Universitat de Lleida

# Estrategias para la detección y control del deoxinivalenol y las fumonisinas en cereales y derivados

*Strategies for the detection and control of deoxynivalenol and fumonisins in cereals and derivatives*

Bernat Borràs Vallverdú

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Universitat de Lleida

# TESIS DOCTORAL

# Estrategias para la detección y control del deoxinivalenol y las fumonisinas en cereales y derivados

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Bernat Borràs Vallverdú

Memoria presentada para optar al grado de Doctor por la Universidad de Lleida
Programa de Doctorado en Ciencia y Tecnología Agraria y Alimentaria

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**During the course of this thesis, a number of articles have been published. They are listed here for reference:**

1. **Borràs-Vallverdú, B.**, Ramos, A. J., Cantero-Martínez, C., Marín, S., Sanchis, V., & Fernández-Ortega, J. (2022). Influence of Agronomic Factors on Mycotoxin Contamination in Maize and Changes during a 10-Day Harvest-Till-Drying Simulation Period: A Different Perspective. *Toxins*, 14(9), 620. https://doi.org/10.3390/toxins14090620
2. **Borràs-Vallverdú, B.**, Marín, S., Sanchis, V., Gatius, F., & Ramos, A. J. (2024). NIR-HSI as a tool to predict deoxynivalenol and fumonisins in maize kernels: A step forward in preventing mycotoxin contamination. *Journal of the Science of Food and Agriculture*, 104(9), 5495-5503. https://doi.org/10.1002/jsfa.13388
3. **Borràs-Vallverdú, B.**, Ramos, A. J., Marín, S., Sanchis, V., & Rodríguez-Bencomo, J. J. (2020). Deoxynivalenol degradation in wheat kernels by exposition to ammonia vapours: A tentative strategy for detoxification. *Food Control*, 118, 107444. https://doi.org/10.1016/j.foodcont.2020.107444
4. **Borràs-Vallverdú, B.**, Ramos, A. J., Marín, S., Sanchis, V., & Rodríguez-Bencomo, J. J. (2022). A new methodology for the analysis of total deoxynivalenol, dissolved and adsorbed on cell walls, in microbiological culture assays. *LWT - Food Science and Technology*, 164, 113684. https://doi.org/10.1016/j.lwt.2022.113684

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3-AcDON - 3-acetyldeoxynivalenol

15-AcDON - 15-acetyldeoxynivalenol

AER - *Aspergillus* ear rot

AFs - Aflatoxins

$AFB_1$ - Aflatoxin $B_1$

$AFB_2$ - Aflatoxin $B_2$

$AFG_1$ - Aflatoxin $G_1$

$AFG_2$ - Aflatoxin $G_2$

$AFM_1$ - Aflatoxin $M_1$

BCA - Biological Control Agent

CSB - Corn Stalk Borer

DON - Deoxynivalenol

DON-3-G - Deoxynivalenol-3-glucoside

ECB - European Corn Borer

EU - European Union

FAO - Food and Agriculture Organization

$FB_1$ - Fumonisin $B_1$

$FB_2$ - Fumonisin $B_2$

FER - *Fusarium* ear rot

FGSC - *Fusarium graminearum* species complex

FHB - *Fusarium* Head Blight

GC - Gas Chromatography

GER - *Gibberella* ear rot

GMOs - Genetically Modified Organisms

HACCP - Hazard Analysis and Critical Control Points

HPLC - High Pressure Liquid Chromatography

HSI - Hyperspectral Imaging

LC - Liquid Chromatography

MMT - Million metric tonnes

MS - Mass Spectrometry

NIR - Near Infrared

NIV - Nivalenol

NTP - Non-thermal Plasma

OTA - Ochratoxin A

PLS - Partial Least Squares

QTL - Quantitative Trait Locus

ROS - Reactive Oxygen Species

RPD - Ratio of Performance to Deviation

UV - Ultraviolet

ZEN - Zearalenone

**Abstract**

Mycotoxins are secondary metabolites produced by fungi that not only are detrimental to human and animal health, but also cause important economic losses. Deoxynivalenol (DON) and fumonisins $B_1$ and $B_2$ ($FB_1$ and $FB_2$) are mycotoxins frequently found in cereals widely consumed worldwide such as wheat or maize. Managing these mycotoxins throughout the food chain is a challenge for both farmers and the food industry. In the present work different strategies for controlling DON, $FB_1$ and $FB_2$ in wheat or maize are studied.

First, the influence of three different agronomic factors (crop diversification, tillage system and nitrogen fertilization rate) on mycotoxin contamination of maize was evaluated. The period between harvesting and drying of this cereal, in which fungal growth and mycotoxin production can still occur, was also studied. Maize planted under direct drilling instead of intensive tillage had lower concentrations of $FB_1$ and $FB_2$, and minimizing the harvest-till-drying period may reduce DON levels.

Second, NIR-HSI was tested as a tool to determine DON, $FB_1$ and $FB_2$ levels in samples of maize kernels. NIR-HSI capacity for classifying samples of maize kernels according to whether they complied or not with the European regulations for these mycotoxins was also investigated. The best DON, $FB_1$ and $FB_2$ regression models presented ratios of performance to deviation (RPDs) of 2.344, 2.018 and 2.301, respectively; and the best DON and $FB_1$+$FB_2$ classification models showed balanced accuracies of 0.899 and 0.773, respectively.

Third, DON-contaminated wheat kernels were treated with ammonia vapours, in order to degrade the mycotoxin. Different ammonia concentrations and temperatures were tested, and the optimal treatment was found to be 4.8 %

$NH_4OH$ and 90 °C. Potential DON degradation products were tentatively identified, and their *in silico* evaluation indicated, in general, lower toxicity and biological effects than for DON.

Fourth and last, a method for analysing total DON (including dissolved and adsorbed DON) in microbiological culture assays was developed. The method is quick, simple, reliable and economical, which makes it suitable as a screening method for searching DON-biodegrading microorganisms.

**Resum**

Les micotoxines són metabòlits secundaris produïts per fongs que no només són perjudicials per a la salut humana i animal, sinó que també causen important pèrdues econòmiques. El deoxinivalenol (DON) i les fumonisines $B_1$ i $B_2$ ($FB_1$ i $FB_2$) són micotoxines que es troben amb freqüència en alguns dels cereals més consumits a nivell mundial, com ara el blat o el panís. Controlar aquestes micotoxines al llarg de la cadena alimentària és un repte tant per als agricultors com per a la indústria alimentària. En aquest treball s'estudien diferents estratègies per a poder controlar el DON, la $FB_1$ i la $FB_2$ en blat o en panís.

En primer lloc, es va avaluar la influència de tres factors agronòmics diferents (la diversificació de cultiu, el sistema de conreu i la freqüència de fertilització nitrogenada) sobre la contaminació per micotoxines en el panís. També es va estudiar el període entre la collita i l'assecat d'aquest cereal, en el qual encara hi pot haver creixement fúngic i producció de micotoxines. El panís plantat utilitzant sembra directa en comptes de llaurat intensiu presentava menor concentració de $FB_1$ i $FB_2$, i minimitzar el període entre la collita i l'assecat pot reduir els nivells de DON.

En segon lloc, es va avaluar el NIR-HSI com a eina per a la determinació dels nivells de DON, $FB_1$ i $FB_2$ en mostres de grans de panís. També es va investigar la capacitat del NIR-HSI per classificar mostres de grans de panís segons si complien o no amb la legislació Europea referent a aquestes micotoxines. Els millors models de regressió per al DON, la $FB_1$ i la $FB_2$ van presentar *RPD*s (*Ratios of Performance to Deviation*) de 2,344, 2,018 i 2,301, respectivament; i els millors models de classificació per al DON i per a la suma de $FB_1$+$FB_2$ van mostrar unes *balanced accuracies* de 0,899 i 0,773, respectivament.

En tercer lloc, es van tractar grans de blat que contenien DON amb vapors d'amoníac, amb l'objectiu de degradar la micotoxina. Es van provar diferents concentracions d'amoníac i temperatures, i es va descobrir que el tractament òptim era a 4,8 % de $NH_4OH$ i 90 °C. Es van identificar temptativament possibles productes de degradació del DON, i la seva avaluació *in silico* va indicar que, en general, presentaven menor toxicitat i efectes biològics que els del DON.

En quart i últim lloc, es va desenvolupar un mètode per analitzar el DON total (incloent el DON dissolt i el DON adsorbit) en assajos de cultius microbiològics. El mètode és ràpid, senzill, fiable i econòmic, el que el fa adequat com a mètode de cribatge per a cercar microorganismes capaços de biodegradar DON.

**Resumen**

Las micotoxinas son metabolitos secundarios producidos por hongos que no sólo son perjudiciales para la salud humana y animal, sino que también causan importantes pérdidas económicas. El deoxinivalenol y las fumonisinas $B_1$ y $B_2$ ($FB_1$ y $FB_2$) son micotoxinas que se encuentran con frecuencia en algunos de los cereales más consumidos a nivel mundial, como el trigo o el maíz. Controlar estas micotoxinas a lo largo de la cadena alimentaria es un reto tanto para los agricultores como para la industria alimentaria. En este trabajo se estudian distintas estrategias para poder controlar el DON, la $FB_1$ y la $FB_2$ en trigo o en maíz.

En primer lugar, se evaluó la influencia de tres factores agronómicos distintos (la diversificación de cultivos, el sistema de laboreo y la frecuencia de fertilización nitrogenada) sobre la contaminación por micotoxinas en el maíz. También se estudió el período entre la cosecha y el secado de este cereal, en el cual todavía puede haber crecimiento fúngico y producción de micotoxinas. El maíz plantado utilizando siembra directa en vez de laboreo intensivo presentaba menor concentración de $FB_1$ y $FB_2$, y minimizar el período entre la cosecha y el secado puede reducir los niveles de DON.

En segundo lugar, se evaluó el NIR-HSI como herramienta para la determinación de los niveles de DON, $FB_1$ y $FB_2$ en muestras de granos de maíz. También se investigó la capacidad del NIR-HSI para clasificar muestras de granos de maíz según si cumplían o no con la legislación Europea referente a estas micotoxinas. Los mejores modelos de regresión para el DON, la $FB_1$ y la $FB_2$ presentaron *RPD*s (*Ratios of Performance to Deviation*) de 2,344, 2,018 y 2,301, respectivamente; y los mejores modelos de clasificación para el DON y para la

suma de $FB_1$+$FB_2$ mostraron unas *balanced accuracies* de 0,899 y 0,773, respectivamente.

En tercer lugar, se trataron granos de trigo que contenían DON con vapores de amoníaco, con el objetivo de degradar la micotoxina. Se probaron diferentes concentraciones de amoníaco y temperaturas, y se descubrió que el tratamiento óptimo era a 4,8 % de $NH_4OH$ y 90 °C. Se identificaron tentativamente posibles productos de degradación del DON, y su evaluación *in silico* indicó que, en general, presentaban menor toxicidad y efectos biológicos que el DON.

En cuarto y último lugar, se desarrolló un método para analizar el DON total (incluyendo el DON disuelto y el DON adsorbido) en ensayos de cultivos microbiológicos. El método es rápido, sencillo, fiable y económico, lo que lo hace adecuado como método de cribado para la búsqueda de microorganismos con capaces de biodegradar DON.

# Chapter 1. Introduction.

## 1.1 Mycotoxins

Organisms morphologically as distant as yeasts, moulds or mushrooms are all fungi that are classified within a kingdom, the *Fungi*. Fungi are eukaryotic organisms, as its cells have a nucleus delimited by a nuclear membrane. Fungi also have a cellular wall made of chitin, and they are heterotrophic, meaning they feed on organic matter. Most fungi grow as multicellular filaments known as hyphae, which extend by growing from their tips. An interconnected network of hyphae is called mycelium. Yet, some species of fungi, yeasts, are unicellular (Morales-Valle, 2011).

Many fungi can produce biologically active substances known as mycotoxins. Mycotoxins are low-molecular-weight secondary metabolites produced by filamentous fungi whose ingestion, inhalation or skin adsorption can cause disease, or even death, in humans, vertebrates and other animals, at low concentrations (Bennett & Klich, 2003; A. J. Ramos et al., 2011).

Mycotoxins can be found in a variety of foods, including cereal grains, oilseeds, nuts, spices, vegetables, fruits and derived products. Mycotoxins can also be found in the milk, eggs or meat of animals that were fed with mycotoxin-contaminated feed. Mycotoxin contamination of food can occur at all stages of the food chain.

The biological function of mycotoxins is not completely understood, but environmental and oxidative stress have strongly been correlated with mycotoxin biosynthesis. The most commonly accepted idea is that the production of mycotoxins and other secondary metabolites by a fungi can help it survive in its ecological niche, where it may have to compete with other organisms (Fox & Howlett, 2008; Reverberi et al., 2010). Common factors affecting mycotoxin

production of a fungal strain include temperature, relative humidity, atmospheric oxygen levels, water activity, pH and growth substrate (Daou et al., 2021; Keller et al., 1997; Taniwaki et al., 2009)

Although more than 300 mycotoxins have been described (Alshannaq & Yu, 2017), not all of them pose a health threat to consumers if we take into account factors such as intrinsic toxicity, contamination levels in certain foods and the populations' usual intake of these foods. Some of the most important mycotoxins in the food and feed industry, the main species that produce them, and the major foods affected can be seen in Table 1.

On the other hand, there is evidence that the incidence of emerging mycotoxins is increasing (Jestoi, 2008; Malachova et al., 2011). Emerging mycotoxins are mycotoxins which are neither routinely determined, nor legislatively regulated (Vaclavikova et al., 2013). Some examples of emerging mycotoxins are enniatins, beauvericin, fusaproliferin, moniliformin, fusaric acid, culmorin, butenolide, sterigmatocystin, emodin and mycophenolic acid (Gruber-Dorninger et al., 2017).

*Table 1 Major mycotoxins, producing species and main foods contaminated* (Alshannaq & Yu, 2017; EFSA Panel on Contaminants in the Food Chain, 2011; Morales-Valle, 2011; Rubinstein & Theumer, 2011).

| **Mycotoxin/s** | **Main producing species** | **Major foods affected** |
|---|---|---|
| $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$ | *Aspergillus flavus* *Aspergillus parasiticus* | Maize, wheat, rice, peanuts, sorghum, pistachios, almonds, nuts, figs, cottonseed, spices |
| $AFM_1$ | Metabolite of $AFB_1$ | Milk, milk products |
| *Alternaria* toxins | *Alternaria alternata* | Spelt, oats, rice, rye, tomato, sunflower, sesame, fruits, vegetables |
| DON | *Fusarium graminearum* *Fusarium culmorum* | Maize, wheat, small grain cereals |
| Ergot alkaloids | *Claviceps purpurea* | Rye, wheat, barley, oats |
| $FB_1$, $FB_2$ | *Fusarium verticillioides* *Fusarium proliferatum* *Fusarium subglutinans* | Maize, maize products, sorghum, asparagus |
| OTA | *Aspergillus ochraceus* *Aspergillus verrucosum* *Aspergillus carbonarius* | Cereals, grapes, wine, raisin, coffee, cocoa |
| Patulin | *Penicillium expansum* | Apples, pears, fruits in general, fruit juices |
| ZEN | *Fusarium graminearum* *Fusarium culmorum* | Maize, wheat, barley, small grain cereals |

## 1.2 Cereal grains

Cereal grains have been essential in the human diet for thousands of years. Globally, it is estimated that the share of dietary energy supplied by cereal grains is around 50 %, this percentage being higher in developing countries (World Health Organization, 2003). Cereal grains are also a source of important nutrients

such as dietary fibre, phenols, vitamins and minerals, but most of those are stripped away if the bran and germ are removed, a common operation done primarily to meet sensory expectations of consumers (Awika, 2011). The most produced cereal grains worldwide in 2021 were maize (1210 MMT), rice (787 MMT) and wheat (771 MMT) (FAO, 2023a). According to the OECD-FAO Agricultural Outlook, in 2021 the majority of the produced rice (81.7 %) and wheat (69.8 %) were directly consumed by humans, while only 12.7 % of the maize went into direct human food supply (OECD/FAO, 2023). Maize principal destination (60.8 %) was animal feed, which indirectly also contributed to human nutrition.

Unfortunately, cereal crops are frequently contaminated with mycotoxin-producing moulds that can have an impact on the quality and safety of the grains.

## 1.3 Important fungal diseases in major cereals and associated mycotoxins

Many of the most important fungal diseases in major cereals are caused by species of the *Fusarium* genera. Different *Fusarium* species normally coexist in the field, but only some of them are pathogenic, especially under specific climatic conditions (Ferrigo et al., 2016).

*Fusarium* Head Blight (FHB), also known as scab, is one of the most problematic fungal diseases for small grain cereals such as wheat, barley, rye or oats (Wegulo et al., 2015). FHB leads to low yields and poor seed quality, and is caused by many co-ocurring species, the most predominant being *F. graminearum* and *F. culmorum* (Dweba et al., 2017; Miller, 1994). The plant is most susceptible to infection at anthesis under warm and high-moisture conditions (Duthie et al., 1986; Osborne & Stein, 2007). The FHB species complex can produce dozens

of different mycotoxins, the most relevant being DON, but also other important mycotoxins such as ZEN, T-2/HT-2 toxins, DON-3-G, 3-AcDON, 15-AcDON or NIV (Johns et al., 2022; Spanic et al., 2023). DON, T-2 toxin, DON-3-G, 3-AcDON, 15-AcDON and NIV all belong to a group of mycotoxins called trichothecenes. All trichothecenes have a common tricyclic 12,13-epoxytrichothec-9-ene core structure (see Fig.1). Based on the substitution at the C-8 position, four types of trichothecenes can be distinguished (A, B, C, and D) (McCormick et al., 2011).

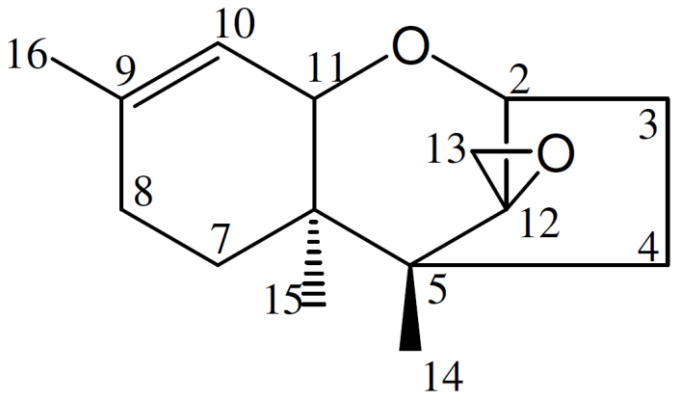


*Fig. 1 Trichothecenes common core structure. Adapted from* McCormick et al. (2011)

*Gibberella* Ear Rot (GER) and *Fusarium* Ear Rot (FER) are important fungal diseases that affect maize, affecting its yield and quality. Similar to FHB, GER and FER are characterized by the co-presence of many fungal species, so GER and FER may be present on maize ears at the same time (Ferrigo et al., 2016). GER, also known as red ear tot, is prevalently caused by species of the *Discolor* section, the predominant ones being *F. graminearum* and *F. culmorum*. In GER, kernels are covered with a pink- to reddish-colored mould, and the major

mycotoxins produced are DON, ZEN and NIV (Ferrigo et al., 2016; Miller, 1994; Reid et al., 1999).

FER, also known as pink ear rot, is mainly caused by species of the *Liseola* section, the most important ones being *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. In FER, kernels are covered with a white- or light pink-colored mould, and the most important mycotoxins produced are $FB_1$, $FB_2$ and moniliformin. FER occurs under warmer and drier conditions than GER (Ferrigo et al., 2016; Miller, 1994; Reid et al., 1999).

Infection of maize kernels can occur through different routes: (i) via germination of spores on the silks, and mycelium growth down to the kernels (which is easier during silking); (ii) via direct injury to the kernels by insects (like the European Corn Borer (ECB)), animals, farm equipment or extreme weather events; or (iii) via systemic transmission from seeds or roots to kernels. The major infection pathway for *F. graminearum* is through silks, but it can also infect via direct injury to the kernels by insects. In contrast, *F. verticillioides* main infection pathway is via insect injury, and silk infection occurs less frequently (Munkvold, 2003; Parsons & Munkvold, 2010, 2012).

Cereals like maize, rice, wheat, barley or sorghum are also susceptible to *Aspergillus* Ear Rot (AER), a fungal disease caused by *Aspergillus* species such as *A. flavus* or *A. parasiticus*. Its growth causes the discoloration of the grains, hinders their drying and diminishes their quality. In addition, they produce mycotoxins known as aflatoxins (AFs), the most common ones being $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ (Kumar et al., 2022; Shabeer et al., 2022).

## 1.4 Deoxynivalenol (DON)

DON, also referred as vomitoxin, is a mycotoxin belonging to the group of type B trichothecenes. The molecular structure of DON can be seen in Fig. 2. DON is a polar organic compound, and presents high resistance to elevated temperatures (Feizollahi & Roopesh, 2022; H. Guo et al., 2020). DON inhibits protein and DNA synthesis in eukaryotic cells. Exposure to DON can induce nausea, vomiting, skin inflammation, leukopenia, diarrhoea, gastroenteritis, haemorrhage in the intestinal tract, lungs and brain, necrosis of bone marrow and lymphoid tissues, kidney and heart lesions and reproductive problems. Experimental animals chronically exposed to DON usually experience decreased weight gain, anorexia and altered nutritional efficiency. Only extremely high DON doses are required to cause death (Pestka, 2007, 2010; Ueno, 1977).

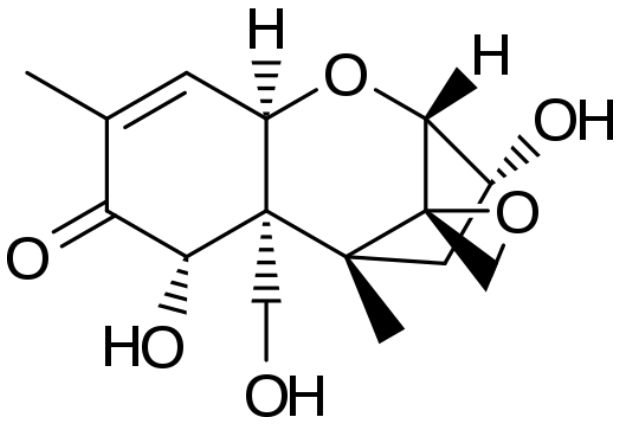


*Fig. 2 Molecular structure of DON*

While it is well-known that trichothecenes inhibit protein and DNA synthesis, the exact mechanism of this inhibition is not completely understood. Type B and C trichothecenes possess an epoxide group attached to $C_{12}$, which is known to be essential for toxicity, but its function may be related to the structural rigidity given

to the molecule rather than the highly reactive nature of epoxides (Foroud et al., 2016).

In the European Union (EU) the maximum content of DON in many foodstuffs is regulated, including wheat kernels and maize kernels, both of which have a limit of 1.75 mg $kg^{-1}$ (European Commission, 2023). The EU also provides guidance values for DON in products intended for animal feed (Commission of the European Communities, 2006a); and recommendations on the prevention and reduction of *Fusarium* toxins in cereal and cereal products (Commission of the European Communities, 2006b).

## 1.5 Fumonisins

A total of 28 fumonisin analogues have been characterized (Rheeder et al., 2002). Among them, fumonisins $B_1$ ($FB_1$) and $B_2$ ($FB_2$) are the most toxic and naturally abundant in maize. The molecular structure of fumonisins $B_1$, $B_2$ and $B_3$ can be seen in Fig. 3. Fumonisins present a strong thermal stability, which makes it challenging to remove them from food and meet edible standards at the same time (Alberts et al., 1990; Humpf & Voss, 2004; Jackson et al., 1996). Fumonisins have a similar structure to that of sphingolipids, structural components in the plasma membranes of eukaryotic cells that modulate fundamental cellular processes. $FB_1$ potently inhibits ceramide synthases, disrupting sphingolipid metabolism and dysregulating cell signaling (Kraft, 2016; Riley & Merrill, 2019). Processes such as cell growth and differentiation, apoptosis and necrosis are disturbed. $FB_1$ also inhibits complex I of the respiratory chain, leading to the overproduction of reactive oxygen species (ROS), which cause oxidative stress. Oxidative stress results in DNA and RNA damage, enhanced lipid peroxidation

and protein damage (Domijan & Abramov, 2011; Galvano, Campisi, et al., 2002; Galvano, Russo, et al., 2002; Kim et al., 2018; X. Wang et al., 2016).

In humans, consumption of fumonisin-contaminated food has been associated with oesophageal cancer and with neural tube defect disease (F. S. Chu & Li, 1994; Gelineau-van Waes et al., 2005; Marasas et al., 2004; Sydenham et al., 1990).

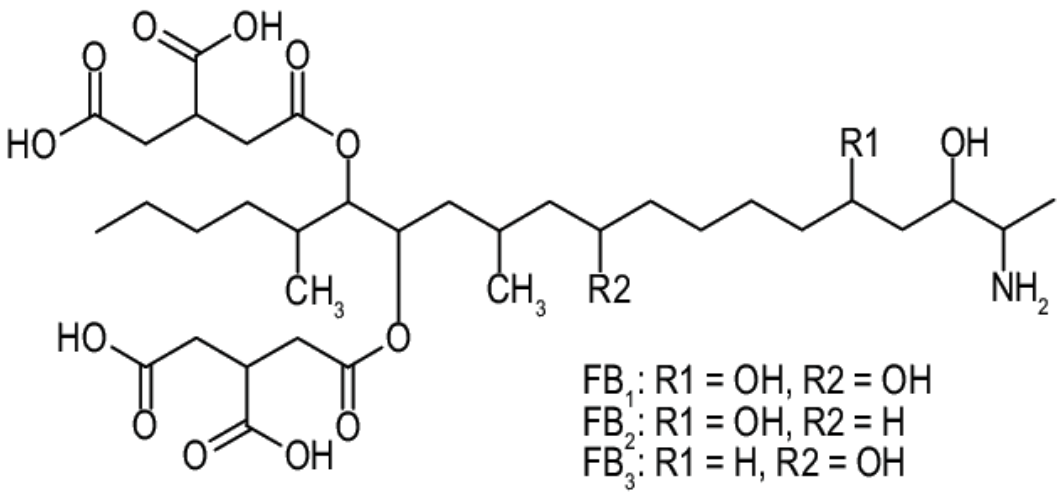


*Fig. 3 Molecular structure of $FB_1$, $FB_2$ and $FB_3$. Adapted from* Dall'Asta et al. (2008)

Numerous *in vivo* and *in vitro* experimental studies have proven that $FB_1$ can cause diverse toxic effects in different animals. $FB_1$ has been shown to cause disease in the nervous, respiratory, digestive, reproductive and immune systems of many animals. Some examples are leukoencephalomalacia in horses, pulmonary edema in piglets and hepatic necrosis in chicks (Gao et al., 2023).

In the EU the maximum content of $FB_1$+$FB_2$ in many foodstuffs is regulated, including maize kernels, which have a limit of 4 mg $kg^{-1}$ (European Commission,

2023). The EU also provides guidance values fumonisins in products intended for animal feed (Commission of the European Communities, 2006a).

## 1.6 Mycotoxins in the cereal food and feed chain: control strategies

Unfortunately, cereal fungal and mycotoxin contamination can occur at all stages of the food and feed chain, both before harvest and after harvest. In this way, mycotoxin control strategies can be classified between pre-harvest and post-harvest strategies. Another criterion to classify mycotoxin control strategies is according to the approach taken to avoid their harmful effect, distinguishing between prevention of contamination, decontamination, and inhibition of absorption of the mycotoxin into the digestive tract.

Efforts should ideally focus on the prevention of the contamination, since once mycotoxins are present on a cereal, it is quite difficult to remove them. Thus, Hazard Analysis and Critical Control Points (HACCP) systems are increasingly being used in the food industry to control mycotoxin contamination in key commodities. HACCP systems are a globally recognized, systematic and science-based approach to food safety that addresses biological, chemical and physical hazards throughout the food chain, from primary production to final consumption (FAO, 2023b). The HACCP focuses on prevention rather than final-product analysis.

Regards mycotoxin decontamination, according to the Food and Agriculture Organization (FAO), an ideal method should meet the following criteria (FAO, 1977):

- Be technically and economically viable
- Destroy, inactivate or remove the mycotoxins

- Not produce or leave toxic or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed decontaminated feed
- Retains the nutritive value and acceptability of the product
- Not significantly alter important technological properties
- Destroys fungal spores and mycelia which could, under favourable conditions, proliferate and form toxins again.

In the EU, the deliberate detoxification by chemical treatments of the mycotoxins $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$, OTA, patulin, DON, ZEN, $FB_1$, $FB_2$, citrinin, ergot sclerotia and ergot alkaloids is forbidden (European Commission, 2023). However, mycotoxin decontamination may be performed on products intended for animal feed. The different acceptability criteria for physical, chemical and (micro)biological detoxification processes on feed have been defined by the EFSA (European Commission, 2015).

A description of different mycotoxin control strategies in cereals, classified between pre- and post-harvest, is presented below.

### 1.6.1 Pre-harvest DON and fumonisin control strategies

#### 1.6.1.1 Cereal breeding

Pre-harvest mycotoxin control strategies may start even before the seed is sown. Many conventional and molecular breeding programmes have focused on obtaining maize and wheat varieties resistant to mycotoxin-producing fungi. Quantitative trait locus (QTL) and genes contributing to resistance to FER and GER in maize have been identified (Giomi et al., 2016; Kebede et al., 2016, 2018; Lanubile et al., 2014; Robertson-Hoyt et al., 2006), as well as maize varieties with resistance to AER and AF production (Betrán et al., 2002; Bhatnagar-Mathur et

al., 2015; Brown et al., 1999; B. Z. Guo et al., 2001). QTL contributing to FHB resistance in wheat have been described too (Anderson et al., 2001; H. Buerstmayr et al., 2002; M. Buerstmayr et al., 2020). Despite the fact that there are many varieties of maize and wheat moderately resistant to the abovementioned fungal diseases, no completely immune genotypes to these diseases have been obtained yet. The achieved amount of resistance is usually limited due to complicated genetics. In addition, in these moderately resistant varieties, the productivity is often compromised, and the resistance traits frequently present heritability problems and are often limited by the environment. An alternative to conventional or molecular breeding for achieving the desired trait is genetically modifying organisms. One option could be to overexpress specific antifungal proteins and metabolites of the plant against fungal colonization. Another option could be to edit the plant genotype so that it has a gene that codes for a compound that can detoxify mycotoxins (Duvick, 2001). In this way, Igawa et al. (2007) developed a transgenic maize line with the *gfzhd101* gene, which encodes an enhanced green fluorescent protein fused to a ZEN-degrading enzyme.

Other more indirect approaches can also contribute to mycotoxin reduction. One example is the Bt maize hybrid. The Bt maize hybrid is a variety that contains the Cry1Ab protein from *Bacillus thuringiensis*, which is able to control the ECB and other stalk-boring pests. As insect injury is a path for fungal infection, maize resistance to insects would indirectly confer resistance to mycotoxin-producing fungi that colonize maize. In a review conducted by Ostry et al. (2010) a total of 23 studies comparing mycotoxin contamination on Bt maize versus mycotoxin

contamination on conventional maize were evaluated, and in 19 it was found that Bt maize was less contaminated with fumonisins, DON and ZEN.

Pericarp composition and thickness may influence the resistance to insects and mycotoxin-producing fungi (B. Z. Guo et al., 1995; Landoni et al., 2020; Sampietro et al., 2009; Tubajika & Damann, 2001), so selection and breeding of varieties with highly-resistant pericarps may be another strategy to fight mycotoxin contamination in cereals.

In a context of global climate change, with increasing temperatures and reduced rainfall, fungal growth and mycotoxin production in cereals may be affected (Medina et al., 2015). Many authors have linked drought and heat stress with higher *Fusarium* and mycotoxin contamination in maize (Abbas et al., 2002; Arino & Bullerman, 1994; Tubajika & Damann, 2001), so breeding and selection of drought- and heat-resistant varieties may help mitigate this problem. It should be considered though, that drought and heat stress are positively related with larger insect populations, which makes it hard to estimate the influence of each of those variables alone (Miller, 2001; Parsons & Munkvold, 2010).

#### 1.6.1.2 Crop management

An adequate crop management can help reduce mycotoxin production in cereals. A description of the factors that play a role in this matter is presented hereunder.

##### 1.6.1.2.1 Sowing and harvesting dates

As previously mentioned, earlier or later sowing and harvesting a crop implies differences in weather conditions and insect populations, which have an effect on mycotoxin contamination. In this way, numerous studies claim that early sowing maize (and thus, early harvesting) reduces the risk of fumonisin contamination

(Abbas et al., 2007; Blandino et al., 2009; Blandino, Reyneri, & Vanara, 2008a; Blandino, Reyneri, Vanara, et al., 2008; Parsons & Munkvold, 2010). More recently, Blandino et al. (2017) designed a 2-years experiment in which they compared the mycotoxin contamination of 3 maize hybrids sowed early (beginning of April) or late (middle of May). They found that a late sown significantly increased the contamination of mycotoxins typically produced by species of *Fusarium* of the *Liseola* section (mainly *F. verticillioides*, *F. proliferatum* and *F. subglutinans*) such as fumonisins, fusaric acid, bikaverin or fusaproliferin. Late sown maize also presented significantly lower grain yield and significantly higher fungal ear rot severity. When the sowing is delayed, higher incidence and severity of the ECB occurs, producing more kernel injuries, which may ease the development of species of *Fusarium* of the *Liseola* section and its mycotoxin production. On the other hand, the effect of sowing time on the contamination of mycotoxins produced by species of *Fusarium* of the *Discolor* section (mainly *F. graminearum* and *F. culmorum*) such as DON, DON-3-G, NIV, ZEN, culmorin and butenolide, was inconclusive. The authors suggest that concentration of these mycotoxins is more related to environmental conditions during maize flowering. Thus, in the 2014 growing season, the contamination of these mycotoxins was significantly higher in early sown maize than in late sown maize, which could be explained by the abundant rainfall and cold temperatures observed that year during the flowering stage of the early sown maize. That is in accordance with the literature (Munkvold, 2003; Parsons & Munkvold, 2010, 2012).

Regarding $AFB_1$ in maize, Jones et al. (1981) performed a two-year study in North Carolina in which they analysed maize from three different cultivars that was

planted either at April or at May. Maize of each treatment was harvested at either at 16-18 %, 22-24 % or 28-30 % moisture. In both years, maize of the same cultivar and harvest moisture always had higher $AFB_1$ concentrations if it was planted in May than if it was planted in April. Other studies have also proven that late-planted maize presents higher contamination by $AFB_1$ (Abbas et al., 2007; Rodriguez-del-Bosque, 1996).

#### 1.6.1.2.2 Irrigation

Jones et al. (1981) observed that, in general terms, non-irrigated maize had a higher percentage of infected ears, $AFB_1$ contamination and lower yield than irrigated maize. Other authors have also observed that non-irrigated maize has a lower yield (Parsons & Munkvold, 2010) and a higher *Fusarium* infection (Arino & Bullerman, 1994) than irrigated maize. It has been suggested that drought stress may compromise host plant defences or lead to increased insect herbivory infection (Jones et al., 1981; Miller, 2001).

#### 1.6.1.2.3 Temperature

Abbas et al. (2002) compared the AFs and fumonisins contamination of different varieties of maize between the years 1998 (under high heat stress) and 1999 (under moderate heat stress). The study was carried out in Mississippi, and all maize varieties were inoculated with *A. flavus*. The effects of drought stress were minimized with supplemental irrigation in both years. High heat stress led to higher contamination of both AFs and fumonisins. Commercial hybrids under high heat stress had average levels of AFs and fumonisins of 4.3 mg $kg^{-1}$ and 11.2 mg $kg^{-1}$, respectively; while the same commercial hybrids under moderate heat stress had average levels of AFs and fumonisins of 6.2 µg $kg^{-1}$ and 2.5 mg $kg^{-1}$, respectively.

#### 1.6.1.2.4 Crop rotation and tillage practices

It is generally accepted that the chances for survival and dispersal of a fungal pathogen in a field after a crop harvest are higher if the following crop is the same or if it hosts the fungal pathogen at issue, than if it doesn't host it. That seems to be particularly true under no tillage, when crop residues are left on the field, turning into fungal pathogen reservoirs (Cotten & Munkvold, 1998; Leplat et al., 2013; Pereyra et al., 2004). The *Fusarium graminearum* species complex (FGSC) is responsible for much of the mycotoxin contamination in cereals such as wheat, maize, rice, barley or oats (Boutigny et al., 2014; Del Ponte et al., 2021). To fight infection by FGSC, strategies like as rotating crops or using different tillage practices can be applied.

##### *1.6.1.2.4.1 Crop rotation*

Alternating between host crops of the FGSC (e.g. wheat, maize, rice, barley, oats) is riskier than alternating between host crops of the FGSC and non-host crops of the FGSC (e.g. soybeans, cotton). Many studies have examined the influence of different preceding crops on the mycotoxin contamination of the following crop using no tillage. Quite a few of those studies have focused on the DON contamination of wheat. Depending on its predecessor crop, DON concentration of wheat could be ordered from largest to smallest in this way: rice > maize > wheat > soybeans > cotton (Dill-Macky & Jones, 2000; Dong et al., 2022; Qiu et al., 2016; Schaafsma et al., 2001; Selvaraj et al., 2015).

More recently, strategies as intercropping or cover cropping have been proposed to fight mycotoxin contamination in crop rotations. Intercropping consists in growing simultaneously two or more crop species, while cover cropping involves cultivating a certain plant species before or after a cash crop. Drakopoulos, Kägi,

et al. (2021) demonstrated that in a maize-wheat rotation under no tillage, the use of white mustard or Indian mustard as intercrops reduced DON in winter wheat by up to 52 % compared with maize grown as a sole crop, while maintaining wheat yield. In the same maize-wheat rotation under no tillage, the use of white mustard, Indian mustard or winter pea as cover crops reduced DON and improved yield in spring wheat by up to 85 % and 25 %, respectively. It should be kept in mind though, that by controlling a particular mycotoxin-producing fungi we may facilitate the infection by a different one. According to Drakopoulos, Sulyok, et al. (2021), if the previous crop is growing pasture instead of maize, barley presents a lower concentration of DON and *F. graminearum* DNA, but also a higher concentration of enniatins and incidence of *Fusarium poae*.

##### *1.6.1.2.4.2 Tillage practices*

Intensive tillage, also known as conventional tillage, usually consists of a primary tillage and a secondary tillage. Primary tillage, generally carried out with a moldboard plough, goes deep into the soil (about 30 cm), turning it over, changing its structure and leaving a rough surface finish. In primary tillage soil is aerated, weeds are killed and almost all crop residues are buried. Secondary tillage acts only on the surface of the soil, leaving a smooth surface finish, required to make an adequate seedbed. Secondary tillage can also be used to incorporate fertilizers, level the surface and control weeds. Sometimes, primary and secondary tillage are combined in a single operation. In no tillage, the opposite approach of intensive tillage, the soil is left completely unaltered: crop residues remain on the surface and seeds are sown directly. Between intensive tillage and no tillage we find minimum tillage, which consists of a minimum soil manipulation,

similar to a secondary tillage, where a considerable proportion of the crop residues are left on the surface of the soil.

There is extensive bibliography arguing that the burial of infected crop residues is likely to reduce the *Fusarium* inoculum for the following crop, making intensive tillage a better choice than no tillage for controlling mycotoxin-producing fungal inoculums (Cotten & Munkvold, 1998; Edwards, 2004; Maiorano et al., 2008). Many of these studies focus on DON contamination of cereals like maize, wheat or barley. According to Mansfield et al. (2005), DON contamination of ensiled maize was lower in maize planted using a moldboard till than in maize planted using no tillage. Obst et al. (1997) stated that using minimum tillage instead of moldboard ploughing after a maize crop could result in a 10-fold increase in the DON concentration of the following wheat crop. Dill-Macky & Jones (2000) observed that DON concentration was lower in wheat planted using moldboard ploughing following maize or wheat than in wheat planted using no tillage following the same crops. Drakopoulos, Sulyok, et al. (2021) detected lower levels of DON and incidence of *F. graminearum* in barley under conventional tillage than in barley under reduced or no tillage. Similarly, Schöneberg et al. (2016) demonstrated that barley from ploughed fields presented lower concentration of DON and incidence of *F. graminearum* than barley from fields under reduced tillage.

However, some recent studies disagree with the predominant view that the risk of DON contamination is lower under conventional tillage than under no tillage. Supronienė et al. (2012) studied the effect of different tillage practices (conventional tillage, reduced tillage and no tillage) on DON contamination in spring and winter wheat, but no clear relationship was observed. Roucou et al.

(2022) collected data from 2032 maize fields located in France between 2004 and 2020, and they found that DON contamination in maize was statistically the same whether the crop residues of the previous year were adequately managed (mainly through soil tillage) or not. Kaukoranta et al. (2019) analysed survey data from 804 spring-oat fields and found that the DON concentration of the oats was the same or lower under non-ploughing than under ploughing.

Regarding fumonisins contamination in maize, the relationship between intensive tillage and a lower mycotoxin contamination is not clear at all. In a three-year study, Marocco et al. (2009) observed that during the first year no-tillage maize presented higher fumonisins incidence and contamination than conventional tillage maize, but this difference could not be seen the second and third years. In another study, Marocco et al. (2008) did not observe differences in the incidence of fumonisins between no-tillage maize and conventional tillage maize. Ono et al. (2011) showed that maize under no tillage had higher fumonisins content than maize under conventional tillage if the preceding crop was oats, but no differences were observed if maize preceded fallow.

It should be noted that intensive tillage alters the soil structure, modifying its physical and chemical properties, which has many impacts (Shah et al., 2017). In no tillage, crop residues protect the soil surface from erosive agents such as water and wind, while in intensive tillage soil surface is exposed to those agents. In intensive tillage, the impact of water drops degrades the soil by breaking down water-stable aggregates, causing soil crusting (Arjmand Sajjadi & Mahmoodabadi, 2015; Pareja-Sánchez et al., 2017). Soil crusting negatively affects seedling emergence, reduces water infiltration rates and water storage capacity, favours runoff, diminishes organic matter and can cause overland flow

(Awadhwal & Thierstein, 1985; Pareja-Sánchez et al., 2017; M. C. Ramos et al., 2019). Soils rich in silt and fine sand and particularly susceptible to soil crusting (M. C. Ramos et al., 2000). In comparison with intensive tillage fields, no tillage fields have higher soil fertility and crop productivity, and also higher microbial diversity and richness, which have been suggested as soil quality indicators (Ceja-Navarro et al., 2010; Degrune et al., 2016; Mathew et al., 2012; Sengupta & Dick, 2015).

#### 1.6.1.2.5 Nitrogen fertilization

The role of nitrogen fertilization in fumonisins contamination of maize is poorly understood, despite the fact that many studies have addressed this issue. Published works show contradictory results (Ariño et al., 2009; Keszthelyi et al., 2022; Madege et al., 2018; Marocco et al., 2008, 2009; Ono et al., 2011). On one side, some researchers argue that plants with an adequate fertilization are likely to have greater health and less abiotic stress, and therefore be less prone to fungal infection and mycotoxin production (Ferrigo et al., 2014). On the other side, it has been reported that nitrogen oversupply is toxic to plants (De Mello Prado, 2021), which could increase virulence of pathogens, leading to higher fumonisins contamination. According to Blandino, Vanara, & Reyneri (2008), a balanced nitrogen fertilization seems to be the best solution to prevent fumonisin contamination in maize.

Although less studied, the influence of nitrogen fertilization on DON and AFs contamination of maize is also unclear (Jones & Duncan, 1980; Keszthelyi et al., 2022; Payne et al., 1989; Reid et al., 2001)

#### 1.6.1.2.6 Plant density

A high plant density increases the risk of mycotoxin contamination for at least two reasons: (i) it stresses plants, increasing their susceptibility to colonization of mycotoxin-producing fungi; and (ii) it leads to a denser foliage, which holds moisture, favouring fungal infection (Munkvold, 2014). Blandino, Reyneri, & Vanara (2008b) observed that maize plots with high plant density (82000 plants $ha^{-1}$) had higher fungal ear rot severity and $FB_1$ contamination than plots with normal plant density (65000 plants $ha^{-1}$) in 3 of the 4 years studied.

#### 1.6.1.2.7 Treatment of infected seeds

Martin & Johnston (1982) studied the influence of the application of different fungicides on *Fusarium*-infested wheat seeds on the vigor of the resulting plants. Benlate-T (benomyl 30 % + thiram 30 %) was the most effective fungicide. In comparison with the control, it did not significantly improve the germination rate, but it significantly increased both the top and root weights and lengths.

More recently, Perczak et al. (2019) analysed the inhibition of mycotoxin production of essential oils against *F. culmorum* and *F. graminearum* in wheat seeds. Essential oils of oregano, cinnamon, palmarosa, orange, spearmint, verbena, fennel and rosewood were tested. In wheat seeds artificially contaminated with *F. culmorum* or *F. graminearum*, application of high concentrations of any of the abovementioned essential oils (5 mL for 25 g of wheat seeds) strongly reduced mycotoxin contamination after a 28-day incubation. All essential oils tested but the orange one reduced the contamination of ZEN, DON, 3-AcDON and fusarenon X by more than 95 % in comparison to the control, in both *Fusarium* species. Buzón-Durán et al. (2020) tested the efficacy of several chitosan oligomers-aminoacid conjugate complexes against *F.*

*culmorum* in spelt. In a 28-day incubation of spelt seeds artificially contaminated with *F. culmorum*, a high concentration of chitosan oligomer-tyrosine conjugate (5 mL of solution at a concentration of 1500 µg/mL, for 25 g of spelt seeds) reduced DON production by 91 % in comparison with the control. The application of this conjugate also lead to a higher germination rate and a lower disease severity than the control. Despite huge mycotoxin reductions were obtained in the studies of Perczak et al. (2019) and Buzón-Durán et al. (2020), the large quantity of the required agents to achieve such reductions makes it application difficult and economically nonviable.

#### 1.6.1.2.8 Application of foliar fungicides

Several studies have been published on the application of foliar fungicides to fight wheat fungal diseases and mycotoxin contamination. Paul et al. (2010) conducted a meta-analysis using 12 years of data from 14 U.S. states to determine the effect of different triazole-based fungicides on spring and winter wheat yield and test weight. They found that almost all fungicides used lead to significant increases in yield and test weight, which may be attributed, at least in part, to a reduction in FHB indexes. Caldwell et al. (2017) investigated the effect of applying different fungicides at an early stage, at a late stage, or both, on winter wheat and spring wheat. Most fungicide treatments significantly increased yield and reduced FHB severity. However, no clear correlation was observed between FHB severity and DON concentrations. Similarly, Martin & Johnston (1982) studied the application of different fungicides to wheat in order to fight FHB. Propiconazole, the fungicide that gave the best results, significantly increased the yield (+34 %) and reduced head blight severity (−41 %) in comparison with the

untreated control, but no statistically significant differences were observed between treatments regarding DON contamination.

