



UNIVERSITAT ROVIRA I VIRGILI

ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

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**UNIVERSITAT
ROVIRA i VIRGILI**

Analytical tools to determine mycotoxins and modified mycotoxins

EUGÈNIA MIRÓ ABELLA



**DOCTORAL THESIS
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Analytical tools to determine mycotoxins and modified mycotoxins

Eugènia Miró Abella

DOCTORAL THESIS

Supervised by

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Department of Analytical Chemistry and Organic Chemistry



UNIVERSITAT ROVIRA I VIRGILI

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UNIVERSITAT ROVIRA I VIRGILI

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WE STATE that the present study, entitled "ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS", presented by EUGÈNIA MIRÓ ABELLA for the award of the degree of Doctor, has been carried out under our supervision at the Department of Analytical Chemistry and Organic Chemistry of this university, and that it fulfils all the requirements to be eligible for the distinction of International Doctor.

Tarragona, 3th September 2018

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I fins aquí ha arribat aquesta etapa. De vegades va bé tancar per a poder continuar. Però també és útil aturar-se un moment per veure amb perspectiva fins on hem arribat. I ara, intentant recordar tot el viscut durant aquests tres anys de tesi, me n'adono de totes les coses que han arribat a passar i de tots els moments viscuts.

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*Perquè hi haurà un dia que no podrem més
i llavors ho podrem tot.*

Vicent Andrés Estellés

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ABSTRACT

Controlling contaminants in food is a priority for human and animal health and one of the major concerns of authorities across Europe and all over the world. Of all the unwanted compounds that can be found in everyday food and feedstuff, mycotoxins are one of the most widely studied contaminants. Mycotoxins are small secondary metabolites produced by filamentous fungi that are commonly found in cereals and cereal derivatives, which have toxicological effects. The central focus of mycotoxin research is divided into two main topics: determining their presence with the development of robust analytical methods, and studying their toxicological effects. Accordingly, the experimental part of this doctoral thesis is following these two central strands.

As mentioned, the major sources of mycotoxin contamination are agricultural products, especially cereals and their derivatives. For this reason, the main objective of the first section of this thesis is to develop new analytical methods to determine the incidence of targeted mycotoxins, including modified mycotoxins, in cereal and cereal derivative samples. These analytical methods also involved the optimisation of the extraction techniques followed by liquid chromatography coupled to tandem mass spectrometry. The mycotoxins found in the analysed food samples over the course of this doctoral thesis demonstrate their prevalence in the food chain of humans and animals. Thus, it is important to establish solid regulations to monitor them, which can be achieved by developing robust, selective and simple analytical methods.

In the second and third sections, there is an evaluation of the consequences of consuming food contaminated by mycotoxins. To achieve this, for the first time, metagenomic research was performed on rat gut samples after two months of treatment with deoxynivalenol at low concentration levels in order to determine whether mycotoxin consumption can trigger any bacterial changes. The optimisation of the analytical methodology for faecal samples was also explored. Then, a preliminary study of a large number of possible derivatives biologically generated by rats after nivalenol and nivalenol-3-glucoside consumption is presented in the third section. This has led to the identification of new compounds to be explored in further research.

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CHAPTER 1. INTRODUCTION

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1.1. Mycotoxins

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Nutrition and food safety are two of the general concerns of human society, which has been adopting more modern points of view over the last few years. Food quality has become a leading issue, either because of the increasing importance attached to local products or because of the presence of less organic contaminants such as pesticides, hormones, additives or mycotoxins.

The presence of fungi in feed grains can produce secondary metabolites, including mycotoxins in the moulds of target food or feedstuffs. Although crops seem apparently healthy, they can contain large amounts of different fungus types and their metabolites. This has caused considerable damage to cereal crops over time, with significant economic consequences. The problem is a serious one since 25% of the world's crops have contaminated moulds, according to the Food and Agriculture Organization of the United Nations (FAO) [1]. The term mycotoxin combines the original Greek word "*mykes*", which means fungus, with "*toxicum*", from Latin which means poisonous. These small toxic secondary metabolites (MW <800 Da) have toxic and/or carcinogenic effects if humans or animals consume them, breathe them in or otherwise come into contact with them. There are around 400 types of known mycotoxins with varying secondary effects. These mycotoxins can be classified depending on the producer fungus, the moment of production, structural characteristics, or toxicological effects [2].

Throughout history, mycotoxins have been related to disease. They were the source of the tenth plague of Egypt and the cause of the "bewitchments" leading to the Salem Witchcraft Trials, among other episodes. More recently, in the 1940s and 1950s, there were episodes of human lethal disease in Russia and Japan, episodes of mould toxicosis and stachybotryotoxicosis in the United States and also a facial eczema disease in New Zealand sheep. In 1961, a huge number of animals in England died after ingesting contaminated feed, which led to the discovery of aflatoxins (AFs) [3,4]. Since then, a considerable amount of research has been done to improve the extraction, detection, quantification and reduction of mycotoxins, guarantee food and feed security and prevent these past circumstances from repeating.

In terms of abundance and toxicology, the most important mycotoxins are produced by the *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium* filamentous fungi. They normally grow at between 10 and 40 °C, in a pH range between 4 and 8, and at water activity levels above 0.70, although these conditions can vary between fungi species [2]. It should be noted that one mycotoxin can be produced by different fungal species or one fungal species can produce several mycotoxins. The most important mycotoxins produced by *Aspergillus* or *Penicillium* are ochratoxin A (OTA) and AFs, of which aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂) are the most prevalent.

Otherwise, the most common *Fusarium* mycotoxins are fumonisins (FBs) – of which fumonisin B₁ (FB₁) and fumonisin B₂ (FB₂) are predominant – zearalenone (ZEA) and trichothecenes, of which the best known are deoxynivalenol (DON) and HT-2 and T-2 toxins. All these mycotoxins have been studied during the present doctoral thesis.

These kinds of fungi are generally associated with the climate and crop stages of different geographical regions. The genera *Fusarium* and *Alternaria* are common in field contaminations, and *Penicillium* and *Aspergillus* are common to crop storage [5]. Consequently, mycotoxins such as FBs and DON are mainly produced before harvest, and AFs and OTA are mainly produced during post-harvest stages (pre-harvest mycotoxins and post-harvest mycotoxins). However, depending on the producer fungus, they can appear in any crop and harvest stage and, depending on when they are produced, they can be reduced in various ways. Pre-harvest mycotoxins can be reduced by applying good agricultural practices (GAP), using control methods, developing resistant varieties of crops, using crop protection chemicals, etc. And post-harvest mycotoxins can be reduced with such strategies as appropriate drying, handling, packaging, storage and transport conditions, the application of detection and detoxification methods, and the removal of damaged grain.

In spite of these factors, they can be found all over the world and in a wide variety of food samples, due to climate change and international commercialisation. This creates a considerable problem since they are present throughout the food chain. Although mycotoxins are most commonly present in cereals and products derived from cereals, they can also be found in dairy products, spices, dried fruits, nuts, coffee, vegetable oils, wine and fruit juices [6,7]. In fact, any processed products manufactured from contaminated raw material can contain mycotoxins. Furthermore, the mycotoxins commonly associated with cereal grains, like AFs, OTA, DON and ZEA, are moderately stable in most food processing systems, such as milling, baking, frying, roasting and boiling, where temperatures are up to 120 °C. They are not eliminated by food processing, although in some cases their concentration is significantly reduced [8]. This leads to their persistence in the food chain.

Thus, bearing in mind that it is practically impossible not to consume them, it is important to evaluate exposure and risk. Exposure is evaluated by monitoring food contamination and food consumption. The assessment of dietary intake and exposure takes into account age and body weight, respectively. Figure 1 shows a scheme of all the parameters considered for exposure assessment. The risks associated with mycotoxin consumption can then be determined by using the data on exposure and toxicology. The characterisation of risk enables mycotoxins to be regulated and values such as tolerable

daily intake (TDI), no observed adverse effect level (NOAEL) and dietary intake to be established. Toxicological data describe the consumption, the inhalation and the cutaneous absorption of these toxins, which can produce a wide variety of diseases known as mycotoxicoses [4]. These mycotoxicoses depend on the toxicity of each mycotoxin, the degree of exposure, and the age and the nutritional stage of humans and animals [9]. They have several common characteristics: they are not transmissible, they are little affected by treatment with drugs or antibiotics, their symptoms are often seasonal and associated with specific foodstuffs, and they are habitually present in food samples suspected of being contaminated. The toxicological effects of the most common mycotoxins have been widely described, and range from nausea and vomiting, to carcinogenic and teratogenic consequences.

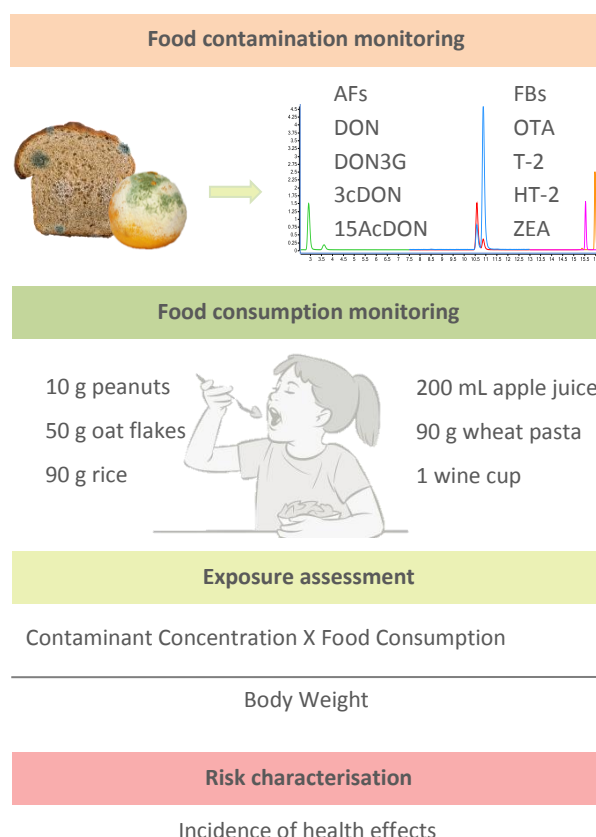


Figure 1. Elements of the risk characterisation.

