by providing a comprehensive evaluation of mycotoxin and fungal contamination in peanuts and their derived products, together with an assessment of consumer exposure resulting from their consumption.

Furthermore, the study aimed to assess the evaluation of mycotoxin concentrations along the production chain and identify critical points where preventive actions could be implemented to reduce exposure of the Beninese population.

My specific contribution to this part of the work was to adapt the multi-mycotoxin LC-MS/MS method to peanut matrices by adding aflatoxins to the method and validating the method which I used for the determination of the mycotoxin contamination in peanuts and peanut-based products collected in Benin.

Experimental section

Study 4: Peanut and peanut-based foods contamination by toxigenic fungi and mycotoxins: Potential risks for Beninese consumers

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Abstract

This study assessed mycotoxin contamination in roasted peanut snacks and *kluiklui* (fried pressed peanut cake), and consumer exposure in southern Benin. Roasted peanut snacks and *kluiklui* were sampled from markets across six municipalities, and their production follow-up was conducted on two sites using different processing methods. Mycotoxins were quantified using UPLC-MS/MS. Aflatoxin B1 predominated, reaching 169 µg/kg in roasted peanut snacks and 2144.64 µg/kg in marketed *kluiklui*. In contrast, just-produced *kluiklui* contained much lower levels (11.73–37.78 µg/kg). The grinding step (using public grinder) was identified as the main contamination point. The significative higher mycotoxin levels in *kluiklui* sampled on markets compared to just produced *kluiklui* are probably due to poor storage conditions. *Kluiklui* consumption poses a significant health risk in Benin. Improved hygiene in public grinders and better storage practices are urgently needed to reduce contamination and protect consumers' health.

Keywords: *Arachis hypogaea*; AFB1; total aflatoxins; OTA; process contribution; *kluiklui*.

Introduction

Mycotoxin contamination of food crops represents one of the most significant threats to global food safety and human health (Eskola et al., 2020). Mycotoxins are toxic secondary metabolites mainly produced by fungal species of the genera *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., and to a lesser extent by *Alternaria* spp., *Claviceps* spp., *Trichoderma* spp. and *Trichothecium* spp. (International Agency for Research on Cancer, 2012b; Ismaiel and Papenbrock, 2015; Battilani et al., 2020; Garello et al., 2024). A single fungal species can produce multiple mycotoxins, potentially leading to the simultaneous contamination of food commodities with several toxins. In addition, successive colonisation by different fungal species can further contribute to multi-mycotoxin contamination (Battilani et al., 2020). To date, over 400 mycotoxins have been identified, but only a limited number are considered of major concern for food safety due to their documented occurrence in food products. These include aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2); ochratoxins, mainly ochratoxin A (OTA) and fumonisins, notably B1 (FUMB1) and B2 (FUMB2) (Yang et al., 2020; Hellany et al., 2023).

Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) and present hepatotoxic and immunosuppressive properties (International Agency for Research on Cancer, 2012a). AFB1 is the most frequently present in contaminated foods and is recognised as the most toxic and carcinogenic. AFB1 has also been associated with growth suppression and weight loss in children (Nejad et al., 2023) and immune system modulation (Bondy, 2008). Ochratoxins are produced by *Aspergillus ochraceus* and some *Penicillium* species, with OTA being the most extensively studied. OTA primarily affects the kidneys and is classified as a possible human carcinogen by the IARC (group 2B) (International Agency for Research on Cancer, 1993). Fumonisins (FUMs), primarily produced by *Fusarium verticillioides* and *Fusarium proliferatum*, are also classified in the group 2B of possible human carcinogens (International Agency for Research on Cancer, 2002). Among them, FUMB1 is the most prevalent and has been associated with both nephrotoxicity and hepatotoxicity in various animal species (Stockmann-Juvala and Savolainen, 2008; Gruber-Dorninger et al., 2017).

The main route of mycotoxin exposure for humans is through ingestion of contaminated food (Li et al., 2025). Fungal contamination and mycotoxin production can occur at any stage of the food production chain, including pre-harvest, harvest, drying, and storage phases (Winter and Pereg,

2019). Crops such as maize, millet, wheat, sorghum, soybean, peanut, and their derived products are among the most susceptible to mycotoxin contamination (El-Sayed et al., 2022).

Peanuts (*Arachis hypogaea*) are one of the most widely grown oilseed crops in the world, cultivated across the intertropical zone (USDA Foreign Agricultural Service, 2024). Peanuts are important in terms of nutritional and economic aspects, particularly in developing countries (Valentine, 2016) where they are processed into a wide range of popular food products (Chang et al., 2013; Variath and Janila, 2017). Peanuts are consumed fresh, boiled, roasted, or processed into paste, oil or fried pressed-peanut cakes (Chang et al., 2013; Adjile et al., 2015; Variath and Janila, 2017). Besides peanut economic and nutritional benefits, peanut and peanut-based foods consumption has potential drawbacks due to their high susceptibility to fungal contamination, particularly by *A. flavus*, which produces aflatoxins (Arya et al., 2016; Bediako et al., 2019).

Weather conditions are favourable to mycotoxin development in peanuts in Africa (Darko, 2016). Studies across West Africa (Senegal, Ghana, Mali, Nigeria, Togo, and Benin) have reported mycotoxin contamination in peanuts and peanut-based foods with mycotoxins (Bankole et al., 2005; Awuah et al., 2009; Adjou et al., 2012; Ezekiel et al., 2012; Waliyar et al., 2015; Watson et al., 2015; Ingenbleek et al., 2019; Tedihou et al., 2019). The reported concentrations widely exceeded the maximum levels established by the Commission Regulation (EU) 2023/915, which are set at 8 µg/kg for AFB1 and 15 µg/kg for total aflatoxins in raw peanuts intended for direct human consumption, and 2 µg/kg and 4 µg/kg, respectively, in peanut-based processed products (European Commission, 2023c). Regular consumption of peanut-based foods may lead to a public health risk.

Also, in West Africa, particularly in Benin, peanut-based foods are primarily obtained through traditional, small-scale processing methods (Videgla et al., 2016). Mycotoxin levels in peanut-based foods may therefore stem from several factors, including the poor quality of peanut seeds used as raw material, inadequate technological processing practices, and/or inappropriate storage conditions of peanut-based foods.

Although a few studies in Benin have reported the presence of mycotoxins in peanut-based foods such as *kluiklui* (fried pressed peanut cake) and peanut oil (Adjou et al., 2012; Ingenbleek et al., 2019), there is a significant lack of research assessing the contribution of processing practices to the contamination of these derived products. This study, therefore, aims to address these gaps by evaluating the occurrence of mycotoxins in peanuts and their derived products.

Material and method

1. *Study Area*

This study was conducted in southern Benin. Benin is located at 6°28′ N and 2°36′ E in West Africa. Benin has a tropical monsoon climate in the south and a tropical wet and dry climate in the north, according to the Koppen-Geiger climate type (Chen and Chen, 2013). Benin is one of the most vulnerable countries in global warming scenarios of +2.7 °C by 2070 (World Bank, 2025). Climate change remains the main driving factor in the production of mycotoxins (Magan et al., 2010; Paterson and Lima, 2010; Nji et al., 2022).

2. *Sampling of Peanut-Based Foods from Beninese Markets*

In order to determine mycotoxin concentrations in peanut-based foods marketed in Southern Benin, a total of 52 samples of peanut-based foods were purchased randomly in municipalities representative of peanut production and/or consumption in southern Benin (Abomey, Aplahoué, Covè, Cotonou, Glazoué, and Ouèssè). Figure 1 shows the sampling areas. The collected samples represent the main peanut-based foods produced and consumed in Southern Benin, and include roasted peanut (n = 27) and *kluiklui* (pressed fried peanut cake) (n = 25). The sampling was performed at production and retail sites (market and supermarket). The samples were ground, packed in sealed containers and stored under dry conditions for further analysis.

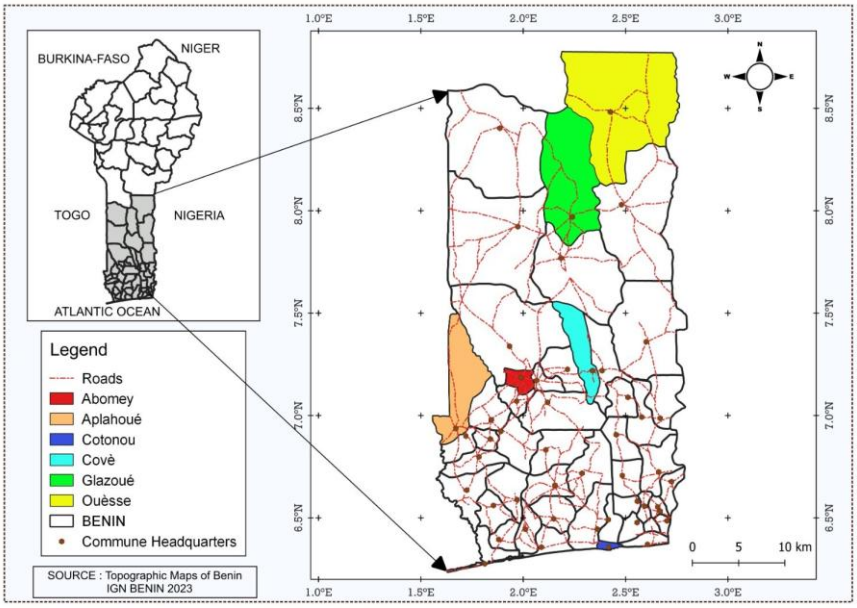

Figure 1. Map of Benin showing the 6 communes of the sampling areas (adapted from a map from the IGN "Institut Géographique National" of Benin using the open source QGIS software, version 3.2).

3. *Follow-Up of Kluiklui and Roasted Peanut Snacks Production and Sampling*

3.1. Experimental Design

Figure 2 and Figure 3 illustrate the process for *kluiklui* and roasted peanut snack production, respectively. *Kluiklui* can be produced by adding or not adding maize flour during the kneading of the peanut paste. About 10 to 20 g of maize flour per kg of peanut paste is used. Regarding roasted peanuts, roasting can be done using a heat transfer material or not.

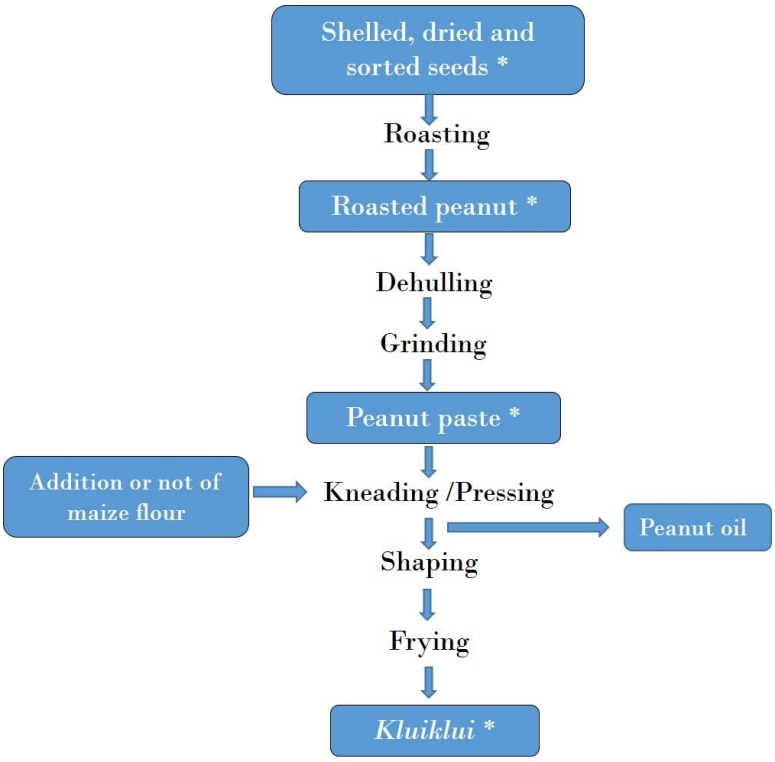

*Figure 2. Flow chart of kluiklui production. *: Samples collected for mycotoxin analysis.*

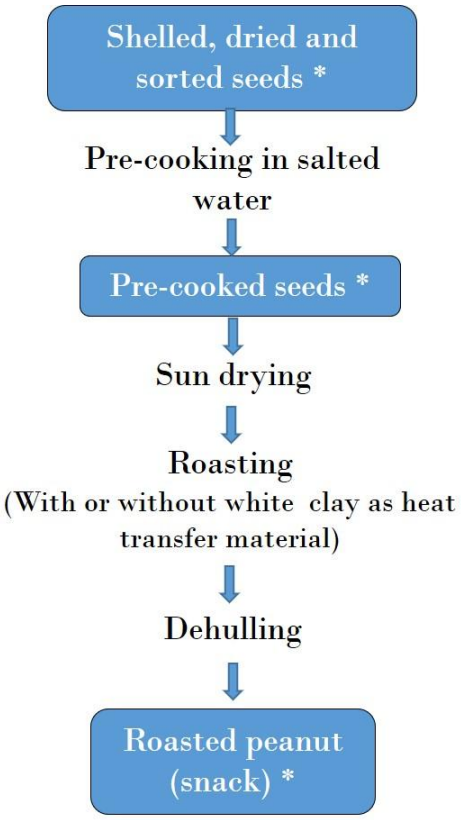

*Figure 3. Flow chart of roasted peanut production. *: Samples collected for mycotoxin analysis.*

The comparison between both processes for *kluiklui* production is particularly relevant, not so much because of the potential role of maize flour as a source of mycotoxins, but rather due to the type of grinder employed. In Covè, the production process without maize flour relies on a grinder dedicated exclusively to peanut grinding, whereas in Aplahoué, the process incorporating maize flour involves the use of a mixed-use grinder. To assess the contribution of peanut processing practices to mould and mycotoxin contamination, *kluiklui* and roasted peanut snacks production follow-up was conducted on 2 producing sites (Covè and Aplahoué). The study considered both processing methods for *kluiklui* (with or without the addition of maize flour) and for roasted peanuts (with or without the use of a heat transfer material). Three independent productions, involving three different processors, were carried out for each product and processing method, resulting in a total of 36 trials (Table 1). The 12 processors were selected based on their experience in processing peanuts into *kluiklui* or roasted peanut snacks.

Table 1. Experimental design applied to the production of kluiklui and roasted peanuts.

Peanut-based foods	Municipality	Processing technology considered	Number of processors	Number of trials
Kluiklui	Covè	Technology excluding maize flour during kneading of the peanut paste	3	9*
	Aplahoué	Technology involving maize flour addition during kneading of the peanut paste	3	9
Roasted peanut snack	Covè	Roasting without heat transfer material	3	9
	Abomey	Roasting with heat transfer material	3	9

** 3 processors per processing technology and 3 trials per processor.*

3.2. Samples Collected During Follow-Up Experiments

The raw peanuts (unshelled dried seeds) were purchased from a single peanut producer based in Covè. The crop was harvested in November 2023 and underwent two post-harvest drying phases: a first sun-drying in the field for 7 days, followed by a second drying at the producer's residence for 14 days. After drying, the seeds were stored unshelled in polypropylene bags.

As mentioned before, *kluiklui* and roasted peanut snack production were performed 3 times by each processor, at intervals of two months between the first and second repetition, and one month between the second and third repetition.

Figure 4 illustrates the distribution of raw material between the 12 selected processors, for one trial. Before each trial, shelling was carried out by the peanut producer using a mechanical sheller. Shelling was stopped once 300 kg of shelled peanuts had been obtained. The shelled seeds were then packaged in 25 kg bags before being distributed to the processors. The 25 kg of shelled seeds were sun-dried and roughly sorted (by visual examination to remove impurities and bad seeds) by each processor before being used for *kluiklui* or roasted peanut snack production.

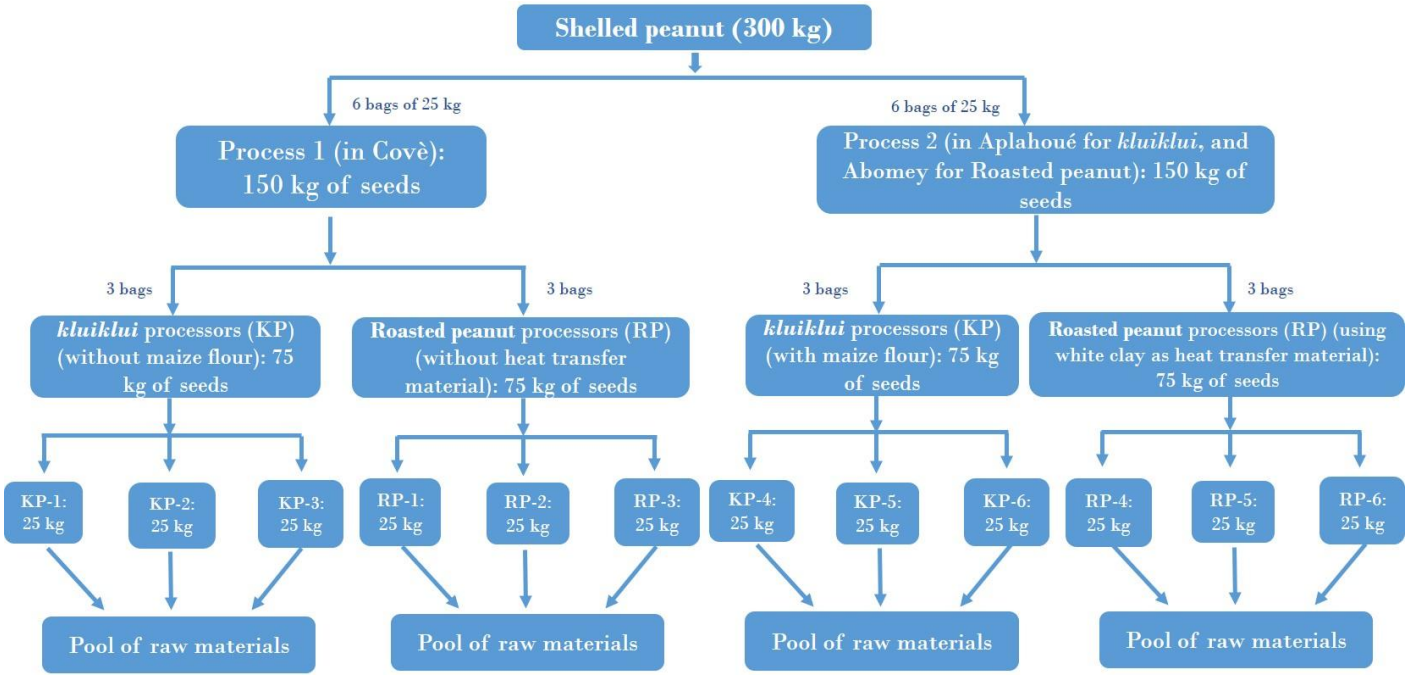

Figure 4. Distribution plan of raw material (shelled peanut) between the 12 processors, for each trial. This trial was performed 3 times.

For mycotoxin analysis, raw peanuts (shelled, dried and sorted seeds) as well as roasted peanuts, peanut paste, maize flour, and *kluiklui* were sampled in the follow-up of *kluiklui* production, while raw peanuts and samples of the final product were collected in the follow-up of roasted peanut snack production.

As the raw material was of the same origin, samples of raw peanuts (shelled, dried and sorted seeds) were pooled. For laboratory analyses, 12 pooled samples of this raw material were created by mixing the 3 samples taken from each trial. Pooling was performed in the laboratory. After freeze-drying and grinding of each individual sample, pools were formed by combining three 50 g subsamples using a blender to obtain a homogeneous mixture. Table S1 presents details of the pooling of raw materials. When pooled raw material samples tested positive for mycotoxins, the corresponding individual samples were analysed, and the resulting data were considered in the present study (Table S1).

During the production of *kluiklui*, roasted peanut samples were collected immediately after partial roasting and cooling, and just before the dehulling and grinding of seeds. Peanut paste was obtained by grinding the roasted seeds using public grinding services. Samples were collected immediately after grinding. The maize flour used in the process was sourced from household stocks and was produced by grinding maize grains purchased from local markets. It was sampled as well. *Kluiklui* samples, produced by frying the shaped and pressed peanut paste, were collected immediately after frying and cooling.

Roasted peanut snacks were obtained by precooking and roasting peanut seeds either with or without a heat transfer material (white clay). Samples of roasted peanut snacks were collected immediately after roasting and cooling of seeds.

4. Determination of Mycotoxins in Peanut-Based Foods Samples

4.1. Standards and Chemicals

Analytical grade standards and solvents were used in this study and sourced from various suppliers. The solvents and reagents included: acetonitrile AR (Biosolve, Dieuze, France), mass spectrometry grade acetonitrile (Biosolve, Dieuze, France), acetic acid AR (Fisher Chemical, Merelbeke, Belgium), MS/MS grade methanol (Biosolve, Dieuze, France), formic acid (Avantor, VWR, Leuven, Belgium), ammonium acetate (Fluka, Mint Street, NC, USA), magnesium sulphate (MgSO4) and sodium chloride (NaCl), both from Roth (Karlsruhe, Germany). Ultrapure water was obtained using a Milli-Q® IQ 7010 purification system (MilliporeSigma, Burlington, MA, USA). Certified analytical standards of mycotoxins were obtained from Romer-Labs (Getzersdorf, Austria) for the following compounds: 15-acetyl-deoxynivalenol (15Ac-DON), 3-acetyl-deoxynivalenol (3Ac-DON), deoxynivalenol (DON), HT-2 toxin, alternariol (AOH), alternariol monomethyl ether (AME), T-2 toxin, zearalenone (ZEN), AF B1, B2, G1 and G2, OTA, ochratoxin B (OTB), FUM B1, FUM B2 and FUM B3, as well as sterigmatocystin. Standards for enniatins A, A1, B, and B1 were purchased from Merck (Merck KGaA, Darmstadt, Germany).