The unclear correlation between FHB severity and DON content may be related to the fungicide main target species. Simpson et al. (2001) applied various fungicide treatments to wheat aimed at reducing FHB, end evaluated the DON concentrations on grain and the populations of different FHB-related *Fusarium* species and *Microdochium nivale*. *M. nivale* (formerly known as *Fusarium nivale*) is a non-mycotoxigenic fungi which also causes FHB (Parry et al., 1995). Simpson et al. (2001) observed that in comparison with the control, treating wheat with azoxystrobin left *Fusarium* population almost unaffected, while it tended to reduce *M. nivale* population, and DON concentration increased. In contrast, treating wheat with tebuconazole generally reduced mycotoxigenic *Fusarium* population while it had little impact on the *M. nivale* population, and DON concentration decreased. Using azoxystrobin, elimination of competitive microbiota probably led to an increased DON production by other *Fusarium* species.

Compared to wheat, literature on the effect of foliar fungicides on fungal diseases and mycotoxin content in maize is not as abundant, and the reported results are contradictory.

He et al. (2023) tested the efficacy of mefentrifluconazole and prothioconazole tebuconazole in maize artificially infected with *F. verticillioides*, and found that both fungicides reduced FER, increased yield and reduced $FB_1+FB_2+FB_3$ in comparison with the control, although mefentrifluconazole performed better. In a two-year study, Andriolli et al. (2016) studied the application of a mix of two commercial formulations (azoxystrobin + cyproconazole and carbendazim) on

two different maize hybrids inoculated 5-6 days after silk emergence with *Fusarium meridionale. F. meridionale* has been described as the most prevalent species in the FGSC in Brazilian maize (Kuhnem et al., 2016). The fungicide was applied at different moments in order to find the optimal application time. Although the fungicide reduced GER severity at any of the moments tested, the application 2 days prior or after inoculation gave the best results. Limay-Rios & Schaafsma (2018) studied the optimal application time for prothioconazole in maize naturally contaminated and maize artificially contaminated with *F. graminearum*. Significant reductions of total DON (DON + DON-3-G + 3-AcDON + 15-AcDON), ZEN and GER rating were observed with at different application times, but the best results were observed 8 days after silk emergence and 4 days after silk emergence, which is in accordance with the results of Andriolli et al. (2016). No significant differences in maize yield and the content of the mycotoxins $FB_1$, $FB_2$, moniliformin, beauvericin, HT-2, T-2 and enniantins were observed between fungicide application and the control. It is worth noting tough, that these mycotoxins are produced by members of the *Liseola* section of the *Fusarium* genus, whose main species is *F. verticillioides*, whose main infection pathway is via insect injury, and the authors did not found any insect damage, most probably because they employed Bt maize hybrids.

Other researchers have published less encouraging results. Blandino et al. (2022) applied prothioconazole + tebuconazole on maize at different growth stages over three growing seasons. They found that in comparison with the control, fungicide application at any of the times tested did not increase yield, and did not reduce ECB incidence, ECB severity, ear rot incidence, ear rot severity, or the concentrations of the mycotoxins DON, $FB_1$+$FB_2$ or moniliformin. In a

similar study, Blandino et al. (2012) applied azoxystrobin + propiconazole on maize at different growth stages over two seasons. In comparison with the control, fungicide application only improved maize yield when applied at GS35 (mid-stem elongation) and GS65 (maize flowering), and no reductions of ECB severity, fungal ear rot severity or $FB_1$+$FB_2$ could be observed in any of the applications.

#### 1.6.1.2.9 Application of foliar insecticides

In a three-year study, Blandino et al. (2022) studied the effect of alpha-cypermethrin, an insecticide against ECB, on the fungal symptoms, infection and mycotoxin content of maize. The insecticide application significantly increased grain yield (+4 %), reduced ECB incidence (−62 %) and severity (−75 %) and reduced ear rot incidence (−54 %) and severity (−68 %). It also significantly reduced the $FB_1$+$FB_2$ (−75 %) and moniliformin (−79 %) contents, but increased the DON content (+60 %). It was also seen that the insecticide application reduced the infection caused by *Fusarium* spp. section *Liseola* and led to an increase in the species belonging to the *Discolor* section. The authors theorize that reduced ECB injuries due to insecticide application also resulted in a significant reduction of species such as *F. verticillioides* and *F. proliferatum*, belonging to the *Liseola* section, as those species usually infect kernels via insect injury. A lower population of *Fusarium* spp. section *Liseola* would explain lower fumonisins and moniliformin contamination, and as a consequence of a "mycobiota inversion" phenomenon, population of species of the *Fusarium* spp. section *Discolor* would have increased, leading to a higher DON contamination.

In a similar study, Scarpino et al. (2018) investigated the impact of the insecticides against ECB alpha-cypermethrin and lambda-cyhalothrin +

chlorantranilipole on the contamination of emerging mycotoxins in maize. As in the study of Blandino et al. (2022), when compared with the control, the insecticide application significantly increased yield and reduced ECB incidence and severity, fungal ear rot incidence and severity and contamination by fumonisins and moniliformin. In comparison with the control, the insecticide-treated maize also presented lower levels of beauvericin, as well as bikaverin, fusaric acid, fusaproliferin and fusarin C, other mycotoxins produced by *Fusarium* spp. section *Liseola*. On the other side, no differences between the control and the maize treated with insecticide could be observed regarding contamination of DON, DON-3-G, 3-AcDON, 15-AcDON, NIV, ZEN, zearalenone-4-sulphate, $\alpha$-zearalenol, $\beta$-zearalenol, aurofusarin, butenolide and culmorin - mycotoxins produced by *Fusarium* spp. sections *Discolor* and *Roseum* -. Only on one trial the mycotoxin contamination caused by the *Discolor* and *Roseum* sections of *Fusarium* spp. was significantly higher in insecticide-treated plots, which could be explained by the environmental conditions (abundant rainfall during the maize flowering and ripening stages), which favored the growth of these species.

Folcher et al. (2009) studied the influence of applying deltamethrine on the mycotoxin contamination of maize and on the populations of ECB and Corn Stalk Borer (CSB) (the first and second most damaging insects in maize grown in France). Overall, insecticide treatment significantly reduced the populations of ECB+CSB and the contamination of $FB_1$+$FB_2$, type A, B and D trichothecenes (including DON) and ZEN and ZEN-related mycotoxins.

In summary, foliar application of insecticides in maize seems to increase yield and reduce the incidence and severity of both ECB and ear rot. Regarding mycotoxin contamination, the use of insecticide leads to a reduction in fumonisins

and other mycotoxins produced by species of *Fusarium* spp. section *Liseola*, but the effect on the contamination of DON, ZEN, or other mycotoxins produced by species of *Fusarium* spp. section *Discolor* or *Roseum* is not yet fully understood.

#### 1.6.1.2.10 Biological Control Agents (BCAs)

Another approach for handling cereal fungal diseases and mycotoxin contamination is the use of BCAs. Action mechanisms of BCAs may be directed to the pathogen (antibiosis, parasitism, cell wall degrading enzymes or competition for space and nutrients) or to the plant (induction of resistance, and biofertilization and/or plant growth promotion) (Chaur-Tsuen, 1998; Köhl et al., 2019; Pertot et al., 2015). Several studies demonstrate BCAs *in vitro* efficacy against cereal fungal diseases and mycotoxin contamination, but fewer do the same on greenhouse or field tests. During pre-harvest, BCAs can be applied primarily in infected crop residues left on the field or in spikelets.

##### *1.6.1.2.10.1 Treatment of infected crop residues left on the field*

In no-till agriculture, maize and wheat crop residues are left on the soil surface, becoming a fungal pathogen reservoir and an important source inoculum for the next crop (Cotten & Munkvold, 1998; Leplat et al., 2013; Pereyra et al., 2004). Application of BCAs at this stage seems to be effective for reducing infection pressure, because BCAs accelerate the crop residues degradation, reducing nutrient availability for fungal growth; and/or because they present antagonistic properties against the pathogen.

Pellan et al. (2021) artificially contaminated wheat straw with *F. graminearum* and treated it with 3 different commercial BCAs (Mycostop® - *Streptomyces griseoviridis* bacteria, Xedavir® - *Trichoderma asperellum* fungus, and Polyversum® - *Pythium oligandrum* oomycete). All 3 BCAs strongly inhibited

perithecia formation by the pathogen (90-99 % inhibition). Cabrera et al. (2020) isolated *Trichoderma* spp. strains from wheat straw samples in order to find a BCA against FHB. The authors found that five *Trichoderma* spp. strains significantly inhibited *F. graminearum* mycelium and perithecia formation in wheat straw inoculated with this pathogen. *Trichoderma atroviride*, the best inhibitor, also proved to produce xylanases (enzymes associated to plant tissue degradation, which would facilitate wheat straw degradation), quitinases (enzymes associated to fungal cell wall polymers degradation), and volatile and soluble antifungal compounds. In a similar study, Inch & Gilbert (2007) demonstrated that five different strains of *Trichoderma harzianum*, including a registered BCA in the US (Plant Shield$^{TM}$), significantly reduced perithecia formation by *F. graminearum* in wheat straw under field conditions.

Other authors have employed earthworms to reduce contamination by fungal pathogens and mycotoxins in crop residues. These animals may act by competing for the nutrients available in crop residues, by feeding on fungal biomass, and/or by burying the crop residues, which reduces fungal survival and chances for the fungus to release spores for head infection.

It is worth mentioning that using earthworms as BCAs against cereal fungal pathogens presents the additional benefit of improving soil properties such as soil structure, soil porosity, soil water retention capacity and root distribution, leading to higher plant growth and health (Briones & Schmidt, 2017; Imaz et al., 2010; Nieminen et al., 2011; Ojha & Devkota, 2014).

Most studies using earthworms to fight fungal and mycotoxin contamination in crop residues use anecic and epigeic species. Anecic earthworms, such as *Lumbricus terrestris* or *Aporrectodea longa*, live below soil level, but explore the

soil surface to find food sources. Epigeic earthworms, such as *Lumbricus rubellus*, don't build burrows, and live amongst decomposing organic matter on the soil surface.

Jorge-Escudero et al. (2021) demonstrated that *L. terrestris*, *A. longa* and *L. rubellus* can reduce wheat straw soil cover, and that *A. longa* and *L. rubellus* can reduce *Fusarium* biomass in wheat straw under suboptimal conditions. According to Wolfarth, Schrader, Oldenburg, Weinert, et al. (2011), *L. terrestris* can reduce soil cover, *Fusarium* biomass and DON in wheat straw. In the same line, Oldenburg et al. (2008) and Schrader et al. (2009) proved that *L. terrestris* efficiently degrades *Fusarium* biomass and DON in wheat straw. In addition, in the studies of Wolfarth, Schrader, Oldenburg, Weinert, et al. (2011) and Oldenburg et al. (2008) it was observed that *L. terrestris* is more attracted to highly *Fusarium*-infected and DON contaminated wheat straw than low infected and contaminated wheat straw. The authors hypothesize that this earthworm prefers the contaminated straw as its N-content and digestibility are increased due to fungal colonization. Previous studies demonstrate that earthworms do not feed at random. Moody et al. (1995) studied the selective consumption of decomposing wheat straw by different earthworms, including *L. terrestris* and *A. longa*, and observed that in general terms, these earthworms preferred early straw decomposers (including *Fusarium*), capable of utilizing water-soluble sugars and cellulose, rather than late stage decomposers, capable of using lignin. In another study by Bonkowski et al. (2000) it was observed that both *L. terrestris* and *L. rubellus* preferred to feed on *F. nivale* over other 8 fungal species of different genera.

In comparison with anecic and epigeic earthworms, little research has been conducted in reference to endogeic earthworms, the ones that live below soil level. Wolfarth et al. (2011) studied the potential of *Aporrectodea caliginosa*, an endogeic earthworm that creates burrows to the soil surface, to degrade *Fusarium* biomass and DON content in wheat straw. The authors concluded that *A. caliginosa* contribution to *Fusarium* biomass and DON degradation was minor, and restricted to belowground straw. Similar results were observed by Wolfarth, Schrader, Oldenburg, Weinert, et al. (2011).

#### *1.6.1.2.10.2 Treatment of spikelets*

Many BCAs studies addressing cereal fungal diseases and mycotoxin contamination are focused on treating infected spikelets. Khan & Doohan (2009) proved that strains of the species *Pseudomonas fluorescens* and *P. frederiksbergensis* reduced DON contamination and grain loss due to FHB in wheat and barley artificially contaminated with *F. culmorum* when inoculated 24 h pre-pathogen inoculation. In comparison with the controls, *P. frederiksbergensis* strain MKB 202 reduced DON levels on wheat and barley by 12 and 21 %, respectively. In the cases of *P. fluorescens* strain MKB 158 or *P. fluorescens* strain MKB 249, DON reductions in wheat and barley were between 74 and 78 %. Palazzini et al. (2007) isolated nine bacterial strains capable of reducing FHB disease severity and DON content of wheat when inoculated together with *F. graminearum* at anthesis. Five of the isolates decreased DON content to undetectable levels.

Despite the promising results of many studies using BCAs against fungal diseases and mycotoxin contamination, it is important to bear in mind that artificial inoculations of BCAs and fungal pathogens in those studies are frequently done

with a higher proportion of BCAs than fungal pathogens, or at most, with a 1 to 1 ratio. It would be interesting to know if similar results would be obtained using a lower ratio of BCAs/fungus in these inoculations, and to know to what extent fungal disease and mycotoxin control is achieved by means of competition for space and nutrients.

### 1.6.2 Post-harvest DON and fumonisin control strategies

#### 1.6.2.1 Physical treatments

##### 1.6.2.1.1 Drying

Usually, recently harvested maize and wheat present a moisture content high enough to allow fungal growth and mycotoxin production. For this reason, after harvesting the cereals it is necessary to dry the grain as soon as possible until it reaches a moisture content that guarantees microbiological stability. Maize is normally harvested at around 18 % moisture, although to ensure no mould development nor mycotoxin production it must be dried to <14 % moisture (Channaiah & Maier, 2014; Richard, 2007). Similarly, wheat is harvested at 16-20 % moisture, but has to be dried to <14.5 % moisture (Magan et al., 2010).

Traditionally, cereals have been dried in the sun. However, this method depends on weather conditions. Inadequate weather conditions for drying can cause a prolonged drying time and, therefore, a greater risk of fungal attack. Some farmers place the cereals to dry directly on the soil surface, which makes them more prone to fungal infections. This can be easily solved by placing a barrier between the crop and the soil. Nowadays, artificial drying is gradually substituting sun drying. Artificial drying provides higher temperatures and faster drying, which

lowers the risk of mycotoxin development, but is more expensive (Daou et al., 2021; Munkvold et al., 2019).

#### 1.6.2.1.2 Cleaning and sorting

Cleaning means removing external materials like dirt and debris from the grains, and sorting means picking out the inferior quality kernels from the healthy ones (Peng et al., 2018). Inferior kernels are those considered undesirable in the food or feed chain (kernels that are broken, small, discoloured, insect-damaged, present visible mould growth or are contaminated by mycotoxins). Dirt, debris and inferior kernels constitute a source of fungal inoculum and mycotoxins. The distribution of mycotoxin contamination in cereals is positively skewed, that meaning most kernels present a low contamination, while few of them are highly contaminated (Chavez et al., 2022; Joshi et al., 2022; Pearson et al., 2010; Rodrigues & Naehrer, 2012; Stasiewicz et al., 2017; Tarazona et al., 2020). Therefore, an adequate sorting will remove a significant quantity of mycotoxins by removing a little portion of the kernels.

Traditionally, cleaning and sorting were done by hand, removing the kernels that were broken, small, discoloured, insect-damaged or presented visible mould growth. Effectiveness of hand-sorting highly depends on the experience of workers. According to Matumba et al. (2015), hand-sorting white maize highly reduced the contamination of NIV, DON, $AFB_1$ and $FB_1$ and other mycotoxins (>90 % for all mycotoxins). Nowadays, in industrialized countries, hand-sorting is economically non-viable in large-scale productions.

Another method of sorting is wet sorting, which consists on eliminating the floating kernels in a liquid, based on their different densities. Low-density grains are usually the result of a fungal infection, and therefore are more probable to contain

mycotoxins. Using a saturated NaCl solution, Huff & Hagler (1985) reduced AF contamination in maize by 74 % discarding only 3 % of the sample. The same authors also achieved high reductions of DON in maize and wheat using a 30 % sucrose solution. Despite the important mycotoxin reductions, wet-sorting is rarely used because it increases the moisture of kernels. Drying the kernels immediately after wet-sorting is highly recommended to prevent fungal growth and mycotoxin contamination.

Alike wet-flotation, mechanical cleaning and sorting is based on density differences between healthy kernels and inferior kernels, dirt or debris. Separation is usually achieved combining mechanical equipment like air separators, sieves, gravity separators and intended cylinders (Peng et al., 2018). Lancova et al. (2008) observed reduced levels of NIV, DON, Ac-DONs, HT-2 and T-2 in wheat after mechanical sorting. Visconti et al. (2004) reported a 23 % decrease in DON concentration in wheat kernels after mechanical cleaning. Other authors have also observed statistically significant reductions in DON concentration in wheat after mechanical cleaning (Tibola et al., 2016).

In the late years, much effort has been put in developing more advanced spectroscopy sorting technology. The principle is to differentiate fungal-infected or mycotoxin-contaminated kernels from healthy kernels based on its distinct interactions with electromagnetic radiation. In this way, it is possible to obtain information about the concentration of various mycotoxins in cereals like maize, wheat, oats or rice using different regions of the electromagnetic spectrum, such as UV, visible, near-infrared (NIR), mid-infrared or a combination of them (Abramović et al., 2007; Aoun et al., 2022; Cheng et al., 2019; Hossain & Goto, 2014; Jia et al., 2020; Kos et al., 2003; Yao et al., 2010). UV, visible, NIR and

mid-infrared are all non-ionizing radiation, meaning they do not cause atoms or molecules to be ionized (Wood & Roy, 2017). Most studies in this area are focused on using NIR spectroscopy to study the mycotoxin contamination of different grains.

##### *1.6.2.1.2.1 NIR spectroscopy*

NIR spectroscopy reveals the interaction between the electromagnetic radiation in the wavelength range from 780 to 2500 nm and the chemical bonds of the analysed sample. When molecules are exposed to radiation in the NIR range, some chemical bonds absorb spectral energy. The rest of chemical bonds reflect or transmit the other beams at different wavelengths, and this radiation is measured by the detector. The intensity of reflection or transmission mainly depends on the energy absorption of molecular overtones and vibrations of chemical bonds in the sample, which provides information about the functional groups in the molecules (Hossain & Goto, 2014; Jia et al., 2020). The main chemical bonds detected in NIR spectra are C-H, N-H, O-H, C-O and S-H. Mycotoxin contaminated kernels present different NIR spectra than healthy kernels, and this can be used to predict the mycotoxin contamination of the kernels (regression models) or to classify them accordingly (classification models). It is worth mentioning that differences between spectra are not due to the mycotoxin concentration of the kernels per se, but due to the chemical and nutritional modifications produced by fungal growth on the surface or inner parts of the grain (Femenias et al., 2022; Freitag et al., 2022).

Mycotoxin predictive models can be developed using NIR spectra with reference data obtained in the laboratory (Agelet & Hurburgh, 2010; Levasseur-Garcia,

2018). Developing a mycotoxin predictive model can be done taking the following steps:

1) Samples used to develop the predictive model are randomly divided into two groups: calibration samples and validation samples (about 70 % and 30 % of samples, respectively). Calibration samples must be representative of the samples that will be routinely analysed once the model is developed.
2) NIR spectra from the calibration samples is recorded.
3) Mycotoxin contamination of the calibration samples is determined with a reference method. The range of mycotoxin concentration in the calibration samples should uniformly cover the desired range of the model.
4) Spectral data from the calibration samples is preprocessed to eliminate baseline noise and drifts. Common preprocessing methods are derivatives, standard normal variation, smoothing or multiplicative scatter correction.
5) A model is developed, establishing a mathematical relationship between the NIR spectra of the calibration samples and its mycotoxin contamination. The most popular regression method is Partial Least Squares Regression (PLS regression). Classification methods can be supervised (defining the characteristics of each group) or unsupervised (spectral similarities and dissimilarities will be used to create groups). A common supervised classification method is SIMCA.
6) The developed model is validated. The model is applied to different samples with known mycotoxin contamination (validation samples) to test its ability for future predictions. If the samples available are not enough to

make two different data sets (one for calibration and another for validation), the model can be internally validated using either cross-validation or leverage correction, although the latter is optimistic.

The performance of a model can be evaluated by many parameters. Some important ones are the coefficient of determination ($R^2$), the root mean square error of prediction (RMSEP) or the ratio of performance to deviation (RPD).

7) Once the model is validated, it can be used routinely to estimate mycotoxin contamination of new samples (prediction).

Mycotoxin analysis by NIR spectroscopy presents many advantages over reference methods. Usually, prediction by NIR is cheaper, as it doesn't require any reagents or any laboratory material rather than the NIR equipment. In addition, reference methods tend to be time-consuming, pollutant and require trained professionals, while NIR analysis is fast, eco-friendly and easy. Finally, NIR is a non-destructive technique, unlike reference methods.

##### *1.6.2.1.2.2 Near Infrared-Hyperspectral Imaging (NIR-HSI)*

A disadvantage of conventional NIR spectroscopy is that is spatially limited. Only a small portion of the sample is scanned, while an average spectrum for the whole sample is given (Sendin et al., 2018). This spectrum may not be representative of the entire sample, especially if it is heterogeneous, as is the case with cereals like wheat or maize. For this reason, NIR-HSI spectroscopy has received raising attention.

NIR-HSI spectroscopy is the combination of NIR spectroscopy with HSI technology. In NIR-HSI spectroscopy not only spectral information is obtained,

but also the spatial position of the acquired spectra. A hyperspectral image of a sample provides thousands of spectra, as one spectrum is taken for each pixel. Hyperspectral images or hypercubes are three-dimensional data sets containing light intensity measurements where two dimensions (x and y) represent the spatial position and the third dimension ($\lambda$) represents the spectral information (Dale et al., 2013). A schematic representation of a hypercube is presented in Fig. 4. HSI-NIR has been used to predict mycotoxin contamination or detect fungal infection in cereals like wheat, maize or oats (X. Chu et al., 2020; Femenias et al., 2020; Teixido-Orries et al., 2023). If measured at each wavelength over a wide enough spectral band, HSI-NIR can also be used to characterize and identify any given material (Shaw & Manolakis, 2002).

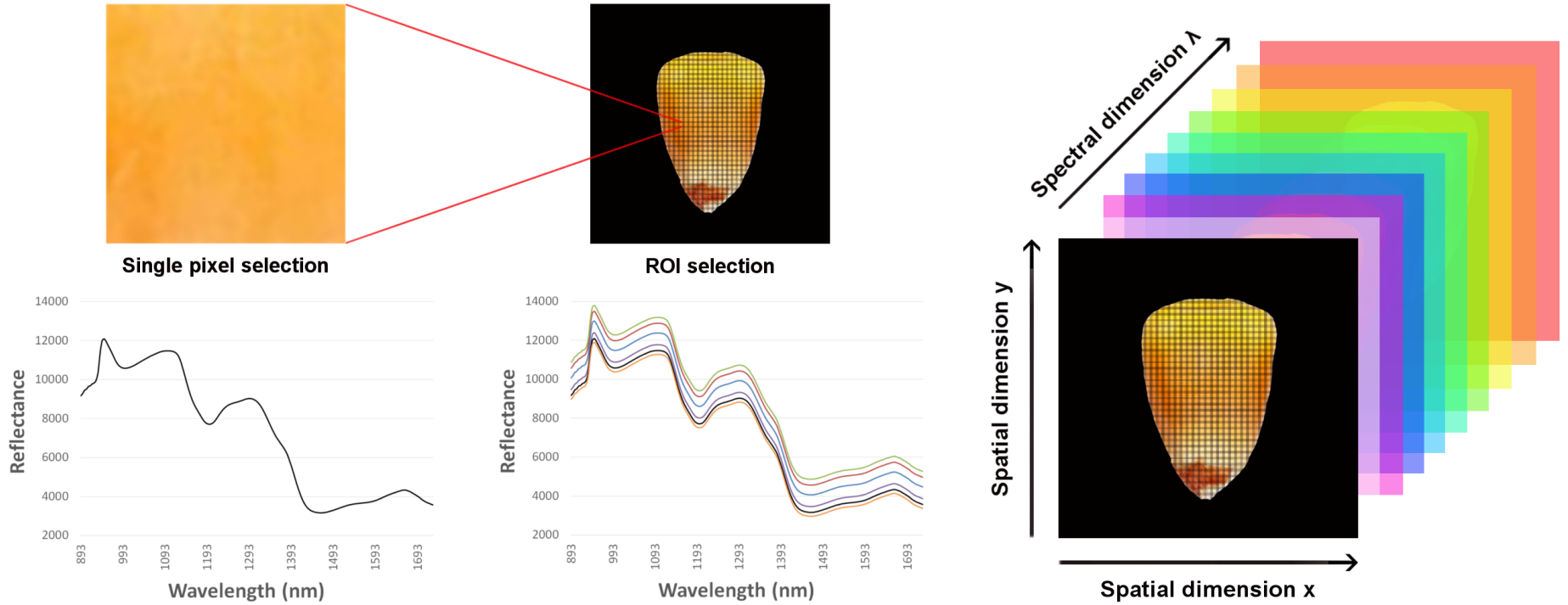


*Fig. 4 Data structure of a hypercube of a maize kernel. Relationship between the spatial dimensions (x, y) and the spectral dimension (λ). ROI: Region of Interest*

#### 1.6.2.1.3 Optimal storage

Although is not a treatment, an optimal storage will prevent fungal growth and mycotoxin production. Optimal storage starts by cleaning the warehouse of residues from the previous stored crop, as those residues often harbour large populations of storage moulds and provide food and a breeding site for storage insects. Insecticidal and antifungal compounds can also be applied at the warehouse before storing the cereal. Introducing uncontaminated cereal is essential to ensure a safe and efficient storage.

The ideal warehouse is a closed structure, weatherproof and with no holes, in order to prevent the entry of rodents and birds (Munkvold et al., 2019). Elements such as an aeration system, temperature sensors and humidity sensors also help fight fungal infection and mycotoxin production. Although slowly, grains respire. In this process, grain carbohydrates are consumed in presence of oxygen, and as a result, heat, water vapour and carbon dioxide are produced. If grains are not aerated, the generated moisture condenses. Therefore, temperature and $a_w$ increase, which further increases the respiration rate (Marcos-Valle et al., 2021). Moisture migration from the grains can also occur due to moisture variability within the grain mass or temperature differences between the warehouse walls and the adjacent grains. If the rise in temperature and $a_w$ is big enough, some species of fungi will be able to grow ($a_w$>0.70) (Mannaa & Kim, 2017). Fungi of the genera *Aspergillus*, *Penicillium* and *Eurotium*, that have a fairly low prevalence in the field, are predominating during the storage (Magan et al., 2003; Mannaa & Kim, 2017). Fungal growth will inevitably be accompanied of more heat and water generation, consequence of the metabolic processes of the fungi. As a result, temperature and $a_w$ will increase again, which could allow the growth of

different type of microorganisms (most yeasts start growing at $a_w$>0.88, and most bacteria at $a_w$>0.91) (Majumdar et al., 2018). Keeping the kernels at a temperature low enough will increase the minimum $a_w$ required for microbial growth. With adequate temperature and humidity monitoring and aeration systems problems in storage can be detected at an early stage, when interventions can still be effective (Daou et al., 2021; Munkvold et al., 2019).

Regular calibration of temperature and humidity sensors is recommended. Incorporation of insect and rodent trapping in the warehouse is also advised.

#### 1.6.2.1.4 Dehulling

Dehulling is a mechanical process in which the outer layer of the kernels is removed. When a healthy kernel is attacked by a mycotoxin-producing fungus, the first part of the kernel to be colonized is the surface. Therefore, most mycotoxins on a kernel are situated in its surface. Hence, by discarding only the outer layers of kernels important mycotoxin reductions can be achieved. After only 15 seconds of pearling, House et al. (2003) removed 66 % of DON in barley discarding only 15 % of the grain mass. Ríos et al. (2009) observed a 45 % DON reduction in wheat by removing 10 % of grain tissue. According to Siwela et al. (2005), a 92 % AF reduction can be obtained by dehulling maize. Matumba et al. (2015) reported significant reductions of type B trichothecenes, AFs, fumonisins and alternariol by dehulling white maize.

#### 1.6.2.1.5 Heat treating

Most mycotoxins, including DON and fumonisins, present considerable thermal stability, although high temperatures can affect them. Despite the concentration of mycotoxins can be reduced by heat treatment, care should be taken, as the resulting compounds may also be toxic (Murphy et al., 1996). Thermal treatments

can be classified as dry processes (e.g. roasting, baking, frying, extrusion) or wet processes (e.g. boiling, steaming, nixtamalization) (Gab-Allah et al., 2023; Odjo et al., 2022). Thermal degradation of a mycotoxin will mostly depend on the temperatures achieved and the treatment duration (Grenier et al., 2014). It should also be considered that thermal treatments can affect the properties of the food such as its color, texture, digestibility or nutrient concentration (Daou et al., 2021). Many studies have examined how different mycotoxins are affected by different heat treatments in a variety of conditions. Vidal et al. (2015) observed DON reductions ranging from 29 to 81 % in wheat bakery products when baked at 160, 180 and 200 °C during 40 minutes, but no reductions could be observed when the temperature was 140 °C. Visconti et al. (2004) and Nowicki et al. (1988) analysed the DON concentration of spaghetti made of wheat before and after boiling them. In both studies, the thermal treatment caused almost no DON reduction, but due to the hydrophilic nature of DON, around half of the toxin present in uncooked spaghetti leached into the water during the boiling process. Mycotoxin reduction during nixtamalization and tortilla baking has also been studied. Mycotoxins can leach into the liquid fraction during washing, cooking or steeping; and mycotoxins present in the germ, tip cap and pericarp are partly removed when nixtamal is washed. Furthermore, high pH and temperature during cooking and baking can result in degradation, modification and/or binding or release of mycotoxins. Many studies conclude that in comparison to raw maize, tortillas show mycotoxin reductions of 50-100 % for AFs and 75-100 % for $FB_1$. Reductions for ZEN, DON and moniliformin have also been observed (Schaarschmidt & Fauhl-Hassek, 2019).

#### 1.6.2.1.6 Irradiation

Irradiating a food means exposing it to ionizing energy, causing its atoms or molecules to be ionized (Wood & Roy, 2017). Examples of ionising radiations are ɣ-rays, X-rays or accelerated electrons. ɣ-rays emmited by $^{60}$Co radioisotopes are the most common ionising radiation used in food due to its deep penetration capacity (Calado et al., 2014). According to a joint report by the FAO, the International Atomic Energy Agency and the World Health Organization (WHO), there's no evidence of adverse effects as a result of food irradiation up to a total dose of 10 kGy (FAO/IAEA/WHO, 1981). Irradiation can help fight mycotoxin contamination in cereals in three different ways: by acting on insects, on mycotoxin-producing fungi or on mycotoxins.

Insects can carry fungal spores and damage both grains and plant tissues, facilitating fungal growth and mycotoxin production. Therefore, killing insects found on cereals through irradiation can reduce fungal load and mycotoxin concentration.

When fungi are irradiated, their DNA is strongly damaged, preventing them from reproducing. Ionizing radiation can damage DNA directly or indirectly (due to the action of oxidative radicals formed by the radiolysis of cellular water) (Farkas, 2006). Aziz et al. (2006) applied ɣ-rays to wheat, barley, maize and sorghum that were contaminated with fungi (1.6-2.6x$10^4$ CFU $g^{-1}$). The application of 5 kGy completely inhibited the growth of any fungi in all cereals. Other studies have also proven irradiation doses in the 2.5-10 kGy range cause important reductions or complete inactivation of fungal population in cereals like wheat, maize, barley or rice (Aziz et al., 2004, 2007; Ferreira-Castro et al., 2007; Maity et al., 2011; J. Wang & Yu, 2010).

Regarding the effect of irradiation on mycotoxins in cereals, published studies show contradictory results. Aziz et al. (2007) applied different irradiation doses to wheat, maize and barley naturally contaminated with $FB_1$ (initial concentrations of 5.8±1.4, 13.2±4.2 and 0.5±0.2 mg $kg^{-1}$, respectively). The authors reported a complete detoxification after a 5 kGy dose in barley and after a 7 kGy dose in maize and wheat. Aziz et al. (1997) irradiated wheat contaminated with DON and ZEN (170 and 35.7 µg $kg^{-1}$, respectively). According to the authors, a 8 kGy dose was enough to complete degrade both toxins from the cereal.

Less optimistic results have been published in other studies. According to D'Ovidio et al. (2007), irradiation levels as high as 100 kGy could not significantly reduce levels of $FB_1$ in whole maize. O'Neill et al. (1993) irradiated whole maize contaminated with DON (33.9±2.76 mg $kg^{-1}$) and 3-AcDON (30.8±2.04 mg $kg^{-1}$). Breakdown of the toxins only began after 20 kGy, and approximately 74 % of DON and 86 % of 3-AcDON remained after 50 kGy. Visconti et al. (1996) irradiated 15 kGy to two batches of maize flour naturally contaminated with fumonisins. Only a decrease of about 20 % could be observed in the $FB_1$ and $FB_2$ levels.

In view of the abovementioned results, irradiation seems to be effective when reducing fungal populations from cereals, but its effectiveness reducing mycotoxin levels remains doubtful.

#### 1.6.2.1.7 Adsorbents

Another strategy for reducing the exposure to mycotoxins is using agents that adsorb them. By doing so, mycotoxins bioavailability is decreased, which leads to a lower mycotoxin uptake as well as distribution to the blood and target organs (Boudergue et al., 2009). Ideally, an adsorbent should keep its binding capacity

unaffected during passage through the gastro-intestinal tract, but usually its efficacy is lower under those conditions.

Adsorbents can be classified according to its origin between organic (yeast and bacterial cell walls, vegetal fibers), inorganic (clays, activated carbon) and synthetic (modified clays, synthetic resins). Different intermolecular interactions can occur in an adsorption process, such as hydrogen binding, Van der Waals forces and electrostatic attraction or repulsion forces (Boudergue et al., 2009).

Many studies have found different adsorbents capable of strongly binding DON or fumonisins *in vitro*, but there's no strong evidence of its efficacy *in vivo*. Polymeric glucomannans added to feed contaminated with DON and other trichothecenes seem to prevent some of the toxin-induced changes in swine metabolism (Diaz & Smith, 2006; Swamy et al., 2002, 2003). Cholestyramine reduces the increase of the sphinganine/sphingosine ratio in urine and kidney of rats fed with fumonisin-contaminated feed (Solfrizzo et al., 2001). For the moment, no feed additives have been approved for binding DON nor fumonisins in the EU. On the other side, in the EU it is allowed to use a specific kind of bentonite (a dioctahedral montmorillonite) as a feed additive in ruminants, poultry and pigs for its AF binding properties (European Commission, 2013b).

#### 1.6.2.1.8 Non-thermal plasma

Non-thermal plasma (NTP) is a novel physical treatment that has recently begun to be tested against mycotoxin-producing fungi and mycotoxins. NTP consists of a fully or partially ionized gas, formed basically by free electrons, ions, photons and atoms in both base and excited states. NTP is usually obtained using an electric discharge or applying microwaves at atmospheric or reduced pressures (Yousefi et al., 2021). The reactive species generated in plasma act rapidly

against both fungi and mycotoxins. In addition, NTP has a rather low impact on quality and needs low energy input (Yousefi et al., 2021). In maize spiked with $AFB_1$ and $FB_1$, Wielogorska et al. (2019) achieved reductions higher than 60 % for both mycotoxins after 10 minutes of plasma exposure. No $FB_1$ degradation products could be found. Other studies have also obtained positive results with spiked $AFB_1$ in hazelnuts (Sen et al., 2019), spiked $AFB_1$ in rice or wheat (Puligundla et al., 2020) or OTA in roasted coffee (Casas-Junco et al., 2019).

#### 1.6.2.2 Chemical treatments

Different chemical treatments have been tested for its capacity to degrade mycotoxins. Like thermal treatments, chemical treatments can transform mycotoxins into compounds that are still toxic. Chemical treatments can also cause alterations in the nutritive value of the treated food. In the EU, chemical detoxification of $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$, OTA, patulin, DON, ZEN, $FB_1$, $FB_2$, citrinin, ergot sclerotia and ergot alkaloids is forbidden (European Commission, 2023). Next, efficacy of the most studied chemical treatments is briefly discussed.

##### 1.6.2.2.1 Ozone

The most studied oxidizing agent capable of degrading mycotoxins is ozone. Ozone disrupts fungal cells by oxidizing sulfhydryl and amino acid groups of enzymes and attacking polyunsaturated fatty acids of the cell wall (Afsah-Hejri et al., 2020). Ozone reduces spore germination, mycotoxin production and insect populations (Afsah-Hejri et al., 2020; McDonough et al., 2011). This agent also decreases mycotoxin concentration, although the mechanism of detoxification remains unclear for some mycotoxins. L. Wang et al. (2016) treated wheat

kernels and whole wheat flour of different moisture contents with ozone. After one hour treatment with 100 mg ozone $L^{-1}$, DON concentration in 11.70 % moisture wheat kernels was reduced by 41.96 % (initial concentration of 3.98 mg $kg^{-1}$). The same treatment in 11.70 % moisture whole wheat flour resulted in a 47.49 % DON degradation. Increased efficiency in flour can be explained by the larger contact area of the cereal with ozone. Ozone has proved to reduce the concentration of other mycotoxins in maize, like AFs (Luo et al., 2014), ZEN and OTA (Qi et al., 2016).

#### 1.6.2.2.2 Sodium bisulfite ($NaHSO_3$) and sodium metabisulphite ($Na_2S_2O_5$)

Sodium bisulfite is a reducing compound that has proven to reduce DON levels in many studies. According to Young (1986), by soaking DON contaminated maize (500 mg $kg^{-1}$) in a 1 % sodium bisulfite solution during 6 days, DON levels were reduced by more than 90 %. Accerbi et al. (1999) soaked DON contaminated wheat (7.3 mg $kg^{-1}$) in a 5 % $SO_2$ equivalent sodium bisulfite solution during 1 hour, and DON concentration fell to 0.8 mg $kg^{-1}$. In a study conducted by Young et al. (1986), after soaking DON contaminated wheat (1 mg $kg^{-1}$) in a 10 % $SO_2$ equivalent sodium bisulfite solution, less than 2 % of the initial DON remained in the cereal. Dänicke et al. (2005) treated DON contaminated wheat (7.6 mg $kg^{-1}$) with 10 g $Na_2S_2O_5$ per kg of wheat for 15 min at 100 °C with saturated steam supply, reducing the DON concentration to 0.28 mg $kg^{-1}$. Treated wheat was fed to piglets, that did show better performance parameters that the piglets fed with non-treated wheat. The inconvenient of sodium bisulfite or sodium metabisulfite treatment is that soaking a cereal leads to increasing its moisture, which facilitates fungal growth.

#### 1.6.2.2.3 Ammonia and ammonium salts

Young (1986) reported that treating DON contaminated maize (1000 mg $kg^{-1}$) with ammonium carbonate at 132 °C during 1 hour lead to a 92 % DON reduction. Young et al. (1986) stated that soaking DON contaminated wheat (1 mg $kg^{-1}$) in a 5 % ammonium hydroxide solution during 24 h caused a 35 % decrease in the toxin concentration.

Park et al. (1992) treated $FB_1$ contaminated maize (86 mg $kg^{-1}$) with 2 % ammonia at 20 °C under 4.08 atm of pressure during 1 h and reached a 79 % $FB_1$ reduction. Norred et al. (1991) treated maize naturally contaminated with $FB_1$ with ammonia in concentrations in the 1-5 % range, at 60 °C and during 4 days, achieving degradations between 41.3 and 50 %.

Moerck et al. (1980) treated yellow dent corn naturally contaminated with $AFB_1$ (200 µg $kg^{-1}$) and $AFB_2$ (35 µg $kg^{-1}$) with $NH_3$. Using 0.5 % $NH_3$ at ambient temperature for 24 h reduced $AFB_1$ and $AFB_2$ levels by 60 and 83 %, respectively. When $NH_3$ concentration was increased up to 2 %, $AFB_1$+$AFB_2$ concentration was reduced to below 20 µg $kg^{-1}$. Gomaa et al. (1997) treated AFs-contaminated maize ($AFB_1$+$AFB_2$+$AFG_1$+$AFG_2$ concentration of 4000 µg $kg^{-1}$) with different ammonia concentrations (0.25-2 %) using two different ammoniation procedures: under atmospheric pressure and ambient temperature for 24 h (AP/AT), and under high pressure (2 bar) at high temperature (121 °C) for 15 min (HP/HT). Overall, higher ammonia concentrations led to higher AFs degradation, and the HP/HT procedure (68.9-99.9 % total AFs degradation) proved to be more efficient than the AP/AT procedure (40.8-90 % total AFs degradation). Brekke et al. (1977) treated maize naturally contaminated with $AFB_1$ at different mycotoxin concentrations and with different moisture levels with different ammonia

concentrations, at different temperatures and during different time treatments. The authors observed that higher $AFB_1$ degradations were achieved using higher ammonia concentrations, higher temperatures and longer treatment times. $AFB_1$ degradation was also higher in maize with higher moisture levels. Initial AF levels affected final AF levels after an ammoniation treatment, but did not influence $AFB_1$ degradation kinetics. Other authors have proven ammonia is useful for destroying AFs in maize (Martinez et al., 1994; Norred, 1982; Nyandieka et al., 2009; Weng et al., 1994) and in other matrices such as sorghum (Hasan, 1996), peanut meal (Gardner Jr. et al., 1971; Mann et al., 1970; Neal et al., 2001; Viroben et al., 1978) or cottonseed meal (Gardner Jr. et al., 1971; Mann et al., 1970). Chelkowski et al. (1981) successfully degraded OTA in maize using ammonia.

If a mycotoxin-contamined cereal lot is ammonia treated with the aim of subsequently feeding it to an animal, proper aeration of the lot must be ensured to remove ammonia odor and prevent it from being rejected by the animal (Brekke et al., 1977; Park, 1993).

Caution must be taken when using a direct treatment with ammonia solution, as it requires soaking and therefore, cereal moisture is increased, which may ease fungal growth.

#### 1.6.2.2.4 Other chemical treatments

Other chemical agents have been proposed for reducing mycotoxin content in cereals. Mixing $FB_1$ and $FB_2$ contaminated maize with a solution of dextrose and sodium bicarbonate ($NaHCO_3$) and autoclaving it leads to the formation of fumonisin B - glucose reaction products. Those fumonisin B - glucose adducts are less toxic to pigs than the original $FB_1$ and $FB_2$ contaminated maize (Fernández-Surumay et al., 2005).

Young (1986) proved that gas concentrations of chlorine >1 % lead to important reductions of DON concentration in maize. Abramson et al. (2005) soaked DON-contaminated barley with 1 M sodium carbonate ($Na_2CO_3$) solution (20 mL 100 $g^{-1}$ barley) and kept it at 80 °C. DON levels were reduced from 18.4 to 1.4 mg $kg^{-1}$ after 1 day, and to under the LOD (0.22 mg $kg^{-1}$) after 8 days. Young et al. (1986) soaked DON contaminated wheat (1 mg $kg^{-1}$) in solutions of different concentrations of hydrogen peroxide, ascorbic acid or hydrochloric acid during 24 hours. DON reductions ranging from 8 to 66 % were achieved. It should be considered that, as previously mentioned, soaking a cereal increases its moisture, which increases the risk of fungal growth.

### 1.6.2.3 Biological treatments

Mainly, microorganisms can help reduce mycotoxin contamination by two means: adsorbing mycotoxins onto their cell walls (which has been discussed in section 1.6.2.1.7) or biodegrading mycotoxins. Mycotoxin biodegradation occurs when cells segregate mycotoxin-degrading enzymes to the environment. Generally, these enzymes are highly specific and can be applied under mild conditions (Lyagin & Efremenko, 2019).

Two approaches can be taken to biodegrade mycotoxins: inoculate with mycotoxin-biodegrading microorganisms, or directly use mycotoxin-degrading enzymes.

Biodegradation of DON has been achieved using both fungi and bacteria. C. He et al. (2008) found a strain of *Aspergillus tubingensis* that biodegraded DON. The biodegradation product was detected, but not identified. The bacteria *Nocardioides* WSN05-2 and *Devosia mutans* 17-2-E-8 are capable of biodegrading DON into 3-epi-DON, a less toxic compound (J. W. He et al., 2015; Ikunaga et al., 2011). A bacterium belonging to the *Agrobacterium-Rhizobium* group has proven capable of biodegrading DON to 3-keto-4-deoxynivalenol, a compound with a much lower immunosuppressive toxicity (Shima et al., 1997). *Eubacterium* BBSH 797 is able to deepoxidate DON, turning it into the non-toxic DOM-1 (Fuchs et al., 2002).

Enzymes capable of degrading DON and fumonisins have been identified. UDP-glycosyltransferases can glycosylate DON and other trichothecenes, reducing its bioavailability and toxicity. UDP-glycosyltransferases with specificity towards DON have been found in barley (Schweiger et al., 2010), rice (Michlmayr et al., 2015), *Arabidopsis thaliana* (Poppenberger et al., 2003) and *Brachypodium distachyon* (Schweiger et al., 2013). Trichothecene 3-O-acetyltransferases capable of transforming DON intro the less toxic form 3-AcDON have been found in a variety of *Fusarium* species (Khatibi et al., 2011). However, glycosylation or 3-O-acetylation of DON is controversial, because the conjugated or acetylated mycotoxin may be hydrolysed and regenerated in the digestive system of animals or humans. Two genes of the bacterium *Sphingopyxis* sp. MTA144 encode for

two proteins that detoxificate $FB_1$ by deesterificating and deaminating it, respectively (Heinl et al., 2010).

Biodegradation of other mycotoxins rather than DON or fumonisins has also been achieved. The enzyme ZEN hydrolase ZenA (ZENzyme®) can convert ZEN to the less estrogenic hydrolysed ZEN. Gruber-Dorninger et al. (2023) fed chickens, rainbow trouts and pigs with feed contaminated with ZEN, feed contaminated with ZEN and supplemented with ZenA or with uncontaminated feed. For each investigated species, animals fed with ZEN-contaminated feed supplemented with ZenA presented lower ZEN and higher hydrolysed ZEN concentrations in their digesta/feces than animals fed with ZEN-contaminated feed only. Molnar et al. (2004) demonstrated that the yeast *Trichosporon mycotoxinivorans* was capable of degrading ZEN and OTA in mineral solutions. Bhatti et al. (2021) observed that supplementation with *T. mycotoxinivorans* reduced oxidative stress and tissue damage of broiler chikens fed with OTA-contaminated feed.

Despite successful biodegradation of DON and fumonisins has been observed in many *in vitro* studies, its applicability on cereals is not as common. To date, not many feed additives with the capacity to biodegrade mycotoxins have been approved in the EU. Two examples are fumonisin esterases (FUMzyme®), to be used in poultry, pigs and avian species feed (European Commission, 2014, 2017a, 2018) and the *Coriobacteriaceae* strain DSM 11798 (Biomin® BBSH® 797), capable of biotransforming trichothecenes, to be used in pigs and avian species feed (European Commission, 2013a, 2017b).