Mycotoxins are commonly found at very low concentrations and in a wide variety of food samples around the world. The chemical composition of food samples is highly complex, and the presence of water, proteins, carbohydrates, sugars and fats, among other things, cause several interferences during the analytical process. Therefore, efficient and accurate methods are necessary to identify mycotoxins in the wide variety of matrices. These methods must also be sensitive enough to quantify them at very low levels since they are usually present in low doses (parts per billion). Thus, rapid and effective analytical methods need to be developed to detect and quantify mycotoxins in food and feed so that they can be removed as far as possible from the food chain and their toxicological effects prevented. The development of suitable analytical methods has become essential to the analysis of mycotoxins and extraction techniques have to be easy, rapid and cheap so that they can be included in routine analysis. They must also be robust enough to detect modified mycotoxins, especially in complex matrices.

The mycotoxins studied in this doctoral thesis were selected for their abundance and toxicological effects. The following sections give a detailed description of these mycotoxins, their producer fungi, toxicological effects (especially in humans), occurrence in food samples, and prevention and regulation.

1.1.1. *Aspergillus* and *Penicillium* mycotoxins

Aspergillus and *Penicillium* are capable of producing such mycotoxins as ochratoxins and AFs, which have dangerous effects in both humans and animals. OTA and AFs are considered to be the most toxic mycotoxins because they have carcinogenic and teratogenic effects. These mycotoxins can be found in a wide variety of food and feed samples, so they have been widely studied by the scientific community and are also discussed in this doctoral thesis.

a) Ochratoxin A

Ochratoxins A, B and C are secondary metabolites produced by filamentous species belonging to the genera *Penicillium* and *Aspergillus*. OTA is the most hazardous [10]. It was first isolated from *A. ochraceus* and chemically characterised in 1965 in corn meal samples [11,12]. To date a wide range of *Aspergillus* species have been shown to produce OTA in foodstuffs, among others *A. niger* and *A. carbonarius* [13,14] (see Table 1). These toxigenic filamentous fungi preferentially grow in hot and wet climatic conditions like South Asia, South America and Africa. Even so, in North Europe and North America two *Penicillium* species grow at low temperatures producing the OTA *P. verrucosum* and *P.*

Table 1. Non-exhaustive list of *Aspergillus* and *Penicillium* producing species.

Mycotoxin	Genus	Specie	Ref.
OTA	<i>Aspergillus</i>	<i>A. ochraceus</i>	[13,14,19]
		<i>A. carbonarius</i>	
		<i>A. niger</i>	
	<i>Penicillium</i>		
		<i>P. verrucosum</i>	
		<i>P. nordicum</i>	
AFs	<i>Aspergillus</i>	<i>A. flavus</i>	
		<i>A. parasiticus</i>	

nordicum [14,15]. Hence, this mycotoxin can be found nearly all over the world and in a wide variety of matrices, principally in insufficiently dried cereals and cereal products, but also in wines, musts and grape juices [16]. They are also present in other commodities such as beer, pork, coffee, peanuts, spices, cocoa, pulses, cow milk and cheeses [13,17,18].

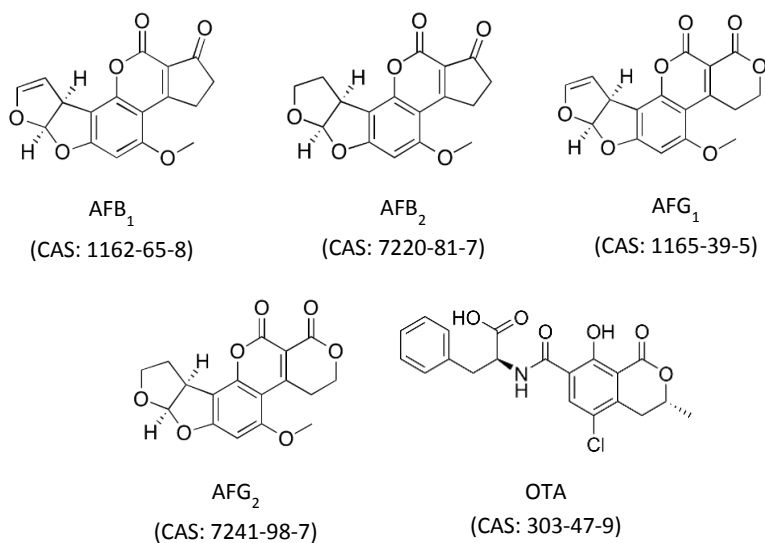


Figure 2. Chemical structures and CAS numbers of OTA and the four AFs studied in this doctoral thesis.

OTA is a cyclic pentaketide and it is regarded as the second most important mycotoxin [2]. Its chemical structure and CAS number are listed in Figure 2. One of the most

important characteristics of this mycotoxin is that it is extremely stable to high temperatures and acidity. For this reason, cooking processes are not enough to completely remove OTA contamination from foodstuffs. This conclusion was drawn from previous research that observed that OTA did not fully degrade: heating wheat to 250 °C was not enough [20], roasting coffee reduced it only by 69% [21] and baking at 200 °C under acidic conditions degraded it slowly but not fully [22].

Moreover, when OTA is consumed by pigs and other animals in contaminated feeds, it is rapidly absorbed and enters the systemic circulation, where it largely binds to plasma proteins, especially to albumin [13]. This is why OTA can be found in internal organs, predominantly in blood, kidneys and liver [23], so it may be present in edible tissues and meat products. It is not excreted easily because of its solubility to fat. In the human body, OTA has been reported to have a long half-life of 35 days after ingestion [24], and *in vivo* experiments reveal that OTA accumulates in the kidneys, which is the cause of its related nephrotoxic properties [17], its main toxic effect. For this reason, kidneys are the main target organ of OTA [25]. Apart from nephrotoxicity, exposure to OTA has also been related to carcinogenicity, teratogenicity, immunotoxicity and possibly neurotoxic properties [17]. The International Agency for Research on Cancer (IARC) has classified some compounds, such as mycotoxins, according to their carcinogenicity in humans and experimental animals [26]. As a possible compound that is carcinogenic to humans, OTA has been assigned to group 2B (see Table 2). This table shows the IARC's carcinogenicity classification for OTA and other important mycotoxins described in the present doctoral thesis.

Table 2. Summary of the IARC classification for mycotoxins.

Category	Description	Mycotoxins	Ref.
Group 1	Carcinogenic to humans	AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂	
Group 2	2A	Probably carcinogenic to humans	
	2B	Possibly carcinogenic to humans	OTA, FB ₁ , FB ₂
Group 3	Not classifiable as carcinogenic to humans	DON, ZEA, T-2, NIV	[26]
Group 4	Probably not carcinogenic to humans		

b) Aflatoxins

Another important *Aspergillus* group of mycotoxins are AFs. AFs are produced by the two species *A. parasiticus* and *A. flavus* (see Table 1). The word aflatoxin comes from the first letter “a” for *Aspergillus*, the following letters “fla” for the species *flavus* and the ending “toxin” [19]. AFs were discovered at the end of the 1950s [9] and are currently the most studied group of mycotoxins, with AFB₁, AFB₂, AFG₁ and AFG₂ being the most important of the approximately 18 AFs that have been chemically characterised. AFs have a difuranocoumarin chemical structure (see Figure 2). They are distinguished and designated according to their fluorescence under ultraviolet light. Thus, AFB₁ and AFB₂ provide blue fluorescence and AFG₁ and AFG₂ provide green fluorescence.

As mentioned above, *Aspergillus* can grow on a wide variety of commodities and under several climatic conditions. Consequently, AFs are as widespread as OTA. Moreover, food can be contaminated by *A. parasiticus* and *A. flavus* during growth, harvest or storage, so it appears extremely unexpectedly. Furthermore, when the aflatoxigenic moulds have disappeared from the substrate, AFs may persist extensively. Thus, the early development of analytical methodologies for extracting, detecting and quantifying AFs was of great significance, as was their incorporation into legislation.

AFs are considered by IARC to be genotoxic and carcinogenic to humans (group 1) [26] as can be seen in Table 2. Of all AFs, AFB₁ is the most common and also the most toxic, in both acute and chronic terms. The carcinogenicity of AFB₁ has been well-established in several animal species, the liver being the primary target organ [27]. As a consequence, AFB₁ is related to a high incidence of hepatocellular carcinoma and in regions with a greater exposure to AFs, the disease occurs more frequently [9,27]. Some clinical manifestations such as vomiting, anorexia, gastrointestinal affections, pulmonary edema, depression, weight loss, haemorrhages and liver necrosis are related to AF exposure [23,27]. Because of these toxic characteristics, there is no threshold dose below which consumption is safe. Consequently, TDI or NOAEL values cannot be suggested without risk.

1.1.2. *Fusarium* mycotoxins

The genus *Fusarium* is an ascomycete fungus, one of the most important genera of fungi and the most predominant toxin producer in cereals from the temperate regions of America, Europe and Asia [28]. Some species of *Fusarium* are widespread plant pathogens with toxic characteristics and feed-grain contamination is commonplace. Additionally,

Table 3. Non-exhaustive list of *Fusarium* producing species.

Mycotoxin	Genus	Specie	Ref.
DON and DON metabolites	<i>Fusarium</i>	<i>F. graminearum</i> <i>F. culmorum</i>	
FBs	<i>Fusarium</i>	<i>F. verticillioides</i> <i>F. moniliforme</i>	
HT-2 and T-2	<i>Fusarium</i>	<i>F. acuminatum</i> <i>F. poae</i> <i>F. sporotrichioides</i> <i>F. langsethiae</i>	[30–32]
ZEA	<i>Fusarium</i>	<i>F. graminearum</i> (<i>F. roseum</i>) <i>F. culmorum</i> <i>F. cerealis</i> <i>F. equiseti</i> <i>F. verticillioides</i> <i>F. incarnatum</i> <i>F. crookwellense</i>	
NIV	<i>Fusarium</i>	<i>F. graminearum</i> <i>F. cerealis</i> <i>F. culmorum</i> <i>F. poae</i>	

Fusarium mycotoxins usually appear together in contaminated matrices to produce co-contaminations. These toxigenic species produce cereal crop diseases that are complicated to control [29] and create serious problems for crops and the economy. The *F. graminearum* species has been the most widely studied since the *Fusarium* secondary metabolites responsible for mycotoxicoses were first identified and characterised at the beginning of the 1960s [30]. Other common mycotoxin producing species are *F. verticillioides*, *F. culmorum* and *F. cerealis* (Table 3 with their most frequent mycotoxins).