4.2. Determination of Mycotoxins

Mycotoxin concentration in marketed *kluiklui* and roasted peanut snacks and in samples from production follow-up were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS), according to the method developed by (Jonard et al., 2025) . A QuEChERS extraction procedure was used for extraction by adding 10 mL of ultrapure water to 5 g of ground sample, accurately weighed into 50 mL polypropylene centrifuge tubes. Then, 10 mL of acetonitrile containing 1% (v/v) acetic acid was added, and samples were homogenised using a Mixer Mill MM400 (Retsch GmbH, Aartselaar, Belgium) at 10 Hz for 10 min. Subsequently, 4 g of anhydrous magnesium sulphate (MgSO4) and 1 g of sodium chloride (NaCl) were added. The tubes were manually shaken and agitated using the Mixer Mill MM400 for 2 min at 10 Hz. The mixtures were then centrifuged at 3000× g for 5 min using a refrigerated centrifuge (Centrifuge 5810 R, Eppendorf, Leipzig, Germany). A 100 µL aliquot of the resulting supernatant was collected and diluted with 900 µL of a 50:50 (v/v) methanol–water solution. The final extracts were transferred into chromatographic vials for

analysis. The LC-MS/MS analyses were carried out using a Waters ACQUITY I-Class UPLC system (Waters, Milford, MA, USA) equipped with a BEH C18 analytical column (2.1 × 50 mm, 1.7 µm particle size; Waters), coupled to a Xevo TQ-xS mass spectrometer (Waters, Milford, MA, USA). Column temperature was maintained at 50 °C. The injection volume was 2 µL, and the flow rate was set at 0.3 mL/min. An elution gradient was applied using mobile phase A consisting of an ammonium acetate-water solution containing 0.1% of formic acid, and mobile phase B composed of methanol with 0.1% of formic acid. The gradient started at 10% B, held for 3 min and increased linearly to 70% over 7 min. Then, the gradient increased again from 10% in 10 s and remained constant for 2 min. Finally, B decreased to 10% in 10 s followed by an equilibration period of 3 min.

Multiple reaction monitoring mode under positive ionisation mode was used on the mass spectrometer. The capillary voltage was set to 3.4 kV, with a desolvation temperature of 400 °C. The desolvation gas flow and cone gas flow were maintained at 800 L/H and 150 L/H, respectively, and the nebuliser pressure was 7 bar.

The software TargetLynx V4.2 (Waters Corporation, Milford, MA, USA) was used to perform chromatographic peak integration and quantification.

4.3. Method Validation

The method was fully validated on wheat. Hence, only a lighter validation was performed on peanuts. Average recoveries for each mycotoxin were evaluated for peanut, peanut paste and maize flour by spiking “blank” samples containing no mycotoxin above the LOD. A total of 6 “blank” samples were extracted and analysed on three different days. Reproducibility and repeatability were thus also evaluated. The limit of detection was determined using a signal to noise ration equal to 10.

5. Data Analysis

Microsoft Excel 2019 was used to perform descriptive statistical analyses. Statistical analyses were done using R version 4.4.2 software. Mycotoxin concentration data were expressed on a wet weight basis. Data were initially tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene’s test, prior to conducting further statistical analyses. Differences between the two groups were analysed using an independent sample t-test or a Wilcoxon nonparametric test. All differences were considered statistically significant at $p < 0.05$.

Results and discussion

1. Method validation

The method previously validated on wheat was successfully transferred to peanuts, peanut paste and maize flour. Despite the higher fat content of peanut-based matrices compared to wheat, no modification of the extraction procedure was required. Since the method had already undergone full validation in wheat, its application to peanuts, peanut-derived products and maize flour was validated using a reduced number of samples.

The limits of detection (LOD) and average recoveries obtained for roasted peanuts, peanut paste and maize flour are presented in Table 2. The LODs achieved by the method were at least ten times lower than the corresponding ML established by European Commission (European Commission, 2023c), demonstrating that the method is sufficiently sensitive to reliably discriminate between compliant and non-compliant samples. Regarding accuracy, recovery values for all investigated mycotoxins exceeded 50 % across the three matrices. According to validation criteria for mycotoxin analysis, recoveries ranging from 50 to 120 % are considered acceptable (European Commission, 2021).

Table 2. Limit of detection (LOD) and recovery of mycotoxin of interest in the different matrices.

Mycotoxin	LOD (µg/kg)	Recovery in roasted peanuts (%)	Recovery in peanut paste (%)	Recovery in maize flour (%)
Aflatoxin B1	0.024	74	69	95
Aflatoxin B2	0.066	69	67	88
Aflatoxin G1	0.16	68	63	94
Aflatoxin G2	0.13	69	67	99
Fumonisin B1	2.4	58	51	100
Fumonisin B2	8.0	67	60	69
Fumonisin B3	6.8	64	56	110
Ochratoxin A	0.5	84	91	120
Ochratoxin B	0.5	82	88	107

The intra-day repeatability and inter-day reproducibility of the method in peanuts are summarized in Table 3. Overall, the coefficients of variation (CV) obtained have demonstrated satisfactory repeatability and reproducibility of the method. For most mycotoxins, the CVs were below 20% which is compliant with European standards (European Commission, 2021). These results indicate good method repeatability and reproducibility and are in agreement with previous studies on mycotoxin analysis in peanuts (Ren et al., 2007; Hidalgo-Ruiz et al., 2019).The CV of reproducibility of FUM B1 was 25.1% but it is still compliant with European standards (European Commission, 2021).

Table 3. Average intra-day repeatability and inter-day reproducibility of mycotoxin of interest in roasted peanuts.

Mycotoxin	Variation coefficient of intra-day repeatability (%)	Variation coefficient of inter-day reproducibility (%)
Aflatoxin B1	5.9	5.0
Aflatoxin B2	5.6	7.9
Aflatoxin G1	4.8	5.1
Aflatoxin G2	6.9	8.6
Fumonisin B1	17.0	25.1
Fumonisin B2	7.8	13.1
Fumonisin B3	2.5	3.7
Ochratoxin A	7.8	10.4
Ochratoxin B	3.1	7.3

In summary, the results confirm that the method exhibits adequate LODs, accuracy, repeatability and reproducibility for the simultaneous determination of the studied mycotoxins in peanuts and peanut-based products.

2. Occurrence of Mycotoxins in Marketed Roasted Peanut Snack and Kluiklui

Figure 5 illustrates the concentration ranges of main mycotoxins quantified in market samples of roasted peanut snacks and *kluiklui* in Benin, i.e., AFB1, AFB2, AFG1, AFG2, and OTA and OTB. The detailed results for all analysed mycotoxins are presented in Supplementary Materials (Tables S2 and S3).

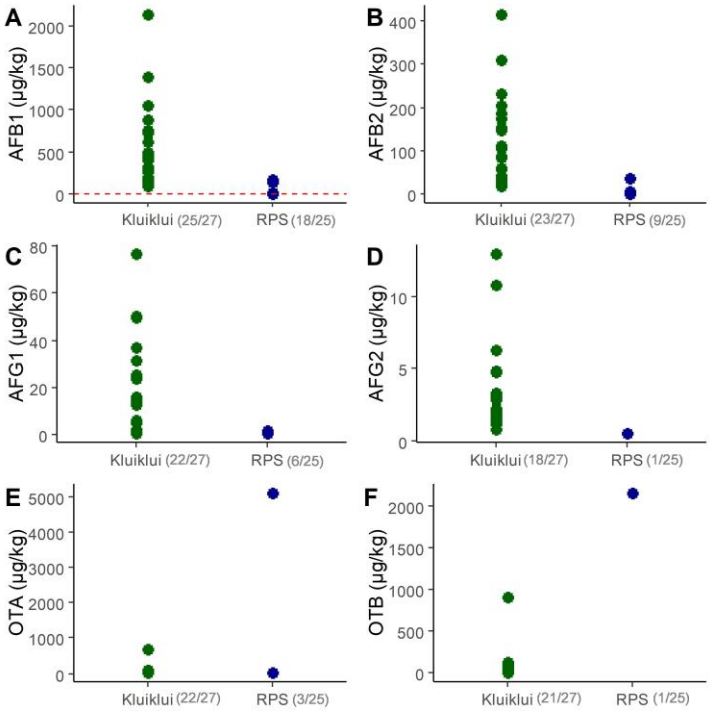

Figure 5. Concentrations of AFB1 (A), AFB2 (B), AFG1 (C), AFG2 (D), OTA (E) and OTB (F) in samples of marketed kluiklui and roasted peanut snacks (RPS). Only samples above limit of quantification (LOQ) are shown. LOQ values are 0.2 µg/kg for AFB1, AFB2, AFG1, AFG2, and 0.5 µg/kg for OTA and OTB. The total number of samples was 27 for kluiklui and 25 for roasted peanut snacks. The number of samples above the LOQ among the total number of samples is mentioned in brackets. In Graph A, the red dotted line represents the AFB1 limit of 2 µg/kg for peanut-based foods, as established by the European Commission (European Commission, 2023c).

AFB1, AFB2, and AFG1 were the most frequently detected mycotoxins in roasted peanut snacks, with respective concentrations ranging from 0.02 to 169.0 µg/kg, 0.07 to 35.3 µg/kg, and 0.16 to 1.7 µg/kg, respectively, and corresponding median concentrations of 1.0, 0.07, and 0.16 µg/kg, respectively. AOH, FUM B1 and B2 were never detected in roasted peanut (Figure 5; Tables S2 and S3). One particular sample (ID: 20230.0S264, from Aplahoué) displayed inexplicably very high concentrations of OTA and OTB (5085 and 2143 µg/kg, respectively) (Figure 5; Tables S2 and S3).

In *kluiklui* samples, AFB1, AFB2, AFG1, AFG2, OTA and OTB were detected in almost all samples (Tables S2 and S3). Their concentration ranges were 89.1–2144.6 µg/kg for AFB1, 0.07–416.4 µg/kg for AFB2, 0.16–76.8 µg/kg for AFG1, 0.13–13 µg/kg for AFG2, 0.4–668 µg/kg for OTA, and 0.4–909 µg/kg for OTB (Tables S2 and S3). The corresponding median upper bound (UB) concentrations were 394.8, 83.9, 12.8, 1.7, 23.3, and 16 µg/kg, respectively. The minimum, median, and maximum total aflatoxins UB concentrations were 0.38, 1.36 and 169.36 µg/kg for roasted peanuts, while they were 112.1, 478.4 and 2644.1 µg/kg for kluiklui (Tables S2 and S3).

Several studies have identified AFB1, AFB2, AFG1, and AFG2 as the main mycotoxins frequently occurring in *kluiklui*, often in conjunction with OTA (Njumbe Ediage et al., 2011; Adjou et al., 2012). According to Adjou et al. (2012), the concentrations of AFB1, AFB2, AFG1, AFG2, and OTA in *kluiklui* marketed in Benin in 2011 reached 455, 491, 100, 88 and 2 µg/kg, respectively. Njumbe Ediage et al. (2011) reported maximum concentrations of 282 µg/kg for AFB1, 31 µg/kg for AFB2, 79 µg/kg for AFG1, 96 µg/kg for AFG2, and 2 µg/kg for OTA in *kluIiklui* samples collected in Benin in 2010. In Nigeria, a neighbouring country of Benin, in 2011, AFB1 and AFG2 concentrations of 2820 and 477 µg/kg, respectively, were reported in *kluiklui* (Ezekiel et al., 2012).

AFB1, AFB2, AFG1 and AFG2 were quantified in roasted peanuts sold in Nigeria, with concentrations reaching up to 165, 26, 20 and 10 µg/kg, respectively (Bankole et al., 2005). In Iran, concentrations of 244, 6.40, 69.25 and 3.45 µg/kg of AFB1, AFB2, AFG1 and AFG2, respectively, have been reported in marketed roasted peanuts in 2015/2016 (Bakherad and Feizy, 2018). Sombie et al. (2018) also reported the presence of AFB1, AFB2, AFG1 and AFG2 in marketed roasted peanut samples, with concentrations reaching up to 1387, 271, 3328 and 742 µg/kg, respectively, in Serra Leone in 2017. None of these studies reported the presence of ochratoxins in roasted peanut snacks, in contrast to the findings of this study. Benin, Nigeria, and Sierra Leone are West African countries with a tropical climate, under the influence of the Atlantic Ocean, and showing regional variations (Chen and Chen, 2013). These countries are very vulnerable to climate change, which favours mycotoxin

contamination in peanuts and peanut-based foods, causing a possible increase in cancer incidence (Yu et al., 2022; World Bank, 2025).

In Europe, no maximal level exists for ochratoxins in peanut-based food, while for aflatoxins, the European Commission set a maximum concentration of 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins (i.e., the sum of AFB1, B2, G1 and G2) in peanut-based food for the final consumer (European Commission, 2023c). Among samples above LOQ, all *kluiklui* samples and 41% of roasted peanut snacks samples had AFB1 concentrations exceeding the established regulatory levels (Figure 5), with maximum levels of AFB1 being 80 and 1000 times higher than the limit, respectively.

3. Contribution of Processing Practices to Mycotoxin Contamination

3.1. Occurrence of Mycotoxins in Raw Peanuts Used for Kluiklui and Roasted Peanut Snacks Production

Table 4 presents mycotoxin concentrations of raw peanuts used for kluiklui and roasted peanut snacks production. Since preliminary analyses revealed mycotoxin contamination of pools 11 and 12, individual samples forming these pools were analysed. Thus, pools 1 to 10, the three individual samples forming pool 11, and the three samples forming pool 12 were analysed, for a total of 16 raw material samples (10 pools and 6 individual samples). Table S4 presents the individual data for the raw material samples analysed. Concentrations of AFB1 and total aflatoxins ranged from 0.02 (Limit of detection (LOD)) to 2.63 µg/kg, and from 0.09 to 2.97 µg/kg, respectively. In case of raw peanuts (to be sorted before human consumption), the European Commission has established maximum levels of 8 µg/kg for AFB1 and 15 µg/kg for total aflatoxins (European Commission, 2023c). Similarly, in Benin, Decree 2007 N°0362 MAEP/D-CAB/SGM/DRH/DP/SA, which sets the maximum levels for contaminants in foodstuffs, has also established the same levels for AFB1 (8 µg/kg) and total aflatoxins (15 µg/kg) (Ministère de l'agriculture, de l'élevage et de la pêche, 2007). Aflatoxin concentrations in raw peanuts used to produce *kluiklui* and roasted peanut snacks in this study are below these European Commission and Benin Government levels. The concentrations of AOH, FUM B1, FUM B2, OTA, and OTB were below the limits of quantification for all analysed raw material samples.

Table 4. Mycotoxin concentrations of raw peanuts used for kluiklui and roasted peanut snacks (n = 18) (µg/kg, UB).

Parameters	AFB1*	AFB2	AFG1	AFG2	AFtot	FUM B1	FUM B2	OTA	OTB
Sample > LOQ**	3	1	0	0	-	0	0	0	0
Min	0.02	0.07	<LOD	<LOD	0.09	<LOD	<LOD	<LOD	<LOD
Med	0.02	0.07	<LOD	<LOD	0.09	<LOD	<LOD	<LOD	<LOD
Max	2.63	0.34	<LOD	<LOD	2.97	<LOD	<LOD	<LOQ	<LOQ
% Sample > EU ML***	0	NA	NA	NA	0	NA	NA	NA	NA

** AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFtot: Total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2), FUM B1: Fumonisin B1, FUM B2: Fumonisin B2, OTA: Ochratoxin A, OTB: Ochratoxin B; ** Sample > LOQ: Number of samples above limit of quantification (AFB1, AFB2, AFG1, AFG2: 0.2 µg/kg; FUM B1, FUM B2: 10 µg/kg; OTA, OTB: 0.5 µg/kg); LOD were 0.02, 0.07, 0.16 and 0.13 µg/kg for AFB1, AFB2, AFG1 and AFG2, respectively; and 0.4 µg/kg for OTA and OTB. The upper bound (UB) concentrations, where left-censored values were replaced by the LOQ or LOD, are presented for AFB1 and AFB2. *** EU ML: Maximum limits of 8.0 and 15.0 µg/kg for AFB1 and Total Aflatoxin for raw peanuts, which are not directly consumed, but subjected to a processing (European Commission, 2023c); N/A, not applicable: No maximum levels (ML) existed for this mycotoxin for peanut-based foods.*

3.2. Kluiklui Processing Practices and Mycotoxins Contamination

The mycotoxin contamination of *kluiklui* samples collected during processing practices is presented in Table 5 and Table S5. All roasted peanut samples (roasting being the first step of the process, see Figure 2) were below the LOD for all the mycotoxins analysed, showing that roasting did not contribute to mycotoxin contamination during *kluiklui* production process.

Table 5. Mycotoxin contamination (µg/kg, UB) in samples collected during kluiklui processing.

Process	Sample	AFB1*	AFB2	AFG1	AFG2	AFtot	AOH	FUM B1	FUM B2	OTA	OTB
Without maize flour (Covè)	Roasted peanut (n=9)	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)	-	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)
	Peanut paste (n=9)	50.56±19.74^{a} (9)	7.82±2.29^{a} (9)	2.20±1.00^{a} (6)	0.33±0.30 (1)	60.91±21.98^{a} -	10.31±4.00 (1)	< LOD (0)	< LOD (0)	3.70±0.11^{a} (7)	1.79±0.13^{a} (6)
	Kluiklui (n=9)	37.78±14.35^{1} (9)	7.37±2.26^{1} (9)	1.60±0.20^{1} (5)	0.23±0.18 (1)	46.99±16.88^{1} -	10.20±3.80 (1)	4.09±2.93 (0)	<LOQ (0)	7.05±3.71^{1} (7)	3.37±1.97^{1} (7)
With maize flour (Aplahoué)	Roasted peanut (n=9)	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)	-	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)	< LOD (0)
	Peanut paste (n=9)	14.65±9.54^{b} (9)	6.58±6.77^{a} (9)	0.25±0.15^{b} (1)	< LOD (0)	21.60±10.08^{b} -	< LOD (0)	< LOD (0)	< LOD (0)	6.13±5.00^{a} (4)	2.94±3.99^{a} (4)
	Maize flour (n=6)	24.11±17.08 (6)	2.61±1.97 (6)	0.42±0.01 (4)	< LOD (0)	27.26±19.06 -	< LOD (0)	991.51±277.26 (6)	333.84±78.72 (6)	2.32±3.71 (2)	0.62±0.46 (2)
	Kluiklui (n=9)	11.73±7.09^{2} (9)	4.14±2.29^{1} (9)	0.24±0.14^{2} (1)	< LOD (0)	16.24±7.87^{2} -	< LOD (0)	31.28±47.84^{1} (3)	14.14±9.50^{1} (2)	6.70±2.71^{1} (5)	4.51±6.09^{1} (5)

** AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFtot: Total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2), AOH: Alternariol, FUM B1: Fumonisin B1, FUM B2: Fumonisin B2, OTA: Ochratoxin A, OTB: Ochratoxin B Upper bound values (UB): concentrations below the limit of quantification (LOQ) or the limit of detection (LOD) were replaced by the LOQ or LOD value. LOQ were 0.2 µg/kg for AFB1, AFB2, AFG1 and AFG2; 10 µg/kg for AOH, FUM B1 and FUM B2; and 0.5 µg/kg for OTA and OTB. LOD were 0.02, 0.07, 0.16, 0.13, 8, 2.4 and 8 µg/kg for AFB1, AFB2, AFG1, AFG2, AOH, FUM B1 and FUM B2, and 0.4 µg/kg for OTA and OTB, respectively. The number of samples above the LOQ is mentioned in brackets. Values within the same column followed by the same letter or number do not differ significantly at the 5% probability level. (n=2).*

On the contrary, aflatoxins were quantified in all peanut paste samples obtained after dehulling and grinding, which are the next steps of the process (Figure 2). Ochratoxins were found as well, but not in all samples, while FUM B1 and B2 and AOH were below the LOD in all peanut paste samples

(Table 5 and Table S5). The mean concentrations of AFB1, total aflatoxins, and OTA were 50.56, 60.91 and 3.70 µg/kg, respectively, for peanut paste from the processing without maize flour; and 14.65, 21.60 and 6.13 µg/kg, respectively, for peanut paste from the processing with maize flour. These findings suggest a contamination occurring during the grinding of roasted peanut seeds, as reported by Ndung'u et al. (2013). Indeed, the grinding is carried out using a public grinder, originally intended for maize or spice grinding. The use of such shared equipment may lead to cross-contamination, as various raw materials of differing hygienic quality are processed in the same grinder. Additionally, as these are public facilities, processors have little control over the sanitary conditions and maintenance of the grinding equipment.

AFB1 and total aflatoxin concentrations were significantly higher in peanut paste samples collected from the process without maize flour; with respective p-values of 0.04718 and 0.0479 for AFB1 and total aflatoxins. During peanut processing without maize flour, conducted in the municipality of Covè, the public grinders used were exclusively dedicated to *kluiklui* production. In contrast, in Aplahoué, the process involving maize flour relied on mixed-use grinders, which were not reserved exclusively for peanuts but were also used for maize grinding. As these grinders in the municipality of Aplahoué are shared with maize processing, a cleaning step is systematically performed prior to peanut grinding, to remove residual maize flour. However, in the municipality of Covè, since the grinders are used exclusively for peanut processing, thorough cleaning is not routinely performed prior to grinding. This could explain the higher level of contamination observed in peanut paste from the process without maize flour. Moreover, the warm and humid climate in southern Benin favours the increase in contamination with fungi and mycotoxins in peanut and ground maize residues from public grinders.

Regarding the maize flour used in *kluiklui* production, all collected samples were contaminated with aflatoxins and FUMs, and showed important variation among samples (Table 5 and Table S5). The mean concentrations were 24.11 µg/kg for AFB1, 27.26 µg/kg for total aflatoxins, 991.57 µg/kg for FUM B1, and 333.84 µg/kg for FUM B2. Contamination of maize by FUMs has also been reported in other studies conducted in Benin, with concentrations of FUM B1 and B2 reaching up to 836 and 221 µg/kg, respectively (Njumbe Ediage et al., 2011), and as high as 14,200 mg/kg and 3750 mg/kg, respectively, in earlier findings (Fandohan et al., 2005).