## 1.7 Mycotoxin analysis in cereals

Mycotoxin analysis in cereals comprises several steps: sampling, homogenization, extraction, clean-up, and finally, separation and detection (Gab-Allah et al., 2023). The samples must be representative of the analysed cereal, and each sample must be crushed and blended, so the analyzed portions can contain the same mycotoxin concentration as the cereal. When choosing an extraction solvent, the physicochemical properties of the analysed mycotoxins and the food sample, the clean-up procedure and the employed separation and detection technique should be considered. Popular clean-up approaches are QuEChERS (quick, easy, cheap, effective, rugged and safe), solid phase extraction columns or immunoaffinity columns. The most common methodology for analysis and quantification of mycotoxins is chromatography, normally liquid chromatography (LC). Immunochemical assays are also used.

The most usual technique to analyse mycotoxins is high pressure liquid chromatography (HPLC). HPLC presents a high sensitivity, selectivity and repeatability, but it's also expensive, requires a previous complex extracting and cleaning step and is time-consuming and pollutant. Depending on the analyte or analytes and the sample matrix, isocratic or gradient elution is chosen. UV or diode-array detectors are frequently used to detect DON and its acetylated forms (Abedi-Tizaki & Zafari, 2015; Yang et al., 2013). Fumonisins lack a property that allows its detection by common detectors, so they are derivatized. In the most frequent fumonisin derivatization process, fumonisins are derivatized with ortho-phthaldialdehyde, forming compounds that can be detected by a fluorescence detector (Belajova & Rauova, 2010; Sydenham et al., 1996).

DON and fumonisins can also be detected using a HPLC coupled to a mass spectrometry (MS) detector (Hickert et al., 2015; Plattner, 1999). MS detectors are used in the case of complex matrices and/or less purified sample extracts (such as in the case of QuEChERS extraction) and multimycotoxin analysis. In addition, high-resolution MS detectors allow identification and/or confirmation of the analytes in complex samples.

Although less usual, DON and fumonisins can also be analysed by gas chromatography (GC) after derivatization, a necessary step to form volatile derivatives (Krska et al., 2007).

The most widespread immunochemical method for analysing mycotoxins is ELISA (Enzyme Linked Immunosorbent Assay). Commercial ELISA kits for analysis of DON and fumonisins are easily available on the market. ELISA is based on the use of enzyme-labelled antibodies or antigens (mycotoxins), so that the conjugates present a quantifiable immunologic and enzymatic activity. Mycotoxin analysis by ELISA is fast, but presents the disadvantages of giving false positives caused by antibodies cross-reactivity and matrix dependence, and giving false negatives due to low sensitivity (Munkvold et al., 2019). Another common immunochemical method to analyse mycotoxins such as DON or fumonisins are lateral flow immunoassays. They employ the same immunoassay principles as ELISA, but the sample is added at the end of a strip and travels by capillary action to the other end. There are zones on the strip with antibodies that react against the antigen (mycotoxin), which allows its detection.

## 1.8 References

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# Chapter 2. Objectives.

The present thesis main objective is to study strategies to minimize mycotoxin contamination, both before and after harvest, in two of the most consumed cereals globally: maize and wheat. Concretely, it has focused on the mycotoxins DON, $FB_1$ and $FB_2$, as they present a high prevalence in these cereals.

The work has been divided into different sub-sections to reach the main objective:

- To study the influence of different agronomic factors on the mycotoxin contamination of maize and to evaluate the changes occurring in maize during a 10-day harvest-till-drying simulation period (Chapter 4).
- To study the potential of NIR-HSI to predict DON, $FB_1$ and $FB_2$ contamination in maize kernels, and to evaluate the capability of this technology to classify maize kernels according to whether they exceed or not the legal limits established in the EU for the abovementioned mycotoxins (Chapter 5).
- To study the DON degradation in wheat kernels by exposition to ammonia vapours and to determine the *in silico* toxicity of the possible DON-derived formed products (Chapter 6).
- To develop a DON analysis method for microbiological culture assays capable of distinguishing between dissolved DON and DON adsorbed on cell walls, useful for the search of DON-biodegrading microorganisms (Chapter 7).

# Chapter 3. Global methodology.

## 3.1 Experimental design

The production of cereals like wheat or maize and their derived products is relatively simple. Cereals are planted and the resulting plants slowly grow. When the new seeds reach both commercial maturity and the desired moisture, they are harvested and dried as soon as possible. Then they are stored, and can go through transformations like dehulling or milling previous to other operations in the food or feed chain. In the EU, sampling and analysis methods for official analysis of cereals such as wheat and maize must follow the Regulation 2023/2782, which means, in the case of analysis methods, complying with a series of specific requirements described in that Regulation (European Commission, 2023). A working plan is presented in Fig. 5, in which different tested strategies to control DON, $FB_1$ and $FB_2$ contamination in wheat or maize through the production process of these cereals are depicted. The general methodology used to develop each of the 4 specific objectives of this thesis, which are described in chapters 4 to 7, is described below.

### Chapter 4

Chapter 4 is divided in two parts. First, the influence of three agronomic factors on the total fungal contamination, *Fusarium* spp. contamination and DON, $FB_1$ and $FB_2$ contamination of maize was studied. The compared factors were:

- Crop diversification: a monocropping long-cycle maize (FAO 700 maturity group) *vs* a legume-maize double cropping, using short-cycle maize (FA 400 maturity group) and vetch.
- Tillage system: intensive tillage (consisting of subsolate, disc harrow and rototiller) *vs* direct drilling.

- N fertilization rate: high (300-400 kg N $ha^{-1}$) *vs* zero (0 kg N $ha^{-1}$).

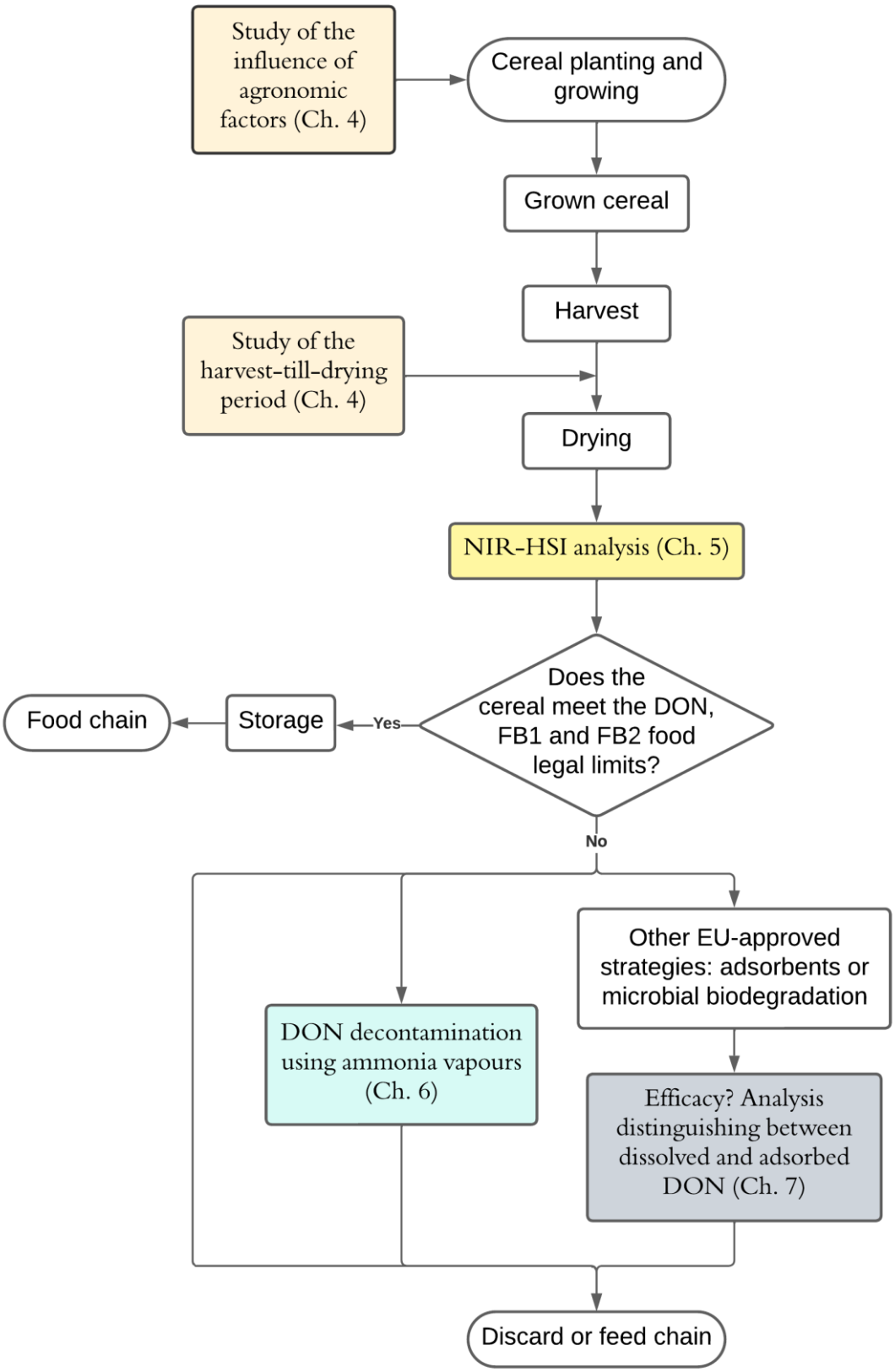


*Fig. 5 Thesis working plan*

Frequently, drying facilities within an area are undersized, and as all maize is harvested in a short time interval, several days may pass between harvesting maize and drying it. Freshly harvested maize has a moisture high enough to allow fungal growth and, therefore, mycotoxin production. In the second part of chapter 4, freshly harvested maize was kept at 15 and 25 °C during a 10 days period, during which changes in moisture, $a_w$, total fungal contamination, *Fusarium* spp. contamination and DON, $FB_1$ and $FB_2$ contamination were monitored.

**Chapter 5**

In chapter 5, NIR-HSI technology was used to develop regression models that can predict the concentration of DON, $FB_1$, $FB_2$ and $FB_1$+$FB_2$ in samples of full maize kernels. Classification models that sort samples of full maize kernels according to whether they meet or not the EU regulations for DON and $FB_1$+$FB_2$ were also developed. In addition, some spectral areas that give information about the contamination by these mycotoxins were identified.

**Chapter 6**

In this chapter, wheat kernels supplemented with DON were exposed to ammonia vapours to test its DON degradation potential. Three different temperatures (65, 90 and 115 °C) and three different $NH_4OH$ concentrations (1.6, 3.2 and 4.8 %) were assayed on wheat kernels fortified with 500 µg $kg^{-1}$ of DON. After determining the optimal DON degradation conditions (90 °C and 4.8 % $NH_4OH$), those were used to study the kinetics of DON degradation and the influence of different initial DON concentration on its degradation. Potential DON-derived formed products were identified and its toxicity was evaluated *in silico*.

**Chapter 7**

Microorganisms can detoxify mycotoxins via biodegradation or via adsorption on cell walls, both of which can co-occur. Distinguishing the two phenomena is important, as adsorption is more easily reversed. Because of the increasing interest in biological detoxification and the very low success rates of biodegradation studies, it was decided to develop a fast, simple, reliable and economic method for quantify both dissolved and adsorbed DON in bacterial culture assays.

Throughout chapters 4, 5 and 6, analysis of DON in wheat and maize, and analysis of $FB_1$ and $FB_2$ in maize have been carried out on multiple occasions. Next, a description of how these analysis were performed is shown.

## 3.2 DON HPLC-DAD analysis of wheat and maize

HPLC-DAD determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex® Gemini C18 column (Torrance, CA, USA) was used (150 × 4.6 mm, 5 µm particle size, 110 Å pore size). Absorbance reading was performed at 220 nm. Three mobile phases were prepared: phase A (methanol:water 10:90, v:v), phase B (acetonitrile:water 20:80, v:v) and phase C (100 % methanol). The gradient applied was as follows: 0 min 100 % A; 10 min 60 % A and 40 % B; 13 min 60 % A and 40 % B; 15 min 100 % C; 25 min 100 % C; 29 min 100 % A until 40 min (for re-equilibrating the column). The flow rate was set at 1 mL $min^{-1}$. The column temperature was 40 °C, and the injection volume was 50 µL. DON retention time was 10.0 min.

Quantification was carried out by using DON calibration curves prepared in methanol:water 10:90, v:v. LOD and LOQ were considered three and ten times the signal of the blank. Recovery, repeatability and reproducibility were calculated using artificially contaminated samples.

For wheat, LOD and LOQ were 11.3 and 37.6 µg $kg^{-1}$. Recovery was assayed per duplicate at three different concentrations (100, 400 and 700 µg $kg^{-1}$). Average recovery values were 92.7 ± 9.7 %. Repeatability and reproducibility of the method were <3.0 % and <16.2 %, respectively.

For maize, LOD and LOQ were 12.6 and 42.0 µg $kg^{-1}$. Recovery was assayed per triplicate at three different concentrations (571, 1143 and 2286 µg $kg^{-1}$ maize). The respective average recoveries and standard deviations were 91.3 ± 14.5, 87.4 ± 13.3 and 81.7 ± 9.5 %.

### 3.3 $FB_1$ and $FB_2$ HPLC-FLD analysis of maize

HPLC-FLD determination of $FB_1$ and $FB_2$ was performed using an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA) coupled with an Agilent 1260 Infinity Fluorescence Detector (FLD). A Phenomenex® Kinetex PFP column (Torrance, CA, USA) was used (150 × 4.6 mm, 5 µm particle size, 110 Å pore size). Excitation and emission were performed at 335 and 460 nm, respectively. Three mobile phases were prepared: phase A (acetonitrile), phase B (methanol) and phase C (0.1 % acetic acid). The gradient applied was as follows: 0 min 15 % A and 85 % C; 10 min 5 % A, 61 % B and 34 % C; 14 min 5 % A, 61 % B and 34 % C; 16 min 5 % A, 72 % B and 23 % C; 20 min 15 % A and 85 % C (for re-equilibrating the column). The flow rate was set at 1.2 mL $min^{-1}$. The column temperature was 40 °C, and the injection volume was 50 µL. $FB_1$ and

$FB_2$ retention times were 15.0 and 17.8 min, respectively. Quantification was carried out by using $FB_1$ and $FB_2$ calibration curves prepared in methanol:water 50:50, v:v. Prior to injection, samples were derivatized. The derivatization mixture (DM) for the analysis of fumonisins was prepared as follows: 40 mg of ortho-phthaldialdehyde was dissolved in 1 mL of methanol and diluted in 10 mL of 0.1 M disodium tetraborate. Then, 50 µL of 2-mercaptoethanol was added and the mixture was vortexed. The prepared mixture was stored in an amber glass vial at 4 °C for a maximum of 7 days. The injector was programmed to draw 37.5 µL of DM and 12.5 µL of the sample to be analyzed, and then we mixed them for 0.3 min before injection.

LOD and LOQ, considered as three and ten times the signal of the blank, were 10.0 and 33.3 µg $kg^{-1}$ for $FB_1$ and 16.0 and 53.3 µg $kg^{-1}$ for $FB_2$, respectively. Recovery was calculated using artificially fumonisin-contaminated maize. Recovery was studied per triplicate at three different fumonisin concentrations: 285 + 285, 570 + 570 and 855 + 855 (µg $FB_1$ + µg $FB_2$) $kg^{-1}$ maize. For $FB_1$, the respective average recoveries and standard deviations were 77.0 $\pm$ 9.5, 86.8 $\pm$ 9.2 and 82.1 $\pm$ 8.7 %. For $FB_2$, those values were 88.6 $\pm$ 27.6, 101.6 $\pm$ 27.7 and 102.6 $\pm$ 21.5 %.

Both the DON analysis method of wheat and maize and the $FB_1$ and $FB_2$ analysis method of maize comply with the recovery performance criteria (average recovery between 70 and 120 %) established in the Regulation (EU) 2023/2782 regarding the confirmatory mycotoxin analysis methods (European Commission, 2023).

## 3.4 References

European Commission. (2023). Commission Implementing Regulation (EU) 2023/2782 of 14 December 2023 laying down the methods of sampling and analysis for the control of the levels of mycotoxins in food and repealing Regulation (EC) No 401/2006. Official Journal of the European Union, L 15.12.2023.

# Chapter 4. Influence of Agronomic Factors on Mycotoxin Contamination in Maize and Changes during a 10-Day Harvest-Till-Drying Simulation Period: A Different Perspective.

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## 4.1 Abstract

Agronomic factors can affect mycotoxin contamination of maize, one of the most produced cereals. Maize is usually harvested at 18 % moisture, but it is not microbiologically stable until it reaches 14 % moisture at the drying plants. We studied how three agronomic factors (crop diversification, tillage system and nitrogen fertilization rate) can affect fungal and mycotoxin contamination (deoxynivalenol and fumonisins $B_1$ and $B_2$) in maize at harvest. In addition, changes in maize during a simulated harvest-till-drying period were studied. DON content at harvest was higher for maize under intensive tillage than using direct drilling (2695 and 474 µg $kg^{-1}$, respectively). We found two reasons for this: (i) soil crusting in intensive tillage plots caused the formation of pools of water that created high air humidity conditions, favouring the development of DON-producing moulds; (ii) the population of *Lumbricus terrestris*, an earthworm that would indirectly minimize fungal infection and mycotoxin production on maize kernels, is reduced in intensive tillage plots. Therefore, direct drilling is a better approach than intensive tillage for both preventing DON contamination and preserving soil quality. Concerning the simulated harvest-till-drying period, DON significantly increased between storage days 0 and 5. Water activity dropped on the 4th day, below the threshold for DON production (around 0.91). From our perspective, this study constitutes a step forward towards understanding the relationships between agronomic factors and mycotoxin contamination in maize, and towards improving food safety.

**Keywords**: maize; deoxynivalenol; fumonisin; tillage system; nitrogen fertilisation; crop diversification; water activity; *Fusarium*; *Lumbricus terrestris*

**Key Contribution**: direct drilling is a better tillage system than intensive tillage; as it not only preserves soil quality; but also helps controlling DON contamination in maize.

## 4.2 Introduction

Maize is one of the most produced cereals worldwide and is used for both human consumption and animal feed. It is estimated that 1,162,352,997 tons of maize were produced in 2020 [1]. Unfortunately, maize is susceptible to toxigenic fungal contamination at all points of its supply chain (pre-harvest, harvest and post-harvest stages) [2,3]. Amongst the most prevalent and toxic fungal metabolites in maize, the mycotoxins fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$) and deoxynivalenol (DON) can be found [2,4]. In maize, fumonisins are primarily caused by *Fusarium verticillioides*, *Fusarium proliferatum* and *Fusarium subglutinans*, while DON is mostly caused by *Fusarium graminearum* and *Fusarium culmorum* [4–7]. Apart from being one of the major causes of economic losses in maize crops, mycotoxin contamination can have a severe impact on human and animal health.

$FB_1$ affects sphingolipid metabolism, causes oxidative stress and can cause damage to cell DNA [8]. In humans, fumonisins have been associated with a higher risk of oesophageal carcinoma [9]. In animals, $FB_1$ ingestion can cause leucoencephalomalacia (LEM) in horses, hepatocarcinogenesis in rats and pulmonary oedema in swine [10]. DON inhibits protein and DNA synthesis in eukaryotic cells, and can induce nausea, emesis, vomiting, skin inflammation, leukopenia, diarrhoea, haemorrhage in the lungs and brain, and the destruction of bone marrow [11,12]. The European Union (EU) regulates the maximum content of DON and the sum of $FB_1$ + $FB_2$ in certain foodstuffs (including maize)

and provides guidance values for those and other mycotoxins in food and feed products [13–15].

Many factors can affect mycotoxin contamination in maize throughout the whole supply chain. Among them, we can find biological factors (susceptibility of the crop), environmental factors (temperature, rainfall, air relative humidity, insects/bird injuries), crop management (planting and harvest dates, tillage practices, fertilization, crop rotation, irrigation), crop harvesting (crop maturity, temperature, moisture, mechanical injury), transportation conditions, time until drying, and proper drying or storage conditions (aeration, temperature, pest/rodent control) [2,4,16].

The accepted commercial moisture for maize harvesting in NE Spain is around 18 %. Sometimes, when the maize is almost ready for harvest, rain can increase the grain moisture, promoting mould proliferation and extending the period before harvesting until moisture reaches commercial standards again. In addition, in some areas drying facilities are undersized. Therefore, as all maize is harvested within an interval of a few days, it is usual to find huge amounts of maize grain outdoors waiting to be processed in the drying plants. This waiting period can sometimes be as long as 10 days. Despite the accepted commercial moisture for maize being about 18 %, it has been reported that to ensure that no moulds can grow in grain nor produce mycotoxins, its maximum moisture content must be no more than 14 % [17,18]. To our knowledge, there is no information about how this waiting period can influence fungal and mycotoxin contamination of the maize.

Hence, the objectives of our study were to: (i) study the impact of several agronomic factors (the crop diversification, the tillage system and the nitrogen (N)

fertilization rate) on total fungal contamination, *Fusarium* spp. contamination and DON, $FB_1$ and $FB_2$ contaminations of recently harvested maize; (ii) simulate the waiting period between maize harvesting and drying for 10 days, and study the influence of waiting time and temperature on the previously mentioned variables.

## 4.3 Materials and methods

### 4.3.1 Climate and Soil Characteristics, Experimental Design and Crop Management

Maize was planted in an experimental field in Agramunt, NE Spain (41°48' N, 1°07' E, 330 m asl). The soil in this area is classified as xerofluvent typic [19]. Many soil characteristics were measured: the average pH of the soils was ($H_2O$, 1:2.5) 8.5; the electrical conductivity (1:5) was 0.15 $dSm^{-1}$; the soil organic carbon (SOC) concentration (0–30 cm) was 8.6 g $kg^{-1}$; the water available holding capacity (between −33 kPa and −1500 kPa) was 10 % (v/v). The climate of the area is semiarid Mediterranean with a continental trend. Climate was monitored with a weather station placed in the experimental field. During the last 30 years, the mean annual precipitation was 442 mm, the mean annual temperature was 14.6 °C, and the mean annual potential evapotranspiration (PET) was 855 mm. The winter is cold, with some days below 0 °C in January. For that, soil temperature does not reach 8 °C until the beginning of April, when the planting date for maize starts. Additionally, the climate imposes hot summers, reaching temperatures over 35 °C in July and August.

The experimental design was a split-plot with 3 blocks. The plots were 50m × 3m = 150 $m^2$ and 4 rows of maize were planted in each plot (rows spaced 73 cm apart). Three agronomic factors were evaluated: the crop diversification, the

tillage system and the N fertilization rate. For the crop diversification, a monocropping long-cycle maize (LC maize) (FAO 700 maturity group, Pioneer's P1570 hybrid) was compared against a legume-maize double cropping, using short-cycle maize (SC maize) (FAO 400 maturity group, Pioneer's P0312 hybrid) as the main crop and vetch (*Vicia sativa* L., var. Prontivesa) as the secondary crop.

In the case of the tillage system, intensive tillage (IT) and direct drilling (DD) were studied. IT consisted of subsolate (35 cm depth), disc harrow and rototiller, while DD consisted of the application of herbicide (1.5 L $ha^{-1}$ of 36 % glyphosate [N-(phosphonomethyl)-glycine]) and sowing directly the seeds into the soil. In reference to the N fertilization rate, a zero N rate (0 N) and high N rate (High N) were evaluated. The rate of mineral fertilization applied was 400 kg N $ha^{-1}$ for LC maize, while it was reduced to 300 kg N $ha^{-1}$ in SC maize because of the possible fixation of the preceding legume crop. N fertilization was distributed between 2 top-dressing fertilizations with ammonium nitrate (34.5 % N), with a rate of 150 kg N $ha^{-1}$ in each one at stages V3–V5 (May in LC maize and June in SC maize) and V7–V8 respectively (June in LC maize and July in SC maize). In addition, for LC maize, a 100 kg N $ha^{-1}$ pre-emergence fertilization was carried out during April with urea (46 % N). The experiment was carried out over 3 years (2019, 2020 and 2021), although the present study was carried out with the third year's harvest. LC and SC maize were seeded in April and June, respectively. Accordingly, its flowering took place in July and August, respectively. Vetch was sown in December. In both maturity groups, the planting rate was 90,000 seeds $ha^{-1}$, with a row spacing of 73 cm. In the case of vetch, the planting density was 267 plants $m^{-2}$. All maize plots received equally a pre-emergence herbicide

treatment with 7 L $ha^{-1}$ of Primextra Gold (Terbuthylazine 18.75 % + S-Metolachlor 31.25 % (SE) w/v). For each tillage system and plant species, the harvest residue was treated differently. In the case of maize and IT, it was integrated into the soil by tillage, whereas in DD, it was chopped and spread on the soil surface. Vetch was harvested for forage at a cutting height of 5 cm, so all the biomass was exported from the plots. The irrigation rate was determined using Dastane's methods [20] for calculating crop water requirements on a weekly basis. Irrigation was carried out by sprinkling, starting in March and ending in October. The amount of irrigation used and mean meteorological conditions in the experimental field, obtained from an on-site weather station, are shown in Fig. 6.

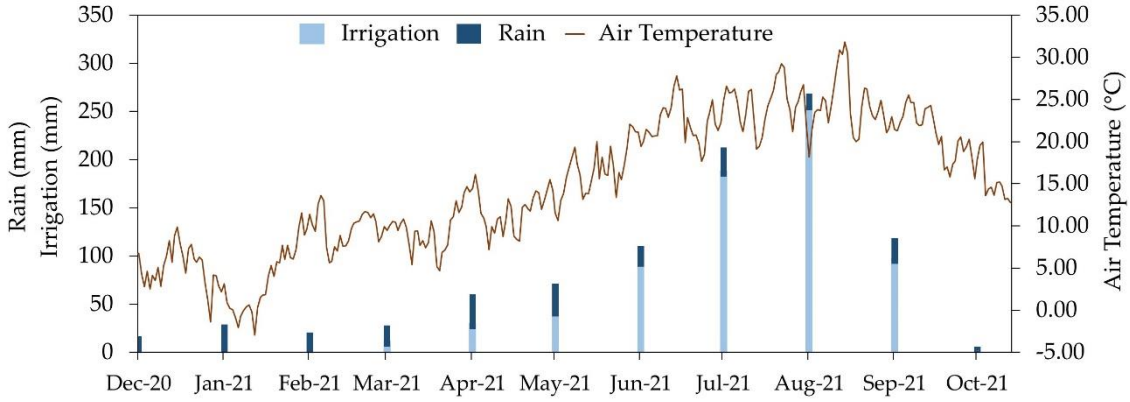


*Fig. 6 Irrigation and meteorological conditions in the experimental field.*

### 4.3.2 Maize Harvesting and Storage

Cob samples of a total of 16 different plots were taken (2 cultivars × 2 tillage systems × 2 N fertilization rates × 2 blocks). Both cultivars were harvested when the maize was close to the commercial moisture (18 %). That was the 21st and 26th of October 2021 for LC and SC maize, respectively. On the harvest day,

around 1.5 kg of maize (approximately 8–10 maize cobs) was sampled from each plot. The different maize cobs were collected throughout the entire plot, being representative of the area of study. As not to alter the microbiota of the samples, the cobs were picked up using different sterile nitrile gloves for each plot. The maize from each plot was deposited and transported in a different sterile plastic bag. In the laboratory, cobs were shelled under sterile conditions in a laminar flow cabinet. The kernels from each plot were split into two different sterile plastic bags, which were kept at different temperatures: 15 or 25 °C, for 10 days. Those specific temperatures were chosen to simulate the average maximum and minimum daily temperatures in the area at the time of harvest.

### 4.3.3 Laboratory Determinations

Different determinations were performed on the harvest day (day 0) and the following days for the maize from each plot. Moisture (%), water activity ($a_w$), total fungal contamination (CFU $g^{-1}$ maize) and *Fusarium* spp. contamination (CFU $g^{-1}$ maize) were determined on days 0, 4, 7 and 10. DON, $FB_1$ and $FB_2$ contamination were determined on days 0, 5 and 10.

#### 4.3.3.1 Moisture

Approximately 15 g of maize kernels were precisely weighed into pre-weighed glass jars. The jars were put in an oven (JP Selecta 210, JP Selecta S.A., Abrera, Spain) at 105 °C for 16 h, and after that period were weighed again. The moisture was calculated according to Equation (1). Three replicates were carried out for each plot, storage time and storage temperature. Average moisture and standard deviation were calculated.

$$Moisture\ (\%) = \frac{W_0 - W_f}{W_0 - W_j} * 100 \quad (1)$$

where $W_0$ is the weight of the glass jar and the maize before drying, $W_f$ is the weight of the glass jar and the maize after drying, and $W_j$ is the weight of the glass jar.

#### 4.3.3.2 Water Activity ($a_w$)

The $a_w$ of whole maize kernels for each plot, storage time and storage temperature was measured using the AquaLab Series 3 TE (AquaLab S.L., Sabadell, Spain). A sample of about 3 g was introduced into the water activity meter, and $a_w$ was properly read.

#### 4.3.3.3 Total Fungal Contamination and *Fusarium* spp. Contamination

One maize sample from each plot, storage time and storage temperature was analysed for total fungal contamination and *Fusarium* spp. contamination. Approximately 20 g of kernels was ground using a disinfected IKA A11 (IKA®-Werke GmbH & Co. KG, Staufen, Germany) mill for 30 s. Ten grams of the resulting flour was weighed in a sterile Stomacher bag with a lateral filter. Then, 90 mL of sterile saline peptone water was added to the bag ($10^{-1}$ dilution). The flour and the saline peptone water were mixed in a laboratory blender (Stomacher 400, Seward Ltd.,Worthing, UK) for 120 s at normal speed. A series of dilutions were prepared based on the filtered extract using saline peptone water (up to the $10^{-6}$ dilution). Then, 0.1 mL of each dilution was plated into Petri plates containing Chloramphenicol Glucose Agar (CGA) (for total fungal contamination) or Malachite Green Agar 2.5 (MGA) (a selective medium for *Fusarium* spp.).

The inoculum was spread across the Petri plates with a Digralsky spreader, and the plates were incubated upside down at 25 °C. Plate readings were performed after 3 days of incubation for CGA plates and 4 days of incubation for MGA plates.

#### 4.3.3.4 DON, $FB_1$ and $FB_2$ Contamination

**Extraction of DON, $FB_1$ and $FB_2$**

One sample from each plot, storage time and storage temperature was analyzed for its DON, $FB_1$ and $FB_2$ content. An amount of 17 g of each sample were ground in a IKA A11 mill for 30 s. Seven grams of ground maize were transferred into a 50 mL Falcon tube for DON analysis, and another 7 g of ground maize were put into another 50 mL Falcon tube for $FB_1$ and $FB_2$ analysis.

**DON Extraction and Sample Preparation**

DON extraction and analysis were based on the study of Borràs-Vallverdú, Ramos, Marín, Sanchis and Rodríguez-Bencomo (2020) [21]. An amount of 1.4 g of NaCl and 40 mL of Milli-Q water were added to the Falcon tube with the ground maize. The mixture was vortexed for 30 s and ultrasound-treated with the Bransonic M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power for 15 min. After that, the Falcon tubes were centrifuged in a Hettich 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 8965 × g for 10 min at 20 °C. The supernatant was vacuum filtered using 90 mm glass microfiber filters (Whatman, Buckinghamshire, UK). DonPrep immunoaffinity columns (Biopharm AG, Darmstadt, Germany) were prepared by adding 10 mL of Milli-Q water. Then, 8 mL of the filtered supernatant was collected and passed through the immunoaffinity column. After that, 1.5 mL of

methanol was added to elute the toxin. Backflushing was done three times, and then another 0.5 mL of methanol was passed through the column. The 2 mL of collected methanolic extract was evaporated at 40 °C (Stuart SBH200D/3 block heater, Cole-Parmer©, Staffordshire, UK) under a gentle stream of $N_2$. The residue was re-suspended in 0.8 mL of MeOH:$H_2O$ 10:90 (v:v), vortexed, filtered through 0.22 µm PTFE filters and analyzed by HPLC-DAD according to the following section.

**DON HPLC-DAD Analysis**

HPLC-DAD determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex® Gemini C18 column (Torrance, CA, USA) was used (150 × 4.6 mm, 5 µm particle size, 110 Å pore size). Absorbance reading was performed at 220 nm. Three mobile phases were prepared: phase A (methanol:water 10:90, v:v), phase B (acetonitrile:water 20:80, v:v) and phase C (100 % methanol). The gradient applied was as follows: 0 min 100 % A; 10 min 60 % A and 40 % B; 13 min 60 % A and 40 % B; 15 min 100 % C; 25 min 100 % C; 29 min 100 % A until 40 min (for re-equilibrating the column). The flow rate was set at 1 mL/min. The column temperature was 40 °C, and the injection volume was 50 µL. DON retention time was 10.0 min. Quantification was carried out by using DON calibration curves prepared in methanol:water 10:90, v:v. LOD and LOQ, considered as three and ten times the signal of the blank, respectively, were 12.6 and 42.0 µg $kg^{-1}$. Recovery was calculated using artificially DON contaminated maize, extracting and analysing the mycotoxins as previously stated. Recovery was studied per triplicate at three

different DON concentrations: 2286, 1143 and 571 µg DON $kg^{-1}$ maize. The respective average recoveries and standard deviations were 81.7 ± 9.5, 87.4 ± 13.3 and 91.3 ± 14.5 %.

**$FB_1$ and $FB_2$ Extraction and Sample Preparation**

Fumonisin extraction and analysis were based on the study of Belajova and Rauova (2010) [22]. An amount of 1.4 g of NaCl and 35 mL of $H_2O$:ACN:MeOH 50:25:25 (v:v:v) were added to the Falcon tube with the ground maize. The mixture was vortexed for 30 s and ultrasound-treated with the Bransonic M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power for 15 min. After that, the Falcon tubes were centrifuged in a Hettich 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 8965 × g for 10 min at 20 °C. The supernatant was vacuum filtered using 90 mm glass microfiber filters (Whatman, Buckinghamshire, UK). The solution to be analyzed was prepared by mixing 3.5 mL of the filtered supernatant with 46.5 mL of PBS in another 50 mL Falcon tube. The whole content of the Falcon tube was passed through a Fumoniprep immunoaffinity column (Biopharm AG, Darmstadt, Germany). After that, 1.5 mL of methanol was added to collect the toxin. Backflushing was done three times, and then 1.5 mL of Milli-Q water was passed through the column. The 3 mL of collected solution was evaporated at 40 °C (Stuart SBH200D/3 block heater, Cole-Parmer©, Staffordshire, UK) under a gentle stream of $N_2$. The residue was re-suspended in 0.8 mL of MeOH:$H_2O$ 50:50 (v:v), vortexed, filtered through 0.22 µm PTFE filters and analyzed by HPLC-FLD according to the following section.

**$FB_1$ and $FB_2$ HPLC-FLD Analysis**

HPLC-FLD determination of $FB_1$ and $FB_2$ was performed using an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA) coupled with an Agilent 1260 Infinity Fluorescence Detector (FLD). A Phenomenex® Kinetex PFP column (Torrance, CA, USA) was used (150 × 4.6 mm, 5 µm particle size, 110 Å pore size). Excitation and emission were performed at 335 and 460 nm, respectively. Three mobile phases were prepared: phase A (acetonitrile), phase B (methanol) and phase C (0.1 % acetic acid). The gradient applied was as follows: 0 min 15 % A and 85 % C; 10 min 5 % A, 61 % B and 34 % C; 14 min 5 % A, 61 % B and 34 % C; 16 min 5 % A, 72 % B and 23 % C; 20 min 15 % A and 85 % C (for re-equilibrating the column). The flow rate was set at 1.2 mL/min. The column temperature was 40 °C, and the injection volume was 50 µL. $FB_1$ and $FB_2$ retention times were 15 and 17.8 min, respectively. Quantification was carried out by using $FB_1$ and $FB_2$ calibration curves prepared in methanol:water 50:50, v:v.

Prior to injection, samples were derivatized. The derivatization mixture (DM) for the analysis of fumonisins was prepared as follows: 40 mg of ortho-phthaldialdehyde was dissolved in 1 mL of methanol and diluted in 10 mL of 0.1 M disodium tetraborate. Then, 50 µL of 2-mercaptoethanol was added and the mixture was vortexed. The prepared mixture was stored in an amber glass vial at 4 °C for a maximum of 7 days. The injector was programmed to draw 37.5 µL of DM and 12.5 µL of the sample to be analyzed, and then we mixed them for 0.3 min before injection.

LOD and LOQ, considered as three and ten times the signal of the blank, were 10.0 and 33.3 µg $kg^{-1}$ for $FB_1$ and 16.0 and 53.3 µg $kg^{-1}$ for $FB_2$, respectively. Recovery was calculated using artificially fumonisin-contaminated maize,

extracting and analysing the mycotoxins as previously stated. Recovery was studied per triplicate at three different fumonisin concentrations: 855 + 855, 570 + 570 and 285 + 285 (µg $FB_1$ + µg $FB_2$) $kg^{-1}$ maize. For $FB_1$, the respective average recoveries and standard deviations were 82.1 ± 8.7, 86.8 ± 9.2 and 77.0 ± 9.5 %. For $FB_2$, those values were 102.6 ± 21.5, 101.6 ± 27.7 and 88.6 ± 27.6 %.

### 4.3.4 Reagents and Chemicals

DON was from Romer Labs (Tulln, Austria). $FB_1$ and $FB_2$ were from Sigma (St. Louis, MO, USA), ortho-phthaldialdehyde was from Merck (Darmstadt, Germany) and 2-mercaptoethanol was from Scharlau (Sentmenat, Spain). Methanol HPLC grade, acetonitrile HPLC gradient grade and NaCl were from Fisher Scientific UK Limited (Loughborough, UK).

CGA was from Biokar (Barcelona, Spain). MGA was prepared in the laboratory according to Castellá et al. (1997) [23]. Peptone was from Biokar (Barcelona, Spain), $KH_2PO_4$ and chloramphenicol were from Scharlau (Sentmenat, Spain) and $MgSO_4 \cdot 7\ H_2O$ was from Quality chemicals (Esparreguera, Spain). Malachite green ($C_{48}H_{50}N_4O_4 \cdot 2C_2H_2O_4$) was from Probus (Badalona, Spain) and agar was from Condalab (Torrejón de Ardoz, Spain).

### 4.3.5 Statistics

Statistical analyses were carried out using the SPSS program for Windows (version 22) (IBM Corp., Armonk, New York, NY, USA; https://www.ibm.com/es-es/analytics/spssstatistics-software, access on 28 August 2022). The significance level was established at 0.05. Descriptive statistics, Principal

Compounds Analysis and multiple-factor ANOVAs were performed. LSD tests were used to evaluate significantly statistical differences among groups in a variable. Graphics were drawn using Microsoft Excel 2013.

## 4.4 Results and discussion

### 4.4.1 Influence of Agronomic Factors on the Maize at Harvest Date

At harvest date (day 0) all the analyzed maize from both maturity groups, N fertilization rates and tillage systems was contaminated with DON (Table 2). On the other hand, only 12.5 % of that same maize samples contained $FB_1$ and $FB_2$. Average concentrations of the contaminated samples were 826 and 196 µg toxin $kg^{-1}$ maize for $FB_1$ and $FB_2$, respectively.

*Table 2 DON contamination in maize at harvest*

| FAO Maturity Group / Cropping System | Fertilization | Tillage system | Average DON Contamination (µg Toxin $kg^{-1}$ Maize) |
|---|---|---|---|
| 400/SC | 0 N | DD | 440 |
| | | IT | 2848 |
| | High N | DD | 566 |
| | | IT | 4406 |
| 700/LC | 0 N | DD | 654 |
| | | IT | 791 |
| | High N | DD | 236 |
| | | IT | 2734 |

*SC: short cycle; LC: long cycle; 0 N: zero nitrogen rate; High N: high nitrogen rate; DD: direct drilling; IT: intensive tillage.*

Multi-factor ANOVAs were carried out to study the impact of the agronomic factors on the response variables at harvest. Neither $FB_1$ nor $FB_2$ concentrations in maize at harvest date were statistically significantly affected by any of the agronomic factors. DON content of the grains at harvest date was statistically significantly affected by the tillage system (see Table 3). Maize planted under IT had higher DON contamination (2695 µg DON $kg^{-1}$ maize on average) than maize planted using DD (474 µg DON $kg^{-1}$ maize on average).

*Table 3 Test of between-subjects effects for DON contamination at harvest date.*

| | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Crop diversification | 3.697 | 1 | 3.697 | 1.292 | 0.289 |
| N fert. rate | 2.574 | 1 | 2.574 | 0.900 | 0.371 |
| Tillage system | 19.729 | 1 | 19.729 | 6.897 | **0.030** |
| Crop diversification x N fert. rate | 0.006 | 1 | 0.006 | 0.002 | 0.964 |
| Crop diversification x Tillage system | 3.260 | 1 | 3.260 | 1.140 | 0.317 |
| N fert. rate x Tillage system | 3.598 | 1 | 3.598 | 1.258 | 0.295 |
| Crop diversification x N fert. rate x Tillage system | 0.216 | 1 | 0.216 | 0.075 | 0.791 |

R Squared = 0.591 (Adjusted R Squared = 0.233). SS: sum of squares; df: degrees of freedom; MS: Mean Square; F: F-value; Sig.: significance value. Bold value is the only statistically significant factor.

It has been reported that residues of crops that were infected with *Fusarium* constitute an inoculum of the fungus for the following crop [24–27]. This inoculum tends to be particularly abundant in the case of maize [28]. Therefore, according to many authors, the removal, destruction or burial of infected crop residues is likely to reduce the *Fusarium* inoculum for the following crop, making IT a better choice than DD for controlling mycotoxin-producing fungal inoculums [24,26,27].

Mansfield, De Wolf and Kuldau (2005) reported that the DON concentration of ensiled maize was lower in maize planted using a moldboard till than in maize planted using no tillage [29]. Dill-Macky and Jones (2000) studied the DON contamination of wheat following corn, wheat or soybean, using different tillage systems [30]. DON levels were lower in wheat planted using moldboard ploughing following corn or wheat in comparison to wheat planted using no tillage following the same crops. No significant differences in DON levels in wheat were observed between the two tillage systems when the previous crop was soybean, as *F. graminearum* is not considered a pathogen of soybeans. Obst, Lepschy-Von Gleissenthall and Beck (1997) stated that the use of minimum tillage instead of mouldboard ploughing after a maize crop could result in a 10-fold increase in DON contamination of the following wheat crop [31]. Schöneberg et al. (2016) demonstrated that barley from fields with ploughed soils showed significantly less *F. graminearum* and DON content than barley from reduced tillage fields, regardless of the previous crop [32]. On the other hand, Roucou, Bergez, Méléard and Orlando (2022), who collected data from a total of 2032 maize fields located in France between 2004 and 2020, found that DON contamination in maize was not significantly different whether the crop residues of the previous year were adequately managed (mostly through soil tillage) or not [33]. Suproniene et al. (2012) studied the effect of different tillage practices (conventional tillage, reduced tillage and no tillage) on mycotoxin contamination in winter and spring wheat, but no clear relationship could be observed [34]. Furthermore, Kaukoranta, Hietaniemi, Rämö, Koivisto and Parikka (2019), who analyzed survey data from 804 spring-oat fields, found that the DON concentration of the oats was the same or higher under ploughing than under non-ploughing

conditions [35]. Our results are closer to those of Kaukoranta et al. (2019), as we found a significantly higher DON contamination in maize planted under IT than in maize planted using DD.

Tillage operations can affect both the soil structure and the crop productivity [36]. Unlike no tillage, IT exposes soil to erosive agents such as wind and water. The impact of water drops induces the degradation of the soil by the breakdown of water-stable aggregates, causing soil crusting [37,38]. Soil crusting negatively affects seedling emergence, reduces water infiltration rates and water storage capacity, favors runoff, diminishes organic matter, and can cause overland flow [36,37,39]. Soils rich in silt and fine sand, such as the one in this study, are highly susceptible to soil crusting [40]. As we observed the presence of pools of water only in IT plots, most probably caused by soil crusting, we hypothesize that in these plots the pools of water created high air humidity conditions, favoring the production of DON by moulds. That would help explain the higher DON contamination in maize planted under IT in comparison to maize planted using DD.

Another hypothesis that supports our results is that tillage affects soil fauna, which in turn can have an impact on *Fusarium* species. Earthworms are known for breaking down organic matter and promoting nutrient cycling along with soil microbiota, and for improving soil structure, soil porosity, soil water retention capacity, root distribution, plant growth and plant health. Frequent tillage adversely affects many earthworm species, especially those linked to the surface layers (epigeics and anecics) [44–47]. When the soil is turned over, earthworms are injured and killed, their burrows are broken, their food sources are buried, and they become exposed to harsh environmental conditions and predators. [44–

46,48,49]. The common earthworm (*Lumbricus terrestris*) is one of the most important anecic earthworms and is capable of incorporating plant litter into the soil and decomposing it. Oldenburg, Kramer, Schrader and Weinert (2008) and Schrader, Kramer, Oldenburg and Weinert (2009) demonstrated that *L. terrestris* accelerates the degradation of *Fusarium* biomass and DON in the wheat straw layer, and that this earthworm is more attracted to highly *Fusarium*-infected and DON contaminated wheat straw than less infected and contaminated wheat straw [50,51]. *L. terrestris* is likely to prefer the contaminated straw as its N-content and digestibility are enhanced due to fungal colonization. Thus, *L. terrestris* most probably reduces *Fusarium* biomass in maize straw too, and consequently, minimizes *Fusarium* infection and DON contamination of maize cobs. Therefore, as the population of *L. terrestris* is smaller in IT plots, a lower DON contamination in maize planted under DD is expected than in maize planted under IT. In our case, we did not sample earthworms during the experiment. However, Santiveri Morata, Cantero-Martínez, Ojeda Domínguez and Angás Pueyo (2004) studied the population of earthworms in the same field where this study was performed, and found that under DD the population of worms was higher than in more aggressive tillage systems [52].

The moisture content of the grains at harvest date was statistically significantly affected by the crop diversification and the tillage system. Moisture was higher in SC maize (21.33 % on average) than in LC maize (16.93 % on average), and higher in maize under IT (20.45 % on average) than in maize planted using DD (17.82 % on average). Likewise, the $a_w$ of the grains was significantly affected by the crop diversification, being greater in SC maize than in LC maize (0.927 and 0.897 on average, respectively). It should be noted, though, that the differences

in moisture and $a_w$ between different maturity groups could easily be modified by the harvesting dates.

It has been reported that no tillage is associated with soil with a higher water holding capacity and higher soil moisture in surface soil layers in comparison with IT [53,54]. Therefore, one might think that the maize kernels obtained from DD-planted maize would have a higher moisture than maize kernels obtained from maize under IT, but that was not the case in our study.

The log of total fungal contamination was significantly affected by the crop diversification (p-value = 0.046), being higher in LC maize (5.28 on average) than in SC maize (4.81 on average).