As has been mentioned above, *Fusarium* mycotoxins are commonly present in crop fields. Of all the mycotoxins produced by *Fusarium* species, this doctoral thesis discusses FBs, ZEA and trichothecenes, because of their considerable prevalence and toxicological effects.

a) *Fumonisin*s

FB₁ and FB₂ are the most common FBs. One of the most representative characteristics of FB₁ and FB₂ is their long hydrocarbon chain (see Figure 3) which contributes to their

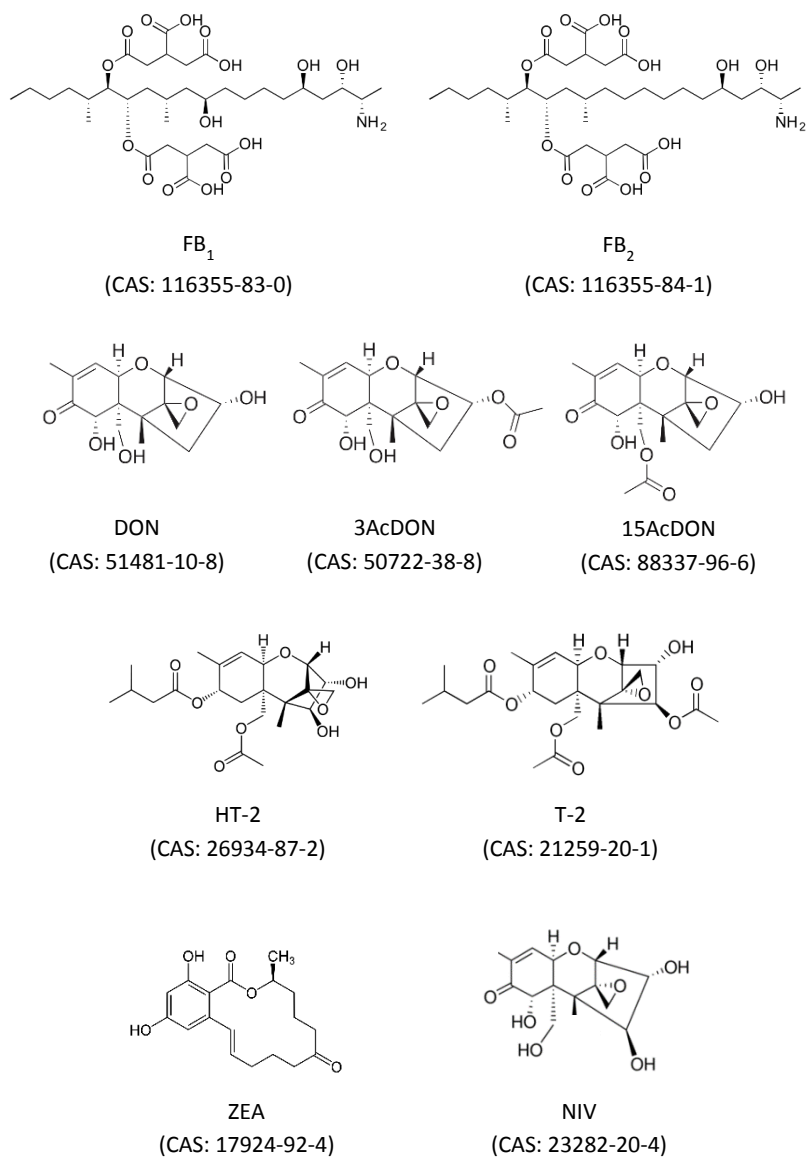


Figure 3. Chemical structures and CAS numbers of the *Fusarium* mycotoxins of interest for the present doctoral thesis.

toxicity [33]. This chemical structure enables FBs to interact with molecular membranes and interfere with the sphingolipid metabolism [34], because they have hydrophilic characteristics unlike most other known mycotoxins. This makes them more difficult to extract and detect. The main difference between FB₁ and FB₂ is the existence of a hydroxyl group in FB₁ (Fig. 3). This makes FB₁ the most toxic Fumonisin and it is classified as possibly carcinogenic to humans (group 2B) by the IARC (Table 2). The effects of FBs ingestion on animals can range from brain lesions in horses to lung edema in swine [30]. The animal species more sensitive to FB₁ consumption are pigs and horses [35]. In humans, FBs are related to oesophageal cancer, especially in the case of FB₁, although these connections have never been completely verified [9]. For this reason, FBs are considered to be cancer promoters, but not mutagenic.

Contaminations by FBs commonly come about during pre-harvest or at the beginning of storage [36], and maize is the matrix in which almost all FB contaminations are produced [35]. Contamination levels can vary drastically between maize samples, especially between maize fractions intended for animal feed and raw maize [35]. Despite this high presence of FBs in maize samples, concentration levels do not increase during storage [36], which makes it easier to control them.

b) Zearalenone

ZEA is another mycotoxin produced by *Fusarium* fungi species and commonly found in maize samples. Its name is a collection of letters from different origins: "Zea-" comes from *Gibberella zae*, which is the name of a producing organism that was the first to be studied; resorcylic acid lactone ("-ral-") is the generic name for these natural products; and finally, "-ene-" and "-one" are the suffixes which indicate the existence of the C-1' to C-2' double bond and the C-6' ketone, respectively [37]. This structure is highly stable, so generally ZEA is not affected by cooking conditions [36]. But the most important characteristic of ZEA is that its chemical structure is similar to that of oestrogens [38]. Thus, ZEA can interact and bind with plant cytoplasmic receptors for oestrogens and act as a plant hormone [37,38], or otherwise bind to the receptors from the membranes of animal cells and cause hyperestrogenism, which leads to reproductive and infertility problems [38]. For instance, swine are especially sensitive to ZEA and can become sterile if concentration levels are sufficiently high [30]. Other clinical symptoms resulting from oestrogen alterations are retention or absence of milk and rectal prolapse in females, and lower testosterone levels and spermatogenesis in males [36]. Despite this, ZEA is not acutely toxic, and because of its lack of teratogenic and mutagenic activity it is not considered to be a human carcinogen by the IARC and is assigned to group 3 (Table 2).

c) *Trichothecenes*

The compounds in group 3, which are not classified as carcinogenic to humans, also include trichothecenes. Native trichothecenes are classified as type A or type B according to their functional groups. Group A is characterised by a functional group other than a carbonyl in C-8 position, and group B is characterised by a carbonyl group in C-8 position. Thus, type A trichothecenes are less polar than type B trichothecenes. Hence, HT-2 and T-2 toxins belong to the type A group, and DON, DON acetylated forms and nivalenol (NIV) to type B. Although the mycotoxins 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyl-deoxynivalenol (15AcDON) are modified forms, they are produced by fungi and are also considered to be native trichothecenes. Other DON secondary metabolites are not considered to be native and are discussed in the following section. Trichothecenes are sesquiterpenoids that can inhibit the synthesis of protein, RNA and DNA, what becomes cytotoxic [33].

Of all the known trichothecenes, DON is the most common and is present in most of the cereal crops (wheat, maize, barley, oats and rye) and processed grains (malt, beer and bread) that have been studied [28]. DON is also known as vomitoxin since acute doses can cause vomiting. The chemical structure of DON, which is presented in Figure 3, has a 12, 13 epoxide group which is largely responsible for its high toxicity. DON is soluble in water and in polar organic solvents and it is highly stable, which means that it can be stored long term and is stable to heat and UV light. Its stability also means that it can withstand several food processing methods, such as milling and heating (up to 350 °C), and enables it to stay in the food chain [39]. Some researchers have been studying the stability of DON and its derivatives through such cooking processes as baking, boiling, frying, steaming and extrusion [40,41]. The reduction in DON depended on pH, the length of cooking and temperature (higher temperatures do not involve greater reductions). Even though boiling provided the most effective degradation, it cannot be considered as a detoxification process. For this reason, DON needs to be stopped from emerging and controlled with regulation limits. The prevention methods are analogous for the main mycotoxins and they are detailed in section 1.1.5.

Three other *Fusarium* secondary metabolites are HT-2 and T-2 toxins and NIV. Much less data has been published on the exposure of these mycotoxins than on those already described. Even so, current legislation determines their maximum allowed concentrations (see section 1.1.5).

The structure of HT-2 and T-2 toxins differs in one functional group: T-2 has an acetyl at C-4 whereas HT-2 does not (Figure 3). Both HT-2 and T-2 are stable at neutral and acidic

pH and are soluble in most organic solvents but not in water. T-2 can be rapidly metabolised to numerous metabolites by hydrolysis, hydroxylation, de-epoxidation, glucuronidation and acetylation, although HT-2 is its main metabolite [42]. They are mainly excreted via urine and bile. They occur mainly in cereal grains, but both are predominantly found in oats and oat products [43], and show apparent synergism when they appear together (co-occurrence) [44]. Once HT-2 and T-2 toxins are consumed, there is no evidence to suggest that they bio-accumulate in animal tissues, and consequently the products of plant origin are the main exposure source of these mycotoxins.

NIV usually co-occurs with DON, since they have a practically identical chemical structure [45]. NIV (see Figure 3) has a hydroxide group at C-4 that is not present in DON. This similar structure means that they have similar chemical and toxicological characteristics. NIV is soluble in organic solvents and slightly soluble in water, and it is commonly found in unprocessed grains like oats, maize, barley and wheat [35].