Regarding the final product of both processing methods, all *kluiklui* samples were contaminated with AFB1 and AFB2. Both OTA and OTB were found in a total of 12 samples out of 18, FUM B1 and B2 in 3 and 2 samples out of the 9 processed with the addition of maize flour, while AOH was not

found in any sample. *Kluiklui* produced without maize flour and with maize flour had mean AFB1 concentrations of 37.78 and 11.73 µg/kg, respectively; and mean total aflatoxin concentrations of 46.99 and 16.24 µg/kg, respectively. AFB1 (37.78 vs. 11.73 µg/kg) and total aflatoxins concentration (46.99 vs 16.24 µg/kg) were significantly higher in *kluiklui* samples produced without the addition of maize flour, compared to those produced with maize flour (Table 5). The corresponding p-values were 0.04787 and 0.04584, respectively, for AFB1 and total aflatoxins. These results are in line with those reported by (Kayode et al., 2013), who observed higher average concentrations of total aflatoxins in peanut-based snacks (620.9 µg/kg) than in peanut/maize-based snacks (12 µg/kg).

The low levels of FUM B1 and FUM B2 found in some samples of *kluiklui* produced using maize flour could be explained by the low amount of maize flour added to the peanut paste (about 10 g of maize flour per kg of peanut paste).

AFB1, total aflatoxins and OTA concentrations were significantly lower in just-produced *kluiklui* (from production follow-up) than marketed *kluiklui* with p-values of 2.158 × 10−6, 1.924 × 10−6 and 0.001096 for AFB1, total aflatoxins and OTA, respectively (Table S7). These findings suggest that inadequate post-production storage conditions may play a significant role in the contamination of *kluiklui* with mycotoxins.

3.3. Roasted Peanut Processing Practices and Mycotoxin Contamination

Table 6 and Table S6 present the contribution of roasting practices (with or without heat transfer material) to mycotoxin contamination in roasted peanut snacks.

Table 6. Mycotoxin concentration in roasted peanut snacks according to the process (µg/kg, UB).

Roasting process	Sample	AFB1*	AFB2	AFG1	AFG2	AF_{tot}	OTA
Without heat transfer material	Raw peanut (n=9)	<LOQ (0)	<LOD (0)	<LOD (0)	<LOD (0)	-	<LOD (0)
	Roasted peanut snack (n=9)	0.50±0.29[a] (3)	0.20±0.09[a] (2)	<LOD (0)	<LOD (0)	1.56±1.14[a] -	<LOD (0)
With heat transfer material	Raw peanut (n=9)	0.35±0.47 (1)	<LOQ (1)	<LOD (0)	<LOD (0)	0.37±0.53 -	<LOQ (0)
	Roasted peanut snack (n=9)	0.12±0.12[a] (1)	0.11±0.04[a] (2)	<LOD (0)	<LOD (0)	0.43±0.51[a] -	<LOD (0)

** AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFtot: Total aflatoxin (AFB1+ AFB2+ AFG1+ AFG2), OTA: Ochratoxin A, OTB: Ochratoxin B. Upper-bound values (UB): concentrations below the limit of quantification (LOQ) or the limit of detection (LOD) were replaced by the LOQ or LOD value. LOQ of 0.2 µg/kg for AFB1, AFB2, AFG1 and AFG2; 10 µg/kg for FUM B1 and FUM B2; and 0.5 µg/kg for OTA and OTB. LOD of 0.02, 0.07, 0.16, 0.13 and 0.4 µg/kg for AFB1, AFB2, AFG1, AFG2 and OTA, respectively. The number of samples above the LOQ is mentioned in brackets. Values within the same column followed by the same letter do not differ significantly at the 5% probability level. The mean concentrations of AFB1 (p = 0.1825) and total aflatoxins (p = 0.1997) were not significantly different in roasted peanut snacks produced using roasting processes with and without heat transfer material. (n=2).*

AFB1 and total aflatoxins concentrations were 0.50 and 1.56 µg/kg, respectively, in roasted peanut snack samples processed without a heat transfer material; whereas concentrations of 0.12 and 0.43 µg/kg were recorded, respectively, in samples processed using a heat transfer material. Roasting is generally expected to reduce mycotoxin levels in peanut seeds, as reported in several studies conducted in Brazil (Martins et al., 2017), Togo (N'pagyendou et al., 2022), Senegal (Diédhiou et al., 2012) and Nigeria (Adeniran et al., 2022). In the present study, the low contamination of peanut seeds by mycotoxins did not allow for an assessment of the effect of roasting processes on the mycotoxin content of roasted peanuts.

There was no AFB1 contamination in the raw seeds used in the process without heat transfer material (<LOQ) and low contamination in those used in the process with heat transfer material (0.35 µg/kg). Despite the absence of mycotoxin contamination in raw peanut seeds used in the roasting process without heat transfer material, AFB1 were detected in the corresponding roasted peanut samples (0.50 µg/kg). Heterogeneity in seed contamination by mycotoxins, as reported by Teixido-Orries et al. (2025), could explain the variability observed in contamination levels between the raw materials used in the two roasting processes. Further studies would be valuable to assess the impact of peanut storage practices, as commonly performed in Benin, on seed contamination by mycotoxins, as well as on their distribution within a batch of seeds.

No significant difference was observed in the concentrations of AFB1 (p-value = 0.1825) and total aflatoxins (p-value = 0.1997) between roasted peanut snacks produced using roasting processes

with and without heat transfer material. The use of a heat transfer material did not contribute to the mycotoxin contamination of the roasted peanuts.

It is also important to note that just-produced roasted peanut snacks do not show significantly different concentrations of mycotoxins compared to those available on the market ($p > 0.05$) (Table S7).

Conclusions

The present study revealed a high level of mycotoxin contamination in peanut-based foods marketed in Southern Benin. AFB1, AFB2, AFG1, AFG2, as well as OTA and OTB, were the predominant mycotoxins detected in roasted peanut snacks and *kluiklui*. Mycotoxin contamination was found to be higher in *kluiklui* samples than in roasted peanut snacks, in marketed and just-produced samples. *Kluiklui* collected from local markets were found to be the most contaminated, compared to those collected immediately after production. The storage conditions of *kluiklui* appear to be a critical factor contributing to its contamination with mycotoxins. During the production of *kluiklui*, the grinding of roasted peanuts in public grinders significantly contributed to its contamination with mycotoxins, especially when the grinding was carried out in grinders exclusively dedicated to peanut processing. In the current context of climate change and global warming, favourable to fungi proliferation, it is essential to improve the storage conditions of *kluiklui* as well as the hygienic quality of the grinding equipment used during its production, in order to limit fungal and mycotoxin contamination. Such improvements are crucial to reduce consumer health risks.

Supplementary materials

***Table S1**: Pooling of raw material (peanut seeds) used for kluiklui and roasted peanut snack processing.*

Trial number	Municipality	Product	Processor	Sample ID	Pools
1	Covè	*Kluiklui*	KP-1	2024-S398	Pool 1
			KP-2	2024-S399	
			KP-3	2024-S400	
		Roasted peanut snack	RP-1	2024-S401	Pool2
			RP-2	2024-S402	
			RP-3	2024-S403	
	Aplahoué	*Kluiklui*	KP-4	2024-S404	Pool 3
			KP-5	2024-S405	
			KP-6	2024-S406	
	Abomey	Roasted peanut snack	RP-4	2024-S407	Pool4
			RP-5	2024-S408	
			RP-6	2024-S409	
2	Covè	*Kluiklui*	KP-1	2024-S410	Pool 5
			KP-2	2024-S411	
			KP-3	2024-S412	
		Roasted peanut snack	RP-1	2024-S413	Pool 6
			RP-2	2024-S414	
			RP-3	2024-S415	
	Aplahoué	*Kluiklui*	KP-4	2024-S416	Pool 7
			KP-5	2024-S417	
			KP-6	2024-S418	
	Abomey	Roasted peanut snack	RP-4	2024-S419	Pool 8
			RP-5	2024-S420	
			RP-6	2024-S421	
3	Covè	*Kluiklui*	KP-1	2024-S422	Pool 9
			KP-2	2024-S423	
			KP-3	2024-S424	
		Roasted peanut snack	RP-1	2024-S425	Pool 10
			RP-2	2024-S426	
			RP-3	2024-S427	
	Aplahoué	*Kluiklui*	KP-4	2024-S428	Pool 11
			KP-5	2024-S429	
			KP-6	2024-S430	
	Abomey	Roasted peanut snack	RP-4	2024-S431	Pool 12
			RP-5	2024-S432	
			RP-6	2024-S433	

Table S2: *Average mycotoxins concentrations of marketed roasted peanut snacks and kluiklui (µg/kg) (n=2).*

Category	Sample ID	AFB1	AFB2	AFG1	AFG2	AOH	FUM B1	FUM B2	FUM B3	OTA	OTB	STE
Roasted peanut snacks	20230.0S259	1.0 ± 0.07	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	< LOQ	< LOQ
Roasted peanut snacks	20230.0S260	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	< LOQ	< LOQ
Roasted peanut snacks	20230.0S261	2.0 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	< LOQ	< LOQ
Roasted peanut snacks	20230.0S263	7.0 ± 0.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	< LOQ	< LOQ
Roasted peanut snacks	20230.0S264	4.0 ± 0.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5085 ± 11	2143 ± 25	< LOQ
Roasted peanut snacks	20230.0S265	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5.0 ± 0.31	< LOQ	< LOQ
Roasted peanut snacks	20230.0S266	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	< LOQ	< LOQ
Roasted peanut snacks	20230.0S269	169 ± 8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.0 ± 0.02	< LOQ	3.0 ± 0.2
Roasted peanut snacks	20230.0S258	2.2 ± 0.02	0.7 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S262	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S267	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S268	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S270	4.7 ± 0.6	1.4 ± 0.06	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S271	0.5 ± 0.01	< LOQ	0.4 ± 0.03	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S272	1.9 ± 0.1	0.6 ± 0.03	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S273	18 ± 2	5.1 ± 0.3	0.4 ± 0.03	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S274	14 ± 1	3.6 ± 0.7	0.9 ± 0.05	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S275	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S276	0.3 ± 0.05	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S277	14 ± 0.2	4.2 ± 0.3	1.0 ± 0.07	0.5 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S278	5.4 ± 0.2	1.9 ± 0.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S279	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S280	0.46 ± 0.04	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S281	132 ± 12	35 ± 2	0.6 ± 0.09	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S282	0.72 ± 0.03	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Roasted peanut snacks	20230.0S283	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Roasted peanut snacks	20230.0S284	6.1 ± 0.05	1.0 ± 0.02	1.7 ± 0.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Kluiklui	20230.0S307	119 ± 1	25 ± 0.2	5.8 ± 0.04	1.6 ± 0.06	<LOD	<LOD	<LOD	<LOD	31 ± 1	22 ± 2	<LOD
Kluiklui	20230.0S308	206 ± 6	43 ± 1	16 ± 0.6	1.9 ± 0.02	<LOD	121 ±	28 ±	<LOD	21 ± 1	14 ± 1	<LOD
Kluiklui	20230.0S309	254 ± 7	57 ± 2	14 ± 0.6	1.2 ± 0.07	<LOD	<LOD	<LOD	<LOD	12 ± 1	7.0 ± 0.5	<LOD
Kluiklui	20230.0S310	498 ± 6	147 ± 22	24 ± 0.7	2.1 ± 0.09	<LOD	< LOQ	< LOQ	<LOD	8.2 ± 0.3	15 ± 1	<LOD
Kluiklui	20230.0S316	618 ± 11	106 ± 1	26 ± 0.4	2.2 ± 0.19	<LOD	<LOD	<LOD	<LOD	2.0 ± 0.05	0.4 ± 0.07	<LOD
Kluiklui	20230.0S317	109 ± 1	25 ± 0.1	5.0 ± 0.07	1.5 ± 0.03	<LOD	<LOD	<LOD	<LOD	27 ± 1	16 ± 1	<LOD
Kluiklui	20230.0S318	478 ± 8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	668 ± 7	909 ± 10	4.0 ± 0.3
Kluiklui	20230.0S319	151 ± 4	40 ± 0.1	14 ± 0.5	3.3 ± 0.09	<LOD	<LOD	<LOD	<LOD	81 ± 6	69 ± 3	<LOD
Kluiklui	20230.0S320	2145 ± 29	416 ± 6	77 ± 1	6.3 ± 0.44	<LOD	<LOD	<LOD	<LOD	24 ± 5	14 ± 1	<LOD
Kluiklui	20230.0S322	879 ± 7	205 ± 1	37 ± 1	4.8 ± 0.16	<LOD	<LOD	<LOD	<LOD	56 ± 6	33 ± 1	<LOD
Kluiklui	20230.0S327	446 ± 5	86 ± 2	5.9 ± 0.5	1.1 ± 0.30	<LOD	<LOD	<LOD	<LOD	24 ± 1	24 ± 1	<LOD
Kluiklui	20230.0S328	1395 ± 6	308 ± 4	6 ± 0.2	1.7 ± 0.1	<LOD	<LOD	<LOD	<LOD	32 ± 1	16 ± 0.1	<LOD
Kluiklui	20230.0S329	320 ± 4	84 ± 1	2 ± 0.1	< LOQ	<LOD	<LOD	<LOD	<LOD	39 ± 1	30 ± 1	< LOQ
Kluiklui	20230.0S330	741 ± 1	151 ± 1	13 ± 0.1	3.1 ± 0.3	<LOD	<LOD	<LOD	<LOD	77 ±1	101 ± 1	<LOD
Kluiklui	20230.0S331	761 ± 12	176 ± 3	32 ± 0.6	4.9 ± 0.04	<LOD	<LOD	<LOD	<LOD	16 ± 1	16 ± 0.1	< LOQ
Kluiklui	20230.0S332	1050 ± 12	233 ± 1	13 ± 0.5	3.0 ± 0.08	<LOD	<LOD	<LOD	<LOD	23 ± 1	18 ± 0.1	< LOQ
Kluiklui	20230.0S333	724 ± 1	154.7	0.6 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	18 ± 1	9.0 ± 0.1	<LOD
Kluiklui	20230.0S334	196 ± 2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	81 ± 1	139 ± 2	4.0 ± 0.2
Kluiklui	20230.0S341	92 ± 0.5	19 ± 1	16 ± 0.6	2.9 ± 0.2	<LOD	<LOD	<LOD	<LOD	1.9 ± 0.1	< LOQ	<LOD
Kluiklui	20230.0S342	759 ± 20	186 ± 1	50 ± 1.7	10.8 ± 0.2	<LOD	<LOD	<LOD	<LOD	38 ± 3	18 ± 1	< LOQ
Kluiklui	20230.0S343	395 ± 4	110 ± 1	50 ± 1.8	13.0 ± 0.1	<LOD	<LOD	<LOD	<LOD	2.6 ± 0.1	1.8 ± 1	<LOD
Kluiklui	20230.0S344	145 ± 2	35 ± 1	1.6 ± 0.1	<LOD	<LOD	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
Kluiklui	20230.0S345	89 ± 2	23 ± 1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Kluiklui	20230.0S347	293 ± 8	62 ± 1	1.9 ± 0.04	0.8 ± 0.3	<LOD	<LOD	<LOD	<LOD	10.1 ± 0.5	8.6 ± 1	<LOD
Kluiklui	20230.0S348	204 ± 1	39 ± 1	2.3 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD
LOD		*0.02*	*0.07*	*0.16*	*0.13*	*8*	*2.4*	*8*	*6.8*	*0.4*	*0.4*	-
LOQ		*0.2*	*0.2*	*0.2*	*0.2*	*10*	*10*	*10*	*10*	*0.5*	*0.5*	*10*

Table S3: *Statistics of main mycotoxins concentrations of marketed peanut-based foods (µg/kg, UB) (n=2).*

Sample	Parameter	AFB_1*	AFB_2***	AFG_1***	AFG_2***	AF_{tot}***	OTA***	OTB***
Roasted peanut (n=27)	Sample > LOQ**	18.0	9.0	6.0	1.0	-	3.0	1.0
	Mean ± SD	14.2±39.9[a]	2.1±6.8[a]	0.3±0.4[a]	0.1±0.1[a]	16.7±44.3[a]	189.0±978.5[a]	79.8±412.3[a]
	Min	0.02	0.07	0.16	0.13	0.38	0.4	0.4
	Med	1.0	0.07	0.16	0.13	1.36	0.4	0.4
	P95	97.8	4.83	1.0	0.13	124.7	4.4	0.5
	Max	169.0	35.3	1.7	0.5	169.36	5085.0	2143.0
	% Sample > EU ML	41.0	N/A***	N/A	N/A	33.0	N/A	N/A
Kluiklui (n=25)	Sample > LOQ**	25.0	23.0	22.0	18.0	-	22.0	21.0
	Mean ± SD	522.6±478.5[b]	109.2±102.8[b]	16.5±19.5[b]	2.7±3.2[b]	651.0±592.0[b]	51.7±130.7[b]	59.3±180.0[b]
	Min	89.1	0.07	0.16	0.13	112.1	0.4	0.4
	Med	394.8	83.9	12.8	1.7	478.4	23.3	16.0
	P95	1326.3	293.6	50.3	9.9	1629.1	80.9	131.4
	Max	2144.6	416.4	76.8	13.0	2644.1	668.0	909.0
	% Sample > EU ML	100.0	N/A	N/A	N/A	100.0	N/A	N/A

UB (Upper bound values): concentrations below the limit of quantification (LOQ) and the limit of detection (LOD) were replaced by the LOQ and LOD values.
**AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFtot: Total aflatoxin (AFB1+ AFB2+ AFG1+ AFG2), OTA: Ochratoxin A, OTB: Ochratoxin B;*
***Sample > LOQ: Number of samples above limit of quantification (AFB1, AFB2, AFG1, AFG2: 0.2 µg/kg; FUM B1, FUM B2: 10 µg/kg; OTA, OTB: 0.5 µg/kg);*
Maximum levels of 2.0 and 4.0 µg/kg for AFB1 and Total Aflatoxin for peanut-based foods [1];
****No maximum levels (ML) existed for this mycotoxin for peanut-based foods. N/A, not applicable.*

***Table S4**: Average mycotoxins concentration in raw peanut (µg/kg) (n=2).*

Trial number	Municipality	Product	Processor	Sample		AFB1	AFB2	AFG1	AFG2	AOH	FUM B1	FUM B2	OTA	OTB
1	Covè	*Kluiklui*	KP-1	Pool 1		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-2											
			KP-3											
		Roasted peanut snack	RP-1	Pool 2		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			RP-2											
			RP-3											
	Aplahoué	*Kluiklui*	KP-4	Pool 3		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-5											
			KP-6											
	Abomey	Roasted peanut snack	RP-4	Pool 4		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			RP-5											
			RP-6											
2	Covè	*Kluiklui*	KP-1	Pool 5		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-2											
			KP-3											
		Roasted peanut snack	RP-1	Pool 6		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			RP-2											
			RP-3											
	Aplahoué	*Kluiklui*	KP-4	Pool 7		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-5											
			KP-6											
	Abomey	Roasted peanut snack	RP-4	Pool 8		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			RP-5											
			RP-6											
3	Covè	*Kluiklui*	KP-1	Pool 9		<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-2											
			KP-3											
		Roasted peanut snack	RP-1	Pool 10		<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			RP-2											
			RP-3											
	Aplahoué	*Kluiklui*	KP-4	Pool 11	2024-S428	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-5		2024-S429	1.5 ± 0.32	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			KP-6		2024-S430	0.9 ± 0.06	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Abomey	Roasted peanut snack	RP-4	Pool 12	2024-S431	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD
			RP-5		2024-S432	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD
			RP-6		2024-S433	2.6 ± 0.2	0.3 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

***Table S5**: Average mycotoxins contamination (µg/kg) in samples collected during kluiklui production (n=2).*

Trial number	Process	Processor	Sample ID	Sample type	AFB1	AFB2	AFG1	AFG2	AOH	FUM B1	FUM B2	OTA	OTB
1	Without maize flour (Covè municipality)	KP-1, 2, 3	Pool 1	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-1	2024-S452	Peanut paste	49 ± 0.5	6.9 ± 0.1	4.5 ± 1.12	< LOQ	29 ± 2	<LOD	<LOD	3.0 ± 0.02	2.4 ± 0.7
			2024-S488	Kluiklui	54 ± 5	7.2 ± 2.1	4.4 ± 0.90	1.1 ± 0.06	28 ± 5	<LOD	<LOD	7.7 ±	5.0 ±
		KP-2	2024-S453	Peanut paste	16 ± 0.2	3.6 ± 0.06	1.2 ± 0.01	<LOD	<LOD	<LOD	<LOQ	5.2 ± 0.1	1.6 ± 0.02
			2024-S489	Kluiklui	38 ± 1	7.1 ± 0.23	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	4.3 ±	1.2 ±
		KP-3	2024-S454	Peanut paste	25 ± 0.5	3.7 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	1.8 ± 0.02	0.6 ± 0.07
			2024-S490	Kluiklui	30 ± 1	7.7 ± 0.6	1.7 ± 0.01	<LOD	<LOD	<LOD	<LOD	7.8 ±	2.3 ±
	With maize flour (Aplahoué municipality)	KP-4, 5, 6	Pool 3	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-4	2024-S456	Peanut paste	36 ± 2.2	6.4 ± 0.2	1.0 ± 0.03	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
			2024-S491	Kluiklui	35 ± 2	6.6 ± 0.15	0.9 ± 0.09	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-5	2024-S455	Peanut paste	2.4 ± 0.5	0.4 ± 0.08	<LOD	<LOD	<LOD	<LOD	<LOD	4.9 ± 0.2	4.2 ± 0.1
			2024-S492	Kluiklui	2.2 ± 0.04	0.5 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	7.41	0.8 ±
		KP-6	2024-S457	Peanut paste	4.6 ± 0.1	0.7 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	8.1 ±	< LOQ
			2024-S493	Kluiklui	2.0 ± 0.07	0.5 ± 0.03	<LOD	<LOD	<LOD	104 ± 4	34 ± 2	8.2 ±	0.7 ±
2	Without maize flour (Covè municipality)	KP-1, 2, 3	Pool 5	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-1	2024-S458	Peanut paste	112 ± 3	18 ± 1	3.0 ± 0.07	< LOQ	<LOD	<LOD	<LOD	6.6 ± 0.1	2.2 ± 0.02
			2024-S494	Kluiklui	2.5 ± 0.01	0.5 ± 0.02	<LOD	<LOD	<LOD	< LOQ	< LOQ	10.3 ±	2.2 ±
		KP-2	2024-S459	Peanut paste	37 ± 2	6.6 ± 0.2	7.5 ± 0.7	1.8 ± 0.01	<LOD	<LOD	<LOD	5.8 ± 0.3	3.2 ± 0.05
			2024-S495	Kluiklui	25 ± 1	4.9 ± 0.22	4.6 ± 0.21	<LOD	<LOD	<LOD	<LOD	5.6 ±	3.1 ±
		KP-3	2024-S460	Peanut paste	42 ± 1	6.3 ± 0.1	0.7 ± 0.01	<LOD	<LOD	<LOD	<LOD	8.6 ± 0.2	4.9 ± 0.08
			2024-S496	Kluiklui	18 ± 1	3.7 ± 0.08	<LOD	<LOD	<LOD	<LOD	<LOD	24 ±	13.7 ±
	With maize flour (Aplahoué municipality)	KP-4, 5, 6	Pool 7	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-4	2024-S524	Maize flour	11 ± 2	1.3 ± 0.01	1.5 ± 0.06	<LOD	<LOD	493 ± 10	165 ± 7	< LOQ	<LOD
			2024-S461	Peanut paste	8.4 ± 0.6	0.8 ± 0.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S497	Kluiklui	3.1 ± 0.16	0.5 ± 0.02	<LOD	<LOD	<LOD	< LOQ	< LOQ	11.2 ±	2.7 ±
		KP-5	2024-S525	Maize flour	2.8 ± 0.05	0.5 ± 0.06	0.2 ± 0.03	<LOD	<LOD	686 ± 13	232 ± 3	3.5 ±	1.0 ±