No effect of the agronomic factors was observed in the log of *Fusarium* spp., $FB_1$ or $FB_2$ contaminations. In this context, Ono et al. (2011) observed no significant differences in the *Fusarium* sp. counts and the fumonisin concentrations between non-tilled and conventional-tilled maize [55]. Similarly, Ariño et al. (2009) found no significant differences in the fumonisin contents of maize planted using minimum tillage and ploughing [56]. Even so, it is necessary to emphasize that the low incidence of $FB_1$ and $FB_2$ contamination on maize at harvest (12.5 %) makes it rather difficult to observe differences in the concentration of these toxins due to agronomic factors.

Regarding how N fertilization can affect fumonisin contamination in maize, previous research has shown contrasting results. A shared vision is that a balanced fertilization is the best approach to minimize fumonisin concentrations, as stress due to N deficiency or high N rates can significantly raise fumonisin levels [55,57–61]. In our study, no differences in fumonisin contamination were observed between 0 N and High N fertilization rates.

### 4.4.2 Correlations between the Studied Variables at the Harvest Date

Principal Component Analysis was performed in search of correlations between response variables at harvest (see correlation matrix heatmap in Fig. 7). The variables studied were moisture, $a_w$, the log of total fungal contamination, the log of *Fusarium* spp. contamination, and the different mycotoxin contaminations (DON, $FB_1$ and $FB_2$). Following the criteria of choosing the principal components with eigenvalues > 1, three principal components were taken, which accounted for 81.32 % of the total variance.

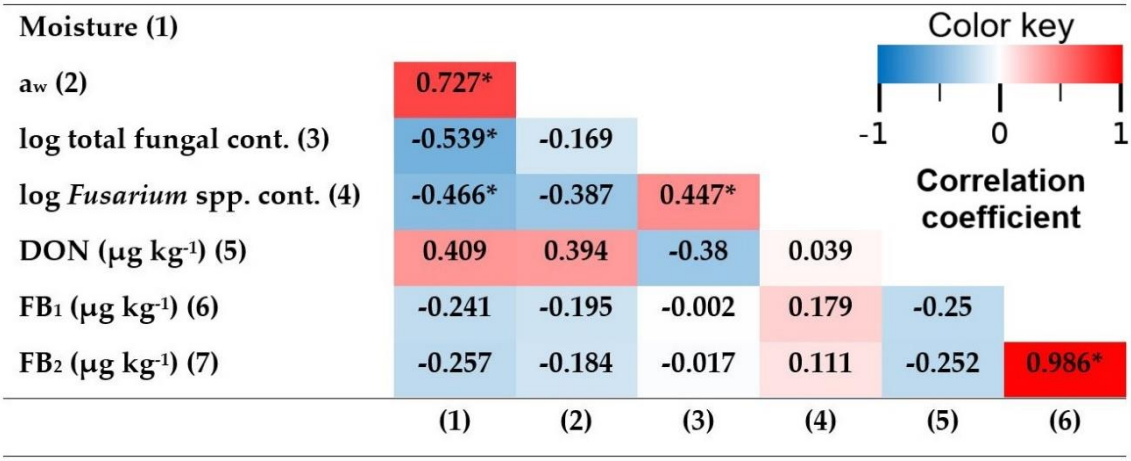


*Fig. 7 Correlation matrix heatmap based on the correlation coefficients from the PCA at harvest date. A darker blue color indicates a stronger negative correlation, while a darker red color indicates a stronger positive correlation.*

** indicates a significant correlation (p-value < 0.05).*

Few variables were significantly correlated. $FB_1$ contamination was significantly positively correlated with $FB_2$ contamination ($r = 0.986$, p-value < 0.001). That is in accordance with the results of Carbas et al. (2021) and Cao et al. (2013), who also found significant positive correlations between $FB_1$ and $FB_2$ contaminations ($r = 0.96$ and $r = 0.99$, respectively) [62,63].

There was a significant positive correlation between the log of total fungal contamination and the log of *Fusarium* spp. contamination, indicating that *Fusarium* spp. is of considerable relevance to total fungal contamination.

Moisture was significantly positively correlated with $a_w$ (r = 0.727, p-value = 0.001), and significantly negatively correlated with the log of total fungal contamination (r = −0.539, p-value = 0.016) and with the log of *Fusarium* spp. contamination (r = −0.466, p-value = 0.035). Cao et al. (2013) also described a significantly negative correlation between moisture and *Fusarium* spp. contamination (r = −0.68, p-value < 0.05) [63].

*Fusarium* spp. contamination at harvest date was not significantly correlated with the concentration of any of the studied mycotoxins (DON, $FB_1$ and $FB_2$) in the same period. This could be explained by there being non-DON/$FB_1$/$FB_2$-producing *Fusarium* spp. strains colonizing our maize, and/or because a higher count of DON/$FB_1$/$FB_2$-producing *Fusarium* spp. at harvest date does not necessarily imply a higher concentration of these mycotoxins. Factors such as $a_w$, temperature and relative humidity can affect mycotoxin production [64,65]. Similarly, Lanza et al. (2017) found no association either between fumonisin levels and the frequency of *Fusarium* spp. in maize kernels [66]. On the other hand, Schöneberg et al. (2016) found that *F. graminearum* was positively correlated with DON content in barley (r = 0.72, p-value < 0.001) [32].

No significant correlations were observed between DON and $FB_1$ or $FB_2$ concentrations. That is consistent with the bibliography, as it has been described that in maize DON is produced primarily by *F. graminearum* and *F. culmorum*, while $FB_1$ and $FB_2$ are mainly produced by *F. verticillioides*, *F. proliferatum* and *F. subglutinans* [4–7].

DON was positively correlated with moisture and $a_w$, but the correlations were not significant (p-values of 0.058 and 0.066, respectively).

### 4.4.3 Effect of Time and Temperature on Maize Moisture, $a_w$, Microbial Counts and Mycotoxin Contamination after Harvest

Multi-factor ANOVAs were carried out to determine the effect of time and temperature (15 or 25 °C) on the studied variables. On one side, moisture, $a_w$ and microbial counts were studied on days 0, 4, 7 and 10. On the other side, mycotoxin contamination was studied on days 0, 5 and 10. All the data are available in a spreadsheet in the Supplementary Materials.

No significant effect of time nor temperature was observed on the moisture, the total fungal contamination or the *Fusarium* spp. contamination during the 10 days of the experiment. By contrast, the variable time significantly affected the evolution of $a_w$ (p-value = 0.001), which dropped on day 4 for both temperatures (Fig. 8). Statistically significant differences were observed between $a_w$ on day 0 and $a_w$ on days 4, 7 and 10.

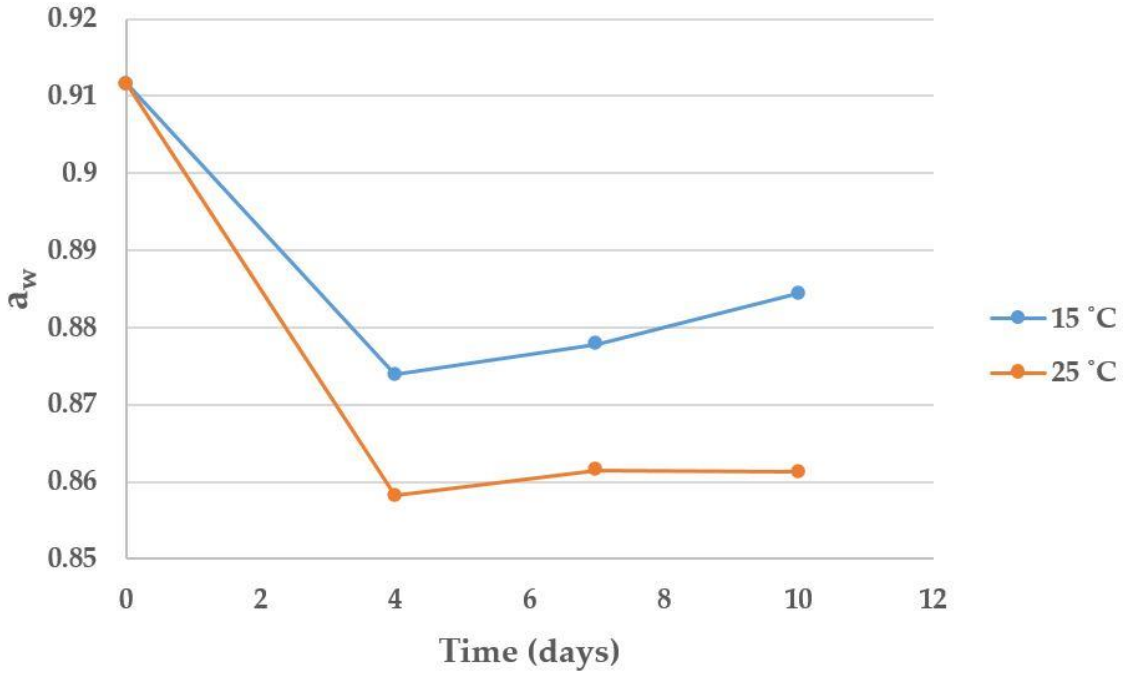


*Fig. 8 Influence of time and temperature on the evolution of $a_w$.*

DON, $FB_1$ and $FB_2$ contaminations were not affected by time or temperature, although in the case of DON time was close to being significant (p-value = 0.078). Thus, statistically significant differences were observed in DON concentrations between days 0 and 5 (p-value = 0.049) but not between days 0 and 10 (p-value = 0.051) or days 5 and 10 (p-value = 0.989) (see Table 4). Regarding $FB_1$ and $FB_2$ contamination, the tendency was the same as that at harvest: a low prevalence of these toxins. On days 5 and 10, only 15.63 and 18.75 % of samples contained at least one of the studied fumonisins. The average contamination of contaminated samples on days 5 and 10 was 1938 and 1709 µg toxin $kg^{-1}$ maize for $FB_1$, and 1068 and 1279 µg toxin $kg^{-1}$ maize for $FB_2$.

*Table 4 Influence of time and temperature on the evolution of DON concentrations (µg DON kg$^{-1}$ maize).*

| Temperature | DON Concentration (µg DON kg$^{-1}$ maize) | | |
|---|---|---|---|
| | Day 0 | Day 5 | Day 10 |
| **15 ºC** | 1584±1932 | 2367±2983 | 2649±2349 |
| **25 ºC** | 1584±1932 | 3771±3597 | 3470±4300 |

Presented values correspond to mean and standard deviation

As an increase in DON concentration was observed in the 0–5 days period, and *Fusarium* spp. counts remained stable during the whole 10 days period, the absence of DON production during the 5–10 days period could be attributed to the drop in $a_w$ during the first 4 days. If $a_w$ levels had remained constant since harvest, DON contamination most likely would have increased continuously. Considering these results, we could say that under the tested temperatures (15 and 25 °C), there are DON-producing *Fusarium* spp. species in maize that can produce DON at an approximate $a_w$ of at least 0.91, while at an $a_w$ of 0.88 they can no longer produce this toxin.

Our results are in line with those obtained by Comerio, Fernández Pinto and Vaamonde (1999), who studied the DON production of *F. graminearum* in wheat at different $a_w$ [67]. They found that at an $a_w$ = 0.925 DON was produced, but not at $a_w$ = 0.900; therefore, the limiting $a_w$ for DON production under those conditions was close to 0.900. Other studies have suggested slightly higher values under similar conditions. Ramirez, Chulze and Magan (2006) studied the DON production of *F. graminearum* on wheat and found mycotoxin production at $a_w$ = 0.95 at the temperatures of 15, 25 and 30 °C, but they did not find DON production at $a_w$ = 0.93 under any temperature [68]. Schmidt-Heydt, Parra, Geisen and Magan (2011) found that *F. culmorum* and *F. graminearum* could produce DON

at an $a_w$ = 0.93 at 25 °C in YES medium after a 9 day incubation, but not at $a_w$ = 0.90 under any of the tested conditions [69].

## 4.5 Conclusions

At harvest, all maize samples were contaminated with DON (1584 ± 1578 µg DON $kg^{-1}$ maize), while only 12.5 % of the maize samples were contaminated with $FB_1$ and $FB_2$ (average contaminations of contaminated samples were 826 and 196 µg toxin $kg^{-1}$ maize, respectively). No effect of the crop diversification or the N fertilization rate was observed on the maize DON contamination. The only agronomic factor that significantly affected the DON content of grains was the tillage system. Maize planted under IT presented a greater DON contamination (2695 µg DON $kg^{-1}$ maize on average) than maize planted using DD (474 µg DON $kg^{-1}$ maize on average). Two main reasons support these results. The first reason is that in IT plots the degradation of the soil resulting from the continuous tillage caused soil crusting, which induced the formation of pools of water, creating high air humidity conditions, which favored the production of DON by producing moulds. The second reason is that the frequent tillage in IT plots causes a decrease in the population of *L. terrestris*. This earthworm is likely to reduce *Fusarium* infection and DON contamination in maize straw. Consequently, maize cobs under DD are expected to be less infected and contaminated. Hence, DD would be a better approach than IT not only in terms of controlling DON contamination, but also from the agronomic point of view. More studies that employ long-term IT and DD plots are needed to assess precisely how the tillage system can influence the mycotoxin contamination of grains.

No significant correlations were found between the log of *Fusarium* spp. contamination at harvest date and the concentration of any of the studied mycotoxins in the same period.

During the 10-day storage, no effect of time or temperature was observed on the moisture, the total fungal contamination, the *Fusarium* spp. contamination or the $FB_1$ and $FB_2$ contaminations. Time affected the evolution of $a_w$, which fell on day 4 for both temperatures. DON concentration on day 5 was significantly higher than on day 0, but there were no significant differences between days 5 and 10. Therefore, it is predictable that continued DON production was held back by the $a_w$ drop in the first 4 days of storage, meaning the minimum $a_w$ for the DON-producing species colonizing our maize to produce this toxin is around 0.91.

To our knowledge, this is the first study that relates soil crusting and the consequent formation of pools of water in maize plots under IT with a higher DON grain contamination in comparison with maize plots under DD, and is also the first work to question how the harvest-till-drying period of maize can affect fungal and mycotoxin contamination.

**Supplementary Materials**: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins14090620/s1. Supplementary Document: Effect of Time and Temperature on Maize Moisture, $a_w$, Microbial Counts and Mycotoxin Contamination after Harvest.

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# Chapter 5. NIR-HSI as a tool to predict deoxynivalenol and fumonisins in maize kernels: A step forward in preventing mycotoxin contamination.

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## 5.1 Abstract

**Background**: Maize is frequently contaminated with deoxynivalenol (DON) and fumonisins $B_1$ ($FB_1$) and $B_2$ ($FB_2$). In the European Union, these mycotoxins are regulated in maize and maize-derived products. To comply with these regulations, industries require a fast, economic, safe, nondestructive and environmentally friendly analysis method.

**Results**: In the present study, near-infrared hyperspectral imaging (NIR-HSI) was used to develop regression and classification models for DON, $FB_1$ and $FB_2$ in maize kernels. The best regression models presented the following root mean square error of cross validation and ratio of performance to deviation values: 0.848 mg $kg^{-1}$ and 2.344 (DON), 3.714 mg $kg^{-1}$ and 2.018 ($FB_1$) and 2.104 mg $kg^{-1}$ and 2.301 ($FB_2$). Regarding classification, European Union legal limits for DON and $FB_1$+$FB_2$ were selected as thresholds to classify maize kernels as acceptable or not. The sensitivity and specificity were 0.778 and 1 for the best DON classification model and 0.607 and 0.938 for the best $FB_1$+$FB_2$ classification model.

**Conclusion**: NIR-HSI can help reduce DON and fumonisins contamination in the maize food and feed chain.

**Keywords**: deoxynivalenol; fumonisins; maize; near-infrared hyperspectral imaging; mycotoxin

## 5.2 Introduction

Maize was the cereal produced in the largest quantity worldwide in 2021, and its total production was estimated at more than 1210 million tonnes.[1] Unfortunately, maize is frequently contaminated with fungi that lead to important diseases such as *Gibberella* ear rot (GER) and *Fusarium* ear rot (FER). GER, also known as red ear rot, is mainly caused by *Fusarium graminearum* and *Fusarium culmorum*, species capable of producing the mycotoxin deoxynivalenol (DON).[2,3] FER, also known as pink ear rot, is mostly caused by *Fusarium verticillioides*, *Fusarium proliferatum* and *Fusarium subglutinans*, species that can produce mycotoxins known as fumonisins.[4,5] Compared with GER, FER occurs under hotter and drier conditions.[6,7] Fumonisins and DON are the mycotoxins with the highest prevalence in maize, which have been estimated at 82 % and 72 %, respectively.[8] Fungal infection and mycotoxin contamination in maize are both economic and health problems. Their consequences include reduced crop value, crop yield loss, animal productivity loss, human health costs or mycotoxin management costs. Mycotoxin-related health problems can be caused by acute or chronic toxicosis. The toxicity of DON stems from its ability to bind to ribosome subunits, thereby inhibiting protein and DNA synthesis in eukaryotic cells.[9,10] Common symptoms of DON poisoning are nausea, emesis, diarrhea, impaired feed intake and growth, skin inflammation, hemorrhage in the lungs and brain and altered immune function.[10,11] Fumonisins can induce oxidative stress and inhibit ceramide synthetase, affecting sphingomyelin metabolism and damaging the integrity of the cell membranes.[12] Exposure to fumonisin $B_1$ ($FB_1$) has been linked to human esophageal cancer.[13,14] Fumonisin ingestion has been reported to cause

hepatocarcinogenesis in rats, neurotoxic disease in horses and pulmonary edema in swine.[15]

In the European Union (EU), the maximum concentration of DON and $FB_1$+fumonisin $B_2$ ($FB_2$) is regulated for some foodstuffs, including maize.[16] In addition, guidance values for these and other mycotoxins are provided for feed products.[17,18]

To comply with the established mycotoxin regulations, the food industry usually analyses samples using immunological methods such as enzyme-linked immunosorbent assay (ELISA) or lateral flow immunoassays. These methods are rapid, but have high rates of false positives (because of cross-reactivity and matrix dependence), high rates of false negatives (because of high detection limits) and present low accuracy overall.[19] The food industry also uses chromatographic methods such as HPLC, which possess low detection limits and high accuracy, but are time-consuming, environmentally harmful and economically costly. Although immunological and chromatographic methods are widely employed, their application for routine analysis is impractical for the abovementioned reasons.

In recent years, near-infrared (NIR) spectroscopy, which uses the 700-2500 nm spectral range to obtain chemical information from samples, was shown to be a promising tool to predict mycotoxin concentrations in different cereals.[20-23] This technique presents many advantages over the usual analysis methods: it is faster, cheaper, safer, nondestructive and eco-friendly. For many years, NIR spectroscopy has been used in quality control in the cereal industry to determine the major constituents of kernels, such as moisture, protein and lipids.[24-26] Mycotoxins are usually found in concentrations in the range of ng $kg^{-1}$ to mg $kg^{-1}$,

much lower than the major constituents of kernels. Although NIR is not sufficiently sensitive to detect mycotoxins directly, it is capable of detecting changes in cereal components such as proteins, carbohydrates or lipids that result from fungal damage at the tissue, cellular and even molecular levels.[27] In other words, by using NIR spectroscopy, mycotoxins can be detected indirectly as a result of the fungal alteration of the kernels, which will cause changes in the spectral signature.

Hyperspectral imaging (HSI) is an emerging technology that combines spectroscopy and conventional imaging to provide both spectral and spatial information from a sample. Each pixel in a hyperspectral image is linked to a spectrum, forming three-dimensional blocks of data known as hypercubes.[28]

In the present study, by using NIR-HSI, we aimed to (i) develop regression models to predict the concentration of the mycotoxins DON, $FB_1$, $FB_2$ and $FB_1$+$FB_2$ in maize; (ii) identify which spectral areas give more information about DON, $FB_1$ and $FB_2$ contamination; and (iii) develop classification models to classify maize samples according to whether they are under or above the EU-established legal limits for the mycotoxins DON, $FB_1$ and $FB_2$.

## 5.3 Materials and methods

### 5.3.1 Experimental design

Ninety-eight maize samples of different cultivars were collected during October 2022. Maize samples were from the Lleida and Girona provinces, located in Catalonia, Spain. None of the maize samples were artificially contaminated or presented any visible signs of fungal infection. After harvesting, samples were stored at 4 °C to avoid spoilage prior to analysis. Samples were analyzed with

NIR-HSI equipment as described below in the section on instrumentation and data acquisition. Afterwards, the mycotoxins DON, $FB_1$ and $FB_2$ were extracted from the samples and analyzed by HPLC-diode array detection (DAD) in accordance with a previous work.[29] Finally, regression and classification models for the different mycotoxins were built as described in the sections on regressin model building section and classification model building.

### 5.3.2 Reagents and chemicals

DON was purchased from Romer Labs (Tulln, Austria). $FB_1$ and $FB_2$ were from Sigma (St. Louis, MO, USA), ortho-phthaldialdehyde was from Merck (Darmstadt, Germany), disodium tetraborate was from Panreac (Montcada i Reixac, Spain) and 2-mercaptoethanol was from Scharlau (Sentmenat, Spain). Methanol HPLC grade, acetonitrile HPLC gradient grade and NaCl were obtained from Fisher Scientific UK Limited (Loughborough, UK), and acetic acid was obtained from VWR (Fontenay sous Bois, France).

### 5.3.3 NIR-HSI: instrumentation and data acquisition

A push-broom hyperspectral imaging system composed of a Pika NIR-320 camera assembled by RESONON Inc. (Boezman, MA, USA) was used. The device consisted of an InGaAS sensor line scan camera with 320 × 256 pixel resolution, a 30 × 30 μm pixel size and a 14-bit resolution A/D spectrograph (Goldeye G-008 SWIR TEC1; Allied Vision Technologies GmbH, Stadtroda, Germany). The spectral resolution was 4.9 nm (168 spectral bands from 893.1 to 1730.7 nm), with 320 pixels of spatial resolution and a frame rate of 520 fps. The objective lens had a 25 mm focal length (F/1.4 SWIR, 0.9–1.7 μm, 21 mm image

format, c-mount) and was positioned 220 mm above the image surface. The illumination unit was composed of a four-halogen lamp lighting system with Lambertian filters fixed on an adjustable tower. The lamps were turned on at least 20 min before image acquisition. The illumination system was powered by a Samplexpower® power converter (SEC-1223CE; Burnaby, BC, Canada), which provided a highly regulated output DC voltage of 13.8 V at 23 A with an AC input of 230 V and 50 Hz. Finally, a motorized linear translation stage with a range of 600 mm was also used, which permitted the scanning of the full sample with the optical systems remaining in a fixed position.

Spectronon Pro (https://resonon.com) (Fig. 9) was used to control Resonon's benchtop for image processing. The raw reflectance readings for each test sample data array were corrected by dividing the dark current-subtracted reflectance by the dark current-subtracted white standard reflectance at each of the corresponding wavelengths (Equation (2)). A dark current intensity image was collected by covering the camera lens before sample scanning to remove dark current noise. Likewise, intensity from a 99 % white reflectance standard made of polytetrafluoroethylene (Spectralon™, SRT-99-120; Labsphere, North Sutton, NH, USA) was collected immediately after the dark current intensity image to correct illumination effects. These two images were applied to subsequent sample intensity images. Reflectance was scaled to effective bit depth.

$$I = \frac{I_0 - I_b}{I_w - I_b} \quad (2)$$

where $I_0$ is the raw hyperspectral image obtained, $I_w$ is the white reference and $I_b$ is the dark current reference. In addition to the dark and absolute reflectance response, the pixel illumination saturation was also adjusted by using camera

controls. The framerate and integration time were established so that no pixel on the image was saturated.

The 98 maize samples were analyzed with the imaging system. Each sample consisted of 17 g of whole maize kernels, which were arranged in a 5.5-cm diameter Petri dish before analysis. Scanning was performed three times per sample, fully redistributing the position of the kernels in the Petri dish in each scan. Therefore, a total of 294 scans were carried out, obtaining 294 hyperspectral images. In all the scans performed, a black tray was used to reduce the background noise on the image and to obtain an accurate pixel selection. In each hyperspectral image, the area of the kernels was accurately selected using the free form pixel selection tool. The mean spectrum of the pixels of each selected area was recorded as a text file for subsequent exporting to the statistical software.

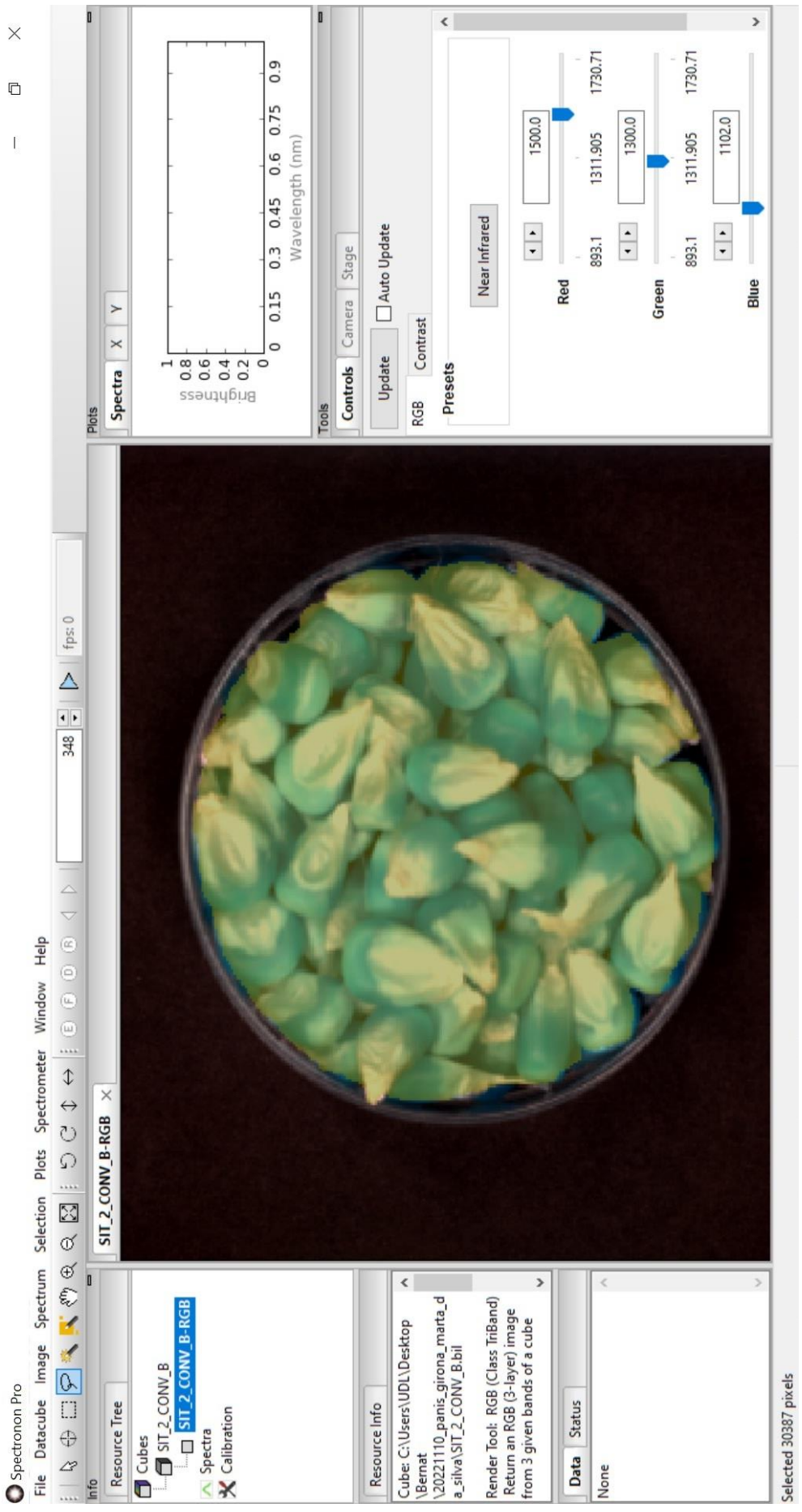


*Fig. 9 Screenshot of the program Spectronon Pro. Analyzing a sample of maize kernels*

### 5.3.4 Regression model building for DON, $FB_1$ and $FB_2$

The regression models were built from the 294 hyperspectral images obtained from 98 the maize samples. Partial least squares (PLS)-1 regression tests were performed using The Unscrambler, version 7.6 SR1 (CAMO, Oslo, Norway) for each of the studied mycotoxins and for the sum of $FB_1$+$FB_2$. Using PLS-1, collinearity problems were avoided.[30] The mean reflectances obtained from each image were considered the explanatory variables (*X*), whereas the DON, $FB_1$, $FB_2$ or $FB_1$+$FB_2$ concentration was the dependent variable (*Y*). The full spectra were employed. Data were used raw or pretreated using full multiplicative scattering correction (MSC), standard normal variate (SNV), Savitzky Golay first derivative, Savitzky Golay second derivative, or combinations of those pretreatments. Both derivatives were calculated using three smoothing points and a second order polynomial. Pretreatment of NIR data is commonly used to reduce baseline variations, dimensionality and noise problems, improving prediction performance.[31,32] Leave-one-out cross-validation was used as the validation method. For each regression model, a maximum of 10% of the original spectra were removed as outliers so that the representativeness of the calibration set was not lost. Outliers were samples that did not properly fit the model, with high leverage and high residual Y variance. The criteria used to choose the optimal number of PLS-factors for each model was the PLS-factor number where the first minimum of the root mean square error of cross validation (RMSECV) occurred. A good model is mainly characterized by a good correlation between its predictions and the reference values. The performance parameters used to evaluate the fitness of the models were the *r*, $R^2$, number of PLS-factors, RMSECV and ratio of performance to deviation (RPD). The RPD was calculated

as the ratio between the SD of the predicted variable and the RMSECV. The best models were considered those with the closest to 1 for *r* and $R^2$ and the lowest RMSECV, RPD and number of PLS factors.

### 5.3.5 Classification model building for DON, $FB_1$ and $FB_2$

The classification models were built from the 294 hyperspectral images obtained from 98 the maize samples. Classification tests were carried out on Quasar, version 1.7.0.[33] We aimed to obtain different classification models to classify maize samples into two categories, depending on whether they were under or above a specific threshold for each mycotoxin or group of mycotoxins studied. The selected thresholds were the EU legal limits for DON and $FB_1$+$FB_2$ for maize kernels intended for human consumption.[16] Classification models were built for DON (under or above 1.75 mg DON $kg^{-1}$ maize), for the sum of $FB_1$+$FB_2$ (under or above 4 mg $FB_1$+$FB_2$ $kg^{-1}$ maize) and for both DON and the sum of $FB_1$+$FB_2$ (samples were considered under the legal limit only if both DON and the sum of $FB_1$+$FB_2$ were under their respective legal limits; otherwise, they were considered above the limit). Four different classification methods (random forest, neural network, logistic regression and *k*-nearest neighbors) were tested to classify maize samples. In the *k*-nearest neighbors classification, five neighbors, Euclidean metrics and uniform weights were used. The full spectra were employed. Data were used raw or pretreated using the same pretreatments that were tested to develop the obtained regression models. Leave-one-out cross-validation was used as the validation method. The best classification models were considered those with higher classification accuracy, sensitivity, specificity and balanced accuracy. Those parameters were calculated from the obtained

confusion matrices (see example in Table 5) according to Equations (3) to (6), respectively.

*Table 5 Example of a confusion matrix*

| | | Predicted | |
|---|---|---|---|
| | | Positive | Negative |
| Actual | Positive | True Positives (TP) | False Negatives (FN) |
| | Negative | False Positives (FP) | True Negatives (TN) |

$$Classification\ accuracy = (TP + TN)/(TP + TN + FP + FN) \quad (3)$$

$$Sensitivity = TP/(TP + FN) \quad (4)$$

$$Specificity = TN/(TN + FP) \quad (5)$$

$$Balanced\ accuracy = (Sensitivity + Specificity)/2 \quad (6)$$

## 5.4 Results and discussion

### 5.4.1 Mycotoxin distribution in maize samples

The mycotoxin distribution of the analyzed maize kernels can be seen in Table 6. Of a total of 98 samples, only six surpassed the EU legal limit of 1.75 mg DON $kg^{-1}$ unprocessed maize.[16] In reference to the EU legal limit of 4 mg $FB_1$+$FB_2$ $kg^{-1}$ unprocessed maize,[16] 24 samples exceeded it. Histograms of DON, $FB_1$, $FB_2$ and $FB_1$+$FB_2$ contamination of the analyzed maize samples can be seen in the Supplementary Figures (Figures S1, S2, S3 and S4) (see Annexes).

*Table 6 Mycotoxin contamination distribution (mg kg$^{-1}$) of the analyzed maize*

| Mycotoxin | Min. | Max. | Mean | SD |
|---|---|---|---|---|
| **DON** | <LOD | 18.622 | 0.947 | 2.462 |
| **$FB_1$** | <LOD | 37.591 | 3.561 | 7.555 |
| **$FB_2$** | <LOD | 27.066 | 2.482 | 5.927 |
| **$FB_1$+ $FB_2$** | <LOD | 63.891 | 6.043 | 13.420 |

### 5.4.2 Overview of the recorded spectra of the samples

To obtain an overview of the potential relationship between the mycotoxin contamination of the samples and their respective recorded spectra, we selected 16 maize samples of different contamination degrees: uncontaminated, DON contaminated, $FB_1$+$FB_2$ contaminated and $FB_1$+$FB_2$ highly contaminated. The spectra of the samples belonging to each class were averaged and represented (Fig. 10). The uncontaminated samples presented higher reflectance values than the contaminated samples in the entire recorded spectral range, especially between 950 and 1400 nm. Little difference was observed between the DON-contaminated, $FB_1$+$FB_2$-contaminated and $FB_1$+$FB_2$ highly contaminated groups.

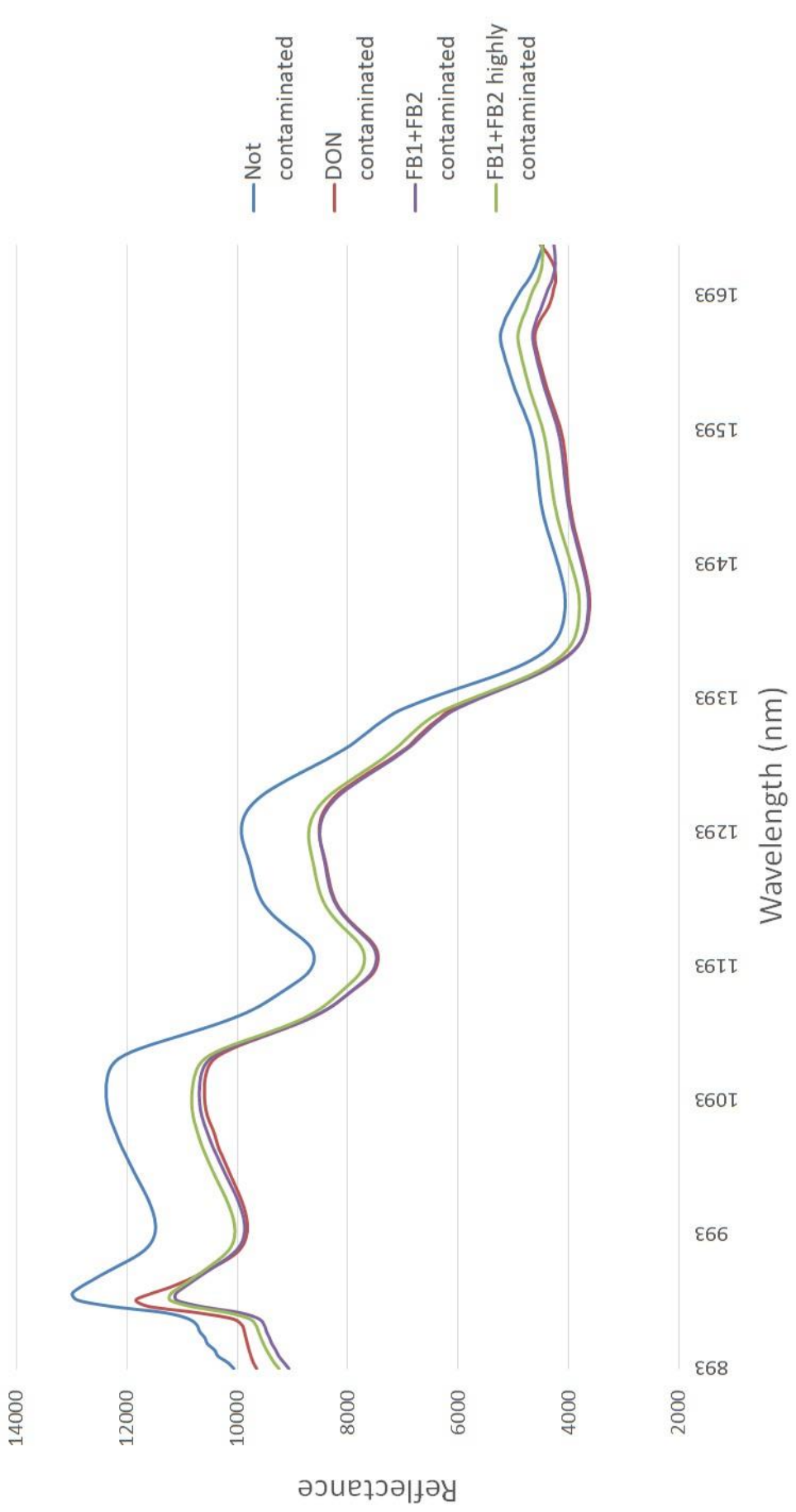


*Fig. 10 Average spectra of samples with different mycotoxin contamination. Average DON, $FB_1$ and $FB_2$ content (mg $kg^{-1}$) for each group: not contaminated: 0.02, <LOD, <LOD; DON contaminated: 8.97, 0.80 and <LOD; $FB_1$+$FB_2$ contaminated: 0.43, 12.15 and 10.00; $FB_1$+$FB_2$ highly contaminated: 1.17, 28.02 and 20.46*

### 5.4.3 Regression models for DON, $FB_1$ and $FB_2$

The performance parameters of the two best regression models obtained for each of the mycotoxins and for the sum of $FB_1$+$FB_2$ can be seen in Table 7. The best regression model for DON was obtained by pretreating the spectra with a full MSC and calculating the first derivative, and the second best model for the same mycotoxin was obtained by applying the same pretreatments in the opposite order. Curiously, in the best regression models for both $FB_1$ and $FB_1$+$FB_2$, no data pretreatment was applied. SNV was the data pretreatment used in the best model to predict $FB_2$ concentration in maize.

Among all the presented models, the optimal number of PLS factors ranged between 15 and 21, except for the best DON prediction model, which needed a total of 24 PLS factors. Similar studies in this field present a comparable number of PLS factors. The best DON model presented a lower RMSECV (0.848 mg $kg^{-1}$) value than the best $FB_1$, $FB_2$ or $FB_1$+$FB_2$ model (3.714, 2.104 and 4.398 mg $kg^{-1}$, respectively), although the RPDs of all models were similar (between 2.018 and 2.344). This can be explained by the different contamination degrees of each mycotoxin, leading to different standard deviations and thus different RMSECVs. In relation to the performance of the reference method, recoveries of DON and $FB_1$+$FB_2$ were previously studied at three different concentrations each.[29] For DON, average recoveries and standard deviations at the concentrations of 2.286, 1.143 and 0.571 mg $kg^{-1}$ were 81.7 ± 9.5, 87.4 ± 13.3 and 91.3 ± 14.5%, respectively. In the case of $FB_1$+$FB_2$, recoveries were studied at the concentrations of 0.855 + 0.855, 0.57 + 0.57 and 0.285 + 0.285 (mg $FB_1$ + mg $FB_2$) $kg^{-1}$. The average recoveries and standard deviations were 82.1 ± 8.7, 86.8 ± 9.2 and 77.0 ± 9.5% for $FB_1$ and 102.6 ± 21.5, 101.6 ± 27.7 and 88.6 ± 27.6%

for $FB_2$. RMSECVs of the best presented models for each toxin are about one order of magnitude higher than the standard deviations of the reference method, confirming the superior precision of HPLC-DAD in front of NIR-HSI. Nevertheless, HPLC-DAD presents the disadvantages of being expensive, time-consuming, environmentally harmful and requiring specialized personnel.

Some authors have conducted similar studies to ours. Gaspardo *et al*.[34] used a Fourier transform (FT)-NIR spectroscope with an integrating sphere (650-2500 nm) to predict the concentration of $FB_1$+$FB_2$ in naturally contaminated ground maize. They determined the concentration of $FB_1$+$FB_2$ using HPLC-fluorescence Detection (FLD). Using PLS regression with a weighted full-cross-validation procedure, they obtained an 18 PLS-factor model with an RMSECV of 2.005 mg $FB_1$+$FB_2$ $kg^{-1}$ and an RPD of 1.2. Tyska *et al*.[35] employed NIR (400-2500 nm) to predict the content of $FB_1$, $FB_2$ and ZEN in naturally contaminated ground maize. They determined the concentrations of $FB_1$ and $FB_2$ using liquid chromatography-tandem mass spectrometry (LC–MS/MS). They used PLS regression with full-cross-validation, testing different data pretreatments, of which the Savitzky Golay second derivative performed best in predicting $FB_1$ and $FB_2$ concentrations. The $FB_1$ model had 18 PLS-factors, an RMSECV of 2.793 mg $kg^{-1}$ and an RPD of 2.028, whereas the $FB_2$ model had 10 PLS-factors, an RMSECV of 1.137 mg $kg^{-1}$ and an RPD of 2.004. They carried out an additional external validation for $FB_1$+$FB_2$ contamination using unknown samples, and the obtained results improved notably compared to those obtained in the full-cross-validation, with RMSEP and RPD values of 0.682 mg $kg^{-1}$ and 3.33, respectively.

*Table 7 Performance parameters of the best regression models obtained for DON, $FB_1$, $FB_2$ and $FB_1$+$FB_2$*

| Toxin | Data pretreatment | *r* | $R^2$ | Number of PLS factors | RMSECV (mg $kg^{-1}$) | RPD |
|---|---|---|---|---|---|---|
| DON | Full MSC + 1st derivative | 0.904 | 0.819 | 24 | 0.848 | 2.344 |
| | 1st derivative + full MSC | 0.839 | 0.704 | 17 | 1.357 | 1.835 |
| $FB_1$ | None | 0.868 | 0.755 | 15 | 3.714 | 2.018 |
| | SNV | 0.832 | 0.693 | 19 | 4.17 | 1.802 |
| $FB_2$ | SNV | 0.901 | 0.812 | 19 | 2.104 | 2.301 |
| | Full MSC | 0.887 | 0.787 | 18 | 2.325 | 2.162 |
| $FB_1$+$FB_2$ | None | 0.901 | 0.813 | 19 | 4.398 | 2.305 |
| | Full MSC | 0.889 | 0.792 | 21 | 5.695 | 2.186 |

RPD is probably the best parameter to compare the performance of different regression models because it takes into account not only its RMSEP, but also its SD. Judging by the RPD, our regression model for $FB_1$+$FB_2$ is better than that presented by Gaspardo *et al*.,[34] and our regression models for $FB_1$ and $FB_2$ are similar to those obtained by Tyska *et al*.[35] in the full-cross-validation. Performance differences between the models of these three studies could be attributed to the following: (i) Gaspardo et *al*.[34] and Tyska *et al*.[35] used a wider spectral range, including spectra belonging to the visible region, and therefore collected more information about the samples; (ii) different data pretreatments were applied in each study; and (iii) we analyzed whole maize kernels, while Gaspardo *et al*.[34] and Tyska *et al*.[35] analyzed ground maize. It is important to consider that grinding maize is an irreversible operation that requiring time and shortening the shelf life of the cereal since it makes nutrients more easily available to microorganisms.

Other comparable studies have been have carried out, but analysing artificially contaminated maize. Bolduan *et al*.[36] used NIR (1100-2500 nm) to determine DON and fumonisins in ground maize that had been previously silk contaminated with *F. graminearum* and *F. verticillioides*. The mycotoxins were determined using an ELISA. SECV and RPD (calculated as SD/SECV) were 0.50 mg $kg^{-1}$ and 2.80 for DON and 1.04 mg $kg^{-1}$ and 1.37 for fumonisins, respectively. The regression model for DON presented by Bolduan *et al*.[36] has a much higher RPD than our model, but it should be taken into account that artificially infecting maize with a pair of fungi will most likely lead to a simplified simulation of the full range of biochemical changes that maize can undergo when infected under natural conditions. On the other hand, their regression model for fumonisins had a much lower RPD than ours. According to Bolduan *et al*.,[36] the lower potential to estimate

the fumonisins content compared to the DON content may be related to the number and distribution of samples in the calibration set and the degree of association between symptomatology and mycotoxin production.

Another study using artificially contaminated maize is that of Miedaner *et al*.[37] They employed NIR (1100-2500 nm) to determine DON and ZEN in ground maize from two different backcross populations. Before grounding, the maize was silk infected with *F. graminearum*. The mycotoxins were analyzed by immunotests. It was found that the DON concentration measured by immunotest and the DON concentration measured by NIR were significantly correlated (*r* of 0.967 and 0.861, for each backcross population).

#### 5.4.3.1 Most relevant spectral areas for the built regression models

For each of the regression models presented, the most relevant spectral areas were determined. To do so, the regression coefficients of each spectral band (explanatory variables) for each regression model were examined. The spectral ranges with higher regression coefficients were those providing more information about mycotoxin contamination, as those were those that presented greater differences between non-contaminated and contaminated maize. Differences in the spectral signature of different samples imply differences in their chemical composition.

We found that all regression models for $FB_1$, $FB_2$ or $FB_1$+$FB_2$ presented three spectral areas of importance in common: 1129-1139 nm, 1168-1183 nm and 1198-1213 nm. Other relevant spectral areas were identified in the fumonisin models, but they were not present in all of them (1057-1067 nm), or their ranges presented slight differences between models (1634-1666 nm, 1671-1687 nm). DON regression models also presented spectral regions of interest in common,

situated at 1120-1125 nm, 1257-1282 nm, 1363-1368 nm, 1465-1486 nm, 1645-1655 nm, 1666-1671 nm and 1682 nm. A graphic representation of the regression coefficients of the presented fumonisin models and DON models can be seen in Supplementary Figures (Fig. S5 and Fig. S6) (see Annexes). All regression coefficients of the cited models are also provided in Supplementary Tables (Table S1) (see Annexes).

### 5.4.4 Classification models for DON, $FB_1$ and $FB_2$

The performance parameters of the two best classification models for each studied mycotoxin or group of mycotoxins can be seen in Table 8*Table 8*. All the models presented in this table were developed using a neural network. The presented metrics range from 0 to 1, representing the worst and the best possible scenarios in each case. Classification accuracy is perhaps the most widely used classification metric. The best DON, $FB_1$+$FB_2$ and DON+$FB_1$+$FB_2$ models gave classification accuracies of 0.986, 0.844 and 0.898, respectively. Although these classification accuracies are considerably high, especially in the case of DON, it is important to take into account that classification accuracy can be a misleading performance parameter when data are imbalanced.[38] Because most of the analyzed samples were under the EU mycotoxin legal limits for both DON and $FB_1$+$FB_2$, the obtained models might have been biased toward the more frequent class, leading to higher specificities and lower sensitivities. This might have happened particularly in the case of DON, where only six samples out of 98 were above the legal limit, whereas, in the case of $FB_1$+$FB_2$, there were 24 samples out of 98 above the legal limit. For this reason, classification accuracy might be an optimistic classification parameter for our data, while balanced accuracy,

which does not take into account the different number of representatives from each class, would be a better classification metric. Thus, the balanced accuracies for the best DON, $FB_1$+$FB_2$ and DON+$FB_1$+$FB_2$ models were 0.889, 0.773 and 0.865, respectively. Among all the presented models, specificity ranged between 0.933 and 1, whereas sensitivity ranged between 0.607 and 0.778. In general terms, this means that the developed models will very rarely misclassify negative samples but sometimes will misclassify positive samples.