Of these trichothecenes, T-2 is more toxic than both DON and NIV, but fungi that can produce DON and NIV are more geographically widespread than those that produce T-2 [30]. Nevertheless, the toxicological effects of DON, HT-2 and T-2, and NIV are similar. At the cellular level, these toxins inhibit protein, RNA and DNA synthesis, they have several effects on membranes and lipid peroxidation, and they can produce immunotoxicity, hematoxicity and apoptosis [46,47]. In terms of clinical symptoms, low doses can reduce growth and feed consumption, diarrhoea, gastroenteritis, leukopenia, haemorrhage and vomiting [48]. But like most mycotoxins, there are many differences between animal species, with pigs being the most sensitive [42,49,50]. Moreover, several researches suggest that male animals are more sensitive, for example, to DON than females [51–54]. This may be explained by the fact that, in males, tissue clearance and urine excretion are slower. The IARC classified the carcinogenicity of *Fusarium* mycotoxins in terms of the producer fungi. Thus, toxins derived from *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. sporotrichioides* are not classified as carcinogenic to humans (group 3), whereas toxins derived from *F. moniliforme* are classified as possibly carcinogenic to humans (group 2).

The final fungi categorisation for the mycotoxins studied in the present doctoral thesis is represented in Figure 4.

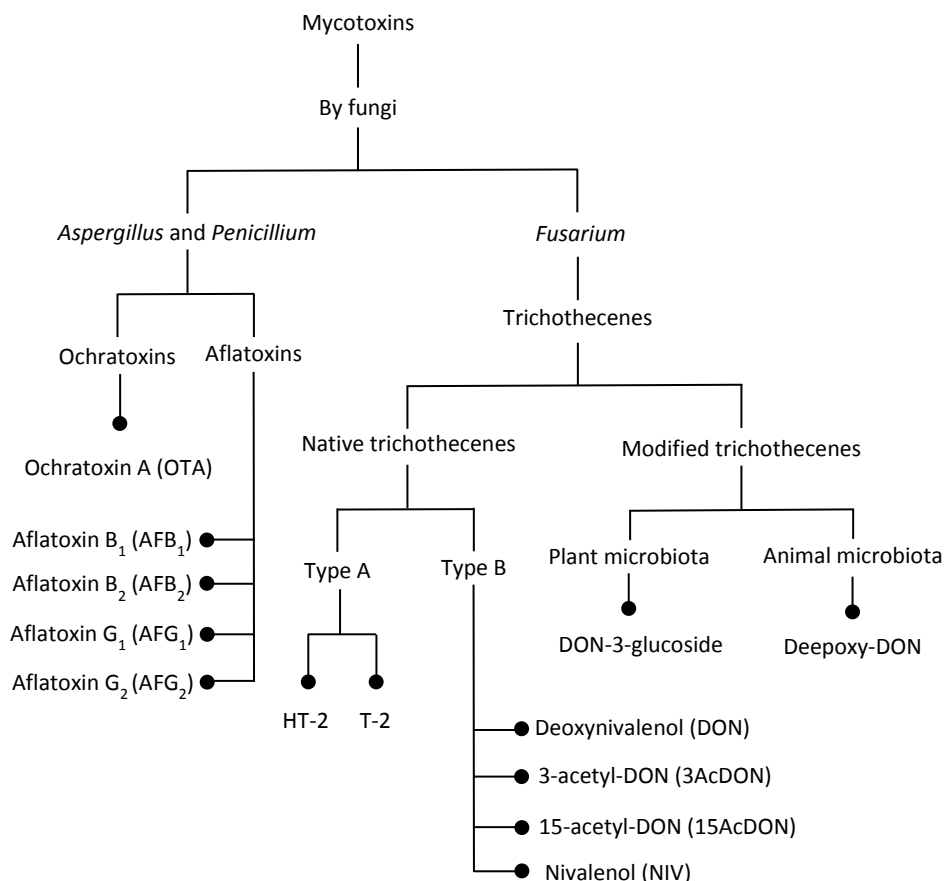


Figure 4. Schematic organisation of the mycotoxins studied in this doctoral thesis.

1.1.3. Modified mycotoxins

The chemical structure of some mycotoxins can be altered and this has led to food, feed and biological samples commonly containing a large number of modified mycotoxins. These chemical transformations can be produced in various ways: for instance, by plant enzymes during detoxification processes, by fermentation enzymes during food processing or by human and animal biological enzymes during the digestion process. Hence, the four major sources of conjugated mycotoxins are fungi, plants, food processing and mammals [55].

Although most of these modified mycotoxins are less toxic than their precursors, they are not usually detected with the conventional analytical techniques for detecting mycotoxins. These conventional analytical techniques encompass the methodologies initially developed only for specific mycotoxins and not for their derivatives. When mycotoxins are determined from samples, the presence of modified mycotoxins can produce either underestimation or overestimation. If the total mycotoxin content of the sample is determined and the modified mycotoxin presence is not determined, the result is underestimation. But if the amount of a particular mycotoxin is determined and the modified mycotoxin signal is confused with the native mycotoxin signal, the result is overestimation. For these reasons, it is important that suitable analytical methodologies be developed so that they can be correctly extracted, detected and quantified.

The term “masked mycotoxins” was introduced for the first time by Gareis *et al.* [56] in 1990 to refer to molecules that cannot be detected by conventional analytical techniques. However, it has changed over the years, and there has been some confusion with the use of the nomenclatures “conjugated” and “masked”. In 2009, Berthiller *et al.* [55] classified the conjugated mycotoxins into two groups: masked mycotoxins – for soluble conjugates – and bound mycotoxins – for insoluble conjugates. In order to standardize the nomenclature and avoid misunderstandings, in 2014 Rychlik *et al.* [57] proposed to limit the definition of “masked mycotoxins” to only the plant metabolites of mycotoxins, which are mainly involved in detoxification processes. Thus, mycotoxin derivatives resulting from thermal modifications or the mammalian metabolism are not masked mycotoxins, but mycotoxin derivatives or modified mycotoxins. Once masked mycotoxins had been defined in this way, four hierarchical levels were proposed as a systematic definition of mycotoxins. Among others, definitions were given for free mycotoxins, mycotoxins covalently or non-covalently bound to the matrix, modified mycotoxins, and modifications produced biologically by fungi, animals or plants, or chemically by thermal procedures. In spite of this, some compounds can belong to more than one classification depending on their origin. This is the case of the acetylated forms of DON (3AcDON and 15AcDON). Although they are regarded as native mycotoxins because they are mainly produced by fungi, sometimes they can be acetylated by plants as a detoxification procedure [58]. Consequently, depending on their origin, 3AcDON and 15AcDON can be regarded as masked mycotoxins equally than deoxynivalenol-3-glucoside (DON3G). Nevertheless, this global definition was proposed by Rychlik *et al.* to harmonise the scientific wording and subsequent legislation, and, as has been mentioned above, to avoid confusion. The present doctoral thesis respects this definition, and the term masked mycotoxins is only used for plant mycotoxin metabolites.

Plant metabolism contains efficient detoxification systems for metabolising phytotoxic and xenobiotic compounds, like mycotoxins, to protect them from pathogens. These metabolic processes, which include chemical modifications and compartmentation, have two main detoxification reactions included in the chemical modifications: phase I and phase II. Phase I reactions, which mainly affect lipophilic compounds, include both hydrolysis and oxidation although oxidation catalysed by the cytochrome P-450 is the most usual [59]. However, reactions in phase I do not usually reduce the toxicity of the native compound. On the other hand, the toxicity of the products obtained from phase II reactions depends on the native compound [59]. These phase II reactions can bind residues from conjugation reactions, such as a glucose, a malonic acid or a glutathione, to functional groups of xenobiotics. These conjugation reactions produce more hydrophilic compounds, which favour the elimination of mycotoxins [60]. In this way, the main conjugation reaction for the mycotoxin DON in mammals is glucuronidation, whereas in poultry it is sulfation [61] and in rats it is sulfonation [62]. Consequently, deoxynivalenol-15-glucuronide is considered to be the main DON metabolite in mammals [61], deoxynivalenol-3-sulfate has been suggested as the main metabolite in poultry [63] and DON-10-sulfonate is the main metabolite in rats [62]. Plant metabolites have also been identified for NIV, T-2, HT-2, ZEA, OTA and FBs. For instance, some plant metabolites from ZEA are zearalenone-14-*O*- β -Glucoside (ZEA-14-Glc) and zearalenone-16-*O*- β -Glucoside (ZEA-16-Glc), although only ZEA-14-Glc has been found in food samples [64].

In addition, when mycotoxins are consumed by animals, their microbial detoxification by specific bacterial strains can also generate different mycotoxin metabolites [65]. For instance, in the case of the mycotoxin DON, the main modified mycotoxin generated after its microbial metabolisation is deepoxy-deoxynivalenol (DOM-1). As has been mentioned above, the 12, 13 epoxide group is largely responsible for the high toxicity of DON. There is a large number of bacteria found in rumen fluid or intestines able to deactivate DON by reduction of this epoxide ring, like the bacterial strain BBSH 797 [65,66]. This reaction is known to take place in the gut of animals in strictly anaerobic conditions, and consequently DOM-1 has been detected in urine and faecal samples from animals treated with DON [67,68]. The chemical structure of DOM-1 and the mycotoxin DON3G is illustrated in Figure 5. In a study performed with DON incubated with gut content and liver homogenate, both from rats, DOM-1 was only found in the gut simulation. This shows that DON is metabolised by microorganisms in the gut, especially from the caudal segments (cecum, colon and rectum) [67]. Furthermore, DOM-1 has been detected even in human milk [69] and cows' milk, as the only form of DON excretion in milk [47], and in the urine of people whose diet was cereal-based [70].