			2024-S462	Peanut paste	4.5 ± 0.04	1.3 ± 0.1	<LOD	<LOD	<LOD	<LOD	<LOD	20 ± 1	18 ± 1
			2024-S498	Kluiklui	3.3 ± 0.08	1.3 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	20 ± 2	33 ± 3
		KP-6	2024-S526	Maize flour	0.7 ± 0.02	0.2 ± 0.01	<LOD	<LOD	<LOD	302 ± 9	101 ± 2	<LOD	<LOD
			2024-S463	Peanut paste	3.8 ± 0.03	0.6 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	20 ± 2	1.7 ± 0.3
			2024-S499	Kluiklui	2.3 ± 0.05	0.5 ± 0.04	<LOD	<LOD	<LOD	39 ± 2	< LOQ	11.6 ± 0.3	1.4 ± 0.08
3	Without maize flour (Covè municipality)	KP-1, 2, 3	Pool 9	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-1	2024-S464	Peanut paste	58 ± 3	6.7 ± 0.7	<LOD	<LOD	<LOD	<LOD	<LOD	1.5 ± 0.02	<LOQ
			2024-S500	Kluiklui	107 ± 2	22 ± 1	0.8 ± 0.09	<LOD	<LOD	< LOQ	<LOD	2.7 ± 0.02	1.9 ± 0.03
		KP-2	2024-S465	Peanut paste	52 ± 6	8.2 ± 0.51	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S501	Kluiklui	22 ± 1	4.2 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-3	2024-S466	Peanut paste	64 ± 3	10.7 ± 0.1	2.4 ± 0.09	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S502	Kluiklui	44 ± 1	9.1 ± 0.3	2.3 ± 0.12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	With maize flour (Aplahoué municipality)	KP-4	2024-S428	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S527	Maize flour	64 ± 5	6.2 ± 0.05	<LOD	<LOD	<LOD	1822 ± 11	602 ± 8	< LOQ	<LOD
			2024-S467	Peanut paste	32 ± 1	5.0 ± 0.22	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S503	Kluiklui	22 ± 1	6.2 ± 0.16	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD	<LOD
		KP-5	2024-S429	Raw peanut	1.5 ± 0.02	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S528	Maize flour	7.9 ± 0.23	0.8 ± 0.02	<LOD	<LOD	<LOD	739 ±	275 ± 10	8.6 ± 0.2	1.1 ± 0.05
			2024-S468	Peanut paste	21 ± 1	41 ± 1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S504	Kluiklui	18 ± 1	17 ± 1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		KP-6	2024-S430	Raw peanut	0.9 ± 0.01	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S529	Maize flour	58 ± 3	6.7 ± 0.31	0.3 ± 0.05	<LOD	<LOD	1907 ±	629 ± 15	<LOD	<LOD
			2024-S469	Peanut paste	18 ± 1	3.0 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S505	Kluiklui	18 ± 1	4.1 ± 0.07	<LOD	<LOD	<LOD	115.79	31 ± 4	<LOD	<LOD
				LOD	*0.02*	*0.07*	*0.16*	*0.13*	*8*	*2.4*	*8*	*0.4*	*0.4*
				LOQ	*0.2*	*0.2*	*0.2*	*0.2*	*10*	*10*	*10*	*0.5*	*0.5*

***Table S6**: Average mycotoxins concentration in roasted peanut snacks (µg/kg) (n=2).*

Trial number	Process	Processor	Sample ID	Sample type	AFB1	AFB2	AFG1	AFG2	AOH	FUM B1	FUM B2	OTA	OTB
1	Roasting without heat transfer material (Covè municipality)	RP-1,2,3	Pool 2	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-1	2024-S509	Roasted peanut snack	0.2 ± 0.01	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-2	2024-S510	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-3	2024-S511	Roasted peanut snack	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Roasting without heat transfer material (Covè municipality)	RP-4,5,6	Pool 4	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-4	2024-S506	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-5	2024-S507	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-6	2024-S508	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
2	Roasting without heat transfer material (Covè municipality)	RP-1,2,3	Pool 6	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-1	2024-S512	Roasted peanut snack	0.2 ± 0.06	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
		RP-2	2024-S513	Roasted peanut snack	< LOQ	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-3	2024-S514	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
	Roasting with white clay as heat transfer material (Abomey municipality)	RP-4,5,6	Pool 8	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-4	2024-S515	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
		RP-5	2024-S516	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
		RP-6	2024-S517	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ
3	Roasting without heat transfer material (Covè municipality)	RP-1	2024-S425	Raw peanut	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S518	Roasted peanut snack	0.49 ± 0.01	0.22 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-2	2024-S426	Raw peanut	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S519	Roasted peanut snack	2.27 ± 0.71	0.59 ± 0.18	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-3	2024-S427	Raw peanut	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

			2024-S520	Roasted peanut snack	0.92 ± 0.01	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Roasting with white clay as heat transfer material (Abomey municipality)	RP-4	2024-S431	Raw peanut	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD
			2024-S521	Roasted peanut snack	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-5	2024-S432	Raw peanut	< LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	< LOQ	<LOD
			2024-S522	Roasted peanut snack	0.7 ± 0.07	0.2 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		RP-6	2024-S433	Raw peanut	2.63 ± 0.11	0.34 ± 0.09	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
			2024-S523	Roasted peanut snack	<LOQ	0.3 ± 0.06	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
				LOD	*0.02*	*0.07*	*0.16*	*0.13*	*8*	*2.4*	*8*	*0.4*	*0.4*
				LOQ	*0.2*	*0.2*	*0.2*	*0.2*	*10*	*10*	*10*	*0.5*	*0.5*

***Table S7**: Mean mycotoxin concentrations in marketed and just-produced peanut-based foods (µg/kg UB).*

Samples		AFB1	AF tot	OTA
Kluiklui	Marketed (n=27)	522.63±478.48[a]	651.03±591.97[a]	52.89±130.28[a]
	Just produced (n=18)	24.76±25.77[b]	31.67±31.27[b]	6.91±6.92[b]
Roasted peanut snacks	Marketed (n=25)	14.26±39.89[1]	16.94±39.89[1]	189.07±978.46[1]
	Just produced (n=6)	0.80±0.77[1]	1.33±0.77[1]	0.50±0.00[1]

Values within the same column followed by the same letter or number do not differ significantly at the 5% probability level. The mean concentrations of AFB1 (p= 2.158e-06), total aflatoxins (p=1.924e-06) and OTA (p=0.001096) were significantly higher in marketed kluiklui. The mean concentrations of AFB1 (p= 0.3789), total aflatoxins (p=0.2184) and OTA (p=0.4264) were not significantly different in marketed and just-produced roasted peanut. (n=2).

Discussion – Perspectives and Conclusions

1. Context of the study

Mycotoxin contamination of crops is a well-established reality, and its occurrence is expected to increase under the influence of climate change. Indeed, geographical patterns of contamination are expected to evolve across Europe, with higher levels of aflatoxin contamination predicted. In addition, increasing precipitation and soil erosion may facilitate the transfer of mycotoxins into rivers and groundwater, potentially affecting drinking water systems (EFSA, 2020). Moreover, higher mycotoxin contamination is anticipated due to the rising frequency of extreme weather events and the expanding distribution of insect populations, which can increase the incidence of crop infections (Kos et al., 2023). This growing threat raises concerns both for consumer health and for the economic sustainability of the food supply chain. By providing analytical tools, this study helps to anticipate and better manage these risks, thereby supporting preventive rather than reactive strategies. Hence, the first part of this work consisted of the development and validation of a multi-mycotoxin analytical method by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS).

The second part of this work aimed to provide an overview of mycotoxin contamination in Belgian wheat as no recent data were available. In addition, this study focused on the comparison of the susceptibility to fungal contamination between wheat varieties, which can provide valuable data to guide production choices and breeding programs, with direct implications for improving food safety along the food chain.

The third aspect of this work addressed the feasibility of sorting processes to reduce mycotoxin concentrations for field- and storage-associated mycotoxins. In the current global context, characterized by resource scarcity and heightened pressure on food systems, exacerbated by geopolitical instability and conflicts such as the war in Ukraine, it becomes essential to propose alternative uses and management strategies for contaminated batches, rather than discarding them entirely. This perspective aligns with the urgent need to minimize food waste while safeguarding public health.

Finally, the analytical method was extended to peanuts which are also highly affected by mycotoxin contamination, particularly aflatoxins. This part of the work demonstrated that the method can be readily adapted to other food matrices and expanded to include additional mycotoxins, thereby enhancing its utility in meeting current and future monitoring needs.

2. Development and validation of a multi-mycotoxin analytical method

The analytical method initially developed in this study was designed to identify and quantify 20 mycotoxins in wheat, both regulated and emerging. On one hand, deoxynivalenol (DON), zearalenone (ZEN), T2 and HT2, ochratoxin A (OTA) are regulated in wheat by the European Regulation (EU) 2023/915 (European Commission, 2023c). On the other hand, 15-acetyl-deoxyivalenol (15Ac-DON), 3-acetyl-deoxynivalenol (3Ac-DON), deoxynivalenol-3-glucoside (DON-3G), alternariol (AOH), alternariol-monomethyl-ether (AME), diacetoxyscirpenol (DAS), ochratoxin B (OTB), moniliformin, citrinin (CIT), enniatins (ENNs) (A, A1, B, and B1), fumonisins B1 (FUM B1), and sterygmatocistin (STE) are not currently regulated in wheat in Europe, and can therefore be considered as emerging. The non-regulated mycotoxins included in the method were selected for their reported occurrence in wheat or because they are considered of interest by the Belgian Federal Agency for the Security of the Food Chain or the European Commission (Comité Scientifique institué auprès de l'Agence Fédérale pour la Sécurité de la Chaîne Alimentaire, 2022; European Commission, 2022).

A successful validation in accordance with the European standard (EU) 2021/808 (European Commission, 2021), was performed for the five regulated mycotoxins, DON , ZEN, T2, HT2 and OTA, and for 8 non-regulated mycotoxins, AOH, AME, OTB, ENNs, and STE. The validation confirmed the trueness, repeatability, and reproducibility of the method for these mycotoxins. Currently, DON is regulated by the European Commission. However, its maximum level was revised in July 2024 and reduced from 1250 to 1000 µg/kg. However, with regard to 3Ac-DON and 15Ac-DON, the recoveries obtained from wheat flour samples were only 59.0% and 50.9%, respectively, which is below the minimum required recovery of 80%. Although these values are lower than the typical acceptance criteria for regulated mycotoxins, the analytical procedure could be used as a screening method for these two modified forms of DON which are not regulated.

Other mycotoxins, including CIT, moniliformin, DAS, FUM B1 and DON-3G, were not further investigated as their recoveries were insufficient. Only CIT is regulated in food supplements based on rice fermented with red yeast *Monascus purpureus* and FUM B1 in maize and maize by-products. While the extraction procedure could be adapted to integrate these compounds into the analytical method, for example by changing the extraction solvent, any adjustment might compromise the recovery rates of other targeted mycotoxins. In addition, NIV and TeA were not detected under the

applied liquid chromatography parameters. Modifying these parameters could allow their detection, however, such adjustments could lead to the loss of other mycotoxins.

The limits of detection (LODs) achieved with this method are consistent with values reported in the literature. Leite et al. (2023) reported comparable LODs in maize using QuEChERS extraction followed by dispersive solid phase extraction with a C18 tube and detection and quantification by UPLC-MS/MS including 23 mycotoxins, most of which are the same as those investigated in the present study. Similarly, Sun et al. (2016) obtained comparable LODs using a similar analytical approach, consisting of a QuEChERS extraction with sodium chloride, anhydrous magnesium sulphate, sodium citrate and sodium hydrogen citrate sesquihydrate followed by detection and quantification by UPLC-MS/MS. Lower LODs were reported by Zhang et al. (2013) in wheat, the extraction was performed in an ultrasonic water bath with a solution of methanol, water and acetic acid and a mix of sodium chloride and magnesium sulphate and the detection and quantification was performed by UPLC-MS/MS. However, their method targeted a smaller number of mycotoxins than the one developed in this study. Overall, LODs reported for DON are generally lower in the literature than those achieved in the present work.

In the context of this work, the primary objective was not to achieve the lowest possible LODs, but rather to ensure accurate, reliable, and repeatable quantification around the maximal levels established by the European legislation (European Commission, 2023c). The method was therefore designed to be fit for regulatory monitoring purposes, where the key question is whether a sample is above or below the legal level, rather than to detect trace amounts far below these thresholds. For the emerging mycotoxins, the calibration range was initially defined arbitrarily. However, it can be adapted according to the concentrations observed, as the objective is to include the maximum number of samples within the calibration range.

A deliberate choice was made to maintain a relatively simple and repeatable analytical method, capable of simultaneously detecting and quantifying a wide range of mycotoxins. Although the extraction procedure could potentially be further optimized to increase recoveries, or the chromatographic and mass spectrometric parameters refined to improve chromatographic separation or ionization efficiency, the performance achieved with the current method is considered sufficient with respect to its intended application. Indeed, even minor modifications to the extraction protocol, chromatographic conditions, or mass spectrometric parameters may have positive or negative effects on sensitivity or specificity, depending on the mycotoxin considered. In a multi-mycotoxin approach, improving performance for one compound may result in reduced

performance for others. For example, the use of Primary Secondary Amine (PSA) enhanced the recovery of aflatoxins (AFs), ZEN, T2 and HT2 but decreased the recoveries of OTA and FUMs (Leite et al., 2023). Consequently, in this study, the method was optimized to achieve a balanced compromise between a procedure that is not overly complex, ensuring satisfactory performance for all mycotoxins and the pursuit of maximal performance for individual mycotoxins. Moreover, as the analytical cost remains essentially unchanged whether 8 or 20 mycotoxins are included, the ability to easily incorporate additional compounds into the method represents a clear advantage, allowing broader monitoring without increasing analytical costs.

In this work, the limit of quantification (LOQ) was initially defined as the lowest non-zero calibration point, provided that the signal-to-noise ratio exceeded 10. However, according to more recent guidelines from the EU Reference Laboratory for mycotoxins and plant toxins, the LOQ should be defined as the lowest analyte concentration that can be measured with acceptable accuracy and statistical certainty (Sloth et al., 2022). Indeed, signal-to-noise measurements are poorly reproducible in complex matrices, whereas validation of accuracy and precision at the lowest calibration level provides a robust and performance-based definition of the quantification limit. Consequently, the LOQ should correspond to the lowest concentration that has been successfully validated. In this study, the lowest validated concentration was half of the maximal level, although lower concentrations could likely be validated if experimentally assessed. Therefore, to comply with these guidelines and to achieve lower LOQs, validation of the lowest calibration level would be required.

No internal standards (IS) were used in this method. Ideally, a good IS should be specific to each analyzed mycotoxin due to their distinct chemical structures. The use of individual IS for each compound would therefore represent a significant additional cost per sample, which is why it was chosen not to include IS in the developed method. The use of IS is not mandatory according to the guidelines of the EU Reference Laboratory for mycotoxins and plant toxins. Moreover, interlaboratory comparison studies such as those organized by an interlaboratory assay (BIPEA) indicate that only about 20% of participating laboratories routinely use IS for multi-mycotoxin analysis. In addition, several measures, presented in study 1, were implemented to ensure reliability and accuracy of the measured concentrations.

By including emerging mycotoxins alongside regulated ones, the method not only anticipates potential future regulatory requirements but also contributes to the development of a robust contamination database. The availability of such data is essential to support exposure assessments,

risk characterization, and, ultimately, informed policy decisions. For example, the scientific committee of the Belgian Federal Agency for the Security of the Food Chain has recommended the inclusion of enniatins in analytical methods, as these mycotoxins, although not currently regulated, are frequently detected in cereals (Comité Scientifique institué auprès de l'Agence Fédérale pour la Sécurité de la Chaîne Alimentaire, 2022). This observation has also been widely reported in the scientific literature (Medina, 2023; dsm-firmenich, 2024). Enniatins were reported in 76% of unprocessed grain samples collected in Europe between 2000 and 2013, with maximum concentrations in cereals-based foods of 42, 125, 832 and 980 µg/kg for ENN A, ENN A1, ENN B and ENN B1 respectively (Gruber-Dorninger et al., 2017). In 2024, ENNs B and B1 were found in around 65% of the cereal samples worldwide, whereas AOH was detected in 44% of the samples and STE in 12% of the samples (dsm-firmenich, 2024).

For the non-regulated mycotoxins, validation was carried out with a limited number of samples, over three days at a single concentration level, as no legal thresholds currently exist. Nevertheless, the results indicated that the extraction and detection of these mycotoxins were also consistent and reliable. A full validation according to the (EU) 2021/808 could be performed in the future if a regulatory threshold is established.

Analytical methods are by nature dynamic and evolving tools, designed to respond to new needs as they arise. Indeed, new mycotoxins can be integrated into the method if they become subject to regulation, and even newly discovered compounds could be added for early monitoring alongside established mycotoxins. Similarly, the calibration range can be adjusted in response to changes in maximal levels, ensuring that food maximum thresholds remain appropriately covered. For non-regulated mycotoxins, central calibration points may also be shifted if the usually observed concentrations are not covered by the current quantification range.

An additional strength of the method is its transferability to other matrices. Although it was initially developed for wheat, its successful application to peanuts demonstrates that it can be extended to other commodities affected by mycotoxins. Such transfer, however, requires a re-evaluation of the recoveries for each mycotoxin and a dedicated validation to ensure accuracy and reliability.

Compared to screening techniques such as ELISA or LFIA, the LC-MS/MS method developed in this work demonstrates greater flexibility. It can be easily adjusted to meet evolving analytical needs and has the advantage of allowing the simultaneous detection and quantification of multiple mycotoxins within a single run. A multi-mycotoxin analytical method not only improves analytical

efficiency but also provides a more comprehensive overview of contamination profiles. Nevertheless, it is important to acknowledge that other advanced analytical approaches can further extend the scope of mycotoxin analysis. In particular, high-resolution mass spectrometry (HRMS) represents a powerful complementary tool to targeted LC-MS/MS methods. High-resolution mass spectrometry instruments, such as Orbitrap-based or time-of-flight mass spectrometers, provide high mass accuracy and high resolving power, allowing the simultaneous detection of a very large number of mycotoxins within a single analysis (Righetti et al., 2016).

One of the major advantages of HRMS lies in its ability to perform non-targeted screening. Unlike triple quadrupole systems, which require predefined transitions for each analyte and whose optimization can be time-consuming, HRMS acquires full-scan data, enabling the detection of both known and unknown contaminants (Righetti et al., 2016; Jensen et al., 2019). This is particularly relevant in the context of mycotoxins, where modified, masked, or emerging mycotoxins may be present in food matrices but are not yet included in targeted methods (Righetti et al., 2016). Furthermore, HRMS could enhance mycotoxin detection in complex food or feed matrices such as forage maize and maize silage (Jensen et al., 2019). High-resolution mass spectrometry also allows retrospective data analysis, meaning that previously acquired datasets can be reprocessed to search for newly identified compounds without the need for re-analysing the samples (Righetti et al., 2016; Jensen et al., 2019; Lindemann et al., 2022). Consequently, HRMS is best considered as a complementary approach rather than a replacement for targeted UPLC-MS/MS methods. While LC-MS/MS remains the reference technique for routine monitoring and regulatory compliance due to its high sensitivity, selectivity, and affordability, HRMS offers clear added value for exploratory studies, surveillance of emerging contaminants, and early detection of compounds that may gain regulatory attention in the future (Lindemann et al., 2022). The combination of both approaches could represent a powerful strategy for comprehensive mycotoxin monitoring.

3. Situation in wheat in Belgium in 2023 and 2024

In Belgium, the most recent studies providing contamination data for wheat are from 2009 and covered 2002 to 2005, 2007 and 2008 harvests and focus exclusively on DON (Audenaert et al., 2009; Isebaert et al., 2009). Since then, no comprehensive dataset covering a broader spectrum of mycotoxins has been generated. This highlights the importance of producing updated and more exhaustive contamination data, not only to assess current exposure levels but also to evaluate temporal trends and the potential emergence of new mycotoxins.