With reference to mycotoxin rapid tests, comprising the most popular analysis methods in the food industry, we could find little information on their sensitivity and specificity when analysing naturally contaminated samples. Aamot et al.[39] tested the accuracy of two ELISA tests and two lateral flow immunoassays for classifying naturally contaminated wheat and oats, using LC-MS/MS as a reference method. The classification thresholds were 1.25 and 1.75 mg $kg^{-1}$ for wheat and oats, respectively. For wheat, the average sensitivity and specificity of the four tests were 0.82 and 0.69, respectively, whereas, in the case of oats, the values were 0.86 and 0.74, respectively. Our best classification model has a sensitivity and a specificity of 0.778 and 1, respectively. Any comparison of the results obtained by Aamot et al.[39] and those obtained by ourselves must be made with caution because different cereals were analysed. Taking that into account, our model presented a slightly lower sensitivity but a much superior specificity than those presented in the abovementioned study. Overall, our model presented a higher balanced accuracy than any of the tests reported by Aamot et al.[39] In addition, NIR-HSI is even faster than ELISA tests or lateral flow immunoassays.

*Table 8 Performance parameters for the best classification models obtained for DON, $FB_1$+$FB_2$ and DON+$FB_1$+$FB_2$*

| Toxin | Data pretreatment | Model | Classification accuracy | Sensitivity | Specificity | Balanced accuracy |
|---|---|---|---|---|---|---|
| DON | 1st derivative + full MSC | Neural Network | 0.986 | 0.778 | 1 | 0.889 |
| | Full MSC + 1st derivative | Neural Network | 0.98 | 0.722 | 0.996 | 0.859 |
| $FB_1$+ $FB_2$ | Full MSC | Neural Network | 0.844 | 0.607 | 0.938 | 0.773 |
| | SNV | Neural Network | 0.84 | 0.607 | 0.933 | 0.77 |
| DON+ $FB_1$+$FB_2$ | Full MSC + 1st derivative | Neural Network | 0.898 | 0.774 | 0.955 | 0.865 |
| | 1st derivative + full MSC | Neural Network | 0.878 | 0.753 | 0.935 | 0.844 |

Similar studies to ours that also classify maize according to its mycotoxin content have been published. Levasseur-Garcia & Kleiber[40] used visible-NIR reflectance spectra (400-2498 nm) to classify 200 g samples of naturally contaminated maize kernels under or above different classification thresholds, both for DON and for fumonisins. The thresholds included the legal limits for unprocessed maize in the EU, 1.75 mg $kg^{-1}$ for DON and 4 mg $kg^{-1}$ for fumonisins. They used various data preprocessing methods and applied three different classification methods (discriminant analysis, quadratic discriminant analysis and Mahalanobis discriminant analysis). The classification model with the highest classification accuracy for DON applying the 1.75 mg $kg^{-1}$ threshold used raw data and quadratic discriminant analysis, giving a classification accuracy, a sensitivity, a specificity and a balanced accuracy of 0.730, 0.764, 0.696 and 0.730, respectively. The classification model with the highest classification accuracy for fumonisins applying the 4 mg $kg^{-1}$ threshold used first order derived data and discriminant analysis, giving a classification accuracy, a sensitivity, a specificity and a balanced accuracy of 0.697, 0.668, 0.720 and 0.694, respectively. These models have a lower classification performance than ours, probably because different classification methods and data pretreatments were used in both studies.

Giacomo & Stefania[41] used an FT-NIR spectrometer (650-2500 nm) to classify maize meal samples according to whether their $FB_1$+$FB_2$ concentration was under or above 4 mg $kg^{-1}$. They determined the concentration of $FB_1$+$FB_2$ using HPLC-FLD. Among the two models that they presented, the better one had a classification accuracy of 0.311. The model was based on 45 samples, with 26 false-positives and 5 false-negatives. This low classification accuracy could be

mainly attributed to the fact that they used PLS as a classification method, whereas it is generally employed to perform regression.

Levasseur-Garcia *et al*.[42] used visible-NIR (400-2498 nm) spectroscopy to classify whole maize kernels according to their $FB_1$ concentration using an unusual approach. They recorded the NIR spectra and measured the fungal biomass [colony-forming units (CFU) $g^{-1}$], ergosterol (mg $kg^{-1}$) and $FB_1$ concentration (mg $kg^{-1}$) of maize samples. Then, they created a decision tree to classify samples depending on whether their $FB_1$ concentration was above or below 4 mg $kg^{-1}$, using ergosterol and fungal biomass as explanatory variables. The attribute that led to the best split was consideration of whether the sample had more or less than $2\times10^5$ CFU $g^{-1}$. Next, NIR spectra were analyzed by principal components analysis, and its first six principal components were used in a quadratic discriminant analysis to determine if the fungal count of the sample was above or below the threshold of $2\times10^5$ CFU $g^{-1}$ (and indirectly, to determine whether the $FB_1$ concentration was above or below 4 mg $kg^{-1}$). On an independent verification, discriminant analysis based on NIR to sort maize samples according to their fumonisin contamination gave a classification accuracy, a sensitivity, a specificity and a balanced accuracy of 0.824, 0.800, 0.833 and 0.817, respectively. Although using a completely different approach than ours, Levasseur-Garcia *et al*.[42] achieved a similar classification performance, with a lower specificity but a higher sensitivity and balanced accuracy.

Other experiments similar to ours have been performed, but using different ranges of the spectra. Kos *et al*.[43] employed FTIR-ATR from 5555 to 12500 nm to classify maize meal according to its DON contamination. They used naturally

contaminated maize and maize artificially contaminated with *F. graminearum*, *F. verticillioides* and *F. culmorum*. DON was determined by LC–MS/MS. Using a bootstrap-aggregated decision tree and a classification threshold of 1.75 mg $kg^{-1}$, they achieved a classification accuracy of 0.73, and eliminating the *F. verticillioides*-infected samples from the model raised the classification accuracy to 0.79, with the sensitivity, specificity and balanced accuracy being 0.92, 0.50 and 0.71, respectively. Kos *et al*.[43] obtained a model with a higher sensitivity than ours but with a poor specificity.

Although many studies use NIR or similar types of radiation to classify maize kernels or maize meals according to their mycotoxin contamination, other studies have focused on analyzing single maize kernels. As has been seen in studies such as that of Pearson *et al*.,[44] the mycotoxin distribution in single maize kernels is positively skewed. Most maize kernels will have low mycotoxin contamination, whereas a small percentage of kernels will be highly contaminated. Thus, by removing only the most contaminated kernels from a batch, large reductions in overall contamination could be achieved. In this way, Chavez *et al*.[45] used UV–visible-NIR (304-1086 nm) to classify naturally contaminated single maize kernels from nine different bulk samples according to their aflatoxin and fumonisin concentrations. Mycotoxin concentrations were determined using ELISA. Algorithms were trained on 70 % of the kernels, whereas the remaining 30 % were used for testing. In the case of fumonisin, using a classification threshold of 2 mg $kg^{-1}$, the best model obtained was a penalized discriminant analysis, with a testing classification accuracy, sensitivity, specificity and balanced accuracy of 0.86, 0.78, 0.87 and 0.825, respectively. In comparison with our best model to classify maize samples according to their $FB_1$+$FB_2$ contamination, Chavez *et al*.[45]

obtained a lower specificity but a higher sensitivity and balanced accuracy. It should be taken into account, however, that single kernel analysis is time-consuming in comparison to batch analysis. In this sense, some investigations have worked to achieve high-speed sorters that could be implemented online. For this to occur, the complexity of the models has to be drastically reduced, which can be done using few wavelengths.

Pearson *et al*.[46] used a diode-array NIR spectrometer (500-1700 nm) to record reflectance spectra of artificially contaminated single maize kernels and then measured their aflatoxin and total fumonisin contamination ($FB_1$+$FB_2$+$FB_3$). The best pair of absorbance bands for separating aflatoxin- and fumonisin-contaminated maize were selected (750 and 1200 nm). Good separation of kernels was achieved only between very high and very low aflatoxin- or fumonisin-contaminated maize. Regarding fumonisin contamination, all kernels with <1 mg $kg^{-1}$ were classified as uncontaminated, and all kernels with >100 mg $kg^{-1}$ were classified as contaminated; however, 95 % of kernels contaminated with 1-10 mg $kg^{-1}$ and 50 % of the kernels with 10-100 mg $kg^{-1}$ were classified as uncontaminated. Then, the selected pair or absorbance bands were applied in a high-speed commercial sorting machine to separate aflatoxin- and fumonisin-contaminated single maize kernels. The sorter was capable of processing 300 kg maize $h^{-1}$, and both naturally and artificially contaminated maize were used. Despite the poor performance of classifying fumonisin-contaminated kernels in the range of 10-100 mg $kg^{-1}$, fumonisin reductions between 75.8 % and 97.5 % were achieved, depending on the maize used and the established thresholds.

## 5.5 Conclusions

In this study, NIR reflectance spectra (893-1731 nm) were used to create regression models to predict the concentration of the mycotoxins DON, $FB_1$, $FB_2$ or $FB_1$+$FB_2$ in maize kernel samples. The best model for predicting DON had an RMSECV and an RPD of 0.848 mg $kg^{-1}$ and 2.344, respectively, whereas for the best $FB_1$ prediction model, those values were 3.714 mg $kg^{-1}$ and 2.018, respectively. For the best $FB_2$ prediction model, the RMSECV and RPD were 2.104 mg $kg^{-1}$ and 2.301, respectively. The best $FB_1$+$FB_2$ regression model had an RMSECV and an RPD of 4.398 mg $kg^{-1}$ and 2.305, respectively.

All regression models for $FB_1$, $FB_2$ or $FB_1$+$FB_2$ presented three spectral areas with higher regression coefficients: 1129-1139 nm, 1168-1183 nm and 1198-1213 nm. DON regression models also had spectral regions with major regression coefficients, specifically at 1120-1125 nm, 1257-1282 nm, 1363-1368 nm, 1465-1486 nm, 1645-1655 nm, 1666-1671 nm and 1682 nm.

Classification models to sort maize samples below or above the EU legal limits for DON and $FB_1$+$FB_2$ were also developed. For DON, the best classification model presented a sensitivity, specificity and balanced accuracy of 0.778, 1 and 0.889, respectively. For the best $FB_1$+$FB_2$ classification model, those values were 0.607, 0.938 and 0.773. An additional type of classification model was created to sort the maize kernel samples depending on whether they complied with both the DON and $FB_1$+$FB_2$ EU limits. The best performing model under these conditions presented a sensitivity, a specificity and a balanced accuracy of 0.774, 0.955 and 0.865, respectively.

As demonstrated, NIR-HSI can help reduce DON, $FB_1$ and $FB_2$ contamination in the maize food and feed chain. Improvements in NIR-HSI equipment, a better

understanding of the relationship between mycotoxin contamination and changes in the spectrum, advances in chemometrics and faster mechanical sorters can be a step forward toward this goal.

## 5.6 Acknowledgements

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## 5.7 Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## 5.8 Supporting information

Supporting information may be found in the online version of this article.

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# Chapter 6. Deoxynivalenol degradation in wheat kernels by exposition to ammonia vapours: A tentative strategy for detoxification.

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## 6.1 Abstract

Deoxynivalenol (DON) is a mycotoxin produced mainly by *Fusarium* species and occurs predominantly in cereal grains such as wheat. Due to its toxic effects, in the European Union DON content in unprocessed cereals and processed cereal-based products for human consumption has been regulated, and recommended maximum limits have been established for animal feed. In this study, a method for degrading DON on wheat kernels, by exposition to ammonia ($NH_3$) vapours, was optimized. Results have shown that with a simple treatment with ammonia vapours at 90 °C (for 2 h), degradations higher than 75 % were achieved in kernels affected by a moderated contamination up to 2000 µg/kg DON. The study of the reaction between DON and $NH_3$ allowed us to tentatively establish the structure of possible degradation products. In addition, *in silico* evaluation indicated, in general, lower toxicity and biological effects for the degradation products than for DON.

**Keywords**: DON; *Fusarium* toxin; Mycotoxin; Chemical treatment; Detoxification; Degradation products

## 6.2 Introduction

Nowadays, the need to control toxins in food (and feed) products is generalized over the world. In the case of mycotoxins, due to their high toxicity, food producers establish strict controls for the raw materials used for food production. Deoxynivalenol (DON) is a trichothecene produced mainly by *Fusarium* species and occurs predominantly in cereal grains such as wheat, barley, oats, rye and maize. DON is the most commonly detected trichothecene in cereal grains and is also frequently found in higher concentrations than other mycotoxins (Shi, Schwab, & Yu, 2019; Stanciu et al., 2019). The consumption of contaminated wheat and wheat-based products appears to be a significant source of human exposure to DON (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018). The main concern regarding DON is its chronic toxicity, which can lead to weight loss, anorexia and loss of nutritional efficiency (Payros et al., 2016; Pestka, 2007). DON is also considered a major cause of economic losses in animal husbandry (Morgavi & Riley, 2007). The European Union (EU) regulation for contaminants in food products limits the DON content in unprocessed cereals and processed cereal-based products between 500 and 1750 µg/kg, lowering the limit to 200 µg/kg in case of processed cereal-based foods and baby foods for infants and young children. Recommendations on DON limits for products intended for animal feeding are also established in the EU, ranging from 900 to 12000 µg/kg (European Community, 2006a, 2006b).

Although in the food industry DON contaminated batches of raw materials are usually discarded, there is an ongoing research focused on the effects of common and novel food processing techniques on the DON content of food products, especially in cereals and derived products (Karlovsky et al., 2016). In relation to

the usual food processing treatments, much attention has been paid to thermal processing, particularly to baking (Wu, Kuča, Humpf, Klímová, & Cramer, 2017). DON degradation has also been studied by other thermal treatments like frying (Moazami Farahany & Jinap, 2011), boiling (Vidal, Bendicho, Sanchis, Ramos, & Marín, 2016) or steaming (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007).

Although for the most common use of cereals and flours a thermal treatment could be acceptable, sometimes the properties of the raw product are desired to remain unchanged. In this way, studies using approaches like UV or pulsed light for degrading DON in cereal grains have been carried out (Chen et al., 2018; Murata, Yamaguchi, Nagai, & Shimada, 2011; Popović et al., 2018). However, taking into account the contamination heterogeneity of cereal grain batches and the limited effect of light treatments in solids (zone of light incidence and shadow zones), these approaches seem more suitable for the treatment of liquid and more transparent food products. Similar limitations can be attributed to other modern techniques such as the cold plasma (Hojnik et al., 2019; Ten Bosch et al., 2017).

As an alternative to these “non-thermal” techniques, the use of chemical compounds in gas state would allow a more intense contact with the product, increasing in this way the effectiveness of the treatments. Wang et al. (2016) applied ozone on 11.8 % moisture content wheat kernels, achieving a 42.0 % DON degradation in 1 h. Although these results are interesting, the use of ozone presents some important limitations related with the “in situ” generation requirements and that it is not possible to store it. In this sense, a more simple approach to treat wheat grains could be the use of ammonia ($NH_3$), that can be easily stored as gas (in pressurized bottles) or in water solution as ammonium

hydroxide. In addition, regarding the safety of the use of ammonia treatments in food processing, it is important to say that ammonium ion is present in the common rising agent ammonium carbonate (E−503), an additive allowed by the EU in processed cereal-based foods (European Community, 2008).

The use of ammonia has been evaluated for degrading mycotoxins, aflatoxins being the most studied ones. Ammonia aflatoxin degradation has been studied in matrices such as corn, wheat, cotton and peanut (Brekke, Peplinski, & Lancaster, 1977; Chelkowski et al., 1981; Gardner Jr., Koltun, Dollear, & Rayner, 1971; Mann, Codifer Jr., Gardner Jr., Koltun, & Dollear, 1970; Weng, Park, & Martinez, 1994). However, studies with other mycotoxins are really scarce. In the case of DON, only very preliminary studies have been published. Thus, Young, Subryan, Potts, McLaren, and Gobran (1986) treated wheat with 5 % ammonium hydroxide solution (600 ml/kg wheat), obtaining a 35 % DON reduction. The effectiveness of the ammoniation process depends on the treatment conditions (temperature, pressure, time) and also on the contaminated product (Samarajeewa, Sen, Cohen, & Wei, 1990). Among those factors, temperature has considerable importance. Young (1986) observed that treating DON-contaminated corn with ammonium carbonate was more efficient at 132 °C (92 % degradation) than at 100 °C or 70 °C (86 % and 12 % degradation, respectively). Other authors have also previously observed that detoxification of other mycotoxins by ammoniation is more effective when temperatures are increased (Brekke et al., 1977; Chelkowski et al., 1981; Weng et al., 1994).

Therefore, the aim of this work was to study the DON degradation on wheat kernels by ammoniation, optimizing the processing conditions of temperature and ammonia concentration. In addition, studies on the impact of the initial DON

concentration and treatment time on DON degradation were carried out. Possible DON degradation compounds were evaluated and its toxicity was *in silico* estimated.

## 6.3 Materials and methods

### 6.3.1 Chemicals

DON was bought from Romer Labs (Tulln, Austria). Methanol HPLC grade, acetonitrile HPLC gradient grade and sodium chloride were from Fisher Scientific UK Limited (Loughborough, UK) and $NH_4OH$ was bought from Scharlab (Barcelona, Spain).

### 6.3.2 Samples and kernel contamination

Wheat kernels (11.24 $\pm$ 0.01 % moisture content), with a DON contamination below 11.3 µg/kg (LOD of the method), were kindly donated by Aragonesa de Harinas S.A. (Regany group). DON contaminated wheat kernels were prepared by adding an aqueous solution of DON to DON-free wheat kernels. To do that, 12.5 g of wheat kernels were put in a 50 ml Falcon tube, and 0.5 ml of DON standard aqueous solution were added to them by pipetting 50 µl of solution a total of 10 times, closing the tube and agitating it between additions to ensure an homogenous distribution of the toxin amongst the kernels. Final moisture content of the kernels increased by approximately 4 % (0.5 ml added to 12.5 g). This procedure was repeated for each fortified sample.

### 6.3.3 System for ammonia treatment of kernels

To study the effect of $NH_3$ treatments on DON-contaminated wheat kernels, the structure sketched in Supplementary Fig. S7 (see Annexes) was designed. Briefly, in a 430 ml canned food glass jar, a glass Petri dish containing $NH_4OH$ solution (2 ml) was placed at the bottom. The DON contaminated wheat kernels (12.5 g) were placed on a wire mesh 5 cm above the Petri dish allowing the contact with the $NH_3$ vapours on the headspace of the jar. The glass jar was hermetically sealed with a jar lid. The jar was heated in a hot air oven (JP Selecta 210, JP Selecta S.A., Abrera, Spain) for a specific temperature and time. After the heat treatment, wheat was left to aerate in a laboratory fume hood for 15 min to remove residual ammonia. Samples were weighted after the treatment in order to control moisture content variations. DON was analysed according to sections 6.3.6 and 6.3.7.

### 6.3.4 Experimental design for DON degradation with ammonia

According to the scarce literature and our preliminary tests, temperature and $NH_4OH$ solution concentration were chosen as the factors for the optimization of DON degradation. The selected temperature range (from 65 to 115 °C) was based on the experiments of Young (1986) and Weng et al. (1994). The DON concentration of kernels was 500 µg/kg. A 3-level-2-factor central composite design (CCD) with face centered axial points ($\alpha=\pm1$) and three replicates of the center point was designed. The conditions for the 11 runs of the CCD and the results for DON degradation (expressed in %) are shown in Table 9. Three different temperatures (65, 90 and 115 °C) and three different $NH_4OH$ concentrations (1.6, 3.2 and 4.8 %) were assayed. $NH_4OH$ concentrations are expressed as % of $NH_4OH$ respect to the wheat sample weight, and were

prepared by pipetting 2 ml of 10, 20 and 30 % $NH_4OH$ stock solution into the Petri dish. The treatment time was established in 2 h based on preliminary tests. All DON degradation values are relative values, and were calculated respect to non-treated samples (reference samples). RSD of reference samples was <2.8 %. In addition, negative controls for each temperature (analysis of the DON-contaminated wheat, replacing $NH_4OH$ solution by water) were conducted. Reference samples and negative controls were done in duplicate.

*Table 9 Central composite design 3-level-2-factors with face centered axial points (AP; α =±1) and three replicates of the center point (C) for the optimization of the treatment for DON degradation. The factors of the experimental design are: Temperature (Temp) and $NH_4OH$ concentration (%)[a]. Samples were fortified with 500 µg/kg of DON.*

| Run | Temp. (ºC) | $NH_4OH$ Conc. (%)[a] | DON degradation (%) |
|---|---|---|---|
| 1 | 65 | 1.6 | 12.78 |
| 2 | 65 | 4.8 | 34.56 |
| 3 | 115 | 1.6 | 48.95 |
| 4 | 115 | 4.8 | 69.73 |
| 5 (AP) | 65 | 3.2 | 25.54 |
| 6 (AP) | 115 | 3.2 | 67.45 |
| 7(AP) | 90 | 1.6 | 39.75 |
| 8(AP) | 90 | 4.8 | 74.57 |
| 9 (C) | 90 | 3.2 | 68.56 |
| 10 (C) | 90 | 3.2 | 66.16 |
| 11 (C) | 90 | 3.2 | 66.62 |

[a] Expressed as % of $NH_4OH$ respect to the wheat sample weight.

Response surface methodology was used to model and optimize DON degradation (%) according to Equation (7), where Y is the value estimated with the model, and $b_{i,j}$ the regression coefficients (0 is the intercept, 1 is temperature

(T) and 2 is $NH_4OH$ concentration) that include the lineal and quadratic effects and the two-way interaction.

$$Y = b_0 + b_1 \cdot T + b_2 \cdot NH_4OH + b_{1,1} \cdot T^2 + b_{2,2} \cdot NH_4OH^2 + b_{1,2}\ T \cdot NH_4OH \quad (7)$$

Taking into account the slow heat transfer into the jar, the evolution of the sample temperature was recorded during the treatment for each temperature assayed. A thermobutton (Datalogger 22E, Plug & Track, Willems, France) was placed amidst the wheat kernels, registering the temperature every minute. The evolution of the temperature vs. time for each treatment can be seen in Supplementary Fig. S8 (see Annexes). Total transmitted heat to the wheat were 1.17 kJ, 2.14 kJ and 3.24 kJ for 65, 90 and 115 °C treatments, respectively. Changes in specific heat of wheat due to different temperature were taken into account (Cao, Li, Zhang, Chen, Li, Zhang, 2010; Jayas & Cenkowski, 2006).

### 6.3.5 Kinetic and effect of toxin concentration studies on DON degradation under optimal conditions

With the optimal DON degradation conditions selected from the data obtained in the experimental design (90 °C and 4.8 % $NH_4OH$), studies on the kinetics of DON degradation and on the effect of DON concentration on its degradation were carried out. All obtained DON degradation values are relative values calculated respect to reference samples.

In the kinetic study, eight glass jars with 12.5 g of contaminated wheat kernels (500 µg/kg DON) were prepared, containing each 4.8 % $NH_4OH$. The jars were held at 90 °C in the hot air oven and were removed from it one by one in different

periods of time (60, 90, 120 and 240 min). Two replicates for each treatment time were carried out.

As for the study of the effect of DON concentration on its degradation, six glass jars were ammonia treated for 2 h at 90 °C, each of them containing also 12.5 g of wheat kernels and 4.8 % $NH_4OH$. The different DON concentrations tested were 200, 500 and 2000 µg/kg. Tests were performed in duplicate.

### 6.3.6 DON extraction from wheat kernels

Samples were prepared according to the study of Zhang et al. (2019) with some modifications. The 12.5 g of wheat were ground in a IKA A11 (IKA®-Werke GmbH & Co. KG, Staufen, Germany) mill during 30 s. 5 g of ground wheat were transferred into a 50 ml Falcon tube, and 1 g of NaCl and 40 ml of milli-Q water were added. The mixture was vortexed for 15 s and ultrasound-treated with the Bransonic M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power during 15 min. After that, the Falcon tubes were centrifuged in a Hettich 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 8965g for 10 min at 20 °C. DonPrep immunoaffinity columns (Biopharm AG, Darmstadt, Germany) were prepared by adding 10 ml of milli-Q water. 8 ml of the supernatant were collected and passed through the immunoaffinity column. After that 1.5 ml of methanol were added to collect the toxin. Backflushing was done three times, and then other 0.5 ml of methanol were passed through the column. The 2 ml of collected methanol were evaporated at 40 °C (Stuart SBH200D/3 block heater, ©Cole-Parmer, Staffordshire, UK) under a gentle stream of $N_2$. The residue was re-suspended in 1 ml of MeOH:$H_2O$ 10/90

(v:v), vortexed, filtered through 0.22 µm PTFE filters and analysed by HPLC-DAD according to section 6.3.7.

### 6.3.7 HPLC-DAD DON analysis

The determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex ®Gemini C18 column (California, USA) was used (150 × 4.6 mm, 5 µm particle size, 110 Å pore size). Absorbance reading was performed at 220 nm. Three mobile phases were prepared: phase A (100 % methanol), phase B (methanol:water 10:90, v:v) and phase C (acetonitrile:water 20:80, v:v). The gradient applied was as follows: 0 min 100 % B; 10 min 60 % B and 40 % C; 13 min 60 % B and 40 % C; 15 min 100 % A; 25 min 100 % A; 29 min 100 % B until 40 min (including the cleaning and reequilibrating of the column). Flow rate was set at 1 ml/min. The column temperature was 40 °C, and the injection volume 50 µl. DON retention time was 10.2 min. LOD and LOQ, considered as three and ten times the signal of the blank, were 11.3 and 37.6 µg/kg. Quantification was carried out by using DON calibration curves prepared in methanol:water 10:90 (v:v). Recovery was assayed in duplicate at three concentrations (100, 400 and 700 µg/kg). Average recovery values were 92.7 $\pm$ 9.7 %. Repeatability and reproducibility of the method were <3.0 % and <16.2 %, respectively.

### 6.3.8 Study of the possible DON-derived formed products

To identify the DON-derived formed product/s the following sample was prepared: an amount of DON standard solution containing 4 µg of pure DON was pippeted into an amber glass vial and evaporated at 40 °C under a gentle stream of $N_2$. The dried vial was then treated with $NH_3$ vapours in a hot air oven like the DON-contaminated wheat kernels in sections 6.3.3 and 6.3.4, but putting only the vial instead of the kernels on the wire mesh. The oven temperature was set at 90 °C, and 4.8 % $NH_4OH$ was added. The vial with DON was treated for a total time of 20 h to ensure total degradation of DON. After that, 1 ml of MeOH:$H_2O$ 10/90 (v:v) was added to the vial to re-suspend the formed products, vortexed, and filtered through 0.22 µm PTFE filters.

After evaluation of the complete DON degradation by injection in the HPLC-DAD system described in section 6.3.7, the degraded sample, and a non-degraded reference sample, were injected in a liquid chromatographic system coupled to a mass spectrometer (LC-MS). The LC-MS system was a Waters Acquity UPLC equipped with a binary pump, an autosampler and a heated column compartment (40 °C). The column was an ACQUITY UPLC® BEH C18 1.7 µm (2.1 × 150 mm) from Waters. The mobile phases were A (water:methanol (50:50)-0.1 % formic acid)) and B (methanol). The system was operated with a flow of 0.35 ml/min and the gradient applied was as follows: 0 min 100 % A; t = 1.5 min 100 % A; t = 3 min 100 % B; t = 3.5 min 100 % B; t = 3.51 min 100 % A. Injection volume was 2.5 µl. The detector was an Acquity TQD tandem quadrupole mass spectrometer. The electrospray ionisation (ESI) source was operated in positive mode. ESI parameters were: desolvation temperature, 300 °C; desolvation gas ($N_2$) flow rate, 800 L/h; cone gas ($N_2$) flow rate, 150 L/h, capillary voltage, 3.5 kV; and

source temperature, 150 °C. The data were acquired from 250 to 350 m/z (MS1-scan mode and collision energy 5 eV). The tentative identification of the degraded products of DON was carried out by studying the possible chemical reactions between DON and $NH_3$ and by searching for the compounds in the chromatograms on the basis of their molecular ions $[M+H]^+$.

### 6.3.9 *In silico* toxicity and biological activity evaluation

For the evaluation of the differences in toxicity and biological activities of the degraded compounds respect to the parental mycotoxin, two web tools were used. Chemical structures and SMILES notations were generated by using ACD labs Chemsketch software (version 2018.2.1). Biological activities and Lipinski's rule were calculated by using the Molinspiration software version 2018.03 (www.molinspiration.com). For the biological activities, higher score values indicate higher activity. The biological activities evaluated were: G protein-coupled receptor (GPCR) ligand; ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor. For evaluating the permeability across the cell membrane of the compounds the Lipinski's rule of five were used. This rule establishes that for a good permeability across the cell membrane the compound must meet: a) molecular weight under 500 Da, b) octanol/water partition coefficient lower than 5 (Log P < 5), c) less than 5 hydrogen bond donors (nitrogen and/or oxygen), and d) less than 10 hydrogen bond acceptors (nitrogen and/or oxygen). The Lipinski's rule establishes that for a compound to be orally active, there must not be more than one violation of this rule (Lipinski, Lombardo, Dominy, & Feeney, 2001). For the evaluation of the toxicity (mutagenic; tumorigenic, irritant, and reproductive system effects) the tool

Osiris property explorer software (www.organicchemistry.org/prog/peo/) were used. This program estimates the toxicity as red (high risk), yellow, and green (low risk or drug-conform behavior).

### 6.3.10 Statistics

One way-ANOVA and least significance difference (LSD) Fisher tests were used to evaluate the effects of the different treatments and sample types. Significance level was established at 0.05 and confidence limits at 0.95. Statistical analyses were carried out by using STATISTICA program for Windows (version 7.1) (StatSoft, Inc., 2005; www.statsoft.com).

## 6.4 Results and discussion

### 6.4.1 Effect of temperature and $NH_4OH$ concentration on DON degradation

The analysis of the results of the experimental design revealed significant effects (ANOVA, $p < 0.05$) for the linear and quadratic components of both temperature and $NH_4OH$ concentration, but not for the two-way interaction between them. Hence, the model was recalculated leaving out of the equation the two-way interaction for greater accuracy. Results of the ANOVA regression coefficients are presented in Supplementary Tables S2 and S3 (see Annexes). Pareto chart is presented in Fig. 11, showing the significant effects affecting DON degradation. As can be seen, the strongest effect was caused by the linear component of temperature, followed by the linear component of $NH_4OH$ concentration and the quadratic component of temperature. The quadratic component of $NH_4OH$ concentration was also significant, but had a considerable lesser relevance in

comparison to the other components. Linear components of both temperature and $NH_4OH$ concentration had a positive effect on DON degradation, while quadratic components of the two variables had the opposite effect. In the corresponding response surface graph (Fig. 12) it can clearly be seen the lineal positive and quadratic negative effects of temperature and $NH_4OH$ concentration. As quadratic components are of lesser importance than lineal ones, DON degradation generally increased with temperature and $NH_4OH$ concentration.

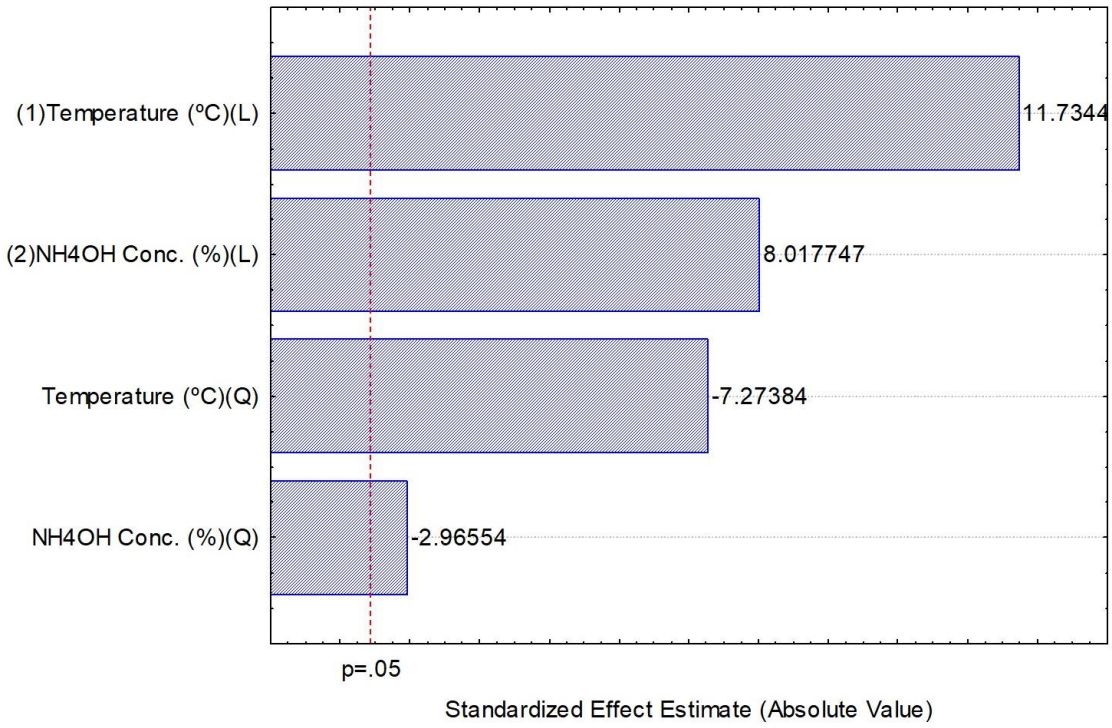


*Fig. 11 Pareto chart of the factors significantly affecting DON degradation. L: linear component. Q: quadratic component.*

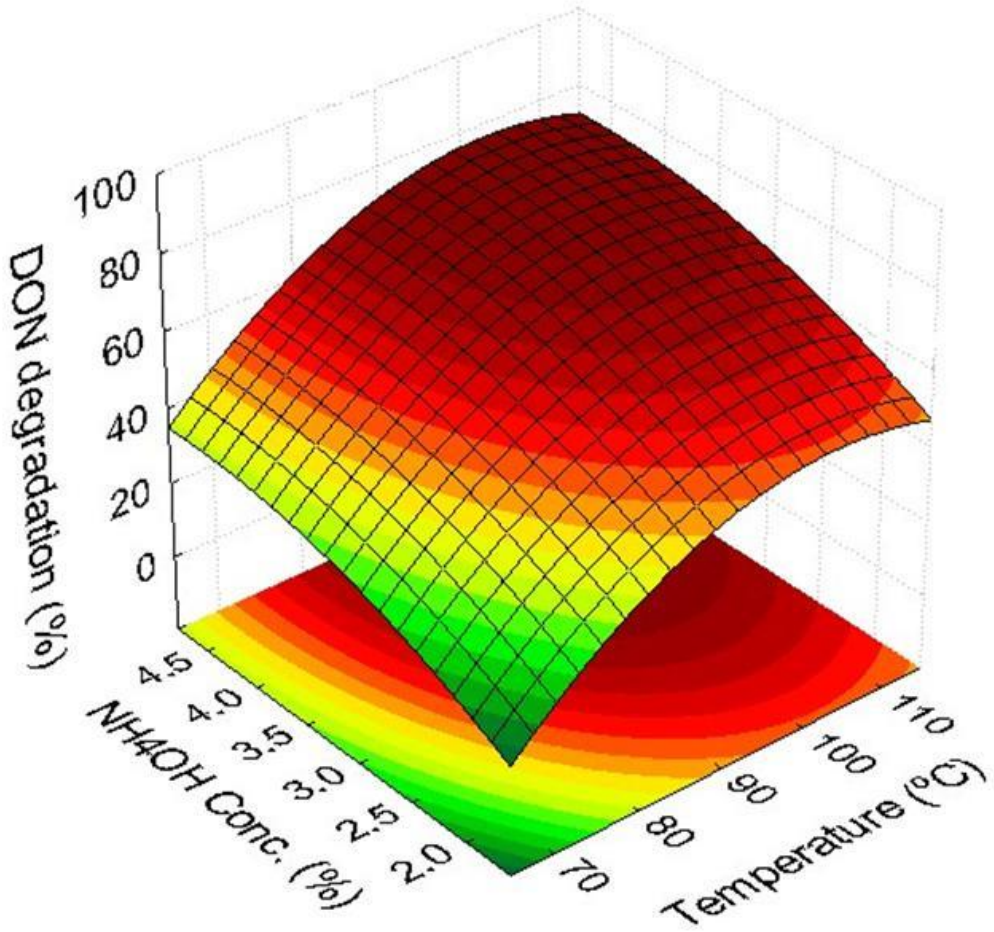


*Fig. 12 Surface plot of the estimated response (DON content reduction (%)) based on the CCD design (effect of temperature and $NH_4OH$ concentration).*

According to the model, highest predicted degradations correspond to treatments of 115 °C with 4.8 % $NH_4OH$ (72.49 % degradation) and 90 °C with 4.8 % $NH_4OH$ (71.62 % degradation). Taking into account the little difference in degradation between treatments, and that heating the media from room temperature to 115 °C is by far more expensive than doing it only up to 90 °C, 90 °C with 4.8 % $NH_4OH$ could be considered the optimal conditions for DON degradation.

Considering that this treatment applies moderate temperatures, the contribution of thermal treatment to DON degradation was evaluated employing two negative controls (DON contaminated wheat samples heated in the oven but with 2 ml of

water instead of 2 ml of $NH_4OH$ solution) for each temperature (65, 90 and 115 °C). The DON degradation percentages at each temperature regarding reference samples (negative controls, relative values) can be seen in Fig. 13. While the 115 °C treatment caused a significant DON degradation (22.05 %), the differences between reference samples and 65 and 90 °C treatments were not significant. Therefore, the degradation observed in ammonia treated samples is mainly caused by the ammonia effect.

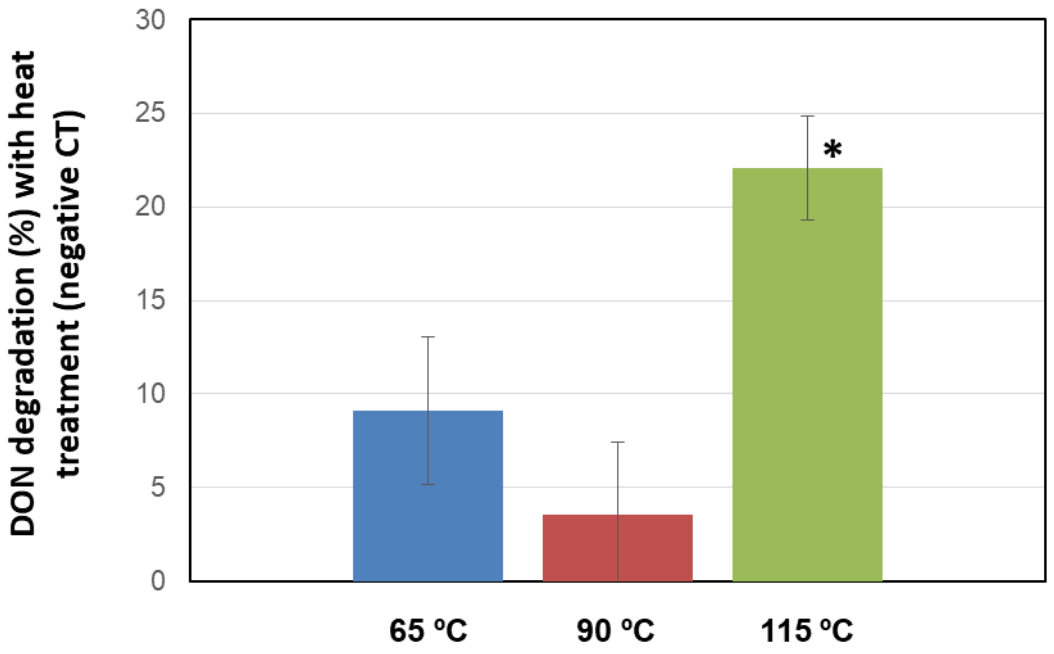


*Fig. 13 DON degradation (%) only due to the thermal treatment (negative control). * Indicates statistical differences in ANOVA respect to reference.*

### 6.4.2 Kinetic and effect of toxin concentration studies on DON degradation under optimal conditions

DON degradation kinetics obtained with the previously optimized conditions are shown in Fig. 14. As can be seen, treating a sample under optimal conditions (4.8 % $NH_4OH$, 90 °C) for a total time of 60 min will lead to a 45.73 % DON reduction. For 120 min, 77.39 % of DON degradation is achieved. Extending the process for another 2 h will increase total DON reduction up to 92.73 %.

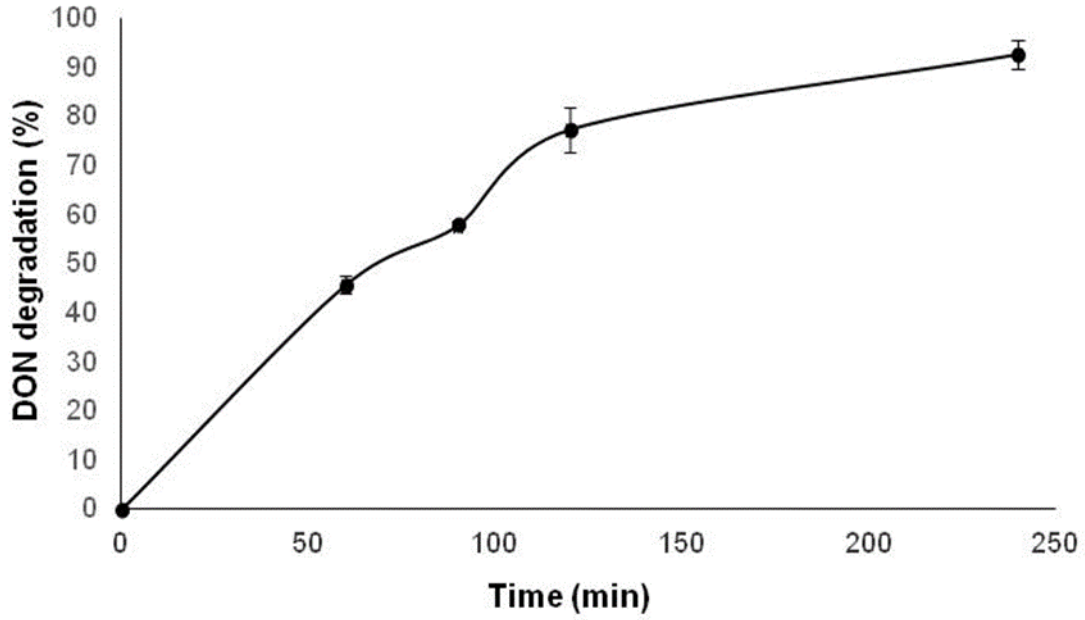


*Fig. 14 Kinetics of DON degradation (%) by the ammonia treatment under optimal conditions (4.8 % $NH_4OH$, 90 °C).*

Regarding the effect of the initial toxin concentration (ranging from 200 to 2000 µg/kg) on its degradation under optimal conditions (4.8 % $NH_4OH$, 90 °C) and 2 h of treatment (Fig. 15), no significant differences were observed between DON initial levels on the treatment effectiveness.

Therefore, under optimal conditions and 2 h of treatment, more than 75 % of DON can be degraded for wheat samples affected with a moderate contamination, probably avoiding the discard of batches of the product. In addition, this treatment followed by proper aeration would minimize the ammonium residues in the sample compared to direct treatments with an ammonium hydroxide solution or with an ammonium salt.

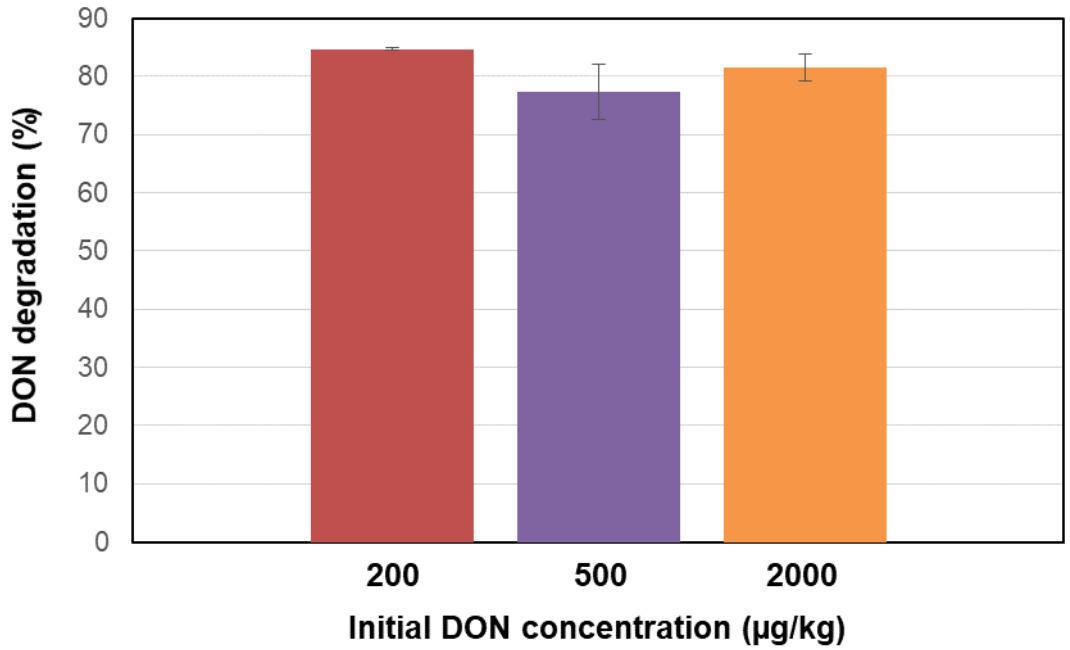


*Fig. 15 Influence of toxin initial concentration on DON degradation under optimal conditions (4.8 % $NH_4OH$, 90 °C) and 2 h of treatment. No significant differences in ANOVA were observed between samples.*

To our knowledge, this is the first study aimed to degrade DON by gaseous ammoniation in wheat kernels. Young et al. (1986) decontaminated DON in wheat but soaking the kernels in liquid ammonium hydroxide (5 % concentration, 600 ml/kg wheat) for 24 h at 22 °C, achieving only a 35 % DON reduction. Other studies using gaseous ammoniation for detoxification of other mycotoxins have been published, yielding similar results to our work. Weng et al. (1994) treated aflatoxin contaminated (354 µg/kg) corn (12 % moisture) with 2 % $NH_3$ at 17 psi

and 121 °C, obtaining a 98.6 % aflatoxin reduction after a 60 min treatment. Gardner Jr. et al. (1971) observed that treating aflatoxin contaminated peanut meal (121 µg aflatoxin/kg, 9 % moisture) with ammonia at 15 psi and 93 °C for 30 min, 80.2 % total aflatoxin degradation was achieved.