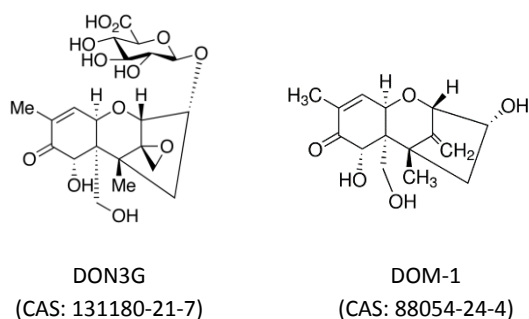


Figure 5. Chemical structures and CAS numbers of the DON derivatives, DON3G and DOM-1.

There is not much toxicological data available for modified mycotoxins, although the very purpose of the process of modification means that these mycotoxins are less toxic than their parents. This has been shown for several modified mycotoxins. The masked mycotoxin DON3G is less virulent for the host than DON because the attached sugar blocks the reactive site of DON [71]. Other modified mycotoxins such as ZEA conjugates were also shown to be less virulent, since the oestrogenicity produced by ZEA is drastically reduced when this mycotoxin is conjugated [64]. The toxicity of several DON sulfonates was also investigated and it was shown that the toxicity of the sulfonated mycotoxins was lower than that of their precursors. Furthermore, DOM-1 is less virulent than DON because the toxic epoxide ring has been removed [72].

However, the risk involved in consuming modified mycotoxins is not that they are toxic but that they may be further chemically modified once they have been consumed. Some modified mycotoxins can undergo hydrolysis immediately after ingestion (for example, 3AcDON and 15AcDON) or in the digestive tract of mammals (for example, DON3G), which releases the native toxin compound [60,73]. For instance, ingested DON3G is almost fully hydrolysed in pigs, although not all the released DON is absorbed [73]. But the hydrolysis in the intestinal tract of mammals may not be the only origin of DON3G hydrolysis. The enzymatic degradation of polysaccharides during food processing can also release DON3G [50]. This may lead to the overestimation of DON concentration levels, and the risk of consuming more mycotoxin content than detected and recommended. Moreover, as can be seen in section 1.1.5., no maximum or guidance values have been set for the great majority of modified mycotoxins due to a lack of information about their toxicological effects.

As a consequence, some studies have evaluated the absorption, distribution, metabolism and excretion of metabolites in animals. One of these evaluated the consumption of ZEA and its naturally occurring metabolites ZEA-14-Glc, ZEA-16-Glc and Zearalenone-14-Sulfate (ZEA-14-S) in pigs [64]. The results demonstrated that these metabolites were easily hydrolysed in the gastrointestinal tract to their native compound, ZEA, and to other unknown metabolites. For this reason, the authors suggested that the guidance or maximum allowed value should be based on the sum of ZEA, ZEA-14-Glc, ZEA-16-Glc and ZEA-14-S.

Over the last few years, several novel modified mycotoxins, especially conjugated ones, have also been identified. Conjugated forms for OTA and NIV have been described in pioneering studies. However, to date, only nivalenol-glucoside has been found in food, more specifically in several grain samples [74]. Schwartz-Zimmermann *et al.* [61,62] identified several DON glucuronides and DON sulfonates as novel and major DON metabolites in urine and faecal samples from animals such as rats, mice, pigs and cows. Regarding sulfonates, the production and characterisation of the DON-, DOM-, and DON3G-sulfonates was useful for their detection mainly in rat faecal samples. The amount in rat faeces was almost 50% of the total DON and DON3G administered, while in urine it was less than 1%. These results suggest that these sulfonates are potential DON metabolites in mammal species [62]. Several DON- and DOM glucuronides have been produced and identified in urine samples from animals treated with DON. Some examples are DON-3-glucuronide and DON-15-glucuronide, which were identified previously, together with DON-7-glucuronide and DON-8,15-hemiketal-8-glucuronide [61,75]. These findings show that current methods for determining DON and its derivatives need to be adapted, since these glucuronides are challenging to detect.

1.1.4. Occurrence data

Several factors favour the occurrence of mycotoxins. These factors can be classified according to the moment of production, as pre-harvest or post-harvest (Figure 6). Among the pre-harvest factors are the field, which is also conditioned by the crop and the fungus present in it, the environmental conditions, such as temperature and humidity, and the harvest itself. Of the post-harvest factors, storage is the most important factor. As mentioned above, the distribution of the mycotoxins around the world will depend on the weather conditions in each region, the season and the most predominant crops. Crops from the same region but with different climate conditions may present differences in mycotoxin contamination levels [76]. The most frequent distribution is *Aspergillus* in tropical and subtropical regions [6,23,36], and *Fusarium*, *Penicillium* and *Alternaria* in temperate regions, although some mycotoxins, such as DON, can also be found in cool

climates [2,6,36]. Thus, in Africa, the Asian subcontinents and Australia, there are mainly AFs and FBs; in North America, AFs, ZEA, OTA and DON; in South America, AFs, FBs, OTA, T-2 toxin and DON; in Eastern Europe, ZEA and DON; and in Western Europe, OTA, ZEA and DON [77].

Mycotoxin contamination raises most concern in developing countries since for various reasons human and animal exposure is greater, especially in infants and young children. The population from developing regions tends to vary little and consume a single cereal [78], which in most of cases is from a highly contaminated crop like maize [79,80]. These cereals tend to be purchased from markets that pay little attention to cereal quality and storage, there is no mycotoxin legislation and there are few tools for determining their presence [78]. Poverty and malnutrition are also factors that contribute to mycotoxin consumption, because cereals are consumed even though they can be seen to be contaminated by fungi. In contrast, in developed countries, the diet is more varied and mycotoxins are more controlled, thanks to the application of regional legislation, which is generally stricter.

Despite this general distribution of fungi, the presence of some mycotoxins can vary considerably between matrices, regions and years. For instance, in the case of T-2 toxin, Binder *et al.* [81] found different median levels between regions and between matrices. In the North and South of Asia, results were similar and the median levels were $309 \mu\text{g kg}^{-1}$ and $314 \mu\text{g kg}^{-1}$, respectively, while in central Europe the median level was $112 \mu\text{g kg}^{-1}$ and in the South of Europe it was $38 \mu\text{g kg}^{-1}$. In the same study [81], huge differences between matrices were found, and the T-2 median level was $51 \mu\text{g kg}^{-1}$ in wheat and $921 \mu\text{g kg}^{-1}$ in barley. It has also been found that the presence of T-2 and HT-2 toxins in oats, wheat and barley also present considerable annual variations [82]. Another example of a mycotoxin is OTA, which varies between different matrices and between countries, since various fungi producers can be present in cool-temperature regions and also in hot and wet regions [82,83]. For instance, the average OTA contamination levels found in cereals and cereal products were very different from those found in beer, which were $0.20 \mu\text{g kg}^{-1}$ and $0.02 \mu\text{g kg}^{-1}$, respectively [17]. Likewise, some mycotoxins are closely related to some matrices. For example, patulin (PAT) is found in most apple samples that have been tested, and particularly in apple juice concentrates [84].

Due to climate change and international trade, mycotoxins can be detected all over the world [2] but intake varies considerably between populations because of different eating habits. For instance, the estimated intake of FB₁ is higher in Africa than in Europe, and the estimated intake of aflatoxin M₁ (AFM₁) is higher in Europe than in Africa [83], because of the higher milk consumption in Europe. Another example of regional

differences is that DOM-1 has been detected in most urine samples from French farmers, but not in UK adults and women from Shanghai [70].

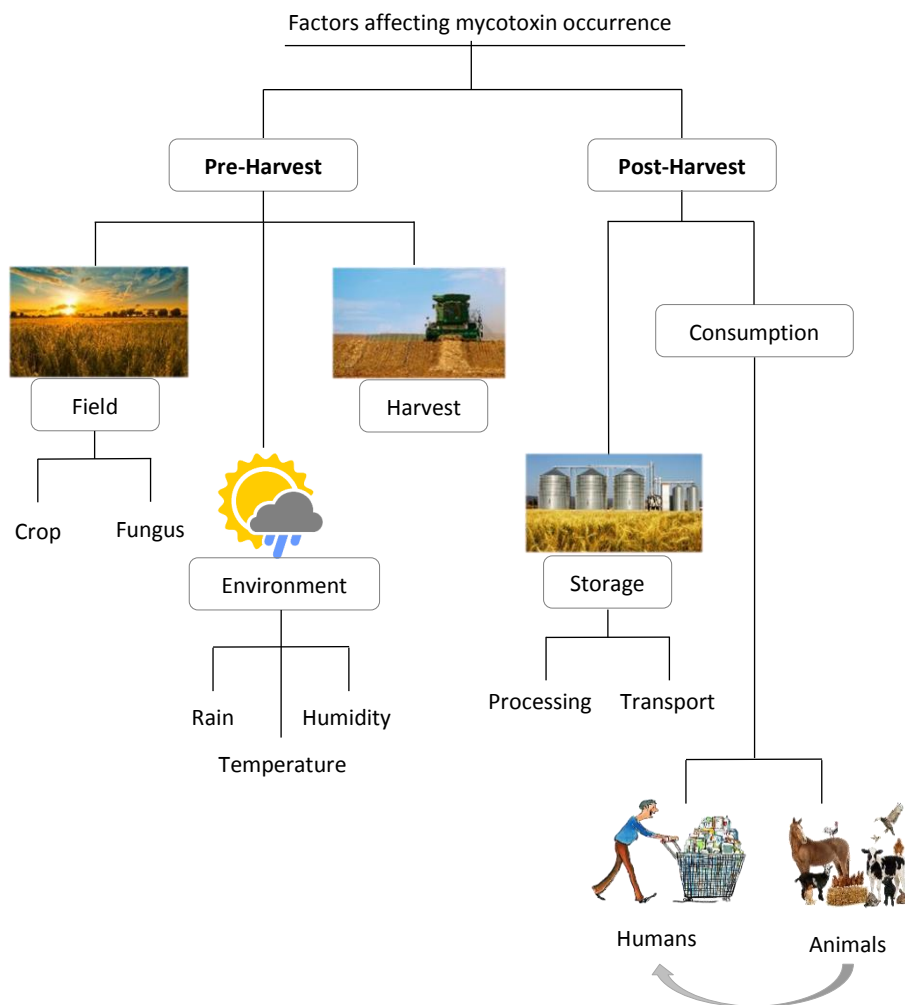


Figure 6. Schematic review of the factors involved in the mycotoxins occurrence.