Overall, the results of this study are consistent with those of previous surveys conducted in Flanders (Belgium) for DON in wheat between 2002–2005, 2007 and 2008, with no striking differences observed (Audenaert et al., 2009; Isebaert et al., 2009). The DON concentration ranges observed by these authors in wheat, from 49 to 1015 µg/kg in 2002, from 1 to 552 µg/kg in 2003, from 0 to 798 µg/kg in 2004, from 0 to 858 µg/kg in 2005, from 110 to 3685 µg/kg in 2007, and from 72 to 2208 µg/kg in 2008, remain comparable to the data of this study obtained for the 2023 and 2024 harvests. Overall, the variability observed for DON reflects strong interannual fluctuations rather than a clear temporal trend. Both ZEN and ENNs were found in the present study but were not investigated in the previous ones. Therefore, the evolution of the occurrence of these mycotoxins could not be assessed. As reported previously, some years appear to be associated with a higher contamination risk, while others are less critical, for example in 2002 more than 22% of the samples contained DON at a concentration higher than 750 µg/kg, whereas only around 4% of the samples contained DON at a concentration higher than 750 µg/kg in 2003 and 2004. In 2005, DON was not detected in around 40% of the samples (Audenaert et al., 2009).

In addition, this variability is clearly illustrated by the comparison between the 2023 and 2024 harvests. The analysis of samples from 2023 and 2024 showed that mycotoxin contamination was higher in 2024 than in 2023, both in terms of the number of positive samples and the concentrations observed. Regarding the contamination profile, the same mycotoxins were detected in both 2023 and 2024, with the exception of ENN A1, which was only observed in 2024. Regulated mycotoxins such as DON and ZEN were detected, as well as ENNs, which were present in most of the 2024 samples. Although ENNs are not currently regulated in Europe, increasing concern has been raised due to their growing occurrence in cereals (Medina, 2023; dsm-firmenich, 2024). These results confirm the relevance of monitoring emerging mycotoxins alongside regulated ones. According to the latest EFSA opinion, acute exposure to ENNs does not raise immediate concerns for human health. However, insufficient data are available to draw conclusions regarding chronic exposure (EFSA, 2014), which underlines the need for continued data generation and surveillance.

The high interannual variability is likely linked to specific local weather events that influence fungal development and mycotoxin production during critical crop development stages, particularly flowering and maturation. For instance, high humidity and rainfall during anthesis can promote *Fusarium* infection, leading to elevated levels of DON and ZEN (Marroquín-Cardona et al., 2014; Perrone et al., 2020; Platzer et al., 2025). As the situation differs from one year to another, the implementation of a long-term surveillance system is essential to better understand the influence

of meteorological factors on fungal infestation and mycotoxin production. Beyond climatic factors, agricultural practices such as crop rotation, soil management, and the handling of previous crop residues also play a crucial role in shaping mycotoxin contamination profiles. In addition, the implementation of accredited quality-control laboratories is an essential component of effective mycotoxin prevention and control strategies. Systematic monitoring throughout the cultivation, harvesting, storage and processing chain would greatly improve the early detection of contaminated batches and support compliance with regulatory limits, both for locally consumed foods and export commodities.

Moreover, varietal differences in resistance to fungal infection were observed. However, to determine which varieties are consistently more susceptible than others, additional samples from multiple harvest years should be analyzed. This would ensure that the observed differences are attributable to varietal characteristics rather than annual environmental conditions and would allow the identification of varieties that are repeatedly more susceptible across years. Differences in mycotoxin concentration among varieties have also been reported by Audenaert et al. (2009) and Isebaert et al. (2009). These findings suggest that mycotoxin content could be considered as an additional criterion in varietal selection, alongside agronomic performance and disease resistance, which is particularly relevant for breeding programs and the release of new varieties.

Currently, Belgium already relies on a pre-harvest alert system in which DON is measured in wheat samples collected from different locations shortly before harvest, allowing the issuance of advisories on whether a given year represents a high-risk situation. Expanding this type of monitoring to other mycotoxins and fungal metabolites could further strengthen early warning systems and improve the anticipation of contamination risks.

While resistance to *Fusarium* head blight has long been assessed in breeding programs, the present results highlight the added value of integrating direct mycotoxin measurements. Field observations of disease symptoms alone are not sufficient to fully characterize varietal resistance, as mycotoxin production does not always correlate perfectly with visible infection. A more comprehensive evaluation of varietal resistance could be achieved by combining agronomic observations with analytical data on mycotoxin contamination.

Regarding the assessment of mycotoxin occurrence in wheat in 2023 and 2024, only two years of data were available. This limited temporal scope restricts the ability to draw strong conclusions about the influence of meteorological conditions, agronomic factors such as previous crops, sowing

dates, and varietal susceptibility. Long-term monitoring would be necessary to confirm whether the observed differences are linked to intrinsic varietal resistance or to year-specific environmental conditions. Similarly, the impact of geographic location on mycotoxin occurrence cannot be fully established within this restricted timeframe.

4. Sorting procedures as a mycotoxin management solution

A first important aspect to consider is the regulatory framework. According to European legislation, the chemical decontamination of cereals intended for food use, as well as the dilution of contaminated batches with non-contaminated ones, are strictly prohibited (European Commission, 2023c). These restrictions are justified by the risk of forming degradation products with unknown or potentially higher toxicity than the parent compounds. Consequently, non-chemical mitigation strategies are required. In this context, sorting represents a relevant and legally acceptable approach to reduce mycotoxin contamination, as it does not modify the chemical structure of mycotoxins and allows the remaining fractions to be valorised for alternative uses, such as animal feed, bioethanol production, or biomethanisation, provided that the applicable regulatory limits for these end uses are respected.

In livestock, the ingestion of contaminated feed can lead to adverse effects such as reduced performance, impaired immune function, reproductive disorders and decreased fertility (Alshannaq and Yu, 2017; Ji et al., 2019; Khan et al., 2024; Pinton et al., 2025). The European Food Safety Authority (EFSA) carries out animal health risk assessments related to the ingestion of mycotoxins in feed. These assessments evaluate mycotoxin adverse effects and species-specific sensitivity, exposure levels, and the potential impact on animal performance and welfare. EFSA has published detailed scientific opinions on several regulated and emerging mycotoxins, including OTA, DON, ZEN, CIT, STE, etc. (EFSA, 2012, 2013, 2017a, 2017b, 2023) For each of these compounds, EFSA reviews toxicological and occurrence data in order to derive health-based guidance values and to characterise the risk for different livestock species. In addition, certain mycotoxins or their metabolites may be transferred from feed to animal-derived products such as meat, milk or eggs. However, the extent of this transfer and its long-term implications are not always well understood and may vary depending on the toxin and the animal species. This uncertainty highlights the need for a precautionary approach in feed management and underlines the importance of carefully controlling mycotoxin contamination in animal feed to protect both animal health and food safety.

Sorting relies on the heterogeneous distribution of mycotoxins within cereal batches and aims to remove the most heavily contaminated kernels. However, its effectiveness is not guaranteed in all situations. When a large proportion of grains are contaminated at levels exceeding the maximum permitted level, the potential improvement achieved through sorting may be limited, resulting in low recovery rates and raising questions about the cost-effectiveness of the process. Moreover, the efficiency of sorting is highly dependent on both the technology used and the type of mycotoxin targeted, as well as on the specific characteristics of the batch.

For DON, sorting generally shows more promising results. This can be explained by the fact that *Fusarium* infections often induce visible and measurable physical changes in kernels, such as discoloration, shrinkage, or reduced density, as widely reported in the literature (Cheli et al., 2013; Karlovsky et al., 2016; Peng et al., 2018). Optical sorting based on the *Fusarium*-damaged kernel (FDK) model can therefore successfully identify and remove the most contaminated grains, leading to a reduction in DON concentration in certain fractions of a batch. Nevertheless, size- and density-based sorting devices often resulted in a redistribution of DON among fractions and typically achieved only modest recovery rates. The results of the present study, together with previous reports (Cheli et al., 2013; Tibola et al., 2016; Peng et al., 2018; Schaarschmidt and Fauhl-Hassek, 2018), confirm that DON reduction through sorting is highly variable and strongly batch-dependent.

It is important to emphasize that grain separation is driven by visible or physical symptoms of fungal infection rather than by the mycotoxins themselves. Indeed, mycotoxins occur at ppb levels and are not directly detectable by visible or infrared sensors. While mycotoxins do not inherently affect kernel size or density, the fungal infection responsible for their production may induce morphological and structural changes that can indirectly enable sorting. This explains why sorting based on *Fusarium* symptoms is particularly effective for DON and raises the question of whether FDK-based models could also be relevant for other *Fusarium*-produced mycotoxins.

For OTA, the situation appears even more challenging. When initial contamination levels are far above the maximum permitted levels, achieving concentrations below the regulatory threshold through sorting alone is particularly difficult. This observation is consistent with the limited efficiency reported in the literature for OTA mitigation by sorting (Duarte et al., 2010; Karlovsky et al., 2016). In the present study, no specific sorting model targeting OTA was developed, as sorting with the near-infrared sorter was performed according to the FDK model. However, these results suggest that future work should focus on the development of dedicated detection models, potentially based on optical or near-infrared technologies. Acquiring spectral data and mycotoxin

measurements at the single-kernel level could help determine whether OTA contamination is associated with detectable physical or spectral features that could be exploited for sorting.

From a practical and implementation perspective, it is important to note that near-infrared sorting equipment is already installed at several storage facilities, where it is primarily used for protein-based segregation of wheat. In addition, NIR and optical sorting systems, such as those developed by companies like BoMill (https://www.bomill.com/, Vintrie, SWEDEN) , already offer the possibility to sort grains based on *Fusarium*-related characteristics (Kautzman et al., 2015). Although NIR sorters represent a substantial investment, their potential for mycotoxin mitigation could be exploited when the equipment is already available for other purposes such as protein-based sorting as mentioned above. In such cases, implementing sorting strategies for mycotoxin risk reduction would mainly require the development and integration of new classification models rather than additional hardware, making this approach readily applicable under real storage conditions.

Although advanced optical and near-infrared technologies are increasingly being investigated for mycotoxin risk mitigation, more conventional grain separation techniques such as size- and density-based sorting are still widely applied in the cereal and seed industry. Their continued use is largely explained by their lower cost and operational simplicity. Even if their efficiency is generally lower and more variable than that of optical sorting systems, these techniques can nevertheless provide a certain degree of risk reduction in practice, particularly when contamination is associated with shrivelled or lightweight kernels.

Beyond technical performance, the economic context plays a crucial role in determining the relevance of sorting strategies. For farmers and storage operators, the primary objective is to increase the value of their batches and improve income. In Belgium, most wheat production is directed towards animal feed or biomethanisation (Delcour et al., 2014), as the price difference between food-grade and feed-grade wheat remains relatively small. This situation incentivises producers to prioritise yield rather than technological or sanitary quality. Moreover, regulatory thresholds for feed are higher than those for food, reducing the economic incentive to invest in costly sorting processes. Cereals therefore remain a low-added-value commodity as long as yields are satisfactory, and sorting is not considered a priority. However, in scenarios of wheat scarcity, such as import disruptions caused by geopolitical conflicts or poor harvests due to adverse climatic conditions, sorting could become a valuable tool to enhance the value of domestic production. In 2021, wheat importation represented more than three times the local production (Services opérationnels du collège des producteurs, 2019). In such contexts, the ability to partially recover

batches and redirect them towards higher-value food uses could contribute to mitigating supply shortages and stabilising markets. Although Belgian wheat is currently consumed only to a limited extent for human food, achieving the required sanitary quality would be essential if domestic production were to be more widely valorised in the future.

During the two years of wheat surveillance conducted in this study, only one sample out of 88 exceeded the European regulatory threshold for mycotoxins in wheat intended for human consumption, and none exceeded the maximum level for mycotoxins in wheat intended for feed. In addition, in 2023and 2024, only a few notifications of high levels of mycotoxin in wheat were reported in the RASFF portal (European Commission, 2023b, 2024a), indicating that sorting is generally not required under current conditions. Nevertheless, investing in the development and optimisation of sorting methods remains a prudent strategy to prepare for years with exceptionally high contamination levels. This anticipation is particularly relevant in the context of climate change, which is expected to increase both the frequency and severity of mycotoxin contamination events (EFSA, 2020; Kos et al., 2023; Medina, 2023; Casu et al., 2024).

Finally, several limitations must be acknowledged regarding the assessment of sorting efficiency. The number of naturally contaminated samples available was limited, which constrained the robustness of the conclusions. In the case of DON, available samples only slightly exceeded the maximum level. Access to large quantities of naturally contaminated wheat remains a major bottleneck, as artificial spiking with pure mycotoxin solutions cannot reliably mimic the effects of fungal infection that are essential for sorting performance. Moreover, sorting efficiency could only be evaluated for two mycotoxins, and sorter parameters were not extensively optimised, as the priority was to obtain sufficient material in each fraction given the limited initial sample quantity.

In addition to physical sorting, other mitigation strategies have been investigated in the literature to reduce mycotoxin contamination in cereals, including chemical and biological approaches, as well as thermal or irradiation treatments. Chemical methods aim to degrade mycotoxins by altering their molecular structures through hydrolysis, oxidation, reduction, ammoniation or alkaline treatments, (Ekwomadu et al., 2021; Khan et al., 2024). However, while these techniques can significantly reduce the concentration of several mycotoxins, they often entail substantial drawbacks. Chemical detoxification may result in the formation of unknown or potentially toxic degradation products, can negatively impact nutritional quality and sensory properties of food, and may pose environmental or safety risks, which limits their acceptance and regulatory approval for food applications (Ben Miri et al., 2024; Khan et al., 2024). Biological detoxification processes using microorganisms or enzymes

offer a more selective and environmentally friendly alternative. Microbial degradation and enzymatic biotransformation have shown promise in breaking down certain mycotoxins into less toxic or structurally modified products (Peng et al., 2018). Nevertheless, the applicability of biological methods remains constrained by uncertainties regarding the safety and toxicity of the resulting metabolites and the limited number of well-characterized degradation pathways for different mycotoxins (Liu et al., 2022). Physical methods such as thermal processing, irradiation, and adsorption have also been explored for mycotoxin reduction, but they are often associated with incomplete elimination and potential alterations to food quality, including nutrient loss, and changes in texture or flavour (Karlovsky et al., 2016). Mycotoxin binders are widely used in animal nutrition as feed additives to reduce the bioavailability of ingested mycotoxins. However, because these adsorbents are not fully specific, they may also bind essential nutrients and vitamins, and their proven efficacy remains limited, particularly for emerging mycotoxins (Kihal et al., 2022; Siri-anusornsak et al., 2023). Compared with these approaches, sorting does not chemically modify the grain and generally preserves the original physical and nutritional properties of the product, making it more acceptable from both a regulatory and consumer perspective. However, as discussed above, its effectiveness varies substantially with batch characteristics and contamination patterns.

5. Expansion of the analytical method scope to peanuts

To conduct a comprehensive risk assessment of peanut and peanut-based product consumption among Beninese consumers, the previously validated analytical method was extended to include an additional matrix and a broader range of mycotoxins. Specifically, AFB1, AFB2, AFG1, and AFG2, as well as FUM B2 and FUM B3, were incorporated into the method. These mycotoxins are typically absent or occur at negligible levels in wheat produced in Northern Europe and were therefore not included in the original method. However, AFs are highly prevalent in peanuts, and FUMs are commonly associated with maize, making their inclusion essential for an accurate exposure and risk evaluation in the Beninese context. According to the RASFF portal, in 2023 and 2024, most of the notifications were for AFB1 in peanuts (European Commission, 2025a, 2025b), highlighting the issue of AFs in peanuts.

Only limited data are available on the occurrence of mycotoxins in Beninese peanuts and peanut-based foods (Adjou et al., 2012; Ingenbleek et al., 2019). The present study provides additional and more recent contamination data, thereby addressing this data gap. Furthermore, by

assessing the contribution of processing practices to mycotoxin contamination, this work also provides novel insights into an area for which information has so far been scarce.

For the extension of the analytical method, recoveries were evaluated for each mycotoxin in both peanuts and peanut paste. As the method had already been fully validated for wheat, a short validation was considered sufficient for the new matrices and was therefore performed on a limited number of samples. Determination of mean recoveries was particularly important, as recovery rates are matrix-dependent and were generally lower in peanuts than in wheat. Nevertheless, the existing extraction protocol proved applicable, as the high lipid content of peanuts did not noticeably interfere with extraction or detection efficiency. A hexane clean-up step was tested to confirm the suitability of the original extraction procedure for lipid-rich matrices. The trueness of the adapted method was further verified through the analysis of reference materials.

Following optimization of the precursor-to-product ion transitions, AFs and FUMs were successfully integrated into the MRM program, demonstrating the flexibility of the analytical method and the limited adjustments required to accommodate additional mycotoxins or new matrices.

Very high aflatoxin concentrations were observed in numerous peanut product samples, which largely exceeded the European regulatory limit of 2 µg/kg for AFB1 in peanut intended for human consumption (European Commission, 2023c), clearly indicating a serious food safety concern.

In general, the results revealed alarmingly high levels of mycotoxin contamination in peanuts and peanut-based products, particularly with AFs and OTA, underscoring the urgent need for strengthened food safety controls and improved management strategies. Cross-contamination during processing appears to be a major risk factor, as peanuts are frequently ground in community or public mills that are not disinfected between batches (Sogbossi Gbétokpanou et al., 2025). In addition, storage conditions play a critical role, with market-sourced samples showing higher contamination levels than samples analysed immediately after preparation. These findings highlight the importance of improving storage practices and hygiene standards in milling operations. However, contamination can also originate from raw materials, leading to downstream contamination of derived products throughout the processing chain.

The results generated in Study 4 also enabled a detailed risk assessment for AFs and OTA, and allowed the assessment of the relation between measured mycotoxins and fungal species present in the different products (Sogbossi Gbétokpanou et al., 2025). The study indicates a high level of

exposure to AFs and OTA through consumption of *kluiklui*, especially marketed products, whereas exposure was considerably lower among consumers of freshly produced *kluiklui* and roasted peanuts, suggesting that mycotoxin production mainly occurs during storage (Sogbossi Gbétokpanou et al., 2025). The estimated liver cancer risks were high, particularly for frequent consumers and individuals with chronic hepatitis B. Margin of exposure values for AFB1 were consistently below the critical threshold of 10,000 for all consumer groups, confirming a health concern (Sogbossi Gbétokpanou et al., 2025). For non-neoplastic effects of OTA, margin of exposure values below 200 in some scenarios also indicated potential risk (Sogbossi Gbétokpanou et al., 2025). To monitor population exposure to mycotoxins, several biomarkers can be employed. At the exposure level, the most widely accepted biomarker is AFB1-lysine adducts in serum albumin, reflecting cumulative exposure over 2 to 3 months, while urinary biomarkers, such as AFM1 and aflatoxin-N7-guanine adducts, can serve as short-term indicators of recent exposure (Mathew et al., 2024).During the production of *kluiklui*, the grinding of roasted peanuts in public grinders was a major source of mycotoxin contamination, and fungal contamination was also higher in *kluiklui* than in raw peanuts, with significantly greater populations of aflatoxigenic fungi, mainly *Aspergillus flavus,* found in kluiklui produced without maize flour. In contrast, *A. chevalieri*, *Cladosporium* spp., and *Talaromyces tumuli* predominated in *kluiklui* prepared with maize flour (Sogbossi Gbétokpanou et al., 2025).

Given that peanuts are a higher-value commodity compared to cereals such as wheat, the development of sorting strategies to remove contaminated kernels could represent a promising mitigation approach. The high proportion of samples exceeding regulatory thresholds further emphasizes the need for alternative strategies to recover contaminated batches. In the context of developing countries, economic constraints must be carefully considered. For aflatoxin contaminated groundnuts, visual sorting is the most widely used mitigation strategy in low-resource settings (Aoun et al., 2020). However, manual visual sorting is labour-intensive and impractical for large grain lots, while optical sorting technologies are generally unaffordable in low-income environments (Aoun et al., 2020). Sorting equipment should therefore be affordable, easy to use, and accessible to local producers, ideally allowing on-site application directly by producers. Low-cost solutions, such as size- or density-based sorting devices, could be prioritised, although their efficiency must first be thoroughly evaluated. The implementation of such strategies could be envisaged in parallel with the use of community mills. A study by Aoun et al. (2020) reported that size sorting was more effective than density sorting in grouping kernels according to AF concentrations, indicating that the exclusion of small and light kernels can significantly reduce AF levels in

groundnut grain. However, only size sorting and visual sorting achieved meaningful reductions in AF contamination (Aoun et al., 2020).

Finally, these results underline the necessity of increasing awareness among producers and consumers. Despite the potential loss of part of the production due to sorting or rejection, such measures are essential to protect public health.

Overall, this work demonstrates that the analytical method is flexible, requiring only limited modifications to address new matrices and mycotoxins. This flexibility is particularly valuable in a global context, where fungal distribution and mycotoxin contamination patterns may change due to climate change. For example, AFs, although not traditionally associated with cereals from Northern Europe, may increasingly pose a risk, as their occurrence has already been reported in Southern Europe despite being previously absent (Marroquín-Cardona et al., 2014; Kos et al., 2023; Casu et al., 2024).

6. Perspectives

The present work provides valuable insights into mycotoxin contamination and sorting efficiency in wheat and highlights several perspectives for further investigation. One important direction for future research concerns the further development of the analytical methodology. Although the method was successfully validated for regulated mycotoxins, extending its validation to non-regulated compounds, such as ENNs, which are increasingly reported in cereals, would be highly relevant. More broadly, the method could be expanded to include additional emerging mycotoxins that may become of regulatory or toxicological interest. The use of HRMS is relevant to detect simultaneously known and unknown compounds, when a large number of compounds need to be analysed, for mycotoxin detection in complex food or feed matrices, and to perform retrospective data analysis. Extension to other food matrices would also be valuable to gain a more comprehensive understanding of mycotoxin contamination across different raw materials. Although single-kernel analysis is labour-intensive, it may serve as a basis for developing spectral models that relate the spectral signatures of individual grains to their mycotoxin concentrations.

In Belgium, long-term monitoring of mycotoxin occurrence in wheat and cereals should be continued in order to build robust datasets covering multiple years and a wide range of climatic conditions. Such long-term data collections would allow a better assessment of the effects of climate change on mycotoxin occurrence in Belgian wheat and could support the development of

predictive risk models integrating meteorological parameters, varietal susceptibility and agronomic practices. These models could help to assess the risk of crop contamination before harvest. Ultimately, they could serve as decision-support tools for farmers, cooperatives, and storage operators, enabling the implementation of preventive measures to reduce contamination risks before harvest or during storage, alongside the pre-harvest warning system already in place for DON.