### 6.4.3 Study of DON-derived formed products and *in silico* biological and toxicological evaluation

The identification of the DON degradation products generated by the ammonia treatments in real wheat samples presents a strong analytical difficulty related to the retention capacity of the immunoaffinity columns, and/or the obtaining of clean samples adequate for the analysis by mass spectrometry. For these reasons, in order to obtain a clear idea of the type of compounds resulting from the reaction of DON and ammonia, several experiments were carried out. These experiments were performed in a similar way of the wheat treatments, by the exposition of a dry vial containing DON to $NH_3$ vapours at 90 °C, obtaining almost a total DON degradation. The HPLC-MS chromatograms, obtained in full scan acquisition mode, are presented in Fig. S9 (see Annexes). For the reference sample DON was detected for m/z 297 and at 0.84 min. For degraded sample clear peaks at m/z 296 (peak time 1.06 min), 294 (peak time 0.88 min), and 312 (peak time 1.12 min) were observed, that would correspond to the degraded products molecular ions, being the less intense peak the first one (m/z 296).

Considering the chemical structure of DON, several points of interaction are possible between DON molecule and $NH_3$. Thus, the carbonyl group, the epoxide ring and the double bond in α,β with carbonyl group are suitable for a nucleophilic attack of the ammonia. In the case of carbonyl group, the $NH_3$ addition would be

followed of a water elimination to form an imine. On the other hand, the nucleophilic attack to the carbon of the epoxide ring (producing the ring-opening), and the attack to the double bond (via Michael addition reaction), would form an amine in both cases. In addition, the tertiary alcohol formed in the case of epoxide ring-opening could suffer dehydration to form an alkene (Carey & Sundberg, 2008).

Therefore, on the basis of these possible chemical reactions, the peak characterized by the ion m/z $[M+H]^+$ = 296, that correspond to the molecular mass 295, can be produced by the formation of an imine (compound A in Supplementary Fig. S10, see Annexes), or by epoxide ring-opening, forming an amine, and dehydration of the tertiary alcohol generated (compound B in Supplementary Fig. S11, see Annexes). On the other hand, the major chromatographic peaks, m/z 312 and 294, require the reaction of multiple ammonia molecules with the DON molecule, being that coherent with the high excess of ammonia respect to the mycotoxin. Thus, the compounds of the molecular ion $[M+H]^+$ 294 would be generated by a first formation of an imine by the reaction of the carbonyl group. After that, the keto-enol tautomerism could produce another carbonyl group and the formation of a second imine group. Both imine groups would be probably enough stable in water solution due to the imine-enamine tautomerism and the conjugated double bonds in the ring. A third addition of ammonia to the ring of epoxide group would produce the ring-opening and the dehydration of the tertiary alcohol generating the compound with molecular weight 293 (compound C in Supplementary Fig. S12, see Annexes). However, the formation of the compound with molecular weight 312 would be explainable by a first Michael addition to the double bond and two subsequent

additions to carbonyl groups (compound D in Supplementary Fig. S13, see Annexes).

In Fig. 16 is shown the chemical structures of DON and the proposed degraded DON compounds. These structures allow to observe that the modifications on the molecule of DON due to the ammonia treatment would be mainly on the epoxide ring and on the carbonyl group and hydroxyl group of the six-carbon ring. In this sense, taking into account that the toxicity of DON is mainly due to the epoxide group (Ehrlich & Daigle, 1987), and that the alteration of the α,β-unsaturated ketone moiety can produce a toxicity decrease (Fruhmann et al., 2014), it can be assumed that the degraded products generated by the ammonia treatment would be less toxic than the parental molecule. In order to evaluate the possible biological activity and toxicity of the generated compounds, in comparison to DON molecule, two *in silico* tests were carried out by using the tools “Molinspiration” and “Osiris”. In Table 10 are shown the results for the estimation of biological activities and the toxicity for the compounds A-D and DON. As can be seen, in general, the biological activities and toxicity of the possible degraded compounds are lower than those of the parental mycotoxin. Thus, except for kinase inhibition, the compounds A, B and C showed, for all the biological activities, lower score (less activity) than the original DON. On the other hand, compound D showed lower score as ion channel modulator, nuclear receptor ligand and enzyme inhibitor than DON. Although the compound D showed, in some cases, higher score than DON molecule, for that compound, and also the compound C, a violation of the Lipinski’s rule of 5 (Lipinski et al., 2001) was observed, so its absorption could be limited compared to DON. Regarding to the evaluation of toxicity, the profiles observed were similar to those for biological activities,

remarking that all degraded compounds present a lower possible effect on the reproductive system than DON.

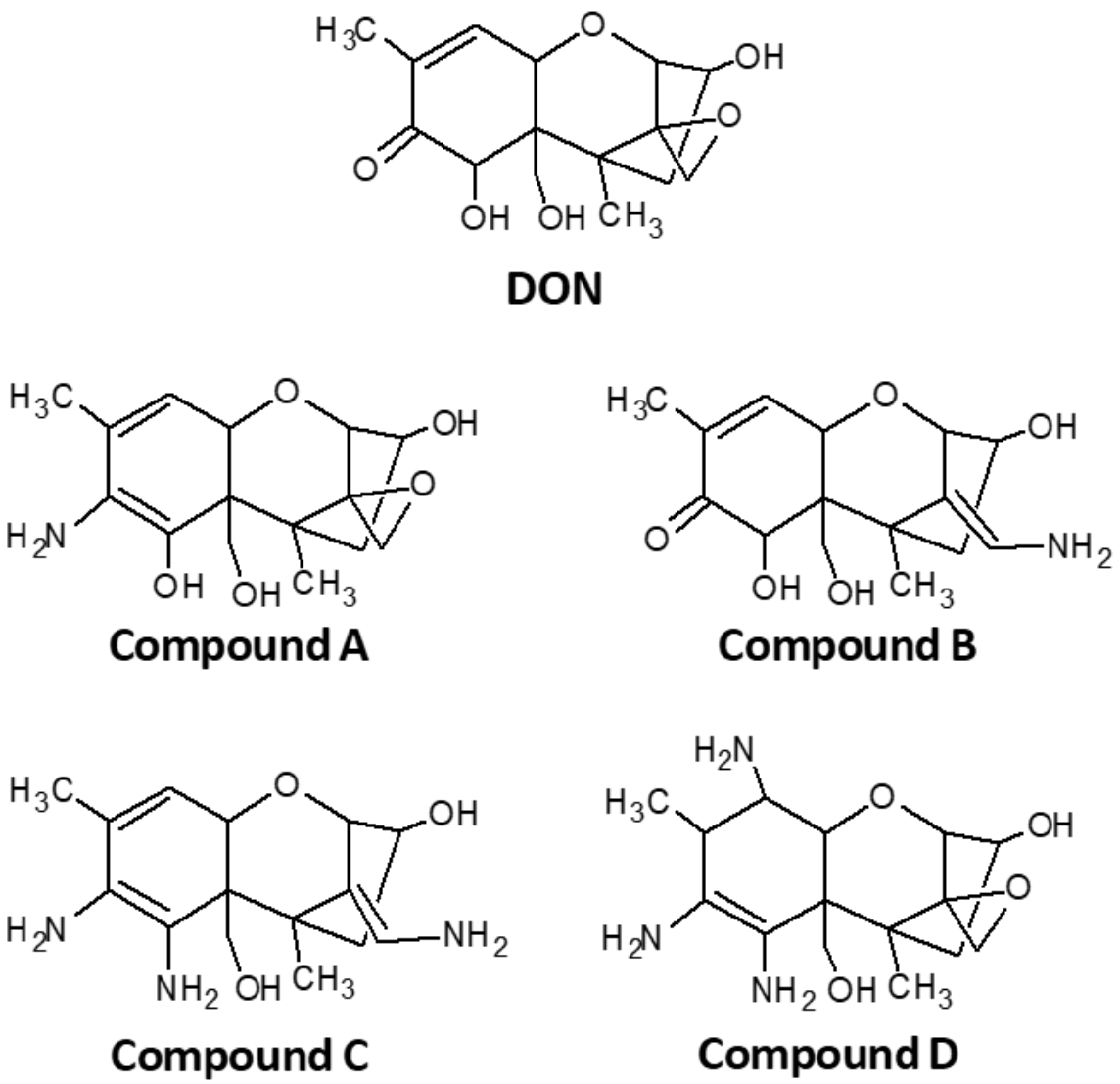


*Fig. 16 Structures of DON and proposed DON degraded compounds resulted from ammonia treatment. Calculated molecular weight: Compounds A and B: 295.33 g/mol; Compound C: 293.37 g/mol; Compound D: 311.38 g/mol.*

## 6.5 Conclusions

A novel and simple method for the chemical degradation of DON in wheat kernels, based on reaction with ammonia vapours, is proposed. Results have shown DON degradations higher than 75 % in kernels affected by a moderated contamination by exposition to ammonia vapours at 90 °C for 2 h. In addition, the type of compounds generated by the reaction between DON and ammonia were studied, concluding that the main DON molecule moieties responsible for the toxic effects would be modified generating less toxic compounds. The proposed degradation strategy could be scaled up easily in the industry, not requiring complex and expensive installations. More research is necessary in order to verify, in real wheat matrices, the DON conversion into the proposed degraded products and to evaluate the toxicity of degraded compounds by *in vitro* and *in vivo* assays.

## 6.6 Acknowledgements

B.B.-V. and J. J R.-B. appreciate the FD pre-doctoral fellowship (PRE2018-085278) of the Spanish Ministry of Science, Innovation and Universities, and the postdoctoral contract from Agrotecnio center, respectively. This work was funded through the project AGL2017-87755-R awarded by the Spanish Ministry of Science, Innovation and Universities.

*Table 10 In silico evaluation of biological activities and toxicity.*

| | DON | Possible Degraded Compounds | | | |
|---|---|---|---|---|---|
| | | Compound A | Compound B | Compound C | Compound D |
| Biological activities[a] | | | | | |
| GPCR ligand | 0.09 | −0.10 | 0.07 | −0.02 | 0.17 |
| Ion channel modulator | 0.2 | 0.04 | 0.1 | −0.02 | 0.01 |
| Kinase inhibitor | −0.60 | −0.54 | −0.44 | −0.30 | −0.18 |
| Nuclear receptor ligand | 0.66 | 0.38 | 0.4 | 0.22 | 0.38 |
| Protease inhibitor | 0.34 | 0.18 | 0.14 | 0 | 0.65 |
| Enzyme inhibitor | 0.5 | 0.48 | 0.41 | 0.38 | 0.43 |
| Lipinski's rule evaluation[a] | | | | | |
| Log P | −0.97 | 0.21 | −1.35 | −0.32 | −1.4 |
| Molecular weight (Da) | 296.32 | 295.33 | 295.33 | 293.37 | 311.38 |
| Acceptor H-Bond(nON) | 6 | 6 | 6 | 6 | 7 |
| Donor H-bond (OHNH) | 3 | 5 | 5 | 8 | 8 |
| Violations Lipinski's rule | 0 | 0 | 0 | 1 | 1 |
| Toxicity Test[b] | | | | | |
| Mutagenic | red | red | green | yellow | red |
| Tumorigenic | red | red | green | yellow | red |
| Irritant | red | red | green | yellow | red |
| Reproductive system Effect | red | green | green | green | green |

[a] *Calculated by using Molinspiration software version 2018.03 (www.molinspiration.com).*

[b] *Calculated by Osiris property explorer software (www.organicchemistry.org/prog/peo/)*

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# Chapter 7. A new methodology for the analysis of total deoxynivalenol, dissolved and adsorbed on cell walls, in microbiological culture assays.

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## 7.1 Abstract

Deoxynivalenol (DON) is a mycotoxin mainly produced by *Fusarium graminearum* and *Fusarium culmorum* and is commonly found in cereals such as wheat, barley, oats, and their derivatives. Scientists have been working on different strategies for DON detoxification, with biological detoxification being an approach with growing interest. When evaluating the use of microorganisms for mycotoxin detoxification, different phenomena can occur, namely, biotransformation (by microorganism metabolism and by interaction with extracellular cell proteins) and adsorption on cell walls, both of which can be present. In this study, a fast, simple, reliable, and inexpensive method for total DON quantification (dissolved and adsorbed) in bacterial culture assays is presented. This method can be used in screenings designed for searching DON-biodegrading microorganisms without requiring the analysis of the metabolites produced. This method has a good recovery (80.2 %), reproducibility (3.2 %) and low limit of quantification (0.60 µg/ml) that allows quantification under a wide range of DON concentrations in microbiological culture assays.

**Keywords**: Deoxynivalenol analysis; Microbiological culture; Bacteria; Adsorption; Biodegradation

## 7.2 Introduction

Mycotoxin contamination of food and feed is a worldwide problem for which there is still no definitive solution, despite the maximum limits that different food regulatory agencies have established. In wheat and barley, the most commonly found mycotoxin is deoxynivalenol (DON) (Wegulo, Baenziger, Hernandez Nopsa, Bockus, & Hallen-Adams, 2015), generating health problems mainly due to chronic exposure (Payros et al., 2016; Pestka, 2007). For this reason, intense research has been carried out considering several approaches for controlling DON levels (prevention, removal, degradation or processing) (Awad, Ghareeb, Böhm, & Zentek, 2010; Karlovsky et al., 2016), one of which that has attracted growing interest is biological detoxification of DON by using selected microorganisms, usually bacteria or bacterial consortia (Muhialdin, Saari, & Meor Hussin, 2020; Yao & Long, 2020; Zhu, Hassan, Lepp, Shao, & Zhou, 2017).

The mechanisms by which microorganisms can detoxify toxins have been studied for different mycotoxins. Thus, interactions with cell walls based on adsorption phenomena and enzymatic transformation are the main mechanisms described in the literature for most mycotoxins (Piotrowska, 2021). In particular, DON biotransformation products have been used to evaluate the transformation percentage of DON (Piotrowska, 2021; Wang et al., 2019; Yu et al., 2010). In addition, Jia, Cao, Liu, and Shen (2021) recently observed similar low DON contents in incubations with bacterial culture and with cell-free culture supernatant, concluding that some extracellular proteins can also play a role in DON detoxification. However, the capacity of bacterial cell walls to bind DON is also well documented (Hassan & Bullerman, 2013; Niderkorn, Morgavi, Pujos-Guillot, Tissandier, & Boudra, 2007). It is clear that in the incubations with

microorganisms, DON biotransformation (if it occurs) will appear concomitant with the adsorption phenomena on cell walls. The importance of distinguishing both effects is related to the possible reversibility of the process, which is more probable in the case of binding to cell walls.

In assays aimed at identifying mycotoxin-biodegrading bacteria, a large number of samples are usually tested, and low success rates are achieved. Different strategies have been followed to identify these bacteria. Some authors have chosen to screen isolated microorganisms by incubation in culture media in the presence of the mycotoxin (Franco, Garcia, Hirooka, Ono, & dos Santos, 2011; Niderkorn, Boudra, & Morgavi, 2006). Niderkorn et al. (2007) screened 202 bacteria and did not observe DON degradation products, with percentages of binding due to adsorption between 15 and 22 %. Yu et al. (2010) used a PCR-DGGE-guided microbial selection process to identify DON-transforming bacteria from chicken intestines. They isolated 196 bacteria, and only 10 of them were found to be capable of transforming DON to DOM-1. Sato et al. (2012) used long time-stressing subculturing processes in minimal media containing the mycotoxin to induce the metabolism of the mycotoxin. From 169 environmental samples, they could only find 13 DON-biodegrading bacteria. Shima et al. (1997) only found a DON-biodegrading bacteria following a similar stress subculturing process; specifically, subcultured samples were subjected to a 2- to 8-day incubation in a medium supplemented with 200 mg of DON/ml up to 14 times. Given the low success rates found, it is necessary to test a large number of isolates to identify a DON-biodegrading strain; thus, having a simple and rapid method is especially convenient. In general, methods aimed at identifying mycotoxin-biodegrading bacteria have been carried out using high levels of

mycotoxin and bacterial inoculum (10–200 µg DON/ml, $10^8$–$10^{10}$ CFU/ml), and the degradation capacity is estimated by the analysis/detection of the remaining mycotoxin and/or the degradation products (Franco et al., 2011; Niderkorn et al., 2007, 2006 and; Sato et al., 2012; Shima et al., 1997). However, in some studies, the possible effect of adsorption of the analytes to the cell walls is not considered, so it is possible that the observed toxin reductions are a result of both biodegradation and adsorption (C. He, Fan, Liu, & Zhang, 2008; Wang et al., 2019; Wilson et al., 2017).

A few studies have included supplementary assays to evaluate the adsorption effect through incubation with inactive cells (Franco et al., 2011) or have opted to try and avoid the adsorption phenomenon instead with a second incubation in a lysis buffer (Völkl, Vogler, Schollenberger, & Karlovsky, 2004). Nevertheless, to perform screening assays with a large number of microorganisms, it is necessary to develop a simple, inexpensive, and rapid method with adequate reproducibility and recovery that allows the quantification of the total amount of analyte/s, including the dissolved and adsorbed fractions.

Therefore, the aim of this work was to establish a fast, simple and reliable method that allows the quantification of the total DON content, even the DON fraction adsorbed on the cell wall of the microorganisms, for its use in screening assays designed for searching DON-biodegrading microorganisms. With this methodology, the DON-biotransformation capacity of a bacteria (or bacteria consortium) can be evaluated by the analysis of DON remaining in the cultures, without the need to evaluate and analyse the metabolites produced.

## 7.3 Materials and methods

### 7.3.1 Chemicals

DON was provided by Romer Labs (Tulln, Austria). Methanol, acetonitrile, ethyl acetate and NaCl were purchased from Fisher Scientific (Loughborough, UK). $K_2HPO_4$, $KH_2PO_4$, $FeSO_4 \cdot 7\ H_2O$ and $CaCl_2 \cdot 2\ H_2O$ were obtained from Scharlau (Sentmenat, Spain). $MgSO_4 \cdot 7\ H_2O$ and $NH_4NO_3$ were obtained from Quality Chemicals (Esparreguerra, Spain). Bouillon MRS and Gelose MRS were purchased from Biokar (Beauvais, France), and L-cysteine was purchased from Aldrich (St. Louis, USA).

### 7.3.2 Bacterial cultures

To minimize the reagents needed, microbial cultures were prepared in sterile 96-well ELISA microplates. Two hundred microlitres of culture media and 50 µl of inoculum (prepared in Man, Rogosa & Sharpe medium supplemented with L-cysteine (2.5 mg/ml), named MRSc) were added to each well of the microplate. Mineral salt medium (MM) according to Sekar, Mahadevan, Sundar, and Mandal (2011) was employed as culture media with slight modifications: 1.73 g $K_2HPO_4$, 0.68 g $K_2HPO_4$, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 4 g NaCl, 0.03 g $FeSO_4 \cdot 7\ H_2O$, 1 g $NH_4NO_3$ and 0.02 g $CaCl_2 \cdot 2\ H_2O$ in 1000 ml of water (pH adjusted to 7.0). A concentrated DON solution was also added to the MM such that the final DON concentration in each well was 30 µg/ml, unless otherwise indicated. DON was the only carbon source in the media, except for some residual MRSc constituents of the inoculum. The microplate was incubated (37 ± 1 °C, 1 week) under anaerobic conditions achieved by employing an oxygen scavenger kit (BD GasPak EZ Anaerobe Container System; Becton, Dickinson and Company; Sparks, USA). To minimize

evaporation, the microplate was sealed with parafilm, and a receptacle of water was left inside the anaerobic jar.

### 7.3.3 DON extraction

A diagram for the DON extraction methodology is shown in Fig. 17. After incubation, the entire content of each well was recovered and transferred to an Eppendorf tube containing 30 mg of NaCl.

Three consecutive extractions with 400 µl of organic solvent were carried out, withdrawing 300, 400 and 400 µl of extracting solvent for the first, second and third extractions, respectively. For each extraction cycle, the Eppendorf tube was vortexed for 30 s, ultrasound-treated for 1 min (to release the DON adsorbed on the cell walls), and vortexed for 30 s again. Therefore, a total of 1100 µl of extraction solvent was collected. Ultrasound was applied using the Bransonic M2800H-E (Branson Ultrasonic SA, Carouge, Switzerland) at maximum power.

The collected extraction solvent was evaporated at 40 °C under a gentle stream of $N_2$. The residue was resuspended in 0.8 ml of methanol:water 10/90 (v:v) (3.2 dilution factor), vortexed, filtered through 0.22-µm PTFE filters and analysed by HPLC-DAD.

### 7.3.4 Selection of the extraction solvent

Three different extraction solvents were tested: ethyl acetate, a 50/50 mixture of ethyl acetate and acetonitrile, and acetonitrile. The performance of the extraction using ethyl acetate but not using salt was also evaluated. Pure MRSc was employed instead of a microbial inoculum prepared in MRSc. Assays were performed in duplicate.

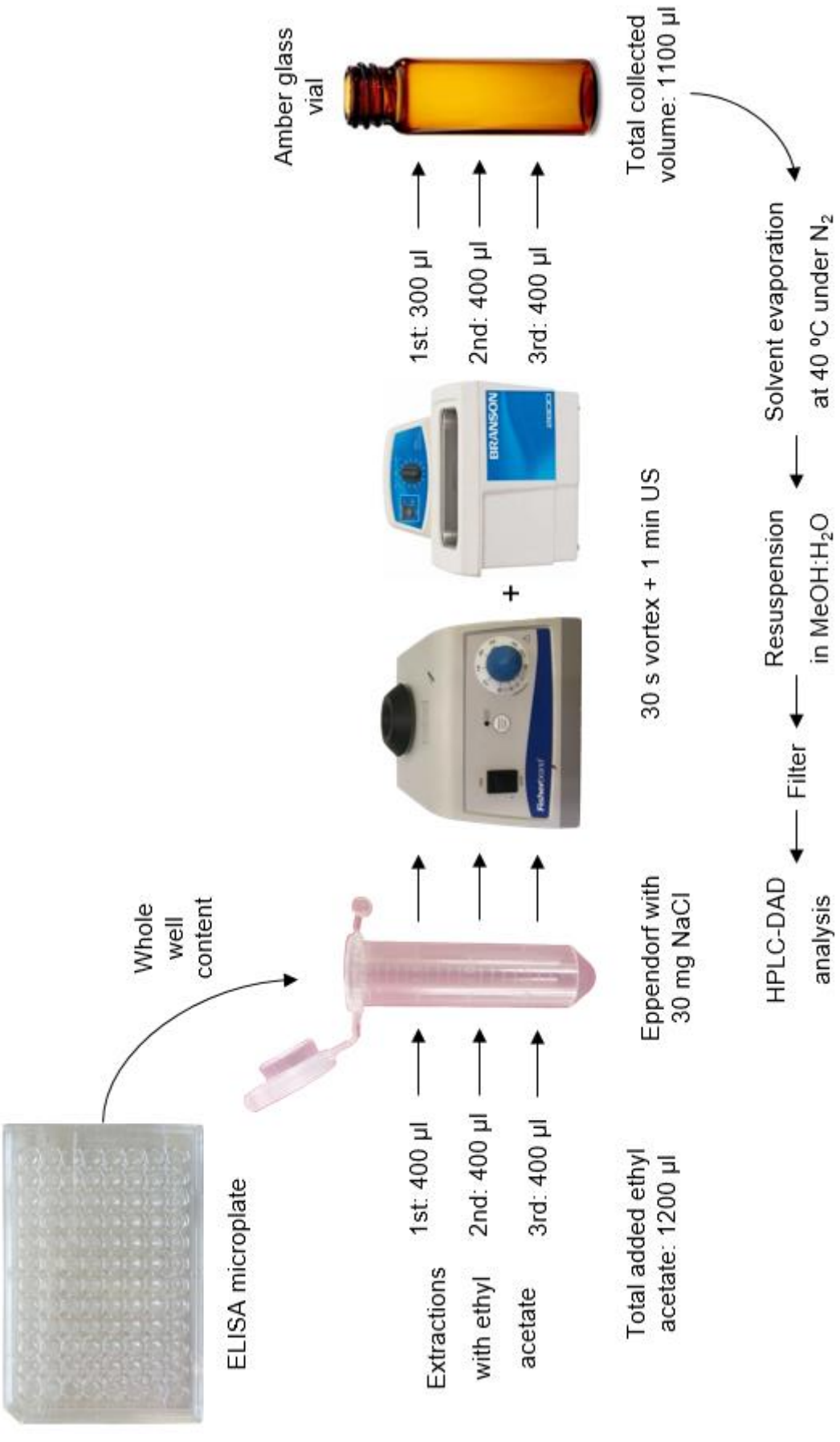


*Fig. 17 DON extraction methodology diagram*

### 7.3.5 DON analysis

HPLC-DAD determination of DON was performed using an Agilent Technologies 1260 Infinity HPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD). A Phenomenex® Gemini C18 column (California, USA) was used (150 × 4.6 mm, 5-µm particle size, 110 Å pore size). Three mobile phases were prepared: phase A (methanol:water 10:90, v:v), phase B (acetonitrile:water 20:80, v:v) and phase C (100 % methanol). The gradient applied was as follows: 0 min 100 % A; 10 min 60 % A and 40 % B; 13 min 60 % A and 40 % B; 15 min 100 % C; 25 min 100 % C; 29 min 100 % A and 40 min 100 % A (11 min were needed to clean and re-equilibrate the column). The flow rate was 1 ml/min, the column temperature was 40 °C, and the injection volume was 50 µl. The DON retention time was 10.0 min (absorbance reading at 220 nm).

### 7.3.6 Testing the method: evaluation of total DON determination

To demonstrate that the proposed method allows the extraction and analysis of the total DON (dissolved and adsorbed on cell walls) from microbial cultures, the following experiment was designed. With the hypothesis that the application of ultrasound during the extraction process releases the DON adsorbed on the cell walls, several samples were prepared and analysed following the methods described above, but half of them were extracted without applying ultrasound.

As microorganisms from the intestinal microbiota have proven to be good DON-biotransforming candidates (Gratz, Duncan, & Richardson, 2013; Guan et al., 2009; Yu et al., 2010), it was decided to use 3 intestinal colonizers: *Lactobacillus fermentum* (LF26), *Staphylococcus hominis* (SH10) and *Enterococcus faecium*

(EF1), all of which were generously provided by the Probilac research group (Complutense University of Madrid, Spain).

The three microorganisms were cultivated in a microplate. Two hundred microlitres of MM and 50 µl of inoculum were mixed in each well, which also contained DON at a final concentration of 10 µg/ml (added to the MM). A control with DON but with no microorganisms was also included (50 µl of pure MRSc was added instead of 50 µl of the inoculum). Three replicates for each microorganism (and for the control) were prepared for each extraction method (with and without the application of ultrasound). The microplate was left to incubate (37 ± 1 °C, 1 week) in anaerobiosis before DON extraction and analysis.

In parallel, concentrations of the three tested microorganisms (CFU/ml) were calculated by plating dilutions of the respective inoculums in MRSc agar, which was the only method for determining the concentration of viable cells in those inoculums. Plates were incubated (37 ± 1 °C, 2 days) in anaerobiosis. Knowing the concentration of the inoculums and the proportion of the MM and inoculum in the wells, the concentration of the tested microorganisms on the well was calculated.

### 7.3.7 Validation of the method

Linearity was checked with a calibration curve of DON standards from five different concentrations (1, 5, 10, 20 and 30 µg/ml) prepared in triplicate. The standards were prepared in methanol:water 10:90 (v:v).

To determine the recovery of the method, cultures of *E. faecium* were prepared as in Section 7.3.2. In this case, cultures with five different DON concentrations (1, 5, 10, 20 and 30 µg/ml) were assayed in triplicate. The concentration of the

inoculum (CFU/ml) was calculated by preparing a dilution series and plating them in Petri plates with MRSc agar in duplicate. Petri plates were incubated (37 ± 1 °C, 2 days) in anaerobiosis. In the case of the DON biodegradation assay, after 1 week of incubation of the microplate, toxin concentrations were determined, and the average recovery from all samples was estimated.

Reproducibility was similarly estimated. Cultures of *E. faecium* were prepared as in Section 7.3.2. at two different DON concentrations (5 and 20 µg/ml) on three different days, in triplicate each day. After 1 week of incubation, DON concentrations were determined, and average repeatability was calculated. The limit of detection (LOD) and quantification (LOQ) were calculated as three and ten times the signal/noise ratio using three samples incubated with *E. faecium* containing 1 µg/ml of DON.

### 7.3.8 Statistics

One-way ANOVA and least-squares difference (LSD) Fisher's tests were performed to evaluate the effects of the different sample types ($p < 0.05$). Statistical analyses were performed using STATISTICA (version 7.1) (StatSoft, Inc., 2005).

## 7.4 Results and discussion

### 7.4.1 Selection of the extraction solvent

The results of DON recovery for each extraction solvent tested are shown in Fig. 18. No significant differences between extraction solvents with added salt were observed in terms of recovery (p value = 0.54); thus, ethyl acetate was selected as the extraction solvent due to its lower boiling point and, thus, faster evaporation.

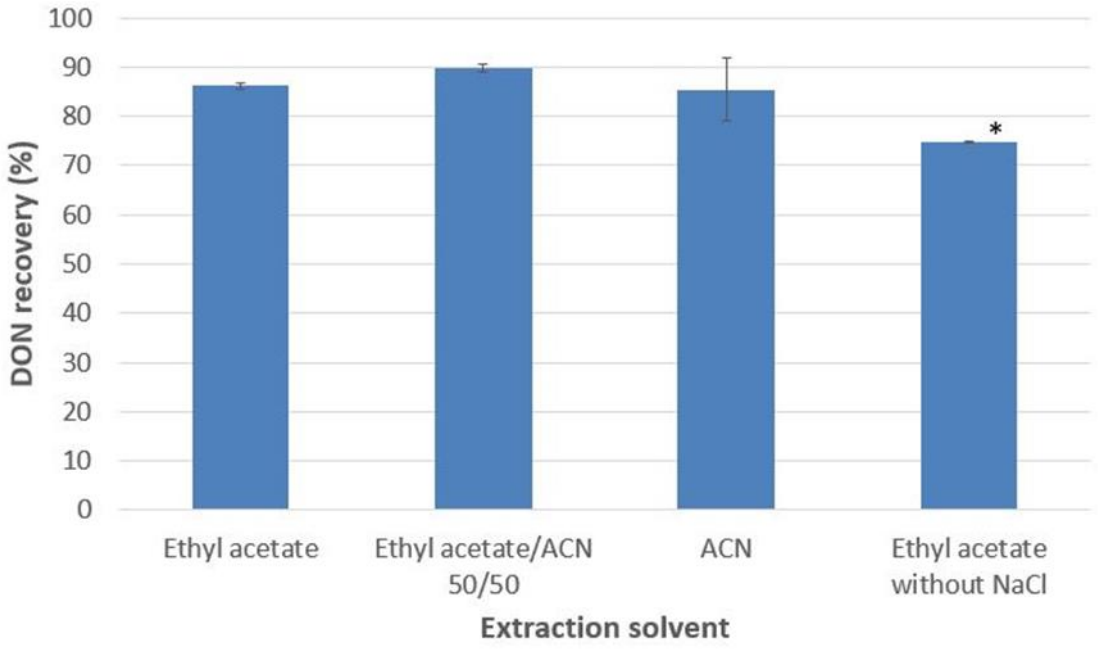


*Fig. 18 DON recovery (%) for each extraction solvent tested (initial DON concentration 30 µg/ml). * Indicates statistical differences in ANOVA respect to the other samples.*

Extraction with ethyl acetate without using salt was discarded, as its recovery was lower than that achieved by adding salt (p value = 0.026). A higher recovery using salt may have been achieved due to the salting-out technique. Salt addition has previously been employed for extracting DON (Hamed, Arroyo-Manzanares,

García-Campaña, & Gámiz-Gracia, 2017; Mariño-Repizo, Goicoechea, Raba, & Cerutti, 2018).

### 7.4.2 Testing the method: evaluation of total DON determination

The DON concentrations in the control and the three tested microorganisms are shown in Table 11. Half of the samples were analysed using the ultrasound-assisted extraction method (US), and the other half were analysed without applying ultrasound (N-US). The CFU/ml of microplate microbial cultures for each microorganism are also shown.

*Table 11 DON concentrations (µg/ml) in samples extracted with (US) and without (N-US) ultrasound-assistance, and CFU/ml of microplate microbial culture.*

| Sample | Extraction method | | CFU/ml |
|---|---|---|---|
| | US | N-US | |
| **Control** | $2.52 \pm 0.06$ | $2.30 \pm 0.25$ | - |
| ***Lactobacillus fermentum*** | $2.54 \pm 0.06^{a}$ | $1.93 \pm 0.15^{b}$ | $1.5 \cdot 10^{7}$ |
| ***Staphylococcus hominis*** | $2.50 \pm 0.09$ | $2.26 \pm 0.16$ | $3.3 \cdot 10^{5}$ |
| ***Enterococcus faecium*** | $2.65 \pm 0.08^{a}$ | $2.38 \pm 0.12^{b}$ | $1.7 \cdot 10^{7}$ |

[a,b] *Means in a row followed by different letters are significantly different at p<0.05 using one-way ANOVA.*

As expected, no significant differences in DON concentrations were observed between the US and N-US control samples (p value = 0.208). Significantly lower DON concentrations were observed for the N-US samples than the US samples in the cases of *L. fermentum* and *E. faecium* (p values of 0.002 and 0.031, respectively). The lower DON concentration in the N-US samples can be explained by the adsorption of the toxin to the cell walls of the bacteria, which is a phenomenon that has been observed in numerous studies (Luo, Liu, Yuan, &

Li, 2020; Yao & Long, 2020). It is also notable that standard deviations are much smaller in US-treated samples than in non-US-treated samples.

In contrast, no significant differences were observed between US and N-US extractions in the case of *S. hominis* (p value = 0.085). This can be explained by the much lower concentration of this inoculum compared with the other two ($10^5$ vs. $10^7$ CFU/ml, respectively). A lower number of microorganisms most likely implies a lower cell wall surface to which DON can adsorb. Lu, Liang, and Chen (2011) demonstrated that the adsorption of zearalenone to bacteria depended on the bacterial concentration, and it is likely that the same phenomenon occurs with DON.

Other authors have used different approaches to try and determine the extent to which biodegradation phenomena are important in mycotoxin detoxification studies. For example, Kosztik, Mörtl, Székács, Kukolya, and Bata-Vidács (2020) chose to centrifuge the cultures for 40 min and analyse the supernatant on one side (20-min shaking extraction) and the cell biomass on the other side (20-min shaking extraction and 10-min centrifugation). The toxin found in the cell biomass corresponded to adsorption phenomena. If the sum of the two concentrations of mycotoxins was lower than the original concentration, it was assumed that the missing toxin had been biodegraded. Although this method works, it is more expensive and time-consuming than the method presented herein because the determination of whether one culture is capable of biodegradation requires the performance of two analyses instead of one, and long wait times are spent centrifuging and shaking.

Völkl et al. (2004) prevented the adsorption of DON to cell walls by using a lysis buffer that contained proteinase K and sodium dodecyl sulfate (SDS). Despite

working, a 60-min incubation at 37 °C and centrifugation were required, again resulting in a long process regarding in comparison with the proposed method.

### 7.4.3 Validation of the method

The results for the validation of the method using ethyl acetate as the extraction solvent are shown in Table 12. For the recovery assay, it was estimated that there were $5.4 \times 10^6$ CFU/ml microplate microbial cultures, and the average recovery rate was approximately 80 %. Overall, this method has proven to exhibit good recovery and reproducibility and is able to evaluate total DON levels over a wide range of concentrations. By testing microbial cultures with a DON concentration of 30 µg/ml, which is much lower than the concentrations used in many other studies (W.J. He et al., 2016; Ikunaga et al., 2011; Shima et al., 1997; Yu et al., 2010), reductions of the toxin of up to 98 % can be studied (based on the obtained LOQ).

*Table 12 Validation parameters for the DON extraction method.*

| | DON concentration (µg/ml) | | | | | Average |
|---|---|---|---|---|---|---|
| | 30 | 20 | 10 | 5 | 1 | |
| Recovery (%) | 80.15 ± 2.12 | 81.34 ± 1.39 | 84.24 ± 0.08 | 83.98 ± 7.56 | 71.25 ± 3.93 | 80.19 ± 5.94 |
| Reproducibility[a] (%) | - | 1.25 | - | 5.19 | - | 3.22 |
| LOD[b] (µg/ml) | 0.18 ± 0.01 | | | | | |
| LOQ[b] (µg/ml) | 0.60 ± 0.02 | | | | | |

*[a] Reproducibility was calculated only for DON concentrations of 20 and 5 µg/ml, in three different days (n=9).*

*[b] LOD and LOQ were calculated using a DON concentration of 1 µg/ml.*

## 7.5 Conclusions

The proposed method for total DON quantification in bacterial cultures can be used for identifying bacteria capable of DON biodegradation (excluding the adsorption effect). It is fast, simple and economical, with good average recovery (80.19 %) and reproducibility (3.22 %), and allows the study of biodegradation phenomena over a wide range of toxin concentrations (1–30 µg/ml).

Considering that biodegradation implies a definitive chemical change in the mycotoxin structure, while adsorption of DON and other mycotoxins can be a reversible process (Adami, Tajabadi Ebrahimi, Bagheri Varzaneh, Iranbakhsh, & Akhavan Sepahi, 2020; Haskard, El-Nezami, Kankaanpää, Salminen, & Ahokas, 2001), the proposed method allows screening studies to focus only on the toxin biotransformation phenomenon and is, therefore, more adequate for use in the search for effective DON-biodegrading microorganisms.

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# Chapter 8. General discussion.

Mycotoxins are secondary fungal metabolites that can contaminate cereals and other plants both in the pre- and post-harvest stages, posing a health risk for humans and animals. Consequently, its concentration has been regulated in many countries, including the EU. Keeping mycotoxin levels under the legal limits is a permanent challenge for cereal producers and the food and feed industries. Mycotoxin control strategies can be classified between prevention of fungal development and mycotoxin contamination, decontamination, and inhibition of absorption of the mycotoxin into the digestive tract. Among them, prevention of the contamination strategies should be prioritized, since once produced, mycotoxins are very difficult to eliminate. In any case, there is no mycotoxin control strategy that alone can guarantee the absence of mycotoxins in cereals, but rather it is necessary to adopt a combination of control strategies throughout the entire food or feed chain to obtain cereals with acceptable mycotoxin concentrations. The present thesis intends to provide knowledge about different control strategies that can be adopted to avoid contamination by DON and fumonisins in wheat and maize. The following sections discuss the most significant results with a global vision.

## 8.1 Influence of agronomic factors on mycotoxin contamination in maize

The influence of three different agronomic factors on mycotoxin contamination of maize at harvest, among other variables, was studied. One agronomic factor was the tillage system: intensive tillage, consisting of subsolate (35 cm depth), disc harrow and rototiller, was compared to direct drilling, which consisted of applying herbicide and sowing the seed directly into the soil. Another agronomic factor was crop diversification: a monocropping long-cycle maize was compared against a

legume-maize double cropping, using short-cycle maize as the main crop and vetch as the secondary crop. The last agronomic factor was N fertilization rate. Two extreme treatments were compared: a zero N fertilization rate and high N fertilization rate (400 kg N $ha^{-1}$ for long cycle maize and 300 kg N $ha^{-1}$ for short cycle maize, because of the possible N fixation of the preceding legume crop).

The DON content of maize at harvest date was only significantly influenced by the tillage system, being higher in intensive tillage plots (average contamination of 2695 µg $kg^{-1}$) than in direct drilling plots (average contamination of 474 µg $kg^{-1}$).

As previously mentioned, and in contrast to our results, numerous published studies investigating cereals that are host to species of the FGSC (like maize wheat or barley) claim that cereals obtained under direct drilling or minimum tillage present a higher DON contamination than the ones obtained under intensive tillage (Dill-Macky & Jones, 2000; Drakopoulos et al., 2021; Mansfield et al., 2005; Obst et al., 1997; Schöneberg et al., 2016). Generally speaking, the authors of the these studies justify their results because it has been described that residues of crops infected with *Fusarium* constitute a fungal inoculum for the following crop (Cotten & Munkvold, 1998; Council for Agricultural Science and Technology, 2003; Edwards, 2004; Maiorano et al., 2008), and the destruction or burial of these residues would reduce the chances of fungal infection and mycotoxin production. For this reason, intensive tillage would be a better option than no tillage for controlling fungal infection and mycotoxin production (Cotten & Munkvold, 1998; Edwards, 2004; Maiorano et al., 2008).

However, there are some works that challenge this hypothesis. Many authors maintain that adopting intensive tillage practices instead of direct drilling or

minimum tillage practices won't led to lower DON contamination in cereals like maize, wheat, barley or oats. Roucou et al. (2022), who analysed data from a total of 2032 maize fields between 2004 and 2020, observed that DON levels of maize were statistically the same whether the crop residues of the previous year were adequately managed (mostly through soil tillage) or not. Supronienė et al. (2012) did not found a clear relationship between different tillage practices (intensive tillage, reduced tillage and direct drilling) and DON contamination of spring and winter wheat. Kaukoranta et al. (2019) analyzed survey data from 804 spring-oat fields, and found that the oat DON concentration was the same or higher under intensive tillage than under direct drilling. Regarding fumonisins in maize, published studies show an unclear correlation between tillage systems and the incidence and concentration of these mycotoxins (Marocco et al., 2008, 2009; Ono et al., 2011).

Coming back to our results, we suggest two reasons that justify a higher DON concentration in maize from intensive tillage plots than in maize from direct drilling plots. On one side, tillage operations can affect the soil structure (Ramos et al., 2019). Under intensive tillage, soil is more exposed to wind and water. The impact of water drops causes the breakdown of water-stable aggregates, leading to soil crusting (Arjmand Sajjadi & Mahmoodabadi, 2015; Pareja-Sánchez et al., 2017). This phenomenon negatively affects multiple soil properties, and can cause overland flow (Awadhwal & Thierstein, 1985; Pareja-Sánchez et al., 2017; Ramos et al., 2019). Due to the fact that we observed the presence of pools of water (see Fig. 19) in plots under intensive tillage but not in plots under direct drilling, we believe it is very likely that plots subjected to intensive tillage were suffering from soil crusting. We hypothesized that pools of water in intensive tillage plots caused

elevated air humidity conditions, which favoured DON production by the moulds present in maize. This would help explain the higher DON concentration in maize grown in plots under intensive tillage versus the maize grown under direct drilling.

*Fig. 19 Pool of water in a maize plot. Available in https://pixabay.com/es/photos/maizal-charco-ma%C3%ADz-camino-de-tierra-2560890/*

On the other side, tillage affects earthworms, especially those living on the surface layers. Earthworms break down organic matter, promoting nutrient cycling along with soil microbiota, and improve several soil properties (Briones & Schmidt, 2017; Imaz et al., 2010; Nieminen et al., 2011; Ojha & Devkota, 2014). Tillage can break earthworms' burrows, bury their food sources, expose them to harsh environmental conditions and predators and injure or kill them (Chan, 2001; Kladivko, 2001; Nieminen et al., 2011; Ojha & Devkota, 2014). Different studies indicate that the earthworms *L. terrestris*, *A. longa* and *L. rubellus* are capable of

reducing not only wheat straw soil cover, which constitutes an important fungal nutrient source, but also the *Fusarium* biomass itself (Jorge-Escudero et al., 2021; Wolfarth et al., 2011) (see a *L. terrestris* individual in Fig. 20). In addition, *L. terrestris* has proven to degrade DON in wheat straw (Oldenburg et al., 2008; Schrader et al., 2009). Furthermore, *L. terrestris* is more attracted to highly *Fusarium*-infected and DON contaminated wheat straw than low infected and contaminated wheat straw, probably due to the increased N-content and digestibility of the first one caused by fungal colonization (Oldenburg et al., 2008; Wolfarth et al., 2011). Despite the studies herein described were performed on wheat fields, it would be expected that maize from fields under intensive tillage had higher DON and *Fusarium* populations than maize from fields under direct drilling, as intensive tillage adversely affects earthworms such as *L. terrestris*, *A. longa* and *L. rubellus*.

A great deal of variables determine mycotoxin contamination of a crop. For whatever reasons, in our study maize was generally not contaminated with fumonisins. Only 12.5 % of maize samples contained $FB_1$ and $FB_2$, and mean concentrations of contaminated samples were 826 and 196 μg $kg^{-1}$ maize, respectively. Neither $FB_1$ nor $FB_2$ concentration of maize was statistically significantly affected by any of the studied agronomic factors, but this lack of influence may be a consequence of the low fumonisins concentration in maize. It would be interesting to know if the obtained results would have been the same if conditions had led to generally higher fumonisin levels.

*Fig. 20 L. terrestris. From Rob Hille - (CC BY-SA 3.0). Available in https://commons.wikimedia.org/wiki/File:Lumbricus_terrestris_R.H_(1).JPG*

It is remarkable that *Fusarium* spp. population at harvest was not significantly correlated to the concentration of any of the studied mycotoxins, but that may be explained by non-DON/$FB_1$/$FB_2$-producing *Fusarium* spp. strains colonizing the studied maize, and/or because higher levels of DON/$FB_1$/$FB_2$-producing *Fusarium* spp. at harvest do not necessarily imply higher concentrations of the cited mycotoxins.

The influence of agronomic factors on the mycotoxin contamination of cereals like maize is an interesting subject and, from my point of view, deserves further study. In our work, the different plots from where we harvested the grain were seeded with the same maize hybrid (and if applicable, with the same vetch), under the same tillage system and supplied with the same type and amount of N fertilizer for 3 years, although the study was performed at the third year's harvest. Similarly, most of the studies on this topic only consider one year's harvest, and information on the crops planted and the tillage systems adopted in the previous years to that harvest is rarely given. It would be interesting to carry out a long-term study about the influence of agronomic factors on mycotoxin contamination of maize, starting from a field that has been left fallow for years, and evaluating not only possible differences in mycotoxin contamination due to the agronomic factors in the first harvest, but also on the following harvests. Keeping track of changes in soil properties and microbial and earthworms populations over time may also be of interest, as it could help to better understand the factors that influence mycotoxin production. Tilling the soil one year may not change properties, microbiota and fauna of soil as much as tilling it multiple years in a row. In a three-year study, Marocco et al. (2009) reported that during the first year direct drilling maize presented higher fumonisins incidence and contamination than intensive tillage maize, but this difference could not be seen the second and third years. Conducting more exhaustive and longer studies in this line would be advisable. Incorporating treatments such as reduced tillage and medium N fertilization would also be interesting.

## 8.2 Changes in maize during a 10-day harvest-till-drying simulation period

When harvesting maize, farmers must not only ensure that it has reached physiological maturity, but also that its moisture does not exceed established commercial standards. The goal of these commercial standards is to incorporate maize into the food or feed chain with the lower moisture levels possible, to hinder possible fungal growth and to make sure that the subsequent drying process is fast and relatively inexpensive. Farmers need to pay special attention to the weather when maize is almost ready for harvest, as rain can increase grain moisture, promoting fungal growth and extending the period prior to maize reaching commercial standards. In northeast Spain, the accepted commercial moisture is around 18 %. Nevertheless, research suggests that to ensure that no moulds can grow nor produce mycotoxins, maize maximum moisture must be 14 % (Channaiah & Maier, 2014; Richard, 2007). In some areas, drying facilities are undersized, and since all maize is harvested within a few days, it is not unusual to find enormous amounts of maize kernels outdoors waiting to be dried (see Fig. 21). This harvest-till-drying period can sometimes last as long as 10 days. Our aim was to study the influence of waiting time and temperature on mycotoxin contamination.