Many occurrence studies reach similar conclusions about the distribution of mycotoxins in food samples [18,29,81–83,85]. They are mainly detected in cereal samples, and are most prevalent in wheat, maize, oat, barley, rye, sorghum and rice. DON is the most detected trichothecene in these cereal samples since it is found in more than 50% of all samples tested [82,83,85]. Of all the different kinds of cereals, DON is most detected in oats [82,83]. DON is also found in high percentages in wheat, maize, barley and rye [81,82]. Other mycotoxins such as FB, ZEA, AFs and OTA are also present in a wide range of samples [81,85].

There is nothing to suggest that organic food is more susceptible to mycotoxin contamination than conventional food [86], despite the fact that of the lack of pesticides in organic agriculture means that the growth of undesirable fungi and rots may be more prevalent. Several studies have determined a direct relation between organic agriculture and a greater presence of mycotoxins and their toxic effects [87–89]. In general, the amounts of mycotoxins observed in organic food samples are higher than in conventional ones, but the differences are not statistically significant [87,88]. On the other hand, studies on plant stress caused by fungicides and herbicides also show an increase in the presence of mycotoxins [90]. Additionally, concentrations of each target mycotoxin varied between organic and conventional food samples [87], and between samples [91,92]. For these reasons, more data is necessary if a direct relation is to be determined between organic agriculture and the presence of mycotoxins.

Although products can contain mycotoxins before they are purchased, the safety of the product after purchase is the responsibility of the individual. Foodstuffs stored at home for a long time are also vulnerable to being contaminated by fungi and events at home cannot be legislated for. It has been demonstrated that food samples with mould may contain mycotoxins, but sometimes it is not clear whether it is sufficient to remove the mouldy area or discard the food entirely. For this reason, the National Food Agency tested several food samples which often go mouldy and studied how fungal metabolites can diffuse into them [93]. To do so, fungal isolates were inoculated and incubated into different food items and the presence of fungal metabolites at different distances from the mouldy surface were analysed. Results showed that mycotoxins do not migrate more than 2 cm into hard cheeses and apples, but they can appear at depths of up to 7 cm in bread and pears. For instance, in the case of fruit it depends on the texture and the water content. This demonstrated that foods have to be handled and stored properly, and people must be aware that if in doubt they should discard mouldy food.

Most food mycotoxin contaminations are multiple. In most of the samples tested by researchers, at least one mycotoxin has been detected [81,85]. However, in a high

percentage of samples more than one mycotoxin has been determined and detected [76,85]. This is known as mycotoxin co-contamination. Co-contamination can come about for three reasons: most fungi can produce different mycotoxins simultaneously, samples can be contaminated by several fungi or final food or feed products are made from different contaminated commodities [94]. The presence of various mycotoxins can produce additive, antagonistic and synergistic interactions, and have several toxic effects. For this reason, these co-contaminations need to be determined and controlled. Some common co-contaminations are T-2, DON and acetylated deoxynivalenol (AcDON) [82], AFB₁ and OTA [81,95], AFs and FBs, 3AcDON and 15AcDON [60,94], T-2 and HT-2 toxins, because they are produced by the same *Fusarium* species on the same metabolic pathway [96], and DON3G and zearalenone-4-glucoside (ZEA4G), because they are both part of the plant's defence mechanism [94].

Apart from the small survey studies mentioned above, the European Food Safety Authority (EFSA) and the Scientific Co-operation on Questions relating to Food (SCOOP) have published several technical occurrence reports for the most prevalent mycotoxins. In these occurrence reports, cereals and cereal-derived products (milling and processed products) are the matrices that have been studied most. They were from different European countries and from different years. The reports demonstrate the prevalence of the various common mycotoxins and the differences between regions, between years and, above all, between common foodstuffs. Table 4 collects the most important contamination results obtained from these technical reports with the TDI of each mycotoxin, which varies considerably for each mycotoxin. Depending on the toxicological effects of each mycotoxin, the TDI values are different. For instance, the TDI for the sum of FB₁ and FB₂ is 2,000 ng kg⁻¹ body weight (bw), for DON, 3AcDON and 15AcDON it is 1,000 ng kg⁻¹ bw and for ZEA it is 250 ng kg⁻¹ bw. The TDI value is lowest for OTA which is 17 ng kg⁻¹ bw but has not been established for AFs because of their genotoxic and carcinogenic characteristics. Results used in these survey studies were provided by national food authorities or similar bodies, research institutions and associations of food and feed business operators, from at least 21 different European countries and for a particular period of time. The analytical results were collected or submitted to the EFSA database. Each country contributed by providing information about different matrices for each target mycotoxin, covering food, feed and unprocessed grains of undefined end-use [35,42,97–99], and cereals, their milling products and processed cereal products [100]. Other samples such as meat products [101] and fruit juices and purees [84] were also studied. Individual occurrence results are reported below.

OTA can be found in a wide range of food samples, and in order to evaluate the consumption and the possible risks of a specific population, the dietary intake needs to

be assessed. To this end, the dietary intake of OTA by the population of the European Community has been assessed in SCOOP task 3.2.7. [101]. The most common commodities were tested and varying percentages of positive samples were found: cereals (55% positive samples), coffee (41%), beer (39%), wine (59%), cocoa-derived products (81.3%), dried fruits (73%), meat products (18%) and spices (52%). The cereals tested were wheat (with 28% of positive samples), corn (13%), oat (30%), rye (53%), barley (24%) and rice (6%). Even though most cocoa-derived products presented OTA contamination, these levels were not high (about $0.2 \mu\text{g kg}^{-1}$). From these results, it was concluded that cocoa and dried fruits are highly susceptible to OTA contamination, and the main contributors to the dietary intake are cereals (50%), followed by wine (13%) and coffee (10%).

In the EFSA's technical report in 2013 [100] the samples tested were cereals and their milling products, and processed cereal products. The results showed that some of the four AFs of interest, AFB₁, AFB₂, AFG₁ and AFG₂, were quantified at 10% of the samples analysed, and in 5.5% of the samples the sum of the four AFs was above $1 \mu\text{g kg}^{-1}$. Of all the samples, the highest concentration levels were found in unspecified grain milling products, in oat milling products, in fine bakery wares and in raw pasta. According to the EFSA, AFB₁ is the most dominant aflatoxin in all the foods tested, and the highest levels were in pistachios and Brazil nuts, followed by figs, peanuts, spices, hazelnuts and almonds [102]. Additionally, the highest concentration of AFB₁ found in feed samples were in raw materials imported from India, other parts of Asia and South America [23].

EFSA's scientific report in 2013 [97] found DON in a high percentage of the samples analysed, of which feed samples were the most contaminated (75.2%) and had the highest concentrations. Feed for poultry (chickens, hens, turkeys and ducks) had the highest levels. In 1.7% of the feed samples, the DON concentration exceeded the maximum guidance values. DON was detected in 43.5% of food samples and in 44.6% of unprocessed grains of undefined end-use. In cereals, DON was quantified in a larger number of samples and at the highest levels in maize, wheat and oat grains and their derivatives. At lower levels, it was quantified in processed cereals such as bread, fine bakery wares, breakfast cereals and pasta. In terms of total chronic exposure to DON, bread and rolls were the main contributors, followed by pasta, fine bakery wares and grain milling products, and infants and children were the most exposed groups.

Table 4. Summary of mycotoxin contaminations in European countries, USA, China and Japan.

Mycotoxin	Tolerable Daily Intake	Matrix*	Dietary intake	Population	Remarks	Ref.
Sum of AFB ₁ , AFB ₂ , AFG ₁ and AFG ₂	For genotoxic and carcinogenic substances, no TDI	Grain and OTA milling products	Average consumers: 0.55 to 1.08 High consumers: 0.82 to 1.90 (ng kg ⁻¹ bw per day)	Irish population	Population with high nut consumption: high levels of AFS	[102]
DON	1,000 ng kg ⁻¹ bw	Maize grains and maize milling products, such as maize-based breakfast cereals	Adults: 180 to 560 Children: 220 to 1,110 (ng kg ⁻¹ bw per day)	European population, mainly French	At the 95 th percentile: Adults: 380 to 1,010 Children: 940 to 2,100 (ng kg ⁻¹ bw per day)	[1,103]
DON3G	Too limited data to include DON3G with the same TDI as DON and AcDON	Wheat, maize and barley	Too limited data for dietary exposure assessment	Samples from Austria, China, Japan and USA	Maximum reported level at 5.4·10 ⁶ ng kg ⁻¹ in wheat	[103]
3AcDON 15AcDON	1,000 ng kg ⁻¹ bw	Wheat, maize and barley	AcDON generally at < 10% than DON	Samples from Austria, China, Japan and USA	Highest levels for 3AcDON and 15AcDON at 1.93·10 ⁵ and 3.65·10 ⁵ in wheat, 2.7·10 ⁴ and 2.36·10 ⁵ in maize and 1.9·10 ⁴ and 300 in barley, respectively (ng kg ⁻¹)	[103]

Table 4. (Cont.).