In addition, wheat is frequently cultivated in association with protein crops, and the impact of such intercropping systems on mycotoxin occurrence remains poorly documented. More generally, climate change is expected to drive shifts in cropping systems and crop species. Extending mycotoxin monitoring to emerging crop matrices in Belgium would therefore be relevant to anticipate future risks.

Further optimisation of sorting technologies also represents a promising research perspective. This could include the combination of different sorting principles in multi-step strategies, as well as fine-tuning sorter parameters to improve overall efficiency, for example, adjusting the hole size for size sorting, or the slope of the densitometric table. In addition, the development of new near-infrared models based on fungal species other than *Fusarium* could improve sorting efficiency for mycotoxins such as ochratoxin A (OTA), for which current approaches remain limited. More broadly, spectral or optical sorting strategies based on secondary fungal symptoms rather than the mycotoxins themselves may enable mitigation of a wider range of contaminants beyond DON and *Fusarium*-related toxins. Infrared spectroscopy already shows strong potential for mycotoxin screening. However, discrimination at aflatoxin guideline levels remains challenging due to very low regulatory limits and complex food matrices (Freitag et al., 2022). In contrast, high classification accuracy has been reported for OTA and DON using NIRS and MIRS (Freitag et al., 2022). Although current methods rely on indirect chemometric prediction, recent advances in deep learning, such as convolutional neural networks, are expected to further enhance the robustness and predictive performance of spectral models (Freitag et al., 2022). It would also be relevant to investigate sorting efficiency across a wider range of contamination levels in order to define theoretical thresholds above which sorting is no longer economically or technically justified. Mycotoxin distribution within a batch is often highly heterogeneous, and contamination patterns can differ substantially between batches. Some batches may contain only a few highly contaminated kernels that disproportionately increase the overall concentration, making sorting highly effective, whereas others may consist of a majority of grains contaminated above the maximum level, for which sorting would bring little benefit. In this context, the development of rapid diagnostic tools capable of characterising the

contamination profile of a batch prior to sorting would be highly valuable. Such tools could rely on infrared technologies, such as the detection of *Fusarium*-damaged kernels using infrared approaches which has already shown promising results.

Beyond technical performance, the economic viability of sorting strategies must be assessed under real field and storage conditions, considering both operational costs and potential gains in batch value. Sorting systems should also be compatible with large throughput requirements, as processing time should not become a limiting factor. Some sorting technologies, such as size sorting or optical and infrared sorting devices, are already implemented in certain storage facilities for wheat protein content segregation, meaning that the necessary equipment is partially available. In such cases, the additional investment required to implement mycotoxin-oriented sorting strategies may be limited to the development of new models rather than new hardware, improving the feasibility of industrial adoption.

Extending sorting trials to other commodities, such as peanuts, would also be highly relevant. Peanuts often show higher contamination levels and frequencies than wheat and represent a higher added value product, making sorting strategies potentially more impactful and economically attractive. This aspect is particularly critical in countries such as Benin or other sub-Saharan countries, where mycotoxin exposure represents a real and significant public health risk, especially for children, who may suffer from growth retardation due to aflatoxin exposure (including during their foetal life) (Gong et al., 2002; Tesfamariam et al., 2022). In this context, there is an urgent need for practical and affordable solutions, including improved hygiene at milling facilities, better drying and storage practices, sorting strategies, and increased awareness among producers and consumers.

Finally, close attention should be paid to the evolution of European regulations on mycotoxins. Anticipating future requirements regarding detection capabilities, threshold levels, and emerging contaminants will be essential to ensure that analytical and sorting strategies remain aligned with regulatory frameworks and market expectations. In this regard, non-targeted HRMS approaches could complement the targeted methods developed in this work by enabling the identification of new, modified, or masked mycotoxins and allowing retrospective data analysis to trace the emergence of new compounds over time.

7. Conclusions

In summary, this work resulted in the development and full validation of a reliable analytical method for the identification and quantification of a broad spectrum of mycotoxins, both regulated and non-regulated, in wheat produced in Belgium. The method fully complies with European performance criteria for regulated compounds. In addition, the method proved to be flexible, with the potential to be extended to other matrices or adapted to include additional mycotoxins that may gain importance in the coming years in cereals in Belgium. In this sense, it constitutes a solid basis that can evolve to meet future analytical and regulatory needs.

The monitoring of wheat samples harvested in 2023 and 2024 generated valuable and up-to-date data on mycotoxin occurrence in Belgian wheat, for which no recent data were available. The study also provided additional insights into the influence of meteorological conditions on contamination levels. In addition, this work highlighted differences in varietal susceptibility to fungal infection, thereby underlining the relevance of varietal selection as a complementary mitigation strategy and the need for multi-year studies to identify consistently more resistant varieties. Furthermore, the frequent detection of emerging mycotoxins confirmed their widespread presence in wheat, reinforcing the importance of monitoring non-regulated compounds alongside regulated ones to achieve a more comprehensive assessment of food safety risks.

This study also explored the potential of sorting technologies to reduce mycotoxin contamination in wheat, with a particular focus on DON and OTA. The results provide a first basis for evaluating sorting efficiency. However, further research is required to establish thresholds of effectiveness and to support the development of decision-support tools that could help farmers and grain operators in practice.

In parallel, the analytical method was successfully transferred to peanuts and peanut-based products. The observation of alarmingly high contamination levels, particularly for AFs and OTA, provides additional data for mycotoxin occurrence in Beninese peanuts and peanut-based products, highlighting a significant public health concern in Benin. These results clearly demonstrate the urgent need for effective risk-reduction strategies, including improved storage conditions, enhanced hygiene during processing, and increased awareness among producers and consumers. Given the higher added value of peanuts compared to wheat, this work also highlights the relevance of exploring sorting approaches as a potential mitigation tool for this commodity.

In conclusion, this study opens the way to several promising research perspectives. Future work should be pursued with a larger number of samples, collected over multiple years, and sorting efficiency should be further studied across varying contamination levels. A full validation of frequently observed emerging mycotoxins, coupled with an extension of sorting studies to other matrices and contaminants, will be crucial to strengthen both scientific knowledge and the practical management of mycotoxin risks along the food and feed chain.

Bibliography

Abdallah, M.F., Ameye, M., De Saeger, S., Audenaert, K., Haesaert, G., 2019. Biological control of mycotoxigenic fungi and their toxins : An update for the pre-harvest approach, in: Mycotoxins : Socio-Economic and Health Impact as Well as Pre- and Postharvest Management Strategies. IntechOpen, London, UK, pp. 59–89. doi:10.5772/intechopen.76342

Abdallah, M.F., De Boevre, M., Audenaert, K., Haesaert, G., De Saeger, S., 2018. Highlight report: Mycotoxins as food contaminants in Africa—challenges and perspectives. Arch Toxicol 92, 2151–2152. doi:10.1007/s00204-018-2203-2

Adeniran, H.A., Ikujenlola, A.V., Nd'janton, G.N., 2022. Effect of processing conditions on the aflatoxin content of kulikuli-A groundnut-based fried snack. Ann Clin Nutr. 5(1): 1023.

Adjile, A., Mongbo, R., Floquet, A., 2015. Les exploitations agricoles familiales arachidières de la commune de Ouessè au centre Bénin : Etat des lieux, typologies et dynamiques des systèmes de cultures. Ahoho, revue de géographie de Lomé 55–67.

Adjou, E.S., Yehouenou, B., Sossou, C.M., Soumanou, M.M., Souza, C.A. de, 2012. Occurrence of mycotoxins and associated mycoflora in peanut cake product (kulikuli) marketed in Benin. Afr. J. Biotechnol 11, 14354–14360. doi:10.5897/AJB12.324

Alshannaq, A., Yu, J.-H., 2017. Occurrence, toxicity, and analysis of major mycotoxins in food. Int. J. Environ. Res. Public Health 14, 632. doi:10.3390/ijerph14060632

Annunziata, L., Stramenga, A., Visciano, P., Schirone, M., De Colli, L., Colagrande, M.N., Campana, G., Scortichini, G., 2017. Simultaneous determination of aflatoxins, T-2 and HT-2 toxins, and fumonisins in cereal-derived products by QuEChERS extraction coupled with LC-MS/MS. Anal Bioanal Chem 409, 5143–5155. doi:10.1007/s00216-017-0462-z

Aoun, M., Stafstrom, W., Priest, P., Fuchs, J., Windham, G.L., Williams, W.P., Nelson, R.J., 2020. Low-cost grain sorting technologies to reduce mycotoxin contamination in maize and groundnut. Food Control 118, 107363. doi:10.1016/j.foodcont.2020.107363

Appell, M., Mueller, A., 2016. Mycotoxin analysis using imprinted materials technology: Recent developments. J AOAC Int 99, 861–864. doi:10.5740/jaoacint.16-0113

Arya, S.S., Salve, A.R., Chauhan, S., 2016. Peanuts as functional food: A review. J Food Sci Technol 53, 31–41. doi:10.1007/s13197-015-2007-9

Audenaert, K., Van Broeck, R., Bekaert, B., De Witte, F., Heremans, B., Messens, K., Höfte, M., Haesaert, G., 2009. *Fusarium* head blight (FHB) in Flanders: Population diversity, inter-species associations and DON contamination in commercial winter wheat varieties. Eur J Plant Pathol 125, 445–458. doi:10.1007/s10658-009-9494-3

Awuah, R.T., Fialor, S.C., Binns, A.D., Kagochi, J., Jolly, C.M., 2009. Factors influencing market participants decision to sort groundnuts along the marketing chain in Ghana. Peanut Sci 36, 68–76. doi:10.3146/PS06-36.1

Ayelign, A., De Saeger, S., 2020. Mycotoxins in Ethiopia: Current status, implications to food safety and mitigation strategies. Food Control 113, 107163. doi:10.1016/j.foodcont.2020.107163

Bakherad, Z., Feizy, J., 2018. Preliminary survey of aflatoxins in Mashhad's roasted red skin peanut kernels during February to May 2016. J. Community Health Res 7, 112–118.

Bakker, M.G., McCormick, S.P., 2019. Microbial correlates of *Fusarium* load and deoxynivalenol content in individual wheat kernels. Phytopathology 109, 993–1002. doi:10.1094/PHYTO-08-18-0310-R

Bankole, S.A., Ogunsanwo, B.M., Eseigbe, D.A., 2005. Aflatoxins in Nigerian dry-roasted groundnuts. Food Chem 89, 503–506. doi:10.1016/j.foodchem.2004.03.004

Battilani, P., Palumbo, R., Giorni, P., Dall'Asta, C., Dellafiora, L., Gkrillas, A., Toscano, P., Crisci, A., Brera, C., De Santis, B., Rosanna Cammarano, R., Della Seta, M., Campbell, K., Elliot, C., Venancio, A., Lima, N., Gonçalves, A., Terciolo, C., Oswald, I.P., 2020. Mycotoxin mixtures in food and feed: Holistic, innovative, flexible risk assessment modelling approach. EFSA Supporting Publications 17, 1757E. doi:10.2903/sp.efsa.2020.EN-1757

Bediako, A.K., Ofori, K., Offei, S.K., Dzidzienyo, D., Asibuo, J.Y., Adu Amoah, R., 2019. Aflatoxin contamination of groundnut (*Arachis hypogaea L.*): Predisposing factors and management interventions. Food Control 98, 61–67. doi:10.1016/j.foodcont.2018.11.020

Behr, A.-C., Fæste, C.K., Azqueta, A., Tavares, A.M., Spyropoulou, A., Solhaug, A., Olsen, A.-K., Vettorazzi, A., Mertens, B., Zegura, B., Streel, C., Ndiaye, D., Spilioti, E., Dubreil, E., Buratti, F.M., Crudo, F., Eriksen, G.S., Snapkow, I., Teixeira, J.P., Rasinger, J.D., Sanders, J., Machera, K., Ivanova, L., Gaté, L., Le Hegarat, L., Novak, M., Smith, N.M., Tait, S., Fraga, S., Hager, S., Marko, D., Braeuning, A., Louro, H., Silva, M.J., Dirven, H., Dietrich, J., 2025. Hazard characterization of the mycotoxins enniatins and

beauvericin to identify data gaps and improve risk assessment for human health. Arch Toxicol 99, 1791–1841. doi:10.1007/s00204-025-03988-3

Belgian Federal Agency for the Safety of the Food Chain (FASFC), 2025. Partie 2 : critères microbiologiques et limites d'action pour les aliments pour animaux, les denrées alimentaires et le matériel de prélèvement (No. 2025/1592/PCCB). Belgian Federal Agency for the Safety of the Food Chain (FASFC), Belgium.

Ben Hassouna, K., Ben Salah-Abbès, J., Chaieb, K., Abbès, S., 2022. Mycotoxins occurrence in milk and cereals in North African countries: A review. Crit. Rev. Toxicol 52, 619–635. doi:10.1080/10408444.2022.2157703

Ben Miri, Y., Benabdallah, A., Chentir, I., Djenane, D., Luvisi, A., De Bellis, L., 2024. Comprehensive insights into ochratoxin A: Occurrence, analysis, and control strategies. Foods 13, 1184. doi:10.3390/foods13081184

Biango-Daniels, M.N., Snyder, A.B., Worobo, R.W., Hodge, K.T., 2019. Fruit infected with *Paecilomyces niveus*: A source of spoilage inoculum and patulin in apple juice concentrate? Food Control 97, 81–86. doi:10.1016/j.foodcont.2018.10.020

Biranjia-Hurdoyal, S., 2025. Mycotoxins in food and feed: Relevance to human and animal health, in: Gupta, A. (Ed.), Prospects of Fungal Biotechnologies for Livestock Volume 1: Fungal Bioengineering in Livestock Health Management. Springer, Cham, pp. 127–148. doi:10.1007/978-3-031-90994-8_5

Bondy, G.S., 2008. Immunological toxicity of mycotoxins. Stewart Postharvest Review 4, 1–6. doi:10.2212/spr.2008.6.7

Bullerman, L.B., Bianchini, A., 2007. Stability of mycotoxins during food processing. Int J Food Microbiol 119, 140–146. doi:10.1016/j.ijfoodmicro.2007.07.035

Cargill, 2024. 2024 Cargill World Mycotoxin Report. Cargill, Incorporated, Minneapolis.

Casu, A., Camardo Leggieri, M., Toscano, P., Battilani, P., 2024. Changing climate, shifting mycotoxins: A comprehensive review of climate change impact on mycotoxin contamination. Compr. Rev. Food Sci. Food Saf 23, e13323. doi:10.1111/1541-4337.13323

CEN, Comité Européen de Normalisation, 2022. Produits alimentaires - Multiméthode de détermination de la teneur en aflatoxines, déoxynivalénol, fumonisines, ochratoxine A, toxine T-2, toxine HT-2 et zéaralénone par CLSM/SM (EN 17641:2022).

CEN, Comité Européen de Normalisation, 2010. Analyse des produits alimentaires - Critères de performance des méthodes validées monolaboratoires d'analyse des mycotoxines (CEN/TR 16059).

Chang, A.S., Sreedharan, A., Schneider, K.R., 2013. Peanut and peanut products: A food safety perspective. Food Control 32, 296–303. doi:10.1016/j.foodcont.2012.12.007

Cheli, F., Pinotti, L., Rossi, L., Dell'Orto, V., 2013. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. LWT - Food Sci. Technol. 54, 307–314. doi:10.1016/j.lwt.2013.05.040

Chen, D., Chen, H.W., 2013. Using the Köppen classification to quantify climate variation and change: An example for 1901–2010. Environ. Dev 6, 69–79. doi:10.1016/j.envdev.2013.03.007

Çiftçi, S., Suna, G., 2022. Functional components of peanuts (*Arachis Hypogaea L.*) and health benefits: A review. Future Foods 5, 100140. doi:10.1016/j.fufo.2022.100140

Comité Scientifique institué auprès de l'Agence Fédérale pour la Sécurité de la Chaîne Alimentaire, 2022. Evaluation du programme d'analyses de l'AFSCA : les mycotoxines dans les denrées alimentaires et les aliments pour animaux.

Darko, C.B., 2016. Effect of storage conditions on *Aspergillus* growth and aflatoxin production in peanuts: A sudy in Ghana. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

De Felice, B., Spicer, L.J., Caloni, F., 2023. Enniatin B1: Emerging mycotoxin and emerging issues. Toxins 15, 383. doi:10.3390/toxins15060383

De Frutos Cachorro, J., Gobin, A., Buysse, J., 2018. Farm-level adaptation to climate change: The case of the Loam region in Belgium. Agricultural Systems 165, 164–176. doi:10.1016/j.agsy.2018.06.007

de Oliveira, A.C.D., Ali, S., Corassin, C.H., Ullah, S., Pereira, K.N., Walsh, J.L., Hojnik, N., de Oliveira, C.A.F., 2025. Application of cold atmospheric plasma for decontamination of toxigenic fungi and mycotoxins: a systematic review. Front Microbiol 15, 1502915. doi:10.3389/fmicb.2024.1502915

De Santis, B., Debegnach, F., Gregori, E., Russo, S., Marchegiani, F., Moracci, G., Brera, C., 2017. Development of a LC-MS/MS method for the multi-mycotoxin determination in composite cereal-based samples. Toxins 9, 169. doi:10.3390/toxins9050169

Delcour, A., Stilmant, D., Burny, P., Rabier, F., Louppe, H., Goffart, J.-P., 2014. État des lieux des flux céréaliers en Wallonie selon différentes filières d'utilisation. Biotechnol. Agron. Soc. Environ.

Dib, A.A., Assaf, J.C., Debs, E., Khatib, S.E., Louka, N., Khoury, A.E., 2023. A comparative review on methods of detection and quantification of mycotoxins in solid food and feed: A focus on cereals and nuts. Mycotoxin Res 39, 319–345. doi:10.1007/s12550-023-00501-6

Diédhiou, P., Ba, F., Kane, A., Mbaye, N., 2012. Effect of different cooking methods on aflatoxin fate in peanut products. Afr. J. Food Sci.

dsm-firmenich, 2024. World Mycotoxin Survey: The global threat - January - December 2024 (No. REP_MTXsurvey_Q4_0525_63898).

Duarte, S.C., Pena, A., Lino, C.M., 2010. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiol 27, 187–198. doi:10.1016/j.fm.2009.11.016

EFSA, 2020. Risk assessment of ochratoxin A in food. EFSA J. 18, e06113. doi:10.2903/j.efsa.2020.6113

EFSA, 2018. Risks to human and animal health related to the presence of moniliformin in food and feed. EFSA Journal 16, e05082. doi:10.2903/j.efsa.2018.5082

EFSA, 2014. Scientific opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA Journal 12, 3802. doi:10.2903/j.efsa.2014.3802

EFSA, 2013. Scientific opinion on the risk for public and animal health related to the presence of sterigmatocystin in food and feed. EFSA Journal 11, 3254. doi:10.2903/j.efsa.2013.3254

EFSA, 2012. Scientific Opinion on the risks for public and animal health related to the presence of citrinin in food and feed. EFSA Journal 10, 2605. doi:10.2903/j.efsa.2012.2605

EFSA, 2011. Scientific opinion on the risks for public health related to the presence of zearalenone in food. EFSA Journal 9, 2197. doi:10.2903/j.efsa.2011.2197

EFSA, Arcella, D., Eskola, M., Gómez Ruiz, J.A., 2016. Dietary exposure assessment to *Alternaria* toxins in the European population. EFSA Journal 14, e04654. doi:10.2903/j.efsa.2016.4654

EFSA, Knutsen, H.-K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Nebbia, C.S., Petersen, A., Rose, M., Roudot, A.-C., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., Dall'Asta, C., Dänicke, S., Eriksen, G.-S., Altieri, A., Roldán-Torres, R., Oswald, I.P., 2017a. Risks for animal health related to the presence of zearalenone and its modified forms in feed. EFSA J. 15, e04851. doi:10.2903/j.efsa.2017.4851

EFSA, Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Nebbia, C.S., Oswald, I.P., Petersen, A., Rose, M., Roudot, A.-C., Schwerdtle, T., Vleminckx, C., Vollmer, G., Wallace, H., De Saeger, S., Eriksen, G.S., Farmer, P., Fremy, J.-M., Gong, Y.Y., Meyer, K., Naegeli, H., Parent-Massin, D., Rietjens, I., van Egmond, H., Altieri, A., Eskola, M., Gergelova, P., Ramos Bordajandi, L., Benkova, B., Dörr, B., Gkrillas, A., Gustavsson, N., van Manen, M., Edler, L., 2017b. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J. 15, e04718. doi:10.2903/j.efsa.2017.4718

EFSA, Maggiore, A., Afonso, A., Barrucci, F., Sanctis, G.D., 2020a. Climate change as a driver of emerging risks for food and feed safety, plant, animal health and nutritional quality. EFSA Supporting Publications 17, 1881E. doi:10.2903/sp.efsa.2020.EN-1881

EFSA, Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Leblanc, J.-C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Marko, D., Oswald, I.P., Piersma, A., Routledge, M., Schlatter, J., Baert, K., Gergelova, P., Wallace, H., 2020b. Risk assessment of aflatoxins in food. EFSA Journal 18, e06040. doi:10.2903/j.efsa.2020.6040

EFSA, Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Leblanc, J.-C., Nebbia, C.S., Nielsen, E., Ntzani, E., Sand, S., Schwerdtle, T., Vleminckx, C., Wallace, H., Gropp, J., Antonissen, G., Rychen, G., Gómez Ruiz, J.Á., Innocenti, M.L., Rovesti, E., Petersen, A., 2023. Risks for animal health related to the presence of ochratoxin A (OTA) in feed. EFSA J. 21, e08375. doi:10.2903/j.efsa.2023.8375

Ekwomadu, T.I., Akinola, S.A., Mwanza, M., 2021. *Fusarium* mycotoxins, their metabolites (free, emerging, and masked), food safety concerns, and health impacts. Int J Environ Res Public Health 18, 11741. doi:10.3390/ijerph182211741

El-Sayed, R.A., Jebur, A.B., Kang, W., El-Demerdash, F.M., 2022. An overview on the major mycotoxins in food products: Characteristics, toxicity, and analysis. J. Future Foods 2, 91–102. doi:10.1016/j.jfutfo.2022.03.002

Erenstein, O., Jaleta, M., Mottaleb, K.A., Sonder, K., Donovan, J., Braun, H.-J., 2022. Global trends in wheat production, consumption and trade, in: Reynolds, M.P., Braun, H.-J. (Eds.), Wheat Improvement: Food Security in a Changing Climate. Springer International Publishing, Cham, pp. 47–66. doi:10.1007/978-3-030-90673-3_4

Eskola, M., Kos ,Gregor, Elliott ,Christopher T., Hajšlová ,Jana, Mayar ,Sultan, and Krska, R., 2020. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%. Crit. Rev. Food Sci. Nutr 60, 2773–2789. doi:10.1080/10408398.2019.1658570

European Commission, 2025a. RASFF Window - Peanuts 2024 [WWW Document]. URL https://webgate.ec.europa.eu/rasff-window/screen/search?searchQueries=eyJkYXRlIjp7InN0YXJ0UmFuZ2UiOiIyMDIzLTEyLTMxVDIzOjAwOjAwLjAwMFoiLCJlbmRSYW5nZSI6IjIwMjQtMTItMzBUMjM6MDA6MDAuMDAwWiJ9LCJjb3VudHJpZXMiOnt9LCJ0eXBlIjp7fSwibm90aWZpY2F0aW9uU3RhdHVzIjp7fSwicHJvZHVjdCI6eyJwcm9kdWN0Q2F0ZWdvcnkiOltbMTg0MjddXX0sInJpc2siOnsiaGF6YXJkQ2F0ZWdvcnkiOltbMjE4MDBdXX0sInJlZmVyZW5jZSI6IiIsInN1YmplY3QiOiJwZWFudXRzIn0%3D (accessed 8.29.25).