*Fig. 21 Pile of maize waiting to be processed outside a drying facility. Available on https://commons.wikimedia.org/wiki/File:Plymouth,_Nebraska_corn_pile_1.JPG*

We split a sample of maize kernels from each plot into two different sterile plastic bags, which were kept at 15 or 25 °C for 10 days. Those temperatures were chosen as representative of the average minimum and maximum daily temperatures in the area at that time of the year. Different determinations were performed throughout the period: moisture, $a_w$, total fungal contamination and *Fusarium* spp. contamination were studied on days 0, 4, 7 and 10; while concentrations of DON, $FB_1$ and $FB_2$ were studied on days 0, 5 and 10. No significant effect of time nor temperature could be observed on moisture, total fungal contamination, *Fusarium* spp. contamination or fumonisins concentration during the 10 days of the experiment. In contrast, time significantly affected the evolution of $a_w$, which dropped at day 4 for both temperatures. DON concentration was not significantly affected by time or temperature, although in the case of time it was a close call (p-value = 0.078). The DON concentration increase between days 0 and 5 was statistically significant (p-value = 0.049), but not between days 0 and 10 (p-value = 0.051). Given this data, we interpreted that as DON concentration increased between the 0-5 days period, and *Fusarium* spp. counts

remained constant throughout the 10 days, the absence of DON production during the last 5 days could be caused by the drop in $a_w$ during the first 4 days. It is very likely that if $a_w$ had remained stable since harvest, DON contamination would have kept increasing. According to our results, there are DON-producing *Fusarium* spp. species in maize that can produce DON at an approximate $a_w$ of at least 0.91, while at an $a_w$ of 0.88 they can no longer produce this mycotoxin. Some authors have reported similar or slightly higher $a_w$ threshold values for DON production in different *Fusarium* species (Comerio et al., 1999; Ramirez et al., 2006; Schmidt-Heydt et al., 2011). Summarizing, we can say that drying maize grain as soon as possible after its harvest is crucial for avoiding additional mycotoxin production. $a_w$ levels below the threshold for DON production must be reached as quickly as possible, and although the best way to do so is by artificial drying, a certain drop in $a_w$ levels can occur under environmental conditions.

Reflecting on this work, I realized there were some differences between the maize harvest and harvest-till-drying period that actually occur, and the ones we simulated. Nowadays, in industrialized countries, maize is not collected and shelled by hand, as we did in our study, but using specialized machinery. This machinery is more aggressive to maize kernels than manual harvesting, and may damage some of the collected kernels. As previously discussed, injuries in the grain may ease fungal infection and mycotoxin contamination. In addition, machinery is not frequently cleaned, and stuck crop residues in it may constitute a fungal reservoir and contaminate recently harvested maize.

Therefore, mechanical harvesting of maize would be desirable for this type of study, as different levels of fungal infection may occur in maize of the same plot depending on whether it is collected manually or mechanically. It is worth

mentioning, though, that mechanical harvesting would be impractical considering the number of different plots to harvest and its size.

In relation to the harvest-till-drying period, we kept maize kernels in sterile plastic bags that were stored at 15 or 25 °C for 10 days. The mass of maize kernels in each bag was less than 1 kg. It could be said that all maize kernels inside a plastic bag were under identical conditions. Moisture exchange between the air inside the bags and the air surrounding the bags could be considered minimal. Environmental humidity of the chamber where the bags were kept was not controlled. Unlike in our study, the piles of maize grain that await outside drying plants are exposed to a wide interval of temperatures and relative humidity, every day being different. Generally, at lower temperatures, relative humidity is higher. In addition, maize in piles outdoors are exposed to other meteorological agents such as rain or wind, and also to the attack of different insects and animals. Meteorological agents, insects and animals can be a source of fungal infection and ease the growth of already infected grain. It is interesting to comment that maize piles can weight multiple tonnes, and in consequence, the drying process of the kernels at the very centre of the maize pile will vary from the one of the kernels outside the maize pile. Kernels outside the maize pile will be more susceptible to changes in meteorological conditions and the attack of insects or animals. In absence of an aeration system, kernels inside the maize pile will generate heat and water vapour as a consequence of respiration, and perhaps moisture migration. This water vapour will condense, increasing $a_w$ and therefore facilitating fungal infection.

In addition, to carry out an adequate sampling of a maize pile, grains from different points of the maize pile should be collected, which is very laborious. Not

to mention that a different maize pile should be made for grains of each of the plots harvested. Besides, meteorological agents like rain could strongly bias the results. Therefore, a simulation under laboratory conditions seems to be the only way. A more feasible option would be harvesting maize like we did, but storing it under controlled dynamic temperature and relative humidity conditions that simulate meteorological conditions that occur in a typical day during the harvest period.

### 8.3 NIR-HSI for predicting DON, $FB_1$ and $FB_2$ in samples of maize kernels and classifying them accordingly

In our study, we proved that NIR-HSI is effective to predict DON and fumonisins in samples of maize kernels. RMSECV (expressed as mg $kg^{-1}$) and RPD of the best prediction models developed for each studied mycotoxin were the following: 0.848 and 2.344 for DON, 3.714 and 2.018 for $FB_1$, 2.104 and 2.301 for $FB_2$ and 4.398 and 2.305 for the sum of $FB_1$ and $FB_2$. Likewise, we demonstrated the potential of NIR-HSI to classify samples of maize kernels according to its DON and fumonisins concentration. Concretely, according to if the samples meet the EU regulations for DON and fumonisins in maize kernels intended for human consumption (1.75 mg DON $kg^{-1}$ maize and 4 mg $FB_1$+$FB_2$ $kg^{-1}$ maize) (European Commission, 2023b). The balanced accuracies of the best classification models were 0.889 for DON, 0.773 for $FB_1$+$FB_2$ and 0.865 for both DON and $FB_1$+$FB_2$ regulations at a time. Other studies have also shown the capacity of NIR-HSI and similar techniques to predict or classify maize depending on its DON and/or fumonisins contamination (Bolduan et al., 2009; Chavez et al., 2022; Kos et al., 2016; Levasseur-Garcia et al., 2015; Levasseur-Garcia & Kleiber, 2015; Miedaner et al., 2015; Pearson et al., 2004; Tyska et al., 2021).

Nonetheless, there is room for improving not only the mycotoxin prediction and classification performance of NIR-HSI, but also its speed.

In our study, we used the free form pixel selection tool for selecting the area of the kernels in each hyperspectral image. Despite the final selections were very accurate, it was a slow process and must be done manually, which precludes the possibility of automation. In preliminary tests, we tried selecting the area of the kernels using the similar spectrum pixels by Euclidean distance selection tool, which is fast and could be automated, but the obtained selections were inaccurate (data not published). Mainly at the edges of maize kernels and in their germ, unwanted pixels were selected and desired pixels were left out. An accurate, fast and easily automatable pixel selection tool would improve the time of analysis.

Even though hyperspectral images can be used to predict mycotoxin contamination in maize and classify it accordingly, NIR-HSI is not sensitive enough to detect mycotoxins *per se*, which are found in very small concentrations. Actually, what NIR-HSI can detect are changes in the major constituents of kernels (namely carbohydrates, lipids and proteins) which result from fungal growth in the outer or inner parts of the grain (Bauriegel et al., 2011; Femenias et al., 2022; Freitag et al., 2022). Those chemical modifications, to a greater or lesser extent, correlate to mycotoxin production. Therefore, it would be interesting to, on the basis of the same hyperspectral images, not only detect DON and fumonisins on maize, but all mycotoxins that could be found in maize. Currently, there are many multimycotoxin liquid chromatography analysis methods for maize published (Frenich et al., 2009; Njumbe Ediage et al., 2011), which could be used as reference methods to develop regression or classification models of multiple mycotoxins using HSI-NIR. The same hyperspectral images

could also be used to detect fungal growth, which is still undesirable even if no mycotoxins are produced.

Considering that the distribution of mycotoxin contamination in cereals is positively skewed, most kernels having a low contamination while a few of them being highly contaminated, focusing on eliminating only the most contaminated would be an adequate strategy. Because of this, further research on single kernel analysis would be advisable.

As previously stated, NIR-HSI is a faster, cheaper, and more eco-friendly analysis method than the widely used HPLC. In addition, it does not require specialized personnel and it is non-destructive. If in the near future NIR-HSI predictions improve to the level of meeting the performance criteria established in the Regulation (EU) 2023/2782 (European Commission, 2023a) (related to recovery, repeatability and reproducibility), consideration could be given to use NIR-HSI as a method that could be taken into account for official mycotoxin analysis.

## 8.4 Deoxynivalenol degradation in wheat kernels by exposition to ammonia vapours

The use of ammonia for decontaminating mycotoxins in cereals is nothing new. Numerous studies using ammonia to detoxify AFs in maize have been published (Brekke et al., 1977; Gomaa et al., 1997; Moerck et al., 1980; Norred, 1982; Nyandieka et al., 2009; Weng et al., 1994). However, little research has been conducted regarding the effect of ammonia on maize or wheat contaminated with fumonisins (Norred et al., 1991; Park et al., 1992) or DON (Young, 1986; Young et al., 1986). For this reason, we decided to investigate on the impact of ammonia vapours on DON-contaminated wheat.

First, we determined the optimal conditions of temperature and $NH_4OH$ concentration for DON degradation of kernels contaminated at 500 µg $kg^{-1}$ in a 2 h treatment. Three different temperatures (65, 90 and 115 °C) and three different $NH_4OH$ concentrations (1.6, 3.2 and 4.8 %) were tested, and 90 °C with 4.8 % $NH_4OH$ was considered the optimal treatment. 115 °C with 4.8 % $NH_4OH$ gave a similar result, but was discarded for being more energetically consuming. The contribution of thermal treatment to DON degradation at each temperature tested was evaluated using negative controls (replacing $NH_4OH$ with water). Only in the 115 °C treatment a significant DON reduction was observed (22.05 %).

Under the determined optimal DON degradation conditions, kinetics of DON degradation in wheat kernels contaminated at 500 µg $kg^{-1}$ were studied. With 60, 120 and 240 min of treatment, respective DON reductions were of 45.73, 77.39 and 92.73 %.

To study if the initial DON concentration played a role in the mycotoxin degradation, wheat kernels inoculated with DON at 200, 500 and 2000 µg $kg^{-1}$ were treated under optimal conditions for 2 hours. No differences in the percentage of DON degradation were observed.

Pure DON was ammonia treated, and possible DON-derived formed products were identified. Its toxicity and biological activities were *in silico* evaluated, using the tools Osiris property explorer and Molinspiration, respectively. In general, the toxicity and biological activities of the possible degraded compounds were lower than those of DON.

Ammonia treatment presents the advantage of being uniform thorough the sample, which is especially important in solid and opaque foods, and taking into account the heterogeneity of mycotoxin contamination. In addition, ammonia can

be easily stored as gas or in water solution, while ozone, another gas which can also be used to decontaminate DON (L. Wang et al., 2016), has to be *in situ* generated, requiring more complex and expensive installations.

After our study, Pasqualotto et al. (2023) treated naturally DON-contaminated wheat kernels with ammonia gas during 7 days, using concentrations in the 0.5-1.5 % range and without heating. They demonstrated that 1 and 1.5 % ammonia concentrations are useful for reducing the DON content of the wheat kernels and that ammonia treatment does not affect the crude protein content of wheat kernels. The authors also performed and *in vivo* test, in which rats were fed during a month with a basal diet, a basal diet + DON-contaminated wheat kernels or with a basal diet + DON-contaminated wheat kernels treated with ammonia at different concentrations. Nine hematological parameters were studied, and in most of them no significant differences could be observed between the blood of the different groups of rats. This may be caused by the low DON concentration of the wheat kernels used, being less than 250 μg $kg^{-1}$, while the European Union DON guidance values for products intended for animal feed range between 900 and 12000 μg $kg^{-1}$ (Commission of the European Communities, 2006). No abnormalities were observed in the histopathological examination of livers and kidneys of rats fed with basal diet + DON-contaminated wheat kernels treated with ammonia.

In the EU, chemical detoxification of $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$, OTA, patulin, DON, ZEN, $FB_1$, $FB_2$, citrinin, ergot sclerotia and ergot alkaloids is forbidden (European Commission, 2023b). The EFSA Panel on Contaminants in the Food Chain assessed the potential application of an ammoniation treatment to reduce the level of AFs on groundnut press cake below the legal limits. A feed

business operator submitted information in support to its claim about the efficacy of the decontamination process, but the panel considered the documents submitted and the literature consulted were insufficient to conclude on the safety and efficacy of the proposed decontamination process (EFSA Panel on Contaminants in the Food Chain, 2021).

On the other side, ammoniation of AF-contaminated maize has been approved in many countries (Kutasi et al., 2021), including some states of the United States (US) (Park, 1993; Wrather et al., n.d.), However, the Food and Drug Administration (FDA) has not approved or sanctioned any ammoniation method for decontaminating AF-contaminated maize (National Grain and Feed Association, 2011; Wrather et al., n.d.). In the US states where maize ammoniation is permitted, the treated cereal can't be distributed in interstate commerce and is subject to labeling and feeding restrictions (Iowa State University, 2012; Wrather et al., n.d.).

Further research is needed on evaluating the *in vivo* toxicity of the compounds formed during ammoniation of DON, especially when it is found at high concentrations.

## 8.5 Analysis of total deoxynivalenol, dissolved and adsorbed on cell walls, in microbiological culture assays

Microorganisms can detoxify mycotoxins via biotransformation (by microorganism metabolism and by interaction with extracellular proteins) and via adsorption on the cell walls. Usually, both phenomena occur at the same time. It is important to bear in mind that adsorption is a more easily reversible process than biotransformation. In many mycotoxin detoxifying studies using

microorganisms, it is unclear to what extent biotransformation or adsorption are responsible for the detoxification (He et al., 2008; G. Wang et al., 2019; Wilson et al., 2017). In research aimed at identifying mycotoxin biotransforming bacteria, usually numerous samples are tested, but success rates are low. For this type of assays, it would be ideal to have a simple, economical and fast analysis method with good recovery and reproducibility, capable of quantifying the total amount of mycotoxin, including the dissolved and adsorbed fractions.

We developed a DON analysis method that meets these requirements, ideal for screening assays searching for DON-biotransforming microorganisms. This method allows to estimate the DON-biotransformation capacity of a culture by analyzing the DON remaining on the culture, without the need to analyse the metabolites produced. The use of ultrasounds allows to free the DON adsorbed on cell walls. The method presented an average recovery of 80.19 %, and an average reproducibility of 3.22 %, allowing the study of DON biotransformation in the 1-30 μg $ml^{-1}$ range.

To our knowledge, few studies have tried to analyze DON decontamination by microorganisms differentiating between biodegradation and adsorption. Franco et al. (2011) grew different strains of lactic acid bacteria. Each culture was divided in three groups according to the received treatment: no treatment (viable bacteria), inactivation by pasteurization (100 °C for 30 min) and inactivation by sterilization (121 °C for 15 min). Then, each cell suspension was centrifuged for 10 min, and the resulting biomass was washed three times with phosphate buffered saline solution and four more times with ultra-pure sterile water. Pellets from all bacterial strains and treatments were then resuspended in a standard DON solution, incubated and analyzed by HPLC. Viable cells might both

biodegrade and adsorb DON, while heat inactivated cells would only be able to adsorb DON. Völkl et al. (2004) prevented DON adsorption in microbial cultures by lysing them using proteinase K and detergent before the mycotoxin extraction. The lysis process required a 60 min incubation before proceding to centrifugation and mycotoxin extraction and analysis.

Kosztik et al. (2020) analyzed the capacity of different *Lactobacillus* strains of biodegrading and adsorbing $AFB_1$ or sterigmatocystin. Microbial cultures including one of the studied mycotoxins were centrifuged for 40 min, and the supernatant and the biomass were analyzed separately. The toxin found in the cell biomass corresponded to adsorption phenomena. If the sum of the two concentrations of mycotoxins was lower than the original concentration, it was assumed that the missing toxin had been biodegraded. Analysis of the supernatant included a 20 min shaking period, and analysis of the biomass included a 20 min shaking period and a 10 min centrifugation.

In comparison with our method, the methods used in the studies of Franco et al. (2011), Völkl et al. (2004) and Kosztik et al. (2020) were more complex, time-consuming and expensive.

## 8.6 References

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# Chapter 9. Conclusions.

There is no single golden-strategy to manage DON and fumonisins contamination in wheat and maize, but using a combination of different approaches will guarantee the production of food and feed with mycotoxin levels within the legal or recommended (and toxicologically acceptable) framework.

The studies framed in this thesis have provided interesting insights to control DON and fumonisins contamination in wheat and maize thorough these cereals production chains. Hereunder conclusions are listed.

With reference to the influence of agronomic factors on mycotoxin contamination in maize and the changes maize undergoes during the harvest-till-drying period:

1. DON contamination in maize planted under intensive tillage is higher than in maize planted under direct drilling. This may be explained partly by tillage operations negatively affecting many soil properties and leading to soil crusting, causing elevated air humidity conditions that may favour DON production by the moulds present in maize.
2. DON contamination in maize is not statistically different whether the nitrogen fertilization applied is high (300-400 kg N $ha^{-1}$) or zero.
3. DON contamination in maize is not statistically different whether a long-cycle maize monocropping system or a double cropping system with vetch and short-cycle maize is adopted.
4. The time lapse between maize harvesting and drying must be as short as possible, because moulds in the cereal can still produce DON until the $a_w$ drops below the threshold production (0.88 aproximately).

With reference to the potential of NIR-HSI to reduce DON, $FB_1$ and $FB_2$ contamination in maize kernels:

5. NIR-HSI can be used to predict the concentration of DON and fumonisins in samples of maize kernels.
6. NIR-HSI can be used to classify samples of maize kernels according to whether they comply or not with the EU legal limits for DON (1.75 mg DON $kg^{-1}$) and fumonisins (4 mg $FB_1$+$FB_2$ $kg^{-1}$)

With reference to the efficacy of ammoniation to treat DON-contaminated wheat kernels and the toxicity of its possible DON-derived formed compounds:

7. Treatment of artificially DON-contaminated wheat kernels with ammonia vapours is effective for degrading the mycotoxin, and the best treatment found was 4.8 % $NH_4OH$ at 90 °C.
8. The initial DON concentration in wheat kernels (200 to 2000 µg $kg^{-1}$ range assayed) did not affect ammonia DON degradation kinetics when treating with 4.8 % $NH_4OH$ at 90 °C for 2 hours.
9. Products derived from the reaction between pure DON and ammonia were tentatively identified. Its toxicity and biological activities were *in silico* evaluated, and in general, were lower than those of DON.

With reference to the analysis of DON in microbiological cultures:

10. A method for analysing total DON in microbiological cultures, including the dissolved and adsorbed fractions, was developed. This method is useful for estimating the DON-biotransformation potential of a culture by analyzing the DON reduction on the media, without analysing any

produced metabolites. It presents the advantages of being simple, economical and fast.

# Chapter 10. Annexes.

## 10.1 Supplementary figures

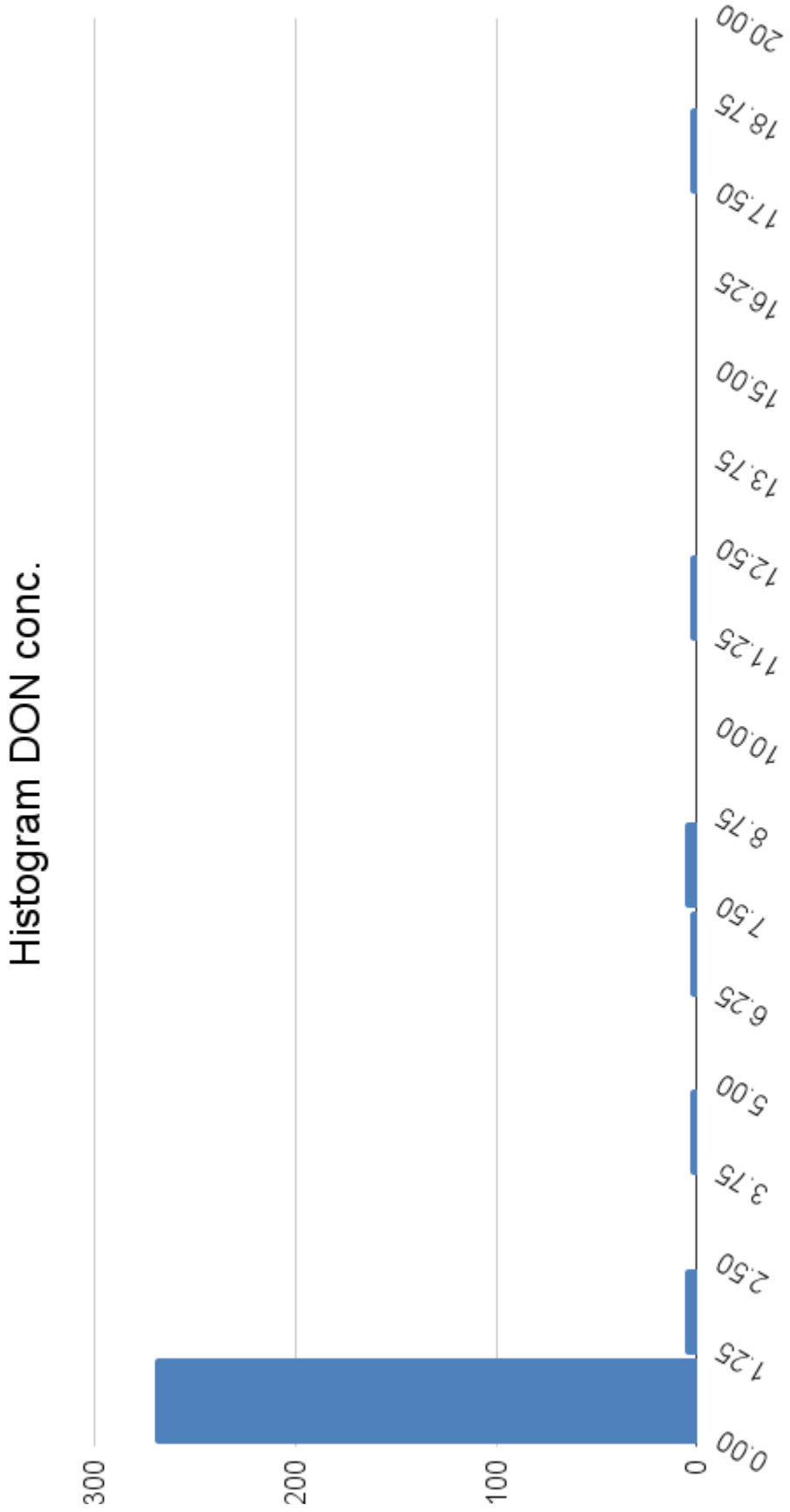


*Fig. S1 Histogram of DON contamination of the analyzed maize samples (mg kg$^{-1}$)*

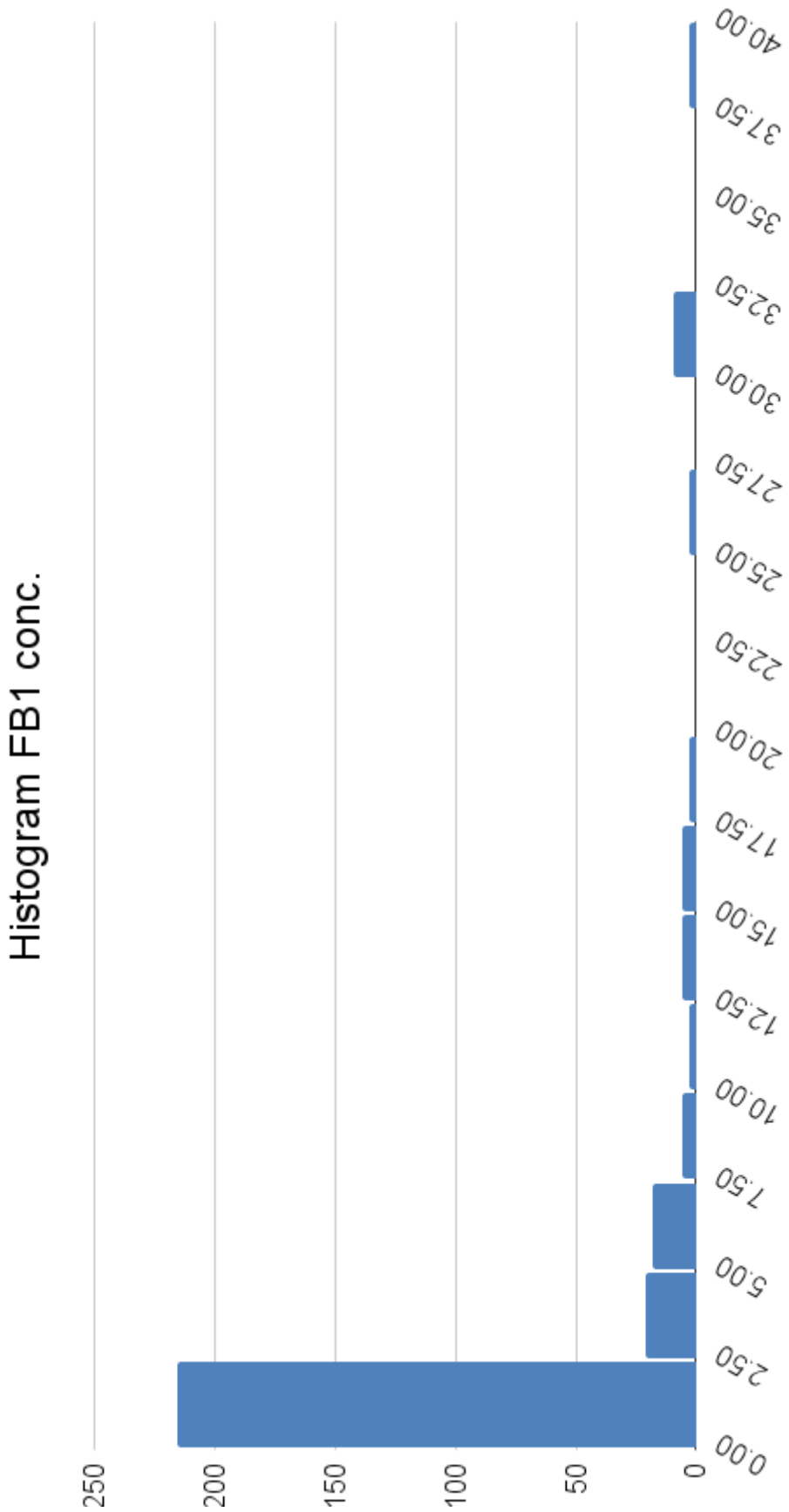


*Fig. S2 Histogram of $FB_1$ contamination of the analyzed maize samples (mg $kg^{-1}$)*

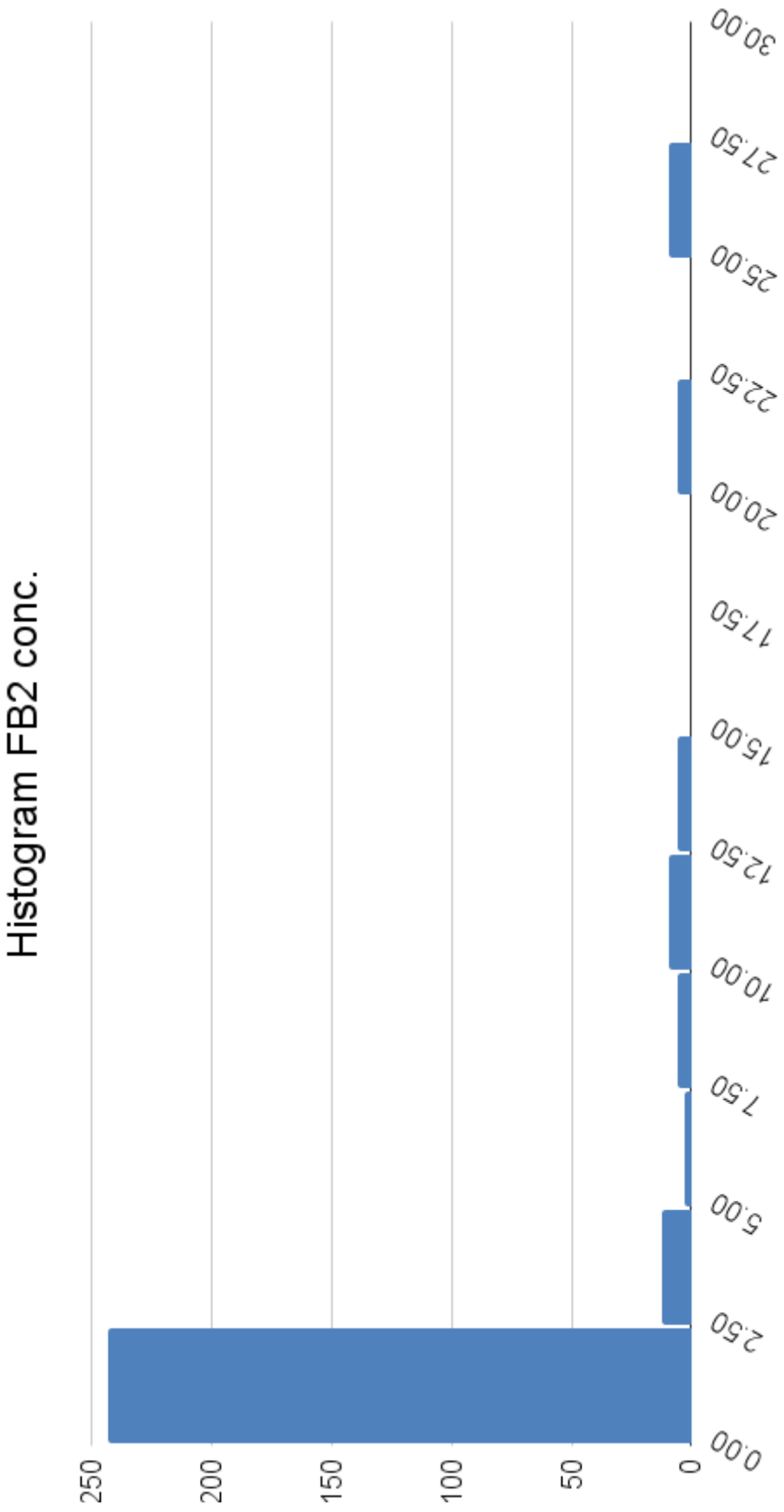


*Fig. S3 Histogram of $FB_2$ contamination of the analyzed maize samples (mg $kg^{-1}$)*

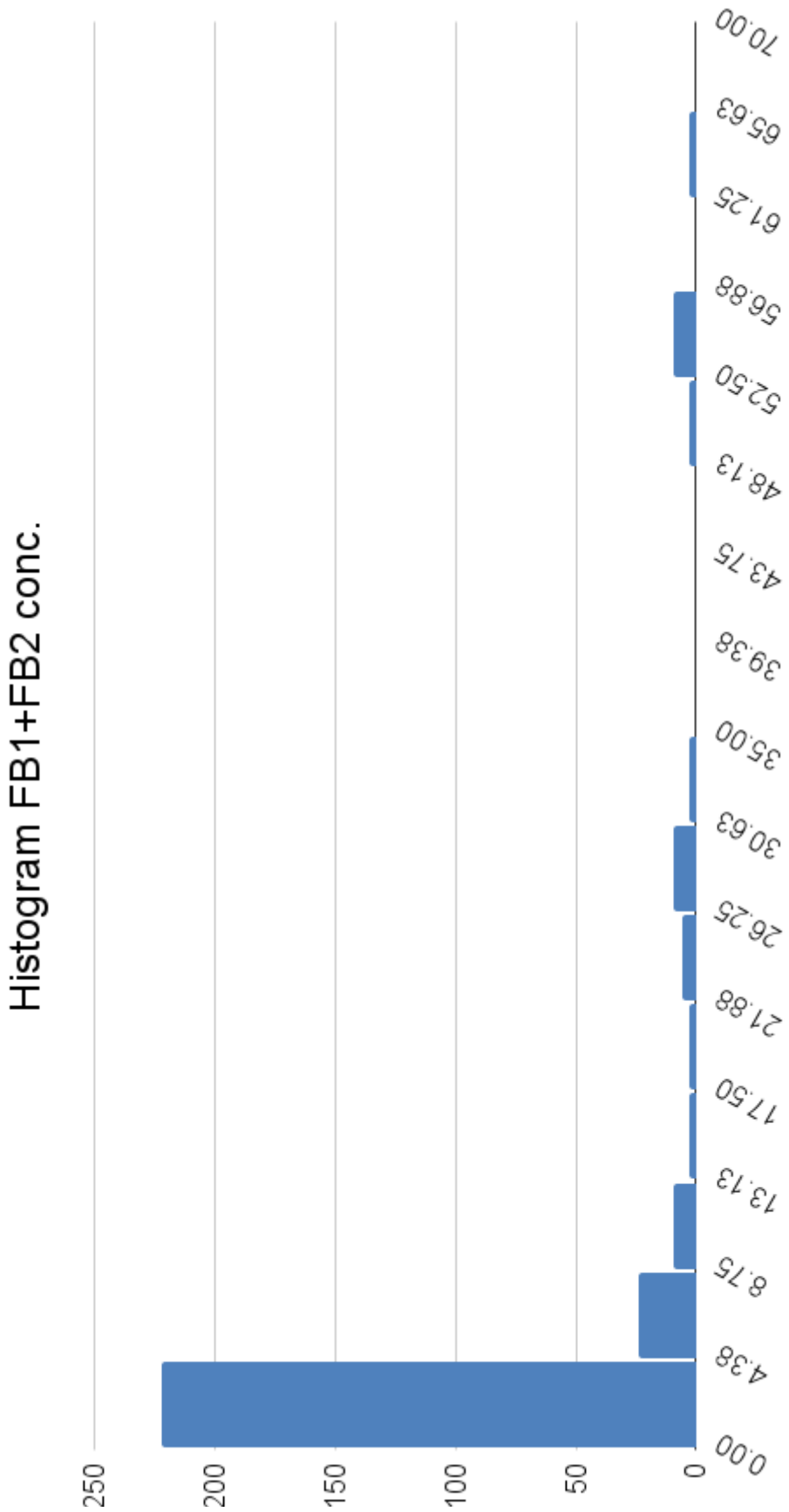


*Fig. S4 Histogram of $FB_1$+$FB_2$ contamination of the analyzed maize samples (mg $kg^{-1}$)*

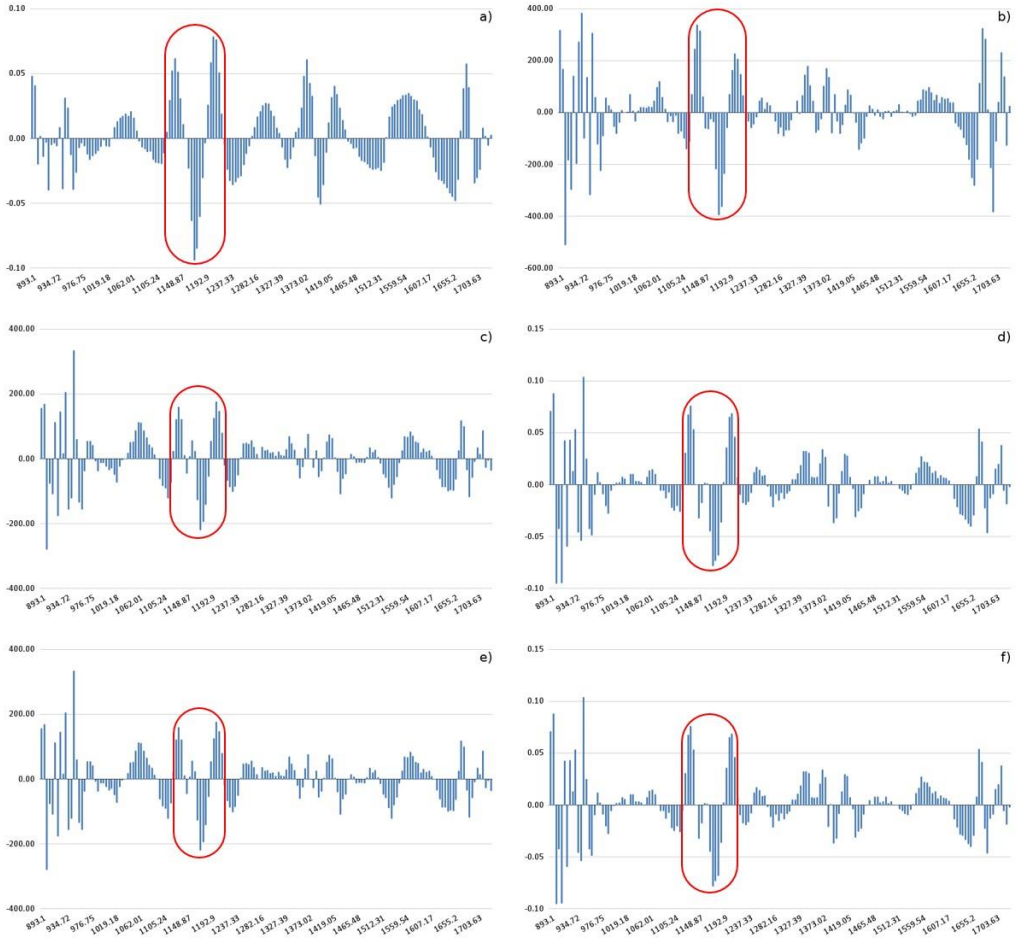


*Fig. S5 Regression coefficients for the different developed fumonisin regression models. The explanatory variables are in the abscissa axis, while their corresponding regression coefficients are on the ordinate axis. a) $FB_1$ model with no data pretreatment; b) $FB_1$ model with SNV; c) $FB_2$ model with SNV; d) $FB_2$ model with full MSC; e) $FB_1$+$FB_2$ model with no data pretreatment; f) $FB_1$+$FB_2$ model with full MSC. Spectral areas of importance are marked in red.*

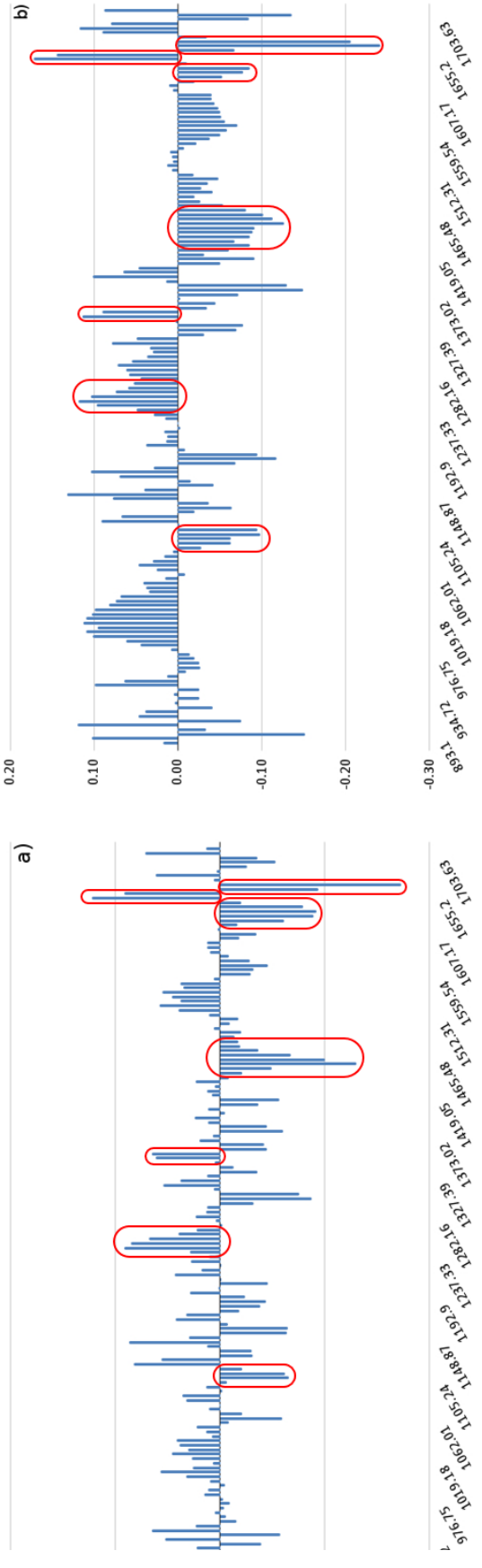


*Fig. S6 Regression coefficients for the different developed DON regression models. The explanatory variables are in the abscissa axis, while their corresponding regression coefficients are on the ordinate axis. a) DON model with full MSC + 1st derivative; b) DON model with 1st derivative + full MSC. Spectral areas of importance are marked in red.*

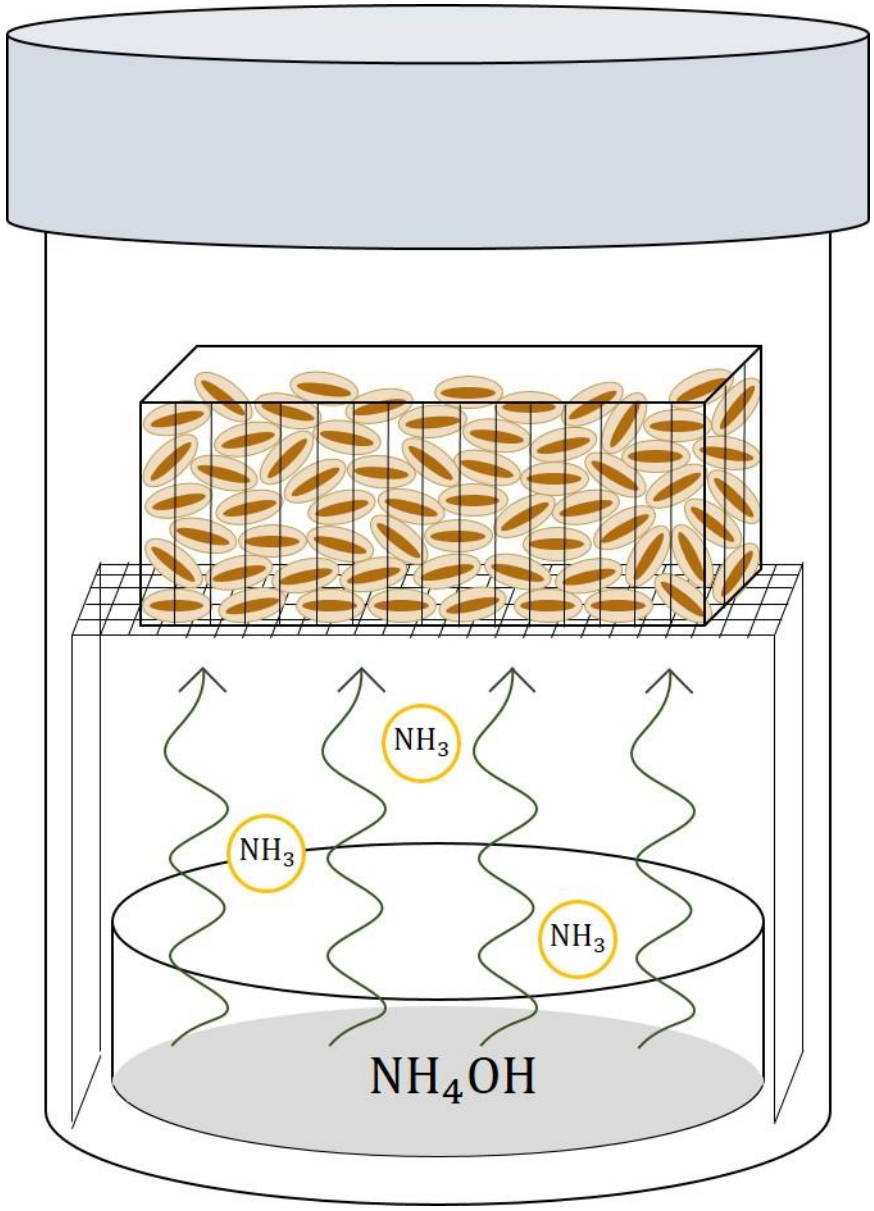


*Fig. S7 Sketch of the structure designed for the treatments of wheat kernels with ammonia.*