Mycotoxin	Tolerable Daily Intake	Matrix*	Dietary intake	Population	Remarks	Ref.
OTA	17 ng kg ⁻¹ bw per day 120 ng kg ⁻¹ bw per week	Cereal and cereal products, wine, fruit juices	From 15 to 60 ng kg ⁻¹ bw per week	Adult European	Tolerable weekly intake (TWI) more appropriate; long half-life of OTA in humans	[17]
Sum FB ₁ and FB ₂	2,000 ng kg ⁻¹ bw per day	Maize grains and maize milling products, such as maize-based snacks	Adults: 30 to 119 Children: 170 to 211 (ng kg ⁻¹ bw per day)	European population, mainly French	At 95 th percentile: Adults: 80 to 230 Children: 540 to 4,390 (ng kg ⁻¹ bw per day)	[1, 104]
Sum HT-2 and T-2	100 ng kg ⁻¹ bw per day	Grains and grain-based foods, such as bread and breakfast cereals	From 3.4 to 18 ng kg ⁻¹ bw per day	Adult European	Dietary exposure lower in elderly population (≥ 65 years), than adults	[42]
ZEA	250 ng kg ⁻¹ bw per day	Grains and grain-based foods, such as bread and fine bakery wares	From 2.4 to 29 ng kg ⁻¹ bw per day	Adult European	Higher exposure in toddlers (12 - 36 months)	[99]
NIV	1,200 ng kg ⁻¹ bw per day	Grains and grain-based foods, such as oats, maize, barley and wheat	From 0.4 to 75 ng kg ⁻¹ bw per day	Adult European	90% tested samples <LOD and <LOQ Higher exposure in toddlers (12 - 36 months)	[35]

* main contributing food categories

An especial scientific opinion about 3AcDON, 15AcDON and DON3G in food and feed was published in the EFSA Journal [105]. These DON derivatives were found in fewer samples and in much smaller concentrations than their precursor. The estimated ratios of 3AcDON, 15AcDON and DON3G with respect to DON were observed to be 10%, 15% and 20%, respectively. Although these co-occurrence ratios varied substantially between different food, feed and grain categories, they are useful for the exposure evaluations. 3AcDON and DON3G were found at higher concentrations in grains and grain-based products, in particular in breakfast cereals for 3AcDON and in grains for human consumption and grain milling products for DON3G. 15AcDON was found in products for special nutritional use, snacks, desserts and other foods, and grains and grain-based products. In feed samples, they were higher in cereal straw and in unprocessed grains of undefined end-use.

The scientific report drawn up by EFSA in 2017 [98] focused on HT-2 and T-2 toxins in food, feed and unprocessed grains of undefined end-use using a considerable amount of analytical data. The highest levels of the sum of both toxins reported in food were in grains for human consumption and in breakfast cereals, in particular in oat-containing commodities (like oat grains and oat cereal flakes). Similarly, the highest levels reported in feed were in oat grains, although animal exposure depended on the animal species. Grain and grain-based products, especially cereal flakes and fine bakery wares, were the foodstuffs which most contributed to the mean chronic dietary exposure. For acute exposure, bread and rolls, fine bakery wares, cereal-based food for infants and young children and cereal flakes were the main contributors.

A scientific opinion on the public health risks of the presence of ZEA in food was also reported by EFSA [99]. ZEA was found at quantifiable levels in 15% of the analytical results provided. The highest concentrations were found in wheat bran, corn and derivatives (like corn flour, cornflakes, corn germ oil and wheat germ oil). A useful observation was made about the importance of the cleaning and selection steps after harvesting, since concentration levels found in the group of unprocessed grains were significantly higher than in the group of grains for human consumption. However, taking into account the dietary exposure, the foodstuffs which most contribute were grains and grain-based foods – especially grains and grain milling products, bread and fine bakery wares – followed by corn germ oil and wheat germ oil.

The co-occurrence and risk of NIV and other mycotoxins from the Mediterranean area were assessed by a study in 2012 [76]. A total of 265 samples from 130 different cereal-based products were analysed and more than half of the samples were contaminated by at least one mycotoxin. Furthermore, of all these positive samples, NIV was detected in

96% of them that demonstrates the high co-occurrence of NIV with other mycotoxins. Quantified concentration levels were between 100 $\mu\text{g kg}^{-1}$ and 961 $\mu\text{g kg}^{-1}$.

All these reports can be useful for assessing the presence of mycotoxins and their consumption in the European population. For instance, the scientific opinions of the EFSA are based on these sampling studies and some scientific literature. The reports can be used to determine which measures prevent mycotoxins from being consumed, or eliminate or reduce their presence. As a result, on the basis of these reports the Commission Regulation listed the maximum levels permitted for food and feedstuffs. They are listed in the following section.

1.1.5. Prevention and regulation

Exposure to mycotoxins may have unwanted adverse effects that can compromise the health status of the consumer. In order to protect public health, it is indispensable for legislation to keep contaminants at toxicologically acceptable levels in both animals and humans. Since the 1960s, when AFs were discovered, many countries established regulations to protect consumers from the presence of certain mycotoxins in food. Many factors can influence the limits stipulated: for example, the availability of toxicological data, food consumption data, occurrence, concentration and distribution data, analytical methodology, and economic factors (commercial and trade interests). In spite of this, maximum permitted levels of some mycotoxins need to be established and an effective method must be implemented to regulate and prevent as far as possible mycotoxin consumption in humans and animals. Thus, during the last 50 years, many countries have established different maximum levels, which are regularly updated.

In conjunction with regulation, prevention strategies are used to prevent and/or reduce mycotoxin consumption. The more important strategies are mainly preventive and focused on pre-, during and post-harvest. The Official Journal of the European Union (OJEU) published the principles for the prevention and reduction of *Fusarium* toxin contamination in cereals [106], which include GAP and Good Manufacturing Practices (GMP) that aim to minimise the occurrence of *Fusarium* mycotoxins in food and feed. The most important factors for contamination control are: crop rotation, crop planning, soil and crop management, choice of variety or hybrid, correct fungicide use, harvesting, drying, storage and transport [106].

Despite the use of prevention methods, it is practically impossible to prevent mycotoxins from occurring, largely because of various factors that are difficult to control. Among these factors, the most influential is the weather and its interaction with the plant

growth stage, especially with variations in DON concentration [107]. For regions in which the weather patterns are similar, mathematical models can be used to predict the appearance of some mycotoxins and pre-harvest risks. Some researchers have been studying the association between climate conditions and the presence of several mycotoxins in oats and maize [108–110]. Higher levels of DON have been observed when it rains during heading/flowering [108], and lower levels during tillering-booting [109]. However, levels of HT-2 and T-2 are higher if it rains during tillering-booting, and lower if rains during heading/flowering [109]. Higher concentrations of DON have been associated with the humidity from inflorescence onwards [108] and higher levels of HT-2 plus T-2 have been associated with the humidity from tillering onwards [109]. Moreover, some researchers have observed significant differences in DON concentrations between samples of wheat grain from the same crop but from different years, and also between samples from the same year but from different geographical areas of the same province [111]. On the basis of these observations, effective models for predicting DON and FBs accumulation have been developed, using weather and insect damage, as variables [107]. These prediction models could be useful for governments, industrial risk managers and farmers.

Maximum levels must be established as strictly as possible bearing in mind their toxicity and whether they can be respected with good agricultural, manufacturing and transport practices. In terms of toxicity, factors such as adverse effects, exposure of the population through dietary intake and the presence of vulnerable population groups, are need to be considered when establishing the legislation. Maximum levels should ensure that products containing contaminants, mycotoxins in this case, should not be commercialised for direct consumption and or for use as an ingredient in foodstuffs.

New advances in scientific knowledge and the improvement in equipment make it possible to implement increasingly stricter levels in different kinds of matrices. However, some mycotoxins do not allow clear regulations. This is the situation, for instance, of T-2 and HT-2 toxins, for which European regulations only give recommended maximum levels, because clear limits are not set [112]. However, the Scientific Panel on Contaminants in the Food Chain (CONTAM panel) of the EFSA established a group TDI for the sum of T-2 and HT-2 [42] (Table 4). The occurrence data available estimates that human exposure to T-2 and HT-2 toxins through the diet is below this TDI in all age groups. This TDI is calculated by determining the NOAEL in animal studies, dividing it by 10 to extrapolate it to humans and finally by 10 again for possible variations between individuals.

There are no international legislation limits for NIV in foodstuffs, since according to the commission regulation, human exposure to NIV is expected to be considerably below the TDI [113]. However, since high concentration levels have been observed [76], its toxicological effects are considerable and it is frequently found in cereal matrices, special attention to this mycotoxin is required.

In 2006, the OJEU published the main mycotoxin regulation, the European Commission (EC) No 1881/2006, in which maximum levels were set for certain contaminants in foodstuffs [114]. Regulation (EC) 1881/2006 sets maximum permitted levels for the most prevalent and toxic mycotoxins in foodstuffs: DON, ZEA, FB₁, FB₂ [114,115], AFs, OTA and PAT [114]. Table 5 gives some examples of these mycotoxins and the maximum levels permitted in some matrices by the 1881/2006 EC legislation and some subsequent variations. The maximum levels permitted in this regulation depend on each mycotoxin and on the matrix. However, it should be noted that this regulation only deals with matrices such as cereals and derivatives, vegetables and milk, which are the foods with the highest levels of mycotoxin contamination. The maximum permitted levels are divided into different groups: unprocessed cereals, cereal grains for direct human consumption, cereal products for human consumption and cereal products for feed and compound feed, among others. In each group there are also differences (see Table 5) between types of cereals and whether these are for infants or young children. The regulation makes special mention of maize products and the particle size. For instance, maximum levels of DON for unprocessed cereals are between 1250 $\mu\text{g kg}^{-1}$ and 1750 $\mu\text{g kg}^{-1}$, and for bread and pasta the levels are 500 $\mu\text{g kg}^{-1}$ and 750 $\mu\text{g kg}^{-1}$, respectively. For processed cereal-based foods and baby foods for infants and young children the highest levels allowed are 200 $\mu\text{g kg}^{-1}$.

The scientific data available demonstrate that milling fractions with small particles were more contaminated by *Fusarium* toxins than those with large particles. For this reason, regulations also classify milling fractions according to whether their particle size is smaller or larger than 500 microns [115] (Table 5).

Table 5. Summary of maximum allowed levels in some matrices by European legislation.