European Commission, 2025b. RASFF Window - Peanuts 2023 [WWW Document]. URL https://webgate.ec.europa.eu/rasff-window/screen/search?searchQueries=eyJkYXRlIjp7InN0YXJ0UmFuZ2UiOiIyMDIyLTEyLTMxVDIzOjAwOjAwLjAwMFoiLCJlbmRSYW5nZSI6IjIwMjMtMTItMzBUMjM6MDA6MDAuMDAwWiJ9LCJjb3VudHJpZXMiOnt9LCJ0eXBlIjp7fSwibm90aWZpY2F0aW9uU3RhdHVzIjp7fSwicHJvZHVjdCI6eyJwcm9kdWN0Q2F0ZWdvcnkiOltbMTg0MjddXX0sInJpc2siOnsiaGF6YXJkQ2F0ZWdvcnkiOltbMjE4MDBdXX0sInJlZmVyZW5jZSI6IiIsInN1YmplY3QiOiJwZWFudXRzIn0%3D (accessed 8.29.25).

European Commission, 2024a. RASFF Window - Results [WWW Document]. URL https://webgate.ec.europa.eu/rasff-window/screen/search?searchQueries=eyJkYXRlIjp7InN0YXJ0UmFuZ2UiOiIyMDIzLTEyLTMxVDIzOjAwOjAwLjAwMFoiLCJlbmRSYW5nZSI6IjIwMjQtMTItMzBUMjM6MDA6MDAuMDAwWiJ9LCJjb3VudHJpZXMiOnt9LCJ0eXBlIjp7fSwibm90aWZpY2F0aW9uU3RhdHVzIjp7fSwicHJvZHVjdCI6eyJwcm9kdWN0Q2F0ZWdvcnkiOltbMTg0MzRdXX0sInJpc2siOnsiaGF6YXJkQ2F0ZWdvcnkiOltbMjE4MDBdXX0sInJlZmVyZW5jZSI6IiIsInN1YmplY3QiOiIifQ%3D%3D (accessed 8.26.25).

European Commission, 2024b. Commission Regulation (EU) 2024/1022 of 8 April 2024 amending Regulation (EU) 2023/915 as regards maximum levels of deoxynivalenol in food, Off. J. Eur. Union, 9.4.2024, 1-4.

European Commission, 2024c. Commission Regulation (EU) 2024/1038 of 9 April 2024 amending Regulation (EU) 2023/915 as regards maximum levels of T-2 and HT-2 toxins in food, Off. J. Eur. Union, 10.4.2024, 1-5.

European Commission, 2023a. Cereals - European Commission [WWW Document]. URL https://agriculture.ec.europa.eu/farming/crop-productions-and-plant-based-products/cereals_en (accessed 8.26.25).

European Commission, 2023b. RASFF Window - Results [WWW Document]. URL https://webgate.ec.europa.eu/rasff-window/screen/search?searchQueries=eyJkYXRlIjp7InN0YXJ0UmFuZ2UiOiIyMDIyLTEyLTMxVDIzOjAwOjAwLjAwMFoiLCJlbmRSYW5nZSI6IjIwMjMtMTItMzBUMjM6MDA6MDAuMDAwWiJ9LCJjb3VudHJpZXMiOnt9LCJ0eXBlIjp7fSwibm90aWZpY2F0aW9uU3RhdHVzIjp7Im5vdGlmaWNhdGlvblN0YXR1cyI6W1sxXV19LCJwcm9kdWN0Ijp7InByb2R1Y3RDYXRlZ29yeSI6W1sxODQzNF1dfSwicmlzayI6eyJoYXphcmRDYXRlZ29yeSI6W1syMTgwMF1dfSwicmVmZXJlbmNlIjoiIiwic3ViamVjdCI6IiIsInBhZ2VTaXplIjoyNX0%3D (accessed 6.19.24).

European Commission, 2023c. Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006, Off. J. Eur. Union, L119, 103-157.

European Commission, 2022. Commission Recommendation (EU) 2022/553 of 5 April 2022 on monitoring the presence of Alternaria toxins in food, Off. J. Eur. Union, L107, 6.4.2022, 90–92.

European Commission, 2021. COMMISSION IMPLEMENTING REGULATION (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC, Off. J. Eur. Union, L180/84, 84-109.

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

European Commission, 2006b. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, Off. J. Eur. Union, L70/12, 12-34.

European Commission, 2002. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed - Council statement, Off. J. Eur. Union, L140, 10-21.

European Commission-Directorate-General for Agriculture and Rural, 2025. Cereals - Gross production by all crops for all member states [WWW Document]. URL https://agridata.ec.europa.eu/extensions/DashboardCereals/CerealsProduction.html (accessed 10.15.25).

Ezekiel, C.N., Sulyok, M., Warth, B., Odebode, A.C., Krska, R., 2012. Natural occurrence of mycotoxins in peanut cake from Nigeria. Food Control 27, 338–342. doi:10.1016/j.foodcont.2012.04.010

Fandohan, P., Gnonlonfin, B., Hell, K., Marasas, W.F.O., Wingfield, M.J., 2005. Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize in Benin, West Africa. Int J Food Microbiol 99, 173–183. doi:10.1016/j.ijfoodmicro.2004.08.012

FAOSTAT [WWW Document], 2025. URL https://www.fao.org/faostat/en/#data/QCL/visualize (accessed 8.29.25).

Feeding the future global population, 2024. Nat Commun 15, 222. doi:10.1038/s41467-023-44588-y

Food and Agriculture Organization of the United Nations, 2025. FAO Cereal Supply and Demand Brief [WWW Document]. WorldFoodSituation. URL https://www.fao.org/worldfoodsituation/csdb/en/ (accessed 6.19.24).

Fraeyman, S., Croubels, S., Devreese, M., Antonissen, G., 2017. Emerging *Fusarium* and *Alternaria* mycotoxins: Occurrence, toxicity and toxicokinetics. Toxins 9, 228. doi:10.3390/toxins9070228

Freire, L., Sant'Ana, A.S., 2018. Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food Chem. Toxicol 111, 189–205. doi:10.1016/j.fct.2017.11.021

Freitag, S., Sulyok, M., Logan, N., Elliott, C.T., Krska, R., 2022. The potential and applicability of infrared spectroscopic methods for the rapid screening and routine analysis of mycotoxins in food crops. Compr. Rev. Food Sci. Food Saf 21, 5199–5224. doi:10.1111/1541-4337.13054

Fu, Y., Yin, S., Zhao, C., Fan, L., Hu, H., 2022. Combined toxicity of food-borne mycotoxins and heavy metals or pesticides. Toxicon 217, 148–154. doi:10.1016/j.toxicon.2022.08.012

Garcia-Cela, E., Gasperini, A.M., 2024. Climate change and mycotoxins: A growing food safety concern. J Consum Prot Food Saf 19, 373–375. doi:10.1007/s00003-024-01528-2

Garello, M., Piombo, E., Buonsenso, F., Prencipe, S., Valente, S., Meloni, G.R., Marcet-Houben, M., Gabaldón, T., Spadaro, D., 2024. Several secondary metabolite gene clusters in the genomes of ten *Penicillium* spp. raise the risk of multiple mycotoxin occurrence in chestnuts. Food Microbiol 122, 104532. doi:10.1016/j.fm.2024.104532

Gastélum-Martínez, E., Compant, S., Taillandier, P., Mathieu, F., 2013. Control of T-2 Toxin in *Fusarium Langsethiae* and *Geotrichum Candidum* co-culture. AIHT 63, 447–456. doi:10.2478/10004-1254-63-2012-2206

Gong, Y.Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P.C., Hall, A.J., Wild, C.P., 2002. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: Cross sectional study. BMJ 325, 20–21. doi:10.1136/bmj.325.7354.20

González-Jartín, J.M., Rodríguez-Cañás, I., Alfonso, A., Sainz, M.J., Vieytes, M.R., Gomes, A., Ramos, I., Botana, L.M., 2021. Multianalyte method for the determination of regulated, emerging and modified mycotoxins in milk: QuEChERS extraction followed by UHPLC–MS/MS analysis. Food Chem. 356, 129647. doi:10.1016/j.foodchem.2021.129647

Gruber-Dorninger, C., Novak, B., Nagl, V., Berthiller, F., 2017. Emerging mycotoxins: Beyond traditionally determined food contaminants. J. Agric. Food Chem. 65, 7052–7070. doi:10.1021/acs.jafc.6b03413

Guo, H., Ji, J., Wang, J., Sun, X., 2020. Co-contamination and interaction of fungal toxins and other environmental toxins. Trends Food Sci. Technol 103, 162–178. doi:10.1016/j.tifs.2020.06.021

Habschied, K., Krstanović, V., Zdunić, Z., Babić, J., Mastanjević, K., Šarić, G.K., 2021. Mycotoxins biocontrol methods for healthier crops and stored products. J Fungi 7, 348. doi:10.3390/jof7050348

Hellany, H., Assaf, J.C., El-Badan, D., Khalil, M., 2023. Quantification, prevalence, and pretreatment methods of mycotoxins in groundnuts and tree nuts: An update. Processes 11, 3428. doi:10.3390/pr11123428

Hidalgo-Ruiz, J.L., Romero-González, R., Martínez Vidal, J.L., Garrido Frenich, A., 2019. Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix. Food Compos. Anal 82, 103228. doi:10.1016/j.jfca.2019.05.011

Houbraken, J., Samson, R.A., Frisvad, J.C., 2006. Byssochlamys: Significance of heat resistance and mycotoxin production, in: Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U. (Eds.), Advances in Food Mycology. Springer US, Boston, MA, pp. 211–224. doi:10.1007/0-387-28391-9_14

Ingenbleek, L., Sulyok, M., Adegboye, A., Hossou, S.E., Koné, A.Z., Oyedele, A.D., Kisito, C.S.K.J., Dembélé, Y.K., Eyangoh, S., Verger, P., Leblanc, J.-C., Le Bizec, B., Krska, R., 2019. Regional Sub-Saharan Africa

total diet study in Benin, Cameroon, Mali and Nigeria reveals the presence of 164 mycotoxins and other secondary metabolites in foods. Toxins 11, 54. doi:10.3390/toxins11010054

Inoue, T., Nagatomi, Y., Uyama, A., Mochizuki, N., 2013. Fate of mycotoxins during beer brewing and fermentation. Biosci. Biotechnol. Biochem. 77, 1410–1415. doi:10.1271/bbb.130027

International Agency for Research on Cancer, 2012a. Chemical agents and related occupations, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.

International Agency for Research on Cancer, 2012b. Improving public health through mycotoxin control, IARC. ed. International Agency for Research on Cancer, Lyon.

International Agency for Research on Cancer, 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, IARC monographs on the evaluation of carcinogenic risks to humans.

International Agency for Research on Cancer (Ed.), 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins: this publication represents the views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 9 - 16 June 1992, IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. IARC, Lyon.

Iqbal, S.Z., 2021. Mycotoxins in food, recent development in food analysis and future challenges; a review. Curr. Opin. Food Sci 42, 237–247. doi:10.1016/j.cofs.2021.07.003

Isebaert, S., De Saeger, S., Devreese, R., Verhoeven, R., Maene, P., Heremans, B., Haesaert, G., 2009. Mycotoxin-producing *Fusarium* species occurring in winter wheat in Belgium (Flanders) during 2002–2005. J phytopathol 157, 108–116. doi:10.1111/j.1439-0434.2008.01443.x

Ismaiel, A.A., Papenbrock, J., 2015. Mycotoxins: Producing fungi and mechanisms of phytotoxicity. Agriculture 5, 492–537. doi:10.3390/agriculture5030492

Janik, E., Niemcewicz, M., Podogrocki, M., Ceremuga, M., Gorniak, L., Stela, M., Bijak, M., 2021. The existing methods and novel approaches in mycotoxins' detection. Molecules 26, 3981. doi:10.3390/molecules26133981

Jensen, T., de Boevre, M., Preußke, N., de Saeger, S., Birr, T., Verreet, J.-A., Sönnichsen, F.D., 2019. Evaluation of high-resolution mass spectrometry for the quantitative analysis of mycotoxins in complex feed matrices. Toxins 11, 531. doi:10.3390/toxins11090531

Jestoi, M., Rokka, M., Järvenpää, E., Peltonen, K., 2009. Determination of *Fusarium* mycotoxins beauvericin and enniatins (A, A1, B, B1) in eggs of laying hens using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Food Chem 115, 1120–1127. doi:10.1016/j.foodchem.2008.12.105

Ji, F., He, D., Olaniran, A.O., Mokoena, M.P., Xu, J., Shi, J., 2019. Occurrence, toxicity, production and detection of *Fusarium* mycotoxin: A review. Food Prod. Process. Nutr 1, 6. doi:10.1186/s43014-019-0007-2

Jonard, C., Chandelier, A., Eylenbosch, D., Pannecoucque, J., Godin, B., Douny, C., Scippo, M.-L., Gofflot, S., 2025. Multi-mycotoxin analyses by UPLC-MS/MS in wheat: The situation in Belgium in 2023 and 2024. Foods 14, 2300. doi:10.3390/foods14132300

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T., Dussort, P., 2016. Impact of food processing and detoxification treatments on mycotoxin contamination. Mycotoxin Res 32, 179–205. doi:10.1007/s12550-016-0257-7

Kautzman, M.E., Wickstrom, M.L., Scott, T.A., 2015. The use of near infrared transmittance kernel sorting technology to salvage high quality grain from grain downgraded due to *Fusarium* damage. Animal Nutrition 1, 41–46. doi:10.1016/j.aninu.2015.02.007

Kayode, O.F., Sulyok, M., Fapohunda, S.O., Ezekiel, C.N., Krska, R., Oguntona, C.R.B., 2013. Mycotoxins and fungal metabolites in groundnut- and maize-based snacks from Nigeria. Food Addit Contam Part B Surveill 6, 294–300. doi:10.1080/19393210.2013.823626

Khan, R., Anwar, F., Ghazali, F.M., 2024. A comprehensive review of mycotoxins: Toxicology, detection, and effective mitigation approaches. Heliyon 10, 28361. doi:10.1016/j.heliyon.2024.e28361

Khodaei, D., Javanmardi, F., Khaneghah, A.M., 2021. The global overview of the occurrence of mycotoxins in cereals: A three-year survey. Curr. Opin. Food Sci 39, 36–42. doi:10.1016/j.cofs.2020.12.012

Kihal, A., Rodríguez-Prado, M., Calsamiglia, S., 2022. The efficacy of mycotoxin binders to control mycotoxins in feeds and the potential risk of interactions with nutrient: A review. J Anim Sci 100, skac328. doi:10.1093/jas/skac328

Kluczkovski, A.M., 2019. Fungal and mycotoxin problems in the nut industry. Curr. Opin. Food Sci. 29, 56–63. doi:10.1016/j.cofs.2019.07.009

Kolawole, O., Siri-Anusornsak, W., Petchkongkaew, A., Elliott, C., 2024. A systematic review of global occurrence of emerging mycotoxins in crops and animal feeds, and their toxicity in livestock. Emerg. Contam 10, 100305. doi:10.1016/j.emcon.2024.100305

Köppen, R., Koch, M., Siegel, D., Merkel, S., Maul, R., Nehls, I., 2010. Determination of mycotoxins in foods: Current state of analytical methods and limitations. Appl Microbiol Biotechnol 86, 1595–1612. doi:10.1007/s00253-010-2535-1

Kos, J., Anić, M., Radić, B., Zadravec, M., Janić Hajnal, E., Pleadin, J., 2023. Climate change—A global threat resulting in increasing mycotoxin occurrence. Foods 12, 2704. doi:10.3390/foods12142704

Kumar, P., Mahato, D.K., Sharma, B., Borah, R., Haque, S., Mahmud, M.M.C., Shah, A.K., Rawal, D., Bora, H., Bui, S., 2020. Ochratoxins in food and feed: Occurrence and its impact on human health and management strategies. Toxicon 187, 151–162. doi:10.1016/j.toxicon.2020.08.031

Leite, M., Freitas, A., Barbosa, J., Ramos, F., 2023. Comprehensive assessment of different extraction methodologies for optimization and validation of an analytical multi-method for determination of emerging and regulated mycotoxins in maize by UHPLC-MS/MS. Food Chem Adv 2, 100145. doi:10.1016/j.focha.2022.100145

Li, K., Cai, H., Luo, B., Duan, S., Yang, J., Zhang, N., He, Y., Wu, A., Liu, H., 2025. Recent progress of mycotoxin in various food products-human exposure and health risk assessment. Foods 14, 865. doi:10.3390/foods14050865

Lindemann, V., Schmidt, J., Cramer, B., Humpf, H.-U., 2022. Detection of mycotoxins in highly matrix-loaded house-dust samples by QTOF-HRMS, IM-QTOF-HRMS, and TQMS: Advantages and disadvantages. Anal Chem 94, 4209–4217. doi:10.1021/acs.analchem.1c04254

Liu, C., Van der Fels-Klerx, H.J., 2021. Quantitative modelling of climate change impacts on mycotoxins in cereals: A review. Toxins 13, 276. doi:10.3390/toxins13040276

Liu, L., Xie, M., Wei, D., 2022. Biological detoxification of mycotoxins: Current status and future advances. Int J Mol Sci 23, 1064. doi:10.3390/ijms23031064

Lombard, M.J., 2014. Mycotoxin exposure and infant and young child growth in Africa: What do we know? Ann Nutr Metab 64, 42–52. doi:10.1159/000365126

Magan, N., Aldred, D., Mylona, K., Lambert, R.J.W., 2010. Limiting mycotoxins in stored wheat. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 27, 644–650. doi:10.1080/19440040903514523

Mahato, D.K., Devi, S., Pandhi, S., Sharma, B., Maurya, K.K., Mishra, S., Dhawan, K., Selvakumar, R., Kamle, M., Mishra, A.K., Kumar, P., 2021. Occurrence, impact on agriculture, human health, and management strategies of zearalenone in food and feed: A review. Toxins 13, 92. doi:10.3390/toxins13020092

Mannaa, M., Kim, K.D., 2017. Influence of temperature and water activity on deleterious fungi and mycotoxin production during grain storage. Mycobiology 45, 240–254. doi:10.5941/MYCO.2017.45.4.240

Marín, S., Ramos, A.J., 2016. Chapter 17 - Molds and mycotoxins in nuts, in: Kotzekidou, P. (Ed.), Food Hygiene and Toxicology in Ready-to-Eat Foods. Academic Press, San Diego, pp. 295–312. doi:10.1016/B978-0-12-801916-0.00017-0

Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60, 218–237. doi:10.1016/j.fct.2013.07.047

Marroquín-Cardona, A.G., Johnson, N.M., Phillips, T.D., Hayes, A.W., 2014. Mycotoxins in a changing global environment – A review. Food Chem. Toxicol. 69, 220–230. doi:10.1016/j.fct.2014.04.025

Martins, L.M., Sant'Ana, A.S., Iamanaka, B.T., Berto, M.I., Pitt, J.I., Taniwaki, M.H., 2017. Kinetics of aflatoxin degradation during peanut roasting. Food Res. Int 97, 178–183. doi:10.1016/j.foodres.2017.03.052

Mathew, G., Pingle, S., S, P., B, R., 2024. Assessing aflatoxin exposure: Exploring types of exposure and diverse biomarkers – A minireview. Curr Fungal Infect Rep 18, 163–171. doi:10.1007/s12281-024-00496-2

McCormick, S.P., Stanley, A.M., Stover, N.A., Alexander, N.J., 2011. Trichothecenes: From simple to complex mycotoxins. Toxins 3, 802–814. doi:10.3390/toxins3070802

Medina, A., 2023. Chapter 22 - Emerging mycotoxin risks due to climate change. What to expect in the coming decade?, in: Knowles, M.E., Anelich, L.E., Boobis, A.R., Popping, B. (Eds.), Present Knowledge in Food Safety. Academic Press, pp. 309–314. doi:10.1016/B978-0-12-819470-6.00053-6

Medina, Á., Rodríguez, A., Magan, N., 2015. Climate change and mycotoxigenic fungi: Impacts on mycotoxin production. Curr. Opin. Food Sci 5, 99–104. doi:10.1016/j.cofs.2015.11.002

Melo, B., Robalo, J., Ramos, F., Sanches Silva, A., 2025. Peanuts (*Arachis hypogaea L.*) and mycotoxins: Analytical approaches, prevalence, and innovative detoxification. Foods 14, 902. doi:10.3390/foods14050902

Michel, S., Steiner, B., Buerstmayr, H., 2024. Efficiency of indirect selection for *Fusarium* head blight resistance and mycotoxin accumulation in winter wheat (*Triticum aestivum* L.). Plant Breed 143, 34–43. doi:10.1111/pbr.13136

Ministère de l'agriculture, de l'élevage et de la pêche, 2007. Arrêté 2007- 0362 /MAEP/D-CAB/SGM//DRH/DP/SA du 30 octobre 2007 portant fixation des teneurs maximales pour certains contaminants dans les denrées alimentaires en République du Benin.