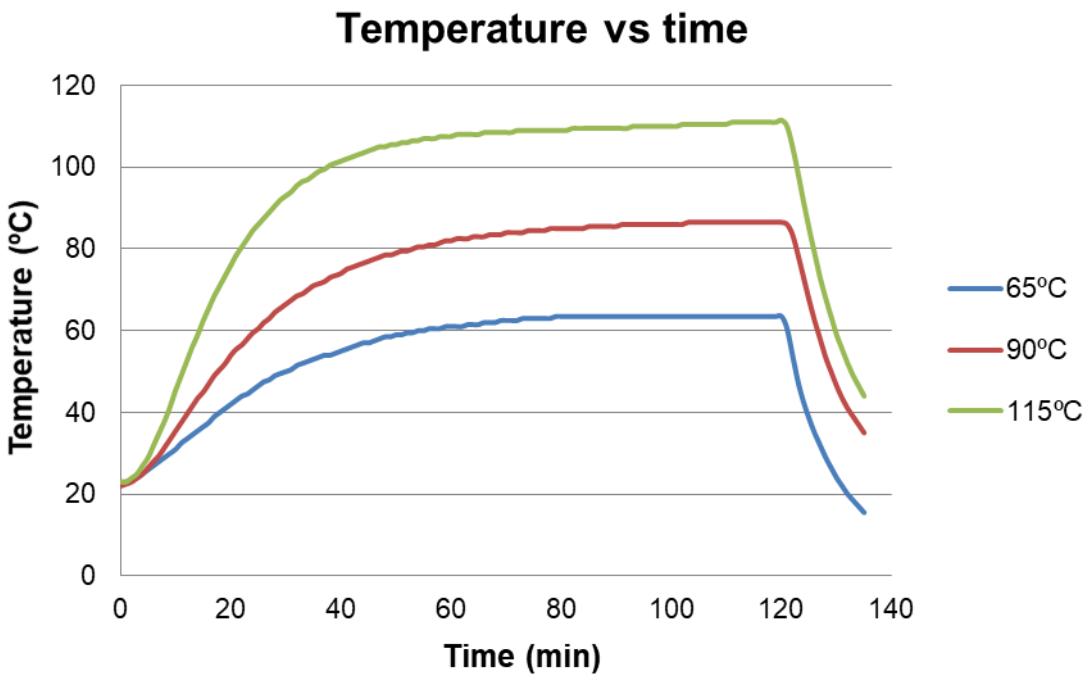


*Fig. S8 Evolution of the temperature of the sample during ammonia treatments.*

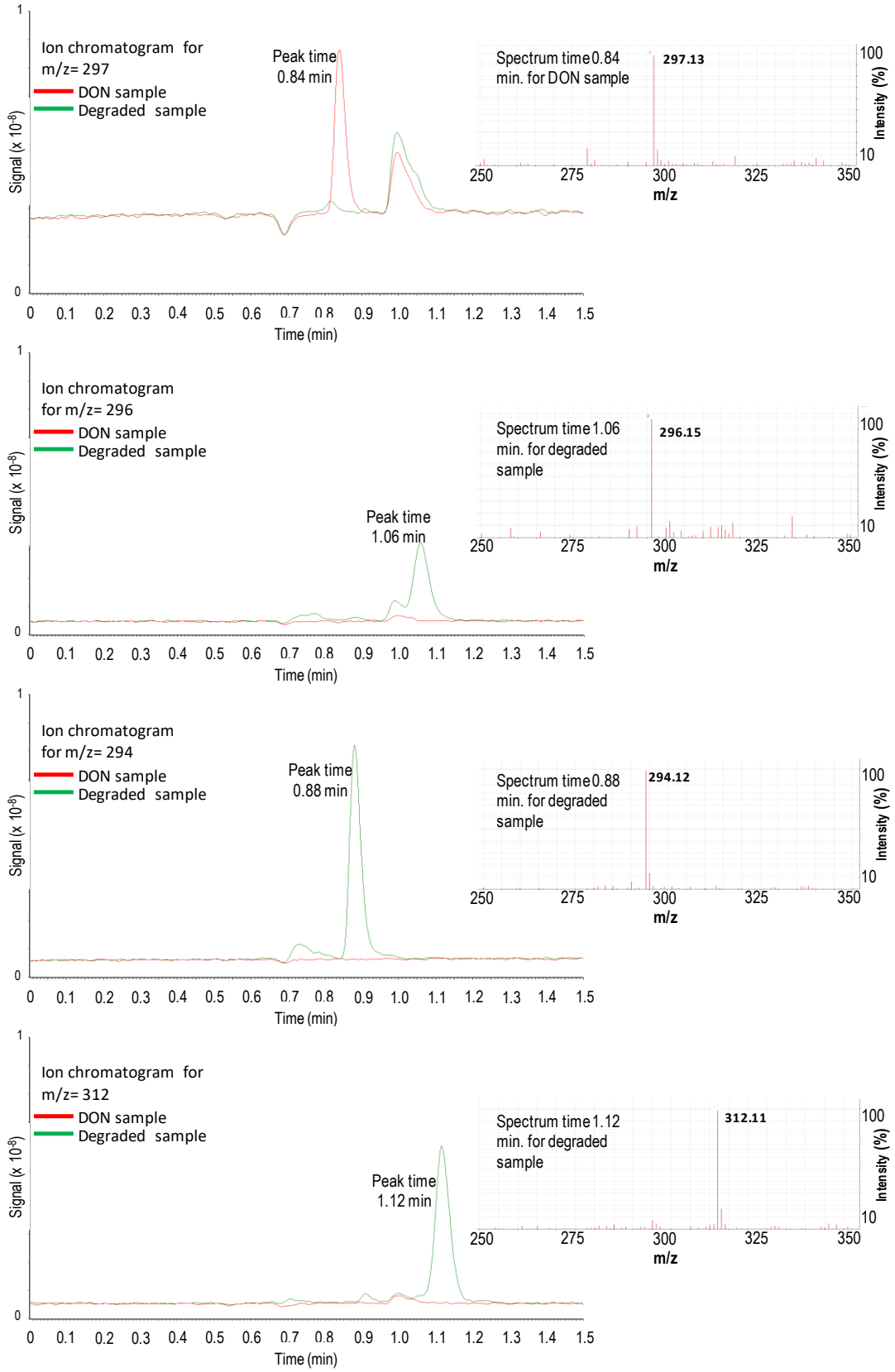


*Fig. S9 Chromatograms obtained by HPLC-MS of samples of DON and degraded DON and the spectra of each peak. Spectra were obtained by subtraction of the background spectra to the peak spectrum.*

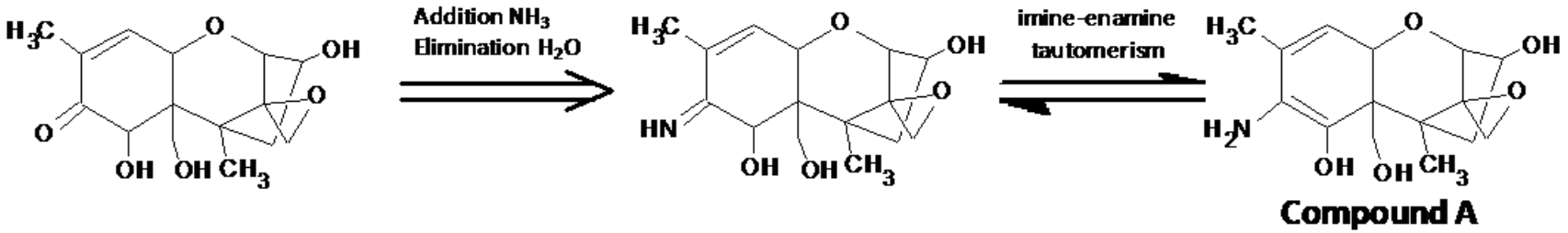


*Fig. S10 Possible chemical reaction between DON and $NH_3$ according to the peaks and molecular ions detected in the chromatograms of degraded sample. Calculated molecular weight of Compound A: 295.33 g/mol.*

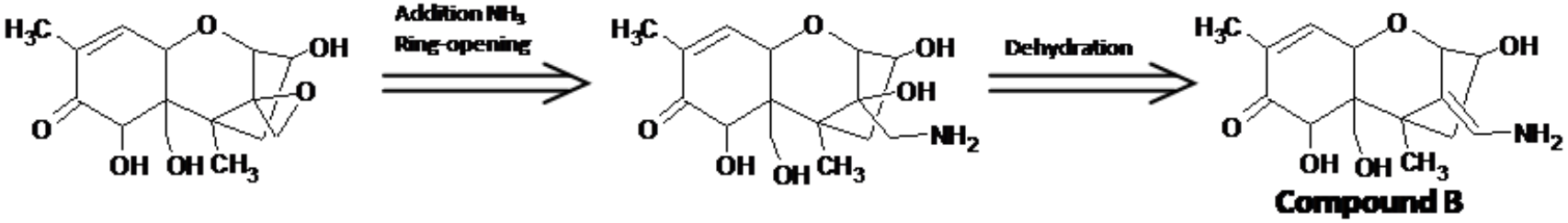


*Fig. S11 Possible chemical reaction between DON and $NH_3$ according to the peaks and molecular ions detected in the chromatograms of degraded sample. Calculated molecular weight of Compound B: 295.33 g/mol.*

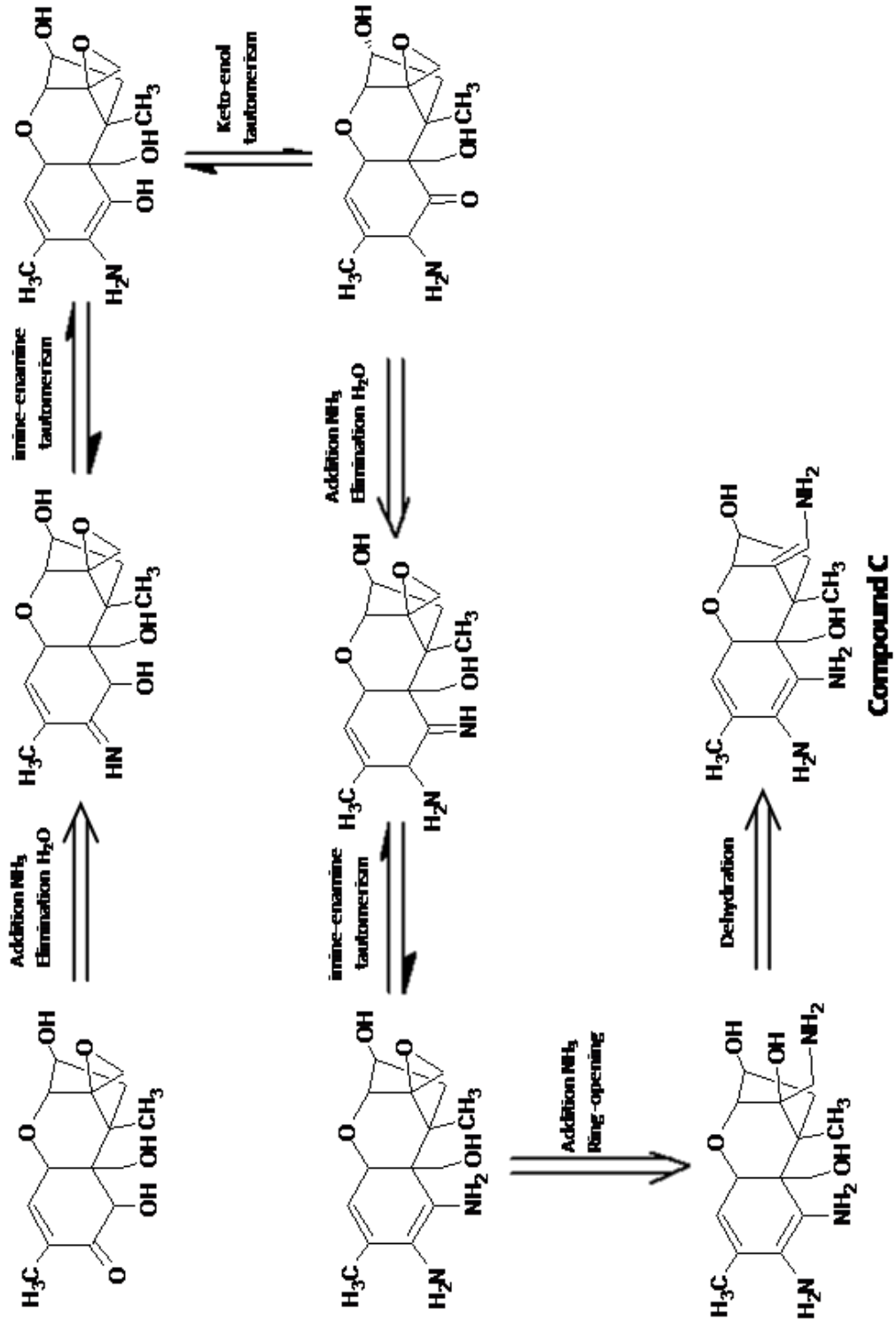


*Fig. S12 Possible chemical reaction between DON and $NH_3$ according to the peaks and molecular ions detected in the chromatograms of degraded sample. Calculated molecular weight of Compound C: 293.37 g/mol.*

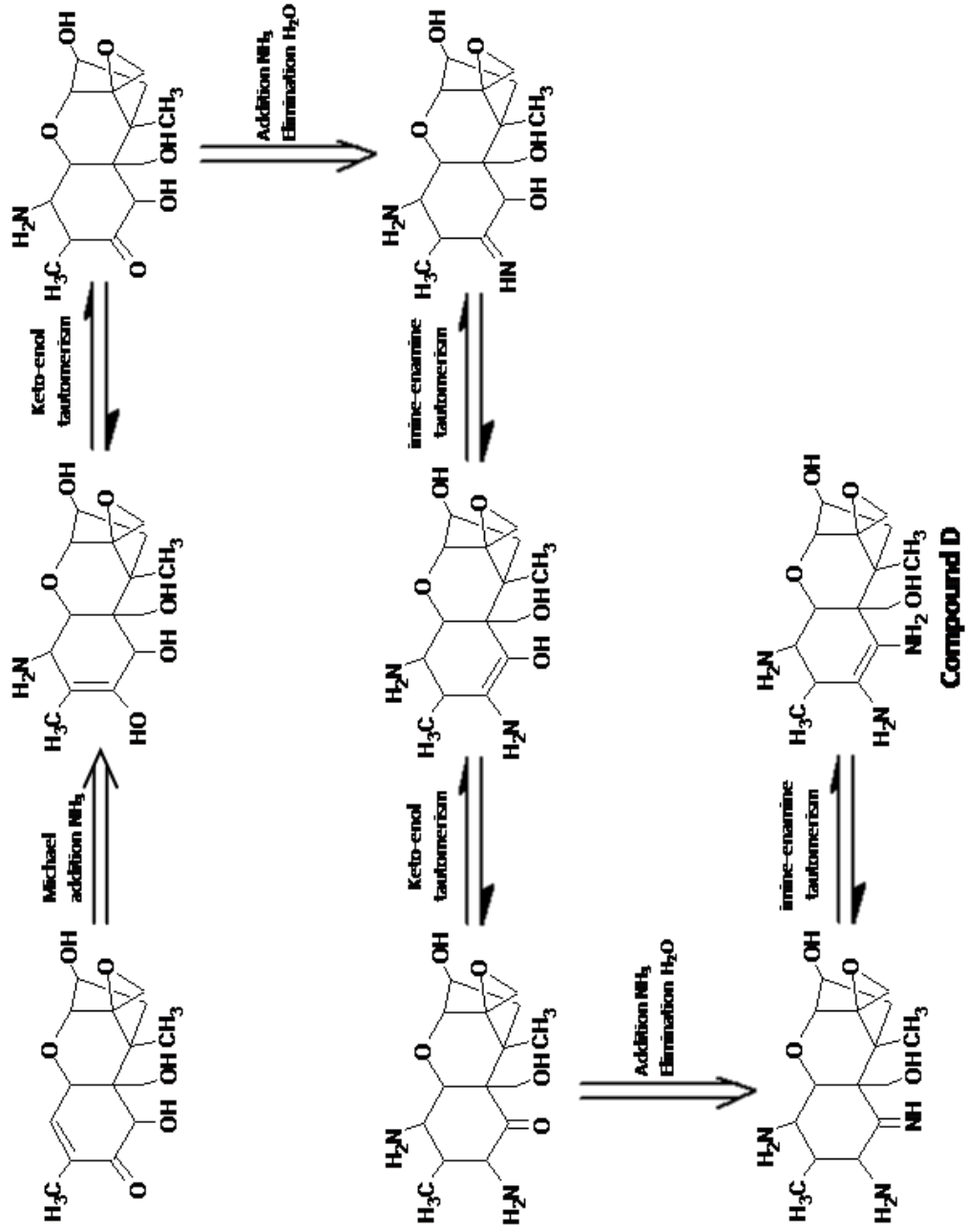


*Fig. S13 Possible chemical reaction between DON and $NH_3$ according to the peaks and molecular ions detected in the chromatograms of degraded sample. Calculated molecular weight of Compound D: 311.38 g/mol.*

## 10.2 Supplementary tables

*Table S1 Regression coefficients of the presented regression models*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 893.1 | 0 | | 4.85E-02 | 3.18E+02 | 157.574 | 7.14E-02 | 2.84E-02 | 3.24E-01 |
| 897.7 | -3.90E-02 | 1.74E-02 | 4.11E-02 | 1.69E+02 | 170.469 | 8.83E-02 | 3.08E-02 | 1.59E-01 |
| 902.31 | 2.24E-02 | 0.103 | -2.00E-02 | -5.11E+02 | -280.231 | -9.57E-02 | -4.13E-02 | -4.75E-01 |
| 906.93 | -1.62E-02 | -0.152 | 2.28E-03 | -1.86E+02 | -77.782 | -4.27E-02 | -4.36E-02 | -8.42E-02 |
| 911.55 | -1.74E-03 | -3.30E-02 | -1.43E-02 | -2.98E+02 | -110.619 | -9.51E-02 | -9.09E-03 | -3.00E-01 |
| 916.17 | 6.49E-02 | 0.12 | -3.29E-03 | 1.41E+02 | 113.233 | 4.31E-02 | 3.32E-03 | 1.80E-01 |
| 920.8 | -1.93E-02 | -7.58E-02 | -4.03E-02 | -1.98E+02 | -176.652 | -5.98E-02 | -1.87E-02 | -1.52E-01 |
| 925.44 | 7.31E-02 | 4.74E-02 | -5.34E-03 | 2.73E+02 | 145.382 | 4.34E-02 | 1.30E-02 | 2.66E-01 |
| 930.08 | -1.08E-02 | 3.89E-02 | -3.66E-03 | 3.86E+02 | 16.452 | 1.32E-02 | 1.73E-02 | 1.28E-01 |
| 934.72 | -3.15E-02 | -4.14E-02 | -6.34E-03 | -1.01E+02 | 205.26 | 5.40E-02 | 7.03E-02 | -9.46E-02 |
| 939.37 | 4.49E-02 | 3.38E-03 | 8.82E-03 | 1.37E+02 | -156.942 | -4.63E-02 | -6.36E-02 | 1.38E-01 |
| 944.03 | -7.80E-02 | -2.59E-02 | -3.92E-02 | -3.19E+02 | -123.227 | -5.42E-02 | -3.36E-02 | -3.14E-01 |
| 948.69 | 0.105 | 5.40E-03 | 3.14E-02 | 3.07E+02 | 335.119 | 0.104 | 0.13 | 0.458 |
| 953.35 | -0.116 | -2.61E-02 | 2.37E-02 | 5.97E+01 | 60.773 | 2.52E-02 | -2.33E-03 | -1.07E-01 |
| 958.02 | 0.129 | 9.88E-02 | -1.27E-02 | -1.23E+02 | -134.723 | -4.30E-02 | -8.51E-02 | -2.45E-01 |
| 962.7 | 4.63E-02 | 6.37E-02 | -3.98E-02 | -2.25E+02 | -156.454 | -4.91E-02 | -7.79E-02 | -1.55E-01 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 967.38 | -3.22E-02 | 1.26E-02 | -2.65E-02 | -9.20E+01 | -39.87 | -9.95E-03 | -5.41E-02 | 3.03E-02 |
| 972.06 | -1.09E-02 | -1.00E-02 | -7.35E-03 | 5.68E+01 | 54.834 | 1.23E-02 | -2.31E-02 | 1.27E-01 |
| 976.75 | 1.10E-02 | -2.62E-02 | -3.75E-03 | 2.77E+01 | 54.942 | 2.72E-03 | 4.87E-03 | 5.21E-02 |
| 981.45 | -7.63E-03 | -2.51E-02 | -6.19E-03 | 1.13E+01 | 43.535 | -9.30E-03 | 2.45E-02 | 1.73E-02 |
| 986.15 | -1.91E-02 | -1.97E-02 | -1.24E-02 | -5.65E+01 | -8.424 | -2.05E-02 | 1.86E-02 | -8.97E-02 |
| 990.85 | -6.42E-03 | -1.48E-02 | -1.65E-02 | -8.30E+01 | -39.127 | -2.78E-02 | 4.38E-03 | -1.01E-01 |
| 995.56 | 2.92E-02 | 7.94E-03 | -1.39E-02 | -3.89E+01 | -13.884 | -6.13E-03 | 6.53E-05 | -2.24E-02 |
| 1000.28 | 2.23E-02 | 4.51E-02 | -1.22E-02 | 1.00E+01 | -12.742 | -9.42E-04 | -1.19E-02 | 2.74E-03 |
| 1004.99 | -9.80E-03 | 6.16E-02 | -9.64E-03 | -1.91E+00 | -24.182 | 1.80E-03 | -2.23E-02 | -9.12E-03 |
| 1009.72 | 1.92E-02 | 0.102 | -6.40E-03 | 3.87E+00 | -34.922 | 2.78E-03 | -3.33E-02 | 1.17E-02 |
| 1014.45 | 6.44E-02 | 0.109 | -1.68E-03 | 7.10E+01 | -30.037 | 7.48E-03 | -4.37E-02 | 6.13E-02 |
| 1019.18 | 0.114 | 9.54E-02 | -6.15E-03 | 8.55E+00 | -49.827 | 5.89E-03 | -4.58E-02 | 6.87E-02 |
| 1023.92 | 5.21E-02 | 0.113 | -6.36E-03 | -3.49E+01 | -73.141 | -2.30E-04 | -4.85E-02 | 8.67E-03 |
| 1028.66 | 1.29E-02 | 0.11 | 8.61E-04 | 8.26E+00 | -24.346 | 1.05E-02 | -2.90E-02 | 6.74E-02 |
| 1033.41 | 5.41E-02 | 0.103 | 8.80E-03 | 2.17E+01 | -4.226 | 1.07E-02 | -1.88E-02 | 4.16E-02 |
| 1038.17 | 9.21E-02 | 9.91E-02 | 1.32E-02 | 2.24E+01 | 1.562 | 3.94E-03 | -1.04E-02 | 2.78E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1042.93 | 6.04E-02 | 8.28E-02 | 1.63E-02 | 1.81E+01 | 18.881 | 3.51E-03 | 3.39E-03 | 2.59E-03 |
| 1047.69 | 7.73E-02 | 7.45E-02 | 1.77E-02 | 2.34E+01 | 52.398 | 2.50E-03 | 2.27E-02 | -1.36E-02 |
| 1052.46 | 8.24E-02 | 6.81E-02 | 1.91E-02 | 2.21E+01 | 54.066 | -4.86E-04 | 3.59E-02 | -1.81E-02 |
| 1057.23 | 1.51E-02 | 3.47E-02 | 1.75E-02 | 4.61E+01 | 87.501 | 7.76E-03 | 5.66E-02 | -1.37E-02 |
| 1062.01 | 2.71E-02 | 3.79E-02 | 2.10E-02 | 9.78E+01 | 113.35 | 1.39E-02 | 6.39E-02 | 1.93E-02 |
| 1066.79 | 4.49E-02 | 4.10E-02 | 1.59E-02 | 1.21E+02 | 112.592 | 1.53E-02 | 6.65E-02 | 3.02E-02 |
| 1071.58 | -1.73E-02 | 1.52E-02 | 6.19E-03 | 5.93E+01 | 87.243 | 1.08E-02 | 6.71E-02 | -1.16E-02 |
| 1076.38 | -0.118 | -8.19E-03 | -2.57E-03 | 1.46E+01 | 66.649 | 5.64E-04 | 6.20E-02 | -4.36E-02 |
| 1081.17 | -4.26E-02 | 2.53E-02 | -7.12E-03 | -3.75E+01 | 45.269 | -5.98E-03 | 4.71E-02 | -8.38E-02 |
| 1085.98 | 2.17E-02 | 4.70E-02 | -8.52E-03 | -1.40E+01 | 35.224 | -5.94E-03 | 3.20E-02 | -6.20E-02 |
| 1090.79 | 2.28E-04 | 3.04E-02 | -1.05E-02 | -3.70E+01 | 13.06 | -1.32E-02 | 1.52E-02 | -6.01E-02 |
| 1095.6 | 6.52E-02 | 1.69E-02 | -1.03E-02 | -1.29E+01 | -0.176 | -7.33E-03 | -3.91E-03 | -1.33E-03 |
| 1100.42 | 7.14E-02 | 6.01E-03 | -1.68E-02 | -8.29E+01 | -62.236 | -2.23E-02 | -2.82E-02 | -4.80E-02 |
| 1105.24 | -4.00E-03 | -2.74E-02 | -1.89E-02 | -7.44E+01 | -83.725 | -2.51E-02 | -4.55E-02 | -2.36E-02 |
| 1110.07 | 2.55E-02 | -6.33E-02 | -1.92E-02 | -1.01E+02 | -91.882 | -2.09E-02 | -4.60E-02 | -5.67E-03 |
| 1114.9 | -1.32E-02 | -6.28E-02 | -1.97E-02 | -1.41E+02 | -122.062 | -2.63E-02 | -5.47E-02 | -3.06E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1119.74 | -0.131 | -9.79E-02 | -1.14E-02 | -1.15E+02 | -74.505 | -6.09E-03 | -4.59E-02 | -1.03E-02 |
| 1124.58 | -0.125 | -9.44E-02 | 5.15E-03 | 7.14E+01 | 23.866 | 3.09E-02 | -3.82E-02 | 1.66E-01 |
| 1129.43 | -4.17E-02 | -2.03E-03 | 2.97E-02 | 2.46E+02 | 122.244 | 6.81E-02 | -3.37E-03 | 2.11E-01 |
| 1134.28 | 0.164 | 9.08E-02 | 5.23E-02 | 3.39E+02 | 160.748 | 7.65E-02 | 3.39E-02 | 1.90E-01 |
| 1139.14 | 0.111 | 6.80E-02 | 6.19E-02 | 3.18E+02 | 122.589 | 5.37E-02 | 7.02E-02 | 1.68E-01 |
| 1144 | -6.25E-02 | -1.98E-02 | 5.16E-02 | 6.34E+01 | 11.533 | 8.65E-05 | 8.05E-02 | -9.10E-02 |
| 1148.87 | -6.04E-02 | -6.40E-02 | 3.12E-02 | -6.29E+01 | -47.048 | -3.23E-02 | 6.42E-02 | -1.69E-01 |
| 1153.74 | 2.48E-02 | -3.73E-02 | 1.14E-02 | -6.53E+01 | 8.237 | -1.79E-02 | 3.65E-02 | -4.84E-02 |
| 1158.62 | 0.173 | 7.79E-02 | -4.30E-04 | -2.56E+01 | 57.131 | 2.02E-03 | 3.44E-05 | 3.41E-02 |
| 1163.5 | 5.98E-02 | 0.132 | -2.35E-02 | -3.73E+01 | 23.969 | 1.47E-03 | -4.19E-02 | 5.46E-02 |
| 1168.39 | -0.128 | 4.07E-02 | -6.37E-02 | -2.18E+02 | -127.467 | -4.50E-02 | -0.112 | -0.111 |
| 1173.28 | -0.13 | -4.20E-02 | -9.40E-02 | -3.96E+02 | -220.392 | -7.86E-02 | -0.147 | -0.223 |
| 1178.18 | -1.56E-02 | -1.57E-02 | -8.49E-02 | -3.65E+02 | -194.88 | -7.33E-02 | -0.135 | -0.207 |
| 1183.08 | 8.38E-02 | 6.98E-02 | -6.06E-02 | -2.36E+02 | -143.021 | -6.86E-02 | -9.47E-02 | -1.71E-01 |
| 1187.99 | 6.45E-02 | 0.104 | -3.04E-02 | -5.97E+01 | -55.499 | -3.66E-02 | -2.89E-02 | -6.80E-02 |
| 1192.9 | -3.67E-02 | 2.86E-02 | -3.85E-03 | 7.04E+01 | 55.979 | 2.34E-03 | 3.64E-02 | 5.79E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1197.81 | -7.65E-02 | -6.87E-02 | 2.61E-02 | 1.65E+02 | 127.018 | 3.64E-02 | 7.68E-02 | 1.64E-01 |
| 1202.74 | -8.81E-02 | -0.117 | 5.89E-02 | 2.29E+02 | 176.447 | 6.54E-02 | 9.88E-02 | 1.90E-01 |
| 1207.66 | -4.82E-02 | -9.53E-02 | 7.86E-02 | 2.07E+02 | 147.41 | 6.87E-02 | 9.08E-02 | 1.66E-01 |
| 1212.59 | 5.70E-02 | -8.83E-03 | 7.65E-02 | 1.48E+02 | 81.183 | 4.61E-02 | 6.17E-02 | 7.33E-02 |
| 1217.53 | 3.50E-03 | 3.76E-02 | 5.09E-02 | 6.65E+01 | -20.089 | 7.71E-03 | 1.81E-02 | -2.95E-02 |
| 1222.47 | -9.23E-02 | 1.36E-02 | 1.93E-02 | 1.72E+01 | -67.752 | -9.91E-03 | -1.80E-02 | -6.81E-02 |
| 1227.42 | -2.93E-03 | 1.28E-02 | -5.01E-03 | -3.50E+01 | -87.534 | -1.78E-02 | -4.62E-02 | -1.04E-01 |
| 1232.37 | 8.67E-02 | 1.66E-02 | -2.42E-02 | -5.98E+01 | -103.151 | -1.96E-02 | -6.84E-02 | -7.54E-02 |
| 1237.33 | 3.46E-02 | -2.53E-03 | -3.31E-02 | -4.69E+01 | -86.568 | -1.65E-02 | -7.14E-02 | -5.24E-02 |
| 1242.29 | -3.00E-03 | -1.04E-03 | -3.59E-02 | -1.95E+01 | -51.396 | -8.08E-03 | -5.90E-02 | -3.81E-02 |
| 1247.25 | 5.48E-02 | 1.48E-02 | -3.36E-02 | 4.54E+01 | 3.865 | 1.22E-02 | -3.73E-02 | 5.10E-02 |
| 1252.23 | 2.04E-02 | 2.93E-02 | -3.06E-02 | 5.70E+01 | 47.269 | 1.75E-02 | -1.48E-02 | 7.85E-02 |
| 1257.2 | 5.80E-02 | 4.95E-02 | -2.94E-02 | 1.39E+01 | 50.228 | 1.45E-02 | -3.58E-03 | 4.41E-02 |
| 1262.18 | 0.183 | 9.70E-02 | -2.07E-02 | 3.85E+01 | 46.945 | 8.96E-03 | 7.56E-03 | 3.28E-02 |
| 1267.17 | 0.169 | 0.119 | -1.19E-02 | 2.76E+01 | 56.597 | 9.26E-03 | 1.48E-02 | 3.68E-02 |
| 1272.16 | 0.136 | 0.104 | -6.22E-03 | -2.44E+00 | 36.982 | -1.69E-03 | 1.65E-02 | 2.08E-03 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1277.16 | 7.90E-02 | 7.43E-02 | 1.94E-03 | -3.56E+01 | 14.719 | -1.14E-02 | 1.56E-02 | -1.98E-02 |
| 1282.16 | 4.49E-02 | 5.95E-02 | 8.73E-03 | -8.28E+01 | 0.695 | -2.20E-02 | 1.97E-02 | -8.45E-02 |
| 1287.16 | -4.38E-03 | 5.23E-02 | 1.69E-02 | -5.76E+01 | 36.591 | -9.06E-03 | 2.65E-02 | -3.95E-02 |
| 1292.17 | 9.10E-03 | 4.51E-02 | 2.11E-02 | -9.15E+01 | 26.405 | -1.57E-02 | 2.51E-02 | -7.26E-02 |
| 1297.19 | 4.65E-02 | 5.88E-02 | 2.56E-02 | -7.03E+01 | 28.303 | -8.19E-03 | 2.88E-02 | -5.14E-02 |
| 1302.21 | 2.62E-02 | 6.20E-02 | 2.73E-02 | -6.85E+01 | 18.55 | -1.36E-02 | 2.31E-02 | -4.93E-02 |
| 1307.23 | 2.43E-02 | 7.21E-02 | 2.72E-02 | -3.00E+01 | 21.624 | -8.88E-03 | 2.04E-02 | -4.01E-03 |
| 1312.27 | -6.53E-02 | 5.44E-02 | 2.15E-02 | 1.66E+00 | 9.969 | -6.73E-03 | 1.56E-02 | 1.15E-02 |
| 1317.3 | -0.175 | 3.71E-02 | 1.77E-02 | 4.59E+01 | 22.977 | 5.44E-03 | 2.22E-02 | 3.89E-02 |
| 1322.34 | -0.152 | 3.05E-02 | 8.28E-03 | -5.59E+00 | 10.967 | 5.23E-03 | 1.13E-02 | -1.61E-02 |
| 1327.39 | 1.19E-02 | 3.35E-02 | 4.26E-03 | 6.78E+01 | 10.285 | 1.14E-02 | 4.97E-03 | 4.11E-02 |
| 1332.44 | 0.108 | 7.93E-02 | -6.89E-03 | 1.47E+02 | 30.844 | 1.93E-02 | 1.68E-02 | 1.09E-01 |
| 1337.49 | 7.56E-02 | 4.90E-02 | -1.67E-02 | 1.81E+02 | 69.11 | 3.28E-02 | 3.06E-02 | 1.55E-01 |
| 1342.55 | 2.48E-02 | -3.14E-02 | -2.29E-02 | 1.05E+02 | 47.357 | 3.27E-02 | 2.80E-02 | 1.20E-01 |
| 1347.62 | -7.20E-02 | -6.97E-02 | -1.60E-02 | 4.53E+01 | 28.855 | 3.10E-02 | 2.54E-02 | 6.96E-02 |
| 1352.69 | -2.57E-02 | -7.73E-02 | -7.17E-03 | -7.92E+01 | -21.036 | 7.85E-03 | 3.54E-02 | -1.11E-01 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1357.76 | 9.61E-03 | 2.33E-03 | 5.13E-03 | -6.99E+01 | -61.173 | 7.23E-03 | 1.45E-02 | -1.33E-01 |
| 1362.84 | 0.122 | 0.114 | 8.32E-03 | -2.56E+01 | -26.662 | 7.94E-03 | 1.52E-02 | -7.83E-02 |
| 1367.93 | 0.13 | 9.05E-02 | 2.39E-02 | 1.02E+02 | 33.733 | 2.06E-02 | 2.80E-02 | 7.70E-02 |
| 1373.02 | -8.89E-02 | -3.47E-02 | 4.85E-02 | 1.71E+02 | 76.369 | 3.42E-02 | 3.88E-02 | 2.28E-01 |
| 1378.11 | -8.47E-02 | -4.49E-02 | 6.11E-02 | 1.38E+02 | -1.142 | 2.69E-02 | 8.41E-03 | 1.63E-01 |
| 1383.21 | 3.98E-02 | -2.38E-03 | 4.30E-02 | -8.04E+01 | -27.864 | -2.14E-02 | 7.16E-03 | -9.42E-02 |
| 1388.31 | 1.33E-02 | -7.24E-02 | 3.31E-02 | 7.18E+01 | 27.273 | 7.23E-04 | -2.28E-02 | 8.71E-02 |
| 1393.42 | -0.121 | -0.149 | -1.37E-02 | -3.52E+01 | -56.95 | -3.69E-02 | -6.58E-02 | -6.61E-02 |
| 1398.54 | -8.92E-02 | -0.13 | -4.57E-02 | -8.27E+01 | -39.199 | -3.26E-02 | -5.73E-02 | -8.40E-02 |
| 1403.66 | 2.19E-02 | 1.39E-02 | -5.09E-02 | -4.79E+01 | 5.331 | -8.98E-03 | -1.70E-02 | -6.64E-02 |
| 1408.78 | 4.88E-02 | 0.102 | -3.60E-02 | 2.97E+01 | 52.78 | 1.33E-02 | 3.11E-02 | -4.01E-02 |
| 1413.91 | -1.06E-02 | 6.49E-02 | -1.12E-02 | 9.06E+01 | 76.12 | 2.98E-02 | 5.53E-02 | 3.89E-02 |
| 1419.05 | 2.34E-02 | 4.71E-02 | 1.25E-02 | 6.81E+01 | 64.713 | 2.79E-02 | 4.82E-02 | 5.09E-02 |
| 1424.18 | -7.31E-02 | -5.03E-02 | 3.20E-02 | -3.68E+00 | 4.518 | 7.76E-03 | 2.27E-02 | 3.79E-02 |
| 1429.33 | -0.113 | -9.11E-02 | 4.05E-02 | -3.96E+01 | -39.953 | -4.08E-03 | -1.45E-02 | 1.64E-02 |
| 1434.48 | 1.62E-02 | -3.10E-02 | 3.44E-02 | -1.44E+02 | -109.531 | -3.13E-02 | -2.66E-02 | -2.10E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1439.63 | 2.52E-02 | -6.02E-02 | 2.40E-02 | -1.19E+02 | -63.251 | -2.57E-02 | -1.42E-02 | -1.54E-02 |
| 1444.79 | 1.05E-02 | -8.59E-02 | 1.41E-02 | -1.01E+02 | -48.788 | -2.32E-02 | -7.38E-03 | -2.97E-02 |
| 1449.95 | 4.68E-02 | -6.78E-02 | 6.96E-03 | -1.71E+01 | -1.108 | -9.16E-03 | 7.85E-03 | 3.30E-02 |
| 1455.12 | -1.75E-02 | -8.55E-02 | -2.33E-03 | 2.90E+01 | 14.732 | -2.00E-04 | 1.48E-02 | 5.04E-02 |
| 1460.3 | -4.23E-02 | -8.88E-02 | -4.25E-03 | 1.46E+01 | 8.508 | 4.95E-03 | 1.41E-02 | 1.43E-02 |
| 1465.48 | -9.80E-02 | -9.12E-02 | -7.71E-03 | -1.24E+01 | -14.402 | -4.40E-04 | 1.93E-02 | -3.28E-02 |
| 1470.66 | -0.261 | -0.127 | -7.36E-03 | 1.27E+01 | -11.2 | 8.39E-03 | 1.89E-02 | -2.60E-02 |
| 1475.85 | -0.201 | -0.113 | -1.45E-02 | -1.72E+01 | -11.831 | 8.05E-03 | 1.09E-02 | -4.97E-02 |
| 1481.04 | -0.136 | -0.102 | -1.76E-02 | -2.52E+01 | -14.353 | 2.52E-03 | 1.28E-02 | -7.24E-02 |
| 1486.24 | -7.27E-02 | -8.16E-02 | -1.86E-02 | 5.63E+00 | 6.799 | 3.94E-03 | 1.67E-02 | -2.39E-02 |
| 1491.44 | -3.87E-02 | -5.45E-02 | -2.01E-02 | 8.23E+00 | 35.305 | 8.12E-03 | 3.45E-02 | -2.58E-03 |
| 1496.65 | -3.51E-02 | -2.63E-02 | -2.28E-02 | -1.82E+01 | 20.083 | 2.03E-03 | 2.84E-02 | -2.44E-02 |
| 1501.86 | -2.83E-02 | -2.02E-02 | -2.41E-02 | 5.65E+00 | 28.364 | 3.60E-03 | 2.24E-02 | 1.26E-03 |
| 1507.08 | -4.04E-02 | -4.15E-02 | -2.38E-02 | 9.51E+00 | 6.508 | 2.08E-05 | 5.38E-03 | 7.20E-03 |
| 1512.31 | 1.22E-02 | -2.77E-02 | -2.31E-02 | 3.38E+01 | -15.03 | 3.69E-04 | -1.62E-02 | 5.87E-02 |
| 1517.53 | -1.98E-02 | -3.60E-02 | -2.50E-02 | 2.97E+00 | -48.903 | -4.30E-03 | -4.21E-02 | 6.03E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1522.77 | -3.51E-02 | -4.86E-02 | -1.88E-02 | 2.56E-01 | -58.227 | -6.11E-03 | -5.57E-02 | 5.41E-02 |
| 1528 | 2.14E-02 | -1.82E-02 | 1.13E-03 | 7.86E+00 | -89.344 | -8.77E-03 | -7.95E-02 | 7.04E-03 |
| 1533.25 | 7.83E-02 | 7.31E-03 | 1.68E-02 | -4.80E+00 | -122.557 | -9.77E-03 | -8.66E-02 | -6.25E-02 |
| 1538.5 | 0.115 | 1.27E-02 | 2.48E-02 | -1.77E+01 | -81.487 | -5.00E-03 | -5.67E-02 | -6.46E-02 |
| 1543.75 | 7.56E-02 | 6.54E-03 | 2.66E-02 | -1.27E+01 | -56.456 | 9.59E-04 | -4.40E-02 | -5.06E-02 |
| 1549.01 | 9.16E-02 | 7.14E-03 | 2.96E-02 | 4.62E+01 | -14.284 | 1.16E-02 | -2.86E-02 | -3.00E-03 |
| 1554.27 | 0.109 | 9.05E-03 | 3.05E-02 | 5.02E+01 | 26.88 | 1.67E-02 | -1.14E-02 | 2.84E-02 |
| 1559.54 | 6.98E-02 | -7.41E-03 | 3.32E-02 | 8.86E+01 | 69.176 | 2.76E-02 | 7.91E-03 | 7.30E-02 |
| 1564.81 | 7.53E-02 | -2.26E-02 | 3.40E-02 | 8.41E+01 | 68.809 | 2.25E-02 | 1.97E-02 | 5.80E-02 |
| 1570.09 | 1.20E-02 | -3.78E-02 | 3.52E-02 | 9.89E+01 | 84.723 | 2.17E-02 | 3.77E-02 | 5.15E-02 |
| 1575.37 | -5.81E-02 | -5.07E-02 | 3.28E-02 | 7.84E+01 | 72.032 | 1.78E-02 | 4.18E-02 | 3.86E-02 |
| 1580.66 | -6.46E-02 | -5.90E-02 | 3.01E-02 | 4.98E+01 | 54.466 | 1.15E-02 | 4.51E-02 | -2.34E-03 |
| 1585.95 | -9.18E-02 | -7.13E-02 | 2.95E-02 | 6.89E+01 | 50.712 | 1.31E-02 | 4.48E-02 | 2.48E-02 |
| 1591.25 | -5.75E-02 | -5.63E-02 | 2.23E-02 | 3.69E+01 | 20.862 | 6.52E-03 | 3.13E-02 | 1.26E-02 |
| 1596.55 | -1.77E-02 | -5.12E-02 | 1.88E-02 | 6.03E+01 | 31.805 | 9.48E-03 | 3.51E-02 | 2.79E-02 |
| 1601.85 | 1.85E-02 | -5.06E-02 | 9.64E-03 | 5.39E+01 | 21.869 | 7.03E-03 | 2.32E-02 | 4.79E-02 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1607.17 | 2.50E-02 | -4.81E-02 | 1.55E-03 | 5.56E+01 | 25.62 | 6.64E-03 | 1.60E-02 | 6.20E-02 |
| 1612.48 | 2.47E-02 | -4.38E-02 | -7.24E-03 | 3.90E+01 | 9.152 | 4.11E-03 | 1.08E-03 | 3.55E-02 |
| 1617.81 | -3.79E-02 | -4.05E-02 | -1.48E-02 | 3.97E+01 | 1.086 | -4.71E-04 | -5.82E-03 | 4.31E-02 |
| 1623.13 | -6.93E-02 | -4.04E-02 | -2.61E-02 | -4.23E+01 | -34.98 | -1.36E-02 | -1.98E-02 | -3.93E-02 |
| 1628.46 | 4.86E-03 | 5.91E-03 | -3.19E-02 | -5.46E+01 | -63.036 | -2.16E-02 | -1.84E-02 | -4.32E-02 |
| 1633.8 | -3.32E-02 | 1.09E-02 | -3.31E-02 | -6.60E+01 | -88.582 | -2.85E-02 | -1.42E-02 | -4.18E-02 |
| 1639.14 | -0.123 | -2.02E-02 | -3.53E-02 | -9.84E+01 | -88.443 | -2.98E-02 | -6.33E-03 | -4.59E-02 |
| 1644.49 | -0.179 | -5.30E-02 | -3.82E-02 | -1.26E+02 | -100.078 | -3.36E-02 | -8.10E-03 | -6.33E-02 |
| 1649.84 | -0.184 | -7.74E-02 | -4.22E-02 | -1.82E+02 | -96.509 | -3.78E-02 | -1.76E-02 | -9.91E-02 |
| 1655.2 | -0.159 | -8.59E-02 | -4.51E-02 | -2.53E+02 | -98.537 | -4.03E-02 | -4.04E-02 | -1.76E-01 |
| 1660.56 | -4.00E-02 | -1.09E-02 | -4.84E-02 | -2.82E+02 | -63.998 | -2.96E-02 | -5.19E-02 | -1.91E-01 |
| 1665.92 | 0.245 | 0.172 | -3.19E-02 | -1.82E+02 | 27.111 | 8.55E-03 | -1.56E-02 | -7.77E-02 |
| 1671.3 | 0.183 | 0.145 | 6.28E-03 | 1.15E+02 | 118.881 | 5.44E-02 | 4.50E-02 | 1.88E-01 |
| 1676.67 | -0.188 | -6.75E-02 | 3.88E-02 | 3.26E+02 | 100.507 | 4.19E-02 | 5.33E-02 | 2.32E-01 |
| 1682.05 | -0.346 | -0.241 | 5.80E-02 | 2.86E+02 | -34.747 | -2.28E-02 | -5.26E-03 | 2.98E-02 |
| 1687.44 | 1.21E-02 | -0.206 | 3.97E-02 | 1.16E+01 | -117.958 | -4.68E-02 | -7.88E-02 | -1.29E-01 |

*Table S1 Regression coefficients of the presented regression models (continued)*

| Wavelength (nm) | Model (Modelled mycotoxin and pretreatment are indicated) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DON. Full MSC + 1st derivative | DON. 1st derivative + full MSC | $FB_1$. No pretreatments | $FB_1$. SNV | $FB_2$. SNV | $FB_2$. Full MSC | $FB_1$+$FB_2$. No pretreatments | $FB_1$+$FB_2$. Full MSC |
| 1692.83 | 0.122 | -3.42E-02 | -7.21E-04 | -2.14E+02 | -59.312 | -1.30E-02 | -6.19E-02 | -9.83E-02 |
| 1698.23 | 5.96E-03 | 8.96E-02 | -3.48E-02 | -3.84E+02 | -9.345 | -9.53E-03 | -1.87E-02 | -1.56E-01 |
| 1703.63 | -5.19E-02 | 0.117 | -3.06E-02 | -1.13E+02 | 34.567 | 1.58E-02 | 4.07E-02 | 4.52E-02 |
| 1709.03 | -0.107 | 8.03E-02 | -2.42E-02 | 4.27E+01 | 16.098 | 2.01E-02 | 4.48E-02 | 6.02E-02 |
| 1714.44 | -7.14E-02 | -8.41E-02 | 8.61E-03 | 2.33E+02 | 87.804 | 3.81E-02 | 7.50E-02 | 2.46E-01 |
| 1719.86 | 0.142 | -0.136 | 1.94E-03 | 1.40E+02 | -28.539 | -6.01E-03 | 3.21E-02 | 6.80E-02 |
| 1725.28 | 2.61E-02 | 8.77E-02 | -5.88E-03 | -1.29E+02 | -5.47 | -1.89E-02 | -1.05E-02 | -1.27E-01 |
| 1730.71 | 0 | | 2.98E-03 | 2.56E+01 | -36.949 | -2.42E-03 | -6.10E-02 | -6.50E-02 |

*Table S2 Analysis of variance (ANOVA) of the central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and $NH_4OH$ concentration ([$NH_4OH$]).*

| | Sum of square | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| *T* | 2137.59 | 1 | 2137.59 | 137.697 | <0.001 |
| *$T^2$* | 821.35 | 1 | 821.35 | 52.909 | <0.001 |
| *[$NH_4OH$]* | 997.94 | 1 | 997.94 | 64.284 | <0.001 |
| *[$NH_4OH$]$^2$* | 136.52 | 1 | 136.52 | 8.794 | 0.025 |
| *Lack of Fit* | 89.90 | 4 | 22.46 | 13.852 | 0.069 |
| *Pure Error* | 3.25 | 2 | 1.62 | | |
| *Total sum square* | 4452.15 | 10 | | | |

*Temperature by $NH_4OH$ linear interaction was excluded due to p value > 0.05. $R^2$=0.9791; $R^2$-Adj=0.9651.*

*Table S3 Regression coefficient results from the data of central composite design for DON degradation. Factors included in the model: linear and quadratic components of temperature (T) and $NH_4OH$ concentration ([$NH_4OH$]).*

| | Regression Coefficients | Standard error | t-value | p-value |
|---|---|---|---|---|
| *Constant* | -290.40 | 30.38 | -9.560 | <0.001 |
| *T* | 5.94 | 0.72 | 8.299 | <0.001 |
| *$T^2$* | -0.029 | 0.004 | -7.274 | <0.001 |
| *[$NH_4OH$]* | 26.41 | 6.27 | 4.213 | 0.006 |
| *[$NH_4OH$]$^2$* | -2.87 | 0.97 | -2.966 | 0.025 |

*Temperature by $NH_4OH$ linear interaction was excluded due to p value > 0.05. $R^2$=0.9791; $R^2$-Adj=0.9651.*