Commodity	Maximum level ($\mu\text{g kg}^{-1}$)						Legislation
	AFB ₁	Σ AFs: B ₁ +B ₂ +G ₁ +G ₂	DON	OTA	Σ FBs: B ₁ +B ₂	ZEA	
Unprocessed cereals	2 ^a	4 ^a	1,250 ^b	5		100 ^c	(EC) 1881/2006 (EU) 165/2010
Processed cereal-based foods and baby foods for infants and young children	0.1		200	0.5	200 ^d	20 ^c	(EC) 1881/2006
Cereals intended for direct human consumption ^e			750	3	1,000 ^f	75	(EC) 1126/2007 (EU) 594/2012
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling			1,750		4,000	350	(EC) 1126/2007
Maize and rice ^g	5	10					(EU) 165/2010
Dried fruit, other than dried figs ^g	5	10					(EC) 1058/2012
Milling fractions of maize with particle size > 500 micron and other maize milling products with particle size > 500 micron not used for direct human consumption			750		1,400	200	(EC) 1126/2007
Milling fractions of maize with particle size \leq 500 micron and other maize milling products with particle size \leq 500 micron not used for direct human consumption			1,250		2,000	300	(EC) 1126/2007

Table 5. (Cont.).

Commodity	Maximum level ($\mu\text{g kg}^{-1}$)					Legislation
	AFB ₁	Σ AFs: B ₁ +B ₂ +G ₁ +G ₂	DON	OTA	Σ FBs: B ₁ +B ₂	
Groundnuts (peanuts) and other oilseeds	8 ^{g, k}	15 ^{g, k}				(EU) 165/2010
	2 ^{h, i}	4 ^{h, i}				
Almonds, pistachios and apricot kernels ^g	12	15				(EU) 165/2010
Hazelnuts and Brazil nuts ^g	8	15				
Tree nuts, other than the tree nuts mentioned above ^g	5	10				(EU) 165/2010
Almonds, pistachios and apricot kernels ^h	8	10				
Hazelnuts and Brazil nuts ^h	5	10				(EU) 165/2010
Tree nuts, other than the tree nuts mentioned above ^h	2	4				
Soluble coffee (instant coffee)				10		(EC) 1881/2006
Maize-based breakfast cereals and maize-based snacks					800	100 (EC) 1126/2007
Spices, including dried spices ^e				15		(EU) 2015/1137

^a All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in the regulation.

^b Excluding durum wheat, oats and maize.

^c Excluding maize.

^d Only processed maize-based foods and baby foods for infants and young children.

^e With some exceptions detailed in the legislation.

^f Only maize.

^g to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs.

^h intended for direct human consumption or use as an ingredient in foodstuffs.

ⁱ with the exception of crude vegetable oils destined for refining and refined vegetable oils.

^k with the exception of groundnuts (peanuts) and other oilseeds for crushing for refined vegetable oil production.

Regarding DON derivatives, no maximum allowed limits are included into the regulation and the same guidelines for DON are applied to these compounds. It is justified due to the co-occurrence with DON and due to the low levels which generally DON derivatives are found [115]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) of $1 \mu\text{g kg}^{-1} \text{bw}$ [1]. And as was done for T-2 and HT-2 toxins, a PMTDI was established for acetylated derivatives, because the JECFA assumes their toxicity to be the same as that of their precursor [1], as does the EFSA [97]. In recent years, EFSA has been working to include in its guidelines DON derivatives and masked DON in food and feed [116].

Regulation (EC) 1881/2006 has been substantially amended on at least 25 occasions to date, with changes in the maximum levels of mycotoxins and other contaminants in foodstuffs. Of all these changes, six were related to mycotoxins. In 2007, the (EC) No 1126/2007 [115] modified different levels of *Fusarium* toxins in maize and maize products (Table 5). OTA maximum levels were also changed in 2010 [117], in 2012 [118] and in 2015 [119], in various foodstuffs of interest. Maximum AF levels were also adjusted in 2010 [120] and in 2012 [121] mainly for groundnuts and dried figs (Table 5).

EC regulates the presence of mycotoxins not only in foodstuffs, but also in feed. It covers all the food destined for animals, even if it is not intended for commercialisation, so as to protect the animals themselves, humans and the environment. There are two guidelines on mycotoxins: one (2002/32/EC [122]) deals with undesirable substances in general which contain AFB₁; and the other (2006/576/EC [123]) is a recommendation on the presence of DON, ZEA, OTA, T-2, HT-2 and FBs in products intended for animal feed. These guidelines also make a special recommendation for maize products, and depend on the type of animal.

Furthermore, the official guidelines on mycotoxin regulation not only set the maximum or recommended levels, but also establish the methods of sampling and analysis for the official control of their levels in foodstuffs [124]. The current regulation specifies such important points as the fact that the sampling method used will depend on the type of matrix or the weight of the sample, which will be influenced by its particle size. In particular, it discusses the case of AFs which are heterogeneously distributed in food products in big particles. In these cases, the samples should be heavier so that they are as representative as food samples with smaller particles.

Once contamination has taken place, the strategies for products intended for animal feed involve the application of several detoxification processes or the use of feed additives. According to Commission Regulation (EU) No 2015/786 [125], a detoxification

process can change the level of contamination of several mycotoxins in feed samples. The contaminated materials can be detoxified by a physical, chemical or (micro) biological detoxification process, which has to be irreversible and should not adversely affect the characteristics and the nature of the feed. Moreover, functional additives can be used to suppress or reduce the absorption of mycotoxins, promote their excretion or modify their mode of action, thus reducing their adverse effects on animal health, according to the Commission Regulation (EC) No 386/2009 [126].

Once contamination has taken place, some decontamination or detoxification approaches can be implemented to remove several mycotoxins from foodstuffs [7,127,128]. Among the physical strategies there are thermal treatments, freezing-defrost processes and UV and gamma treatments, as well as easier treatments like sorting, cleaning, milling and steeping. Gamma radiation has been reported to be a useful tool for eliminating OTA [127], and short-term treatments at elevated temperatures (150 °C) reduced PAT concentrations by about 20% [129]. Sorting and cleaning are suitable treatments because high mycotoxin concentrations are in the surface tissues of cereal grains [7].

Chemical strategies included the use of adsorbent materials to adsorb mycotoxins, such as montmorillonite clay, sodium bentonite and sepiolite [130–132]; chemical agents to degrade or extract mycotoxins, such as fungicides, pesticides and insecticides; chemical solvents like ethanol, dichloromethane and ascorbic acid; microbiological strategies, such as certain enzymes, yeasts, bacteria or microbiological processes, like fermentations in beer, wine, cider and perry [66,127,129], or the ozonisation method [127]. Previous studies have documented the efficacy of these chemical strategies. For instance, during an intestinal fluid simulation, 1% of montmorillonite clay absorbed 98% to 99.5% of AFs and sodium bentonite and sepiolite absorbed almost all of the AFs present [132]. Moreover, the bacterium *Gliocladium roseum* has shown that it can detoxify ZEA in 80-90% yields by ring opening with subsequent decarboxylation of the mycotoxin [133]. However, several chemical detoxification strategies have harmful consequences, as has been demonstrated in some animal experiments [128,131]. Thus, the most suitable chemical strategies for detoxification should be selected according to the mycotoxin of interest and the final purpose.

Some feed additives have been developed and revised by EFSA, which is responsible for determining if the proposed feed additives do not have adverse effects on animal health, human health or the environment. Two examples are the microorganism strain DSM 11798 of the *Coriobacteriaceae* family [134] and the fumonisin esterase produced by *Komagataella pastoris* (DSM 26643) [135]. The former can reduce the 12,13-epoxide

group, thus reducing DON and the trichothecenes with similar structures, from contaminated feed, whereas the latter can degrade FBs in contaminated feed for all avian species.

Thus, the monitoring of the levels described by the legislation, the use of GAP, GAM and predictive models, and the application of detoxification processes or feed additives should ensure minimum mycotoxin consumption and the preservation of food safety.

UNIVERSITAT ROVIRA I VIRGILI

ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

1.2. Determination of mycotoxins

UNIVERSITAT ROVIRA I VIRGILI

ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

Food safety needs to be improved by determining the presence of regulated mycotoxins at low concentration levels in complex matrices. To determine mycotoxins, the general procedure is to extract the analytes from the matrix and then detect and quantify them using various separation and detection techniques.

Due to the low concentrations at which those mycotoxins can occur, a wide variety of extraction techniques have been developed for extracting mycotoxins from food samples: for example, solid liquid extraction (SLE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and pressurised liquid extraction (PLE). But although these techniques often extract the target mycotoxins efficiently, they also extract high percentages of interferences. These interferences may have a matrix effect (ME) on the signal intensity if they are subsequently analysed by LC-MS. For this reason, more selective extraction techniques need to be developed or a clean-up step should be added after the extraction. More selective extraction techniques such as the enzyme linked immunosorbent assay (ELISA) or extractions with water as the extraction solvent have also been developed for the extraction of mycotoxins although solid-phase extraction (SPE) and dispersive SPE (dSPE) as clean-up steps are the preferred methods. The improvement of these extraction techniques, towards greater selectivity, robustness and reliability, makes it easier to apply and respect the legislative levels, which are increasingly more precise while favouring food safety.

Nevertheless, because of the complexity and the heterogeneity of food samples, such pre-treatments as lyophilisation, grinding, sifting and homogenisation are also required before extraction. Mycotoxins are mostly found at higher concentrations in regions near to mould. Thus, mycotoxins are not generally distributed throughout the sample, so an efficient homogenisation step is fundamental if results are to be reliable. In fact, all the above pre-treatments are the only way to ensure the representativeness of the samples.

Once the sample has been pre-treated with the extraction procedure, the separation and detection techniques are also important for determining mycotoxins at low concentrations. The most commonly used technique for separating and identifying mycotoxins is liquid chromatography (LC). LC is the preferred technique because of the need for a single method for detecting multiple mycotoxins simultaneously. Gas chromatography (GC) and thin-layer chromatography (TLC) are two techniques that are also used for determining mycotoxins.

Of the detection methods, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are the most common for determining mycotoxins, and have become

established as the main methods not only for experimental research but also for routine analysis.

The following two sections are a brief evaluation of the most common techniques for the extraction and determination of the target mycotoxins in this doctoral thesis.

1.2.1. Sampling and extraction techniques

Sampling is possibly the most important step in the analytical method to determine the natural presence of mycotoxins. In sampling, the two types of uncertainties are accuracy and precision, and the sampling procedures have to focus on obtaining high accuracy