Mitrović, I., Čanak, P., Živanov, S.T., Farkaš, H., Vasiljević, M., Ćujić, S., Zorić, M., Mitrović, B., Mitrović, I., Čanak, P., Živanov, S.T., Farkaš, H., Vasiljević, M., Ćujić, S., Zorić, M., Mitrović, B., 2025. *Trichoderma harzianum* in biocontrol of maize fungal diseases and relevant mycotoxins: From the laboratory to the field. J Fungi 11. doi:10.3390/jof11060416

Modrzewska, M., Błaszczyk, L., Stępień, Ł., Urbaniak, M., Waśkiewicz, A., Yoshinari, T., Bryła, M., Modrzewska, M., Błaszczyk, L., Stępień, Ł., Urbaniak, M., Waśkiewicz, A., Yoshinari, T., Bryła, M., 2022. *Trichoderma* versus *Fusarium*—Inhibition of pathogen growth and mycotoxin biosynthesis. Molecules 27. doi:10.3390/molecules27238146

Moraes, W.B., Madden, L.V., Gillespie, J., Paul, P.A., 2023. Environment, grain development, and harvesting strategy effects on zearalenone contamination of grain from *Fusarium* Head Blight-affected wheat spikes. Phytopathology 113, 225–238. doi:10.1094/PHYTO-05-22-0190-R

Mousavi Khaneghah, A., Fakhri, Y., Gahruie, H.H., Niakousari, M., Sant'Ana, A.S., 2019. Mycotoxins in cereal-based products during 24 years (1983–2017): A global systematic review. Trends Food Sci. Technol 91, 95–105. doi:10.1016/j.tifs.2019.06.007

Ndung'u, J.W., Makokha, A.O., Onyango, C.A., Mutegi, C.K., Wagacha, J.M., Christie, M.E., Wanjoya, A.K., 2013. Prevalence and potential for aflatoxin contamination in groundnuts and peanut butter from farmers and traders in Nairobi and Nyanza provinces of Kenya. J. Appl. Biosci 65, 4922–4934. doi:10.4314/jab.v65i0.89579

Nejad, B.G., Mostafaei, Z., Rezaabad, A.B., Mehravar, F., Zarei, M., Dehghani, A., Estabragh, M.A.R., Karami-Mohajeri, S., Alizadeh, H., 2023. A systematic review with meta-analysis of the relation of aflatoxin B1 to growth impairment in infants/children. BMC Pediatrics 23, 1–15. doi:10.1186/s12887-023-04275-9

Nelson, R., 2016. Sorting technologies to rehabilitate toxic maize. Outlooks Pest Manag 27, 247–251. doi:10.1564/v27_dec_02

Nešić, K., Habschied, K., Mastanjević, K., 2023. Modified mycotoxins and multitoxin contamination of food and feed as major analytical challenges. Toxins 15, 511. doi:10.3390/toxins15080511

Nji, Q.N., Babalola, O.O., Ekwomadu, T.I., Nleya, N., Mwanza, M., 2022. Six main contributing factors to high levels of mycotoxin contamination in African foods. Toxins 14, 318. doi:10.3390/toxins14050318

Njumbe Ediage, E., Diana Di Mavungu, J., Monbaliu, S., Van Peteghem, C., De Saeger, S., 2011. A validated multianalyte LC–MS/MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. J. Agric. Food Chem. 59, 5173–5180. doi:10.1021/jf2009364

N'pagyendou, L., Rassimwaï, P., Bodjona B. P. I Tchaou, Banfitebiyi, G., Essohouna, A., Simplice, K.D., 2022. Impact of traditional transformation processes on the level of aflatoxin B1 in groundnut seeds sold in northern Togo. IJNRLS 9, 22–30. doi:10.5281/ZENODO.7104795

Ostry, V., 2008. Alternaria mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. World Mycotoxin Journal 1, 175–188. doi:10.3920/WMJ2008.x013

Pascari, X., Ramos, A.J., Marín, S., Sanchís, V., 2018. Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. Food Res Int 103, 121–129. doi:10.1016/j.foodres.2017.07.038

Paterson, R.R.M., Lima, N., 2010. How will climate change affect mycotoxins in food? Food Res. Int, Climate Change and Food Science 43, 1902–1914. doi:10.1016/j.foodres.2009.07.010

Peng, W.-X., Marchal, J.L.M., van der Poel, A.F.B., 2018. Strategies to prevent and reduce mycotoxins for compound feed manufacturing. Anim. Feed Sci. Technol 237, 129–153. doi:10.1016/j.anifeedsci.2018.01.017

Pereira, V.L., Fernandes, J.O., Cunha, S.C., 2014. Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis. Trends Food Sci. Technol 36, 96–136. doi:10.1016/j.tifs.2014.01.005

Perincherry, L., Lalak-Kańczugowska, J., Stępień, Ł., 2019. *Fusarium*-produced mycotoxins in plant-pathogen interactions. Toxins 11, 664. doi:10.3390/toxins11110664

Pernica, M., Martiník, J., Boško, R., Černá, S., Svoboda, Z., Benešová, K., Běláková, S., 2025. Mycotoxins in brewing and malting: is every sample contaminated with mycotoxins? Mycotoxin Res 41, 239–248. doi:10.1007/s12550-024-00579-6

Perrone, G., Ferrara, M., Medina, A., Pascale, M., Magan, N., 2020. Toxigenic fungi and mycotoxins in a climate change scenario: Ecology, genomics, distribution, prediction and prevention of the risk. Microorganisms 8, 1496. doi:10.3390/microorganisms8101496

Pettersson, H., Åberg, L., 2003. Near infrared spectroscopy for determination of mycotoxins in cereals. Food Control, World Mycotoxin Forum, May 2001 14, 229–232. doi:10.1016/S0956-7135(03)00011-2

Pinton, P., Terciolo, C., Payros, D., Oswald, I.P., 2025. Mycotoxins hazard: The European view. Curr. Opin. Food Sci 63, 101306. doi:10.1016/j.cofs.2025.101306

Pitt, J.I., Hocking, A.D., 2022. Fungi and Food Spoilage. Springer International Publishing, Cham. doi:10.1007/978-3-030-85640-3

Pitt, J.I., Miller, J.D., 2017. A concise history of mycotoxin research. J. Agric. Food Chem. 65, 7021–7033. doi:10.1021/acs.jafc.6b04494

Platzer, A., Cherkaoui, Y., Novak, B., Schatzmayr, G., 2025. Investigating the correlations between weather factors and mycotoxin contamination in corn: Evidence from long-term data. Toxins 17, 77. doi:10.3390/toxins17020077

Pleadin, J., Frece, J., Markov, K., 2019. Mycotoxins in food and feed. Adv Food Nutr Res 89, 297–345. doi:10.1016/bs.afnr.2019.02.007

Qin, X., Xie, B., Zong, X., Yu, X., Feng, Y., 2023. Selective production, relationship and controversy between *Monascus* pigments and citrinin. Food Bioscience 56, 103233. doi:10.1016/j.fbio.2023.103233

Ren, Y., Zhang, Y., Shao, S., Cai, Z., Feng, L., Pan, H., Wang, Z., 2007. Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. J Chromato A 1143, 48–64. doi:10.1016/j.chroma.2006.12.064

Righetti, L., Paglia, G., Galaverna, G., Dall'Asta, C., 2016. Recent advances and future challenges in modified mycotoxin analysis: Why HRMS has become a key instrument in food contaminant research. Toxins 8, 361. doi:10.3390/toxins8120361

Rychlik, M., Humpf, H.-U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., Lorenz, N., 2014. Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. Mycotoxin Res 30, 197–205. doi:10.1007/s12550-014-0203-5

Ryu, D., Hanna, M.A., Bullerman, L.B., 1999. Stability of zearalenone during extrusion of corn grits. J. Food Prot 62, 1482–1484. doi:10.4315/0362-028X-62.12.1482

Sarmast, E., Fallah, A.A., Jafari, T., Mousavi Khaneghah, A., 2021. Occurrence and fate of mycotoxins in cereals and cereal-based products: A narrative review of systematic reviews and meta-analyses studies. Curr. Opin. Food Sci 39, 68–75. doi:10.1016/j.cofs.2020.12.013

Schaarschmidt, S., Fauhl-Hassek, C., 2018. The fate of mycotoxins during the processing of wheat for human consumption. Compr. Rev. Food Sci. Food Saf 17, 556–593. doi:10.1111/1541-4337.12338

Services opérationnels du collège des producteurs, 2019. Commission Grandes cultures - Orge brassicole - Plan de développement stratégique 2019-2028. Collègue des producteurs, Gembloux.

Shi, X.-C., Zhou, Y.-X., Yang, J.-B., Mei, D.-Z., Xu, L.-Y., Wang, S.-Y., Laborda, P., 2025. Biocontrol mechanisms of *Paecilomyces* spp. for the management of fungal plant diseases: A review. Pest Management Science 81, 4200–4219. doi:10.1002/ps.8872

Singh, J., Mehta, A., 2020. Rapid and sensitive detection of mycotoxins by advanced and emerging analytical methods: A review. Food Sci Nutr 8, 2183–2204. doi:10.1002/fsn3.1474

Siri-anusornsak, W., Meneely, J., Greer, B., Vangnai, K., Mahakarnchanakul, W., Elliott, C., Petchkongkaew, A., Kolawole, O., 2023. *In vitro* assessment of commercial multi-mycotoxin binders to reduce the bioavailability of emerging mycotoxins in livestock. Emerging Contaminants 9, 100256. doi:10.1016/j.emcon.2023.100256

Sloth, J.J., Givelet, L., Amlund, H., Mol, H., Mulder, P., de Nijs, M., Fromberg, A., Duedahl-Olesen, L., Grandby, K., Nguyen, K.H., Pedersen, M., Schächtele, A., Zwickel, T., Riemenschneider, C., Tschiggfrei, K., 2022. Guidance document on the estimation of LOQ for measurements in the field of contaminants in feed and food.

Smith, M.-C., Madec, S., Coton, E., Hymery, N., 2016. Natural co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. Toxins 8, 94. doi:10.3390/toxins8040094

Sogbossi Gbétokpanou, C., Jonard, C., Mehinto, O.A., Gofflot, S., Adjéniya, M.J.M., Iko Afe, O.H., Anihouvi, D.G., Boutaleb, S., Bragard, C., Azokpota, P., Mahillon, J., Sindic, M., Scippo, M.-L., Madodé, Y.E., Douny, C., 2025. Peanut and peanut-based foods contamination by toxigenic fungi and mycotoxins: Potential risks for Beninese consumers. Toxins 17, 532. doi:10.3390/toxins17110532

Soleimany, F., Jinap, S., Abas, F., 2012. Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. Food Chem 130, 1055–1060. doi:10.1016/j.foodchem.2011.07.131

Solfrizzo, M., Gambacorta, L., Bibi, R., Ciriaci, M., Paoloni, A., Pecorelli, I., 2018. Multimycotoxin analysis by LC-MS/MS in cereal food and feed: Comparison of different approaches for extraction, purification, and calibration. J. AOAC Int 101, 647–657. doi:10.5740/jaoacint.17-0339

Sombie, J.I.N., Ezekiel, C.N., Sulyok, M., Ayeni, K.I., Jonsyn-Ellis, F., Krska, R., 2018. Survey of roasted street-vended nuts in Sierra Leone for toxic metabolites of fungal origin. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 35, 1573–1580. doi:10.1080/19440049.2018.1475753

Sowmya, K.L., Ramalingappa, B., 2023. A review Mycotoxins: Extraction, characterization and analysis. International Journal of Biotechnology and Mycrobiology 5, 61–66.

Stead, S., Roberts, D., Gledhill, A., de Rijk, T., Van Poucke, C., De Saeger, S., 2014. The development of a sensitive multi-residue LC-MS/MS method for the quantitative determination of mycotoxins in animal feedstuffs and silage using Xevo TQ-S. Waters.

Stockmann-Juvala, H., Savolainen, K., 2008. A review of the toxic effects and mechanisms of action of fumonisin B1. Hum Exp Toxicol 27, 799–809. doi:10.1177/0960327108099525

Sun, J., Li, W., Zhang, Y., Hu, X., Wu, L., Wang, B., 2016. QuEChERS purification combined with ultrahigh-performance liquid chromatography tandem mass spectrometry for simultaneous quantification of 25 mycotoxins in cereals. Toxins 8, 375. doi:10.3390/toxins8120375

Sun, L.-H., Lei, M., Zhang, N.-Y., Zhao, L., Krumm, C.S., Qi, D.-S., 2014. Hepatotoxic effects of mycotoxin combinations in mice. Food Chem. Toxicol 74, 289–293. doi:10.1016/j.fct.2014.10.020

Tedihou, E., Hell, K., Aziato, K., Nyaku, A., 2019. Évaluation de la prévalence des aflatoxines dans les produits d'arachide au Togo. Bulletin de la Recherche Agronomique du Bénin (BRAB), Numéro Spécial Productions Végétales, Animales et Halieutiques, Économie Rurale, Sociologie Rurale, Agronomie, Environnement, Développement Durable & Sécurité Alimentaire de l'Institut Togolais de Recherche Agronomique (ITRA) 129–136.

Teixido-Orries, I., Molino, F., Castro-Criado, B., Jodkowska, M., Medina, A., Marín, S., Verheecke-Vaessen, C., 2025. Mapping variability of mycotoxins in individual oat kernels from batch samples: Implications for sampling and food safety. Toxins 17, 34. doi:10.3390/toxins17010034

Tesfamariam, K., Gebreyesus, S.H., Lachat, C., Hanley-Cook, G.T., Roro, M., Mengistu, Y.G., Endris, B.S., Belachew, T., Kolsteren, P., De Saeger, S., De Boevre, M., Argaw, A., 2022. Chronic aflatoxin exposure during pregnancy is associated with lower fetal growth trajectories: a prospective cohort from the Butajira Nutrition, Mental Health, and Pregnancy (BUNMAP) Study in rural Ethiopia. Am J Clin Nutr 116, 1634–1641. doi:10.1093/ajcn/nqac280

Tian, F., Woo, S.Y., Lee, S.Y., Park, S.B., Im, J.H., Chun, H.S., 2022. Mycotoxins in soybean-based foods fermented with filamentous fungi: Occurrence and preventive strategies. Compr. Rev. Food Sci. Food Saf 21, 5131–5152. doi:10.1111/1541-4337.13032

Tibola, C.S., Fernandes, J.M.C., Guarienti, E.M., 2016. Effect of cleaning, sorting and milling processes in wheat mycotoxin content. Food Control 60, 174–179. doi:10.1016/j.foodcont.2015.07.031

Tola, M., Kebede, B., 2016. Occurrence, importance and control of mycotoxins: A review. Cogent Food Agric. 2, 1191103. doi:10.1080/23311932.2016.1191103

Turner, N.W., Bramhmbhatt, H., Szabo-Vezse, M., Poma, A., Coker, R., Piletsky, S.A., 2015. Analytical methods for determination of mycotoxins: An update (2009–2014). Anal Chim Acta 901, 12–33. doi:10.1016/j.aca.2015.10.013

Udomkun, P., Wiredu, A.N., Nagle, M., Bandyopadhyay, R., Müller, J., Vanlauwe, B., 2017. Mycotoxins in Sub-Saharan Africa: Present situation, socio-economic impact, awareness, and outlook. Food Control 72, 110–122. doi:10.1016/j.foodcont.2016.07.039

Uhlig, S., Ivanova, L., 2004. Determination of beauvericin and four other enniatins in grain by liquid chromatography–mass spectrometry. J. Chromatogr. A 1050, 173–178. doi:10.1016/S0021-9673(04)01360-3

USDA Foreign Agricultural Service, 2024. Oilseeds: World markets and trade [WWW Document]. URL https://www.fas.usda.gov/data/oilseeds-world-markets-and-trade-09122025 (accessed 10.9.25).

Vaičiulienė, G., Jovaišienė, J., Falkauskas, R., Paškevičius, A., Sutkevičienė, N., Rekešiūtė, A., Sorkytė, Š., Baliukonienė, V., Vaičiulienė, G., Jovaišienė, J., Falkauskas, R., Paškevičius, A., Sutkevičienė, N., Rekešiūtė, A., Sorkytė, Š., Baliukonienė, V., 2024. Exploring the efficacy of using *Geotrichum* fermentans, *Rhodotorula rubra*, *Kluyveromyce marxiamus*, clay minerals, and walnut nutshells for mycotoxin remediation. Toxins 16. doi:10.3390/toxins16060281

Valentine, H., 2016. The role of peanuts in global food security, in: Stalker, H.T., F. Wilson, R. (Eds.), Peanuts. AOCS Press, pp. 447–461. doi:10.1016/B978-1-63067-038-2.00017-4

Van Der Fels-Klerx, H.J., Liu, C., Battilani, P., 2016. Modelling climate change impacts on mycotoxin contamination. WMJ 9, 717–726. doi:10.3920/WMJ2016.2066

Vargas Medina, D.A., Bassolli Borsatto, J.V., Maciel, E.V.S., Lanças, F.M., 2021. Current role of modern chromatography and mass spectrometry in the analysis of mycotoxins in food. Trends Anal. Chem 135, 116156. doi:10.1016/j.trac.2020.116156

Variath, M.T., Janila, P., 2017. Economic and academic importance of peanut, in: Varshney, R.K., Pandey, M.K., Puppala, N. (Eds.), The Peanut Genome, Compendium of Plant Genomes. Springer International Publishing, Cham, pp. 7–26. doi:10.1007/978-3-319-63935-2_2

Vasapollo, G., Sole, R.D., Mergola, L., Lazzoi, M.R., Scardino, A., Scorrano, S., Mele, G., 2011. Molecularly imprinted polymers: Present and future prospective. Int J Mol Sci 12, 5908–5945. doi:10.3390/ijms12095908

Videgla, E.G., Floquet, A., Mongbo, R., Garba, K., Tossou, H.S., Toukourou, F., 2016. Liens à l'origine et qualité spécifique d'un produit de l'artisanat agroalimentaire du Bénin – le kluiklui d'Agonlin. Cah. Agric. 25, 35003. doi:10.1051/cagri/2016016

Waliyar, F., Umeh, V.C., Traore, A., Osiru, M., Ntare, B.R., Diarra, B., Kodio, O., Vijay Krishna Kumar, K., Sudini, H., 2015. Prevalence and distribution of aflatoxin contamination in groundnut (*Arachis hypogaea* L.) in Mali, West Africa. Crop Protection 70, 1–7. doi:10.1016/j.cropro.2014.12.007

Wang, C., Huang, Y., Wang, Y., Zhang, J., Guo, M., Chen, C., Zhang, H., Yue, B., Kong, D., Luo, J., Yang, M., 2024. Developments and trends in mycotoxin analysis: A review with bibliometric research. Microchemical J 207, 111774. doi:10.1016/j.microc.2024.111774

Wang, X., Liu, C., Van Der Fels-Klerx, H.J., 2022. Regional prediction of multi-mycotoxin contamination of wheat in Europe using machine learning. Food Res Int 159, 111588. doi:10.1016/j.foodres.2022.111588

Watson, S., Diedhiou, P.M., Atehnkeng, J., Dem, A., Bandyopadhyay, R., Srey, C., Routledge, M.N., Gong, Y.Y., 2015. Seasonal and geographical differences in aflatoxin exposures in Senegal. World Mycotoxin J 525–532. doi:10.3920/WMJ2014.1824

WHO (World Health Organization), 2023. Mycotoxins [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/mycotoxins (accessed 5.19.25).

Winter, G., Pereg, L., 2019. A review on the relation between soil and mycotoxins: Effect of aflatoxin on field, food and finance. Eur. J. Soil Sci 70, 882–897. doi:10.1111/ejss.12813

World Bank, 2025. Benin country climate and development report [WWW Document]. World Bank. URL https://www.worldbank.org/en/country/benin/publication/benin-country-climate-and-development-report (accessed 10.16.25).

Xie, L., Chen, M., Ying, Y., 2016. Development of methods for determination of aflatoxins. Crit Rev Food Sci Nutr 56, 2642–2664. doi:10.1080/10408398.2014.907234

Yang, Y., Li, G., Wu, D., Liu, J., Li, X., Luo, P., Hu, N., Wang, H., Wu, Y., 2020. Recent advances on toxicity and determination methods of mycotoxins in foodstuffs. Trends Food Sci. Technol 96, 233–252. doi:10.1016/j.tifs.2019.12.021

Yu, P., Xu, R., Yang, Z., Ye, T., Liu, Y., Li, S., Abramson, M.J., Kimlin, M., Guo, Y., 2022. Cancer and ongoing climate change: Who are the most affected? ACS Environ Au 3, 5–11. doi:10.1021/acsenvironau.2c00012

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc 15, 129–144. doi:10.1016/j.jscs.2010.06.006

Zhang, H., Ahima, J., Yang, Q., Zhao, L., Zhang, X., Zheng, X., 2021. A review on citrinin: Its occurrence, risk implications, analytical techniques, biosynthesis, physiochemical properties and control. Food Res Int 141, 110075. doi:10.1016/j.foodres.2020.110075

Zhang, J.-M., Wu, Y.-L., Lu, Y.-B., 2013. Simultaneous determination of carbamate insecticides and mycotoxins in cereals by reversed phase liquid chromatography tandem mass spectrometry using a quick, easy, cheap, effective, rugged and safe extraction procedure. J. Chromatogr. B 915–916, 13–20. doi:10.1016/j.jchromb.2012.12.016

Zingales, V., Fernández-Franzón, M., Ruiz, M.-J., 2020. Sterigmatocystin: Occurrence, toxicity and molecular mechanisms of action - A review. Food Chem Toxicol 146, 111802. doi:10.1016/j.fct.2020.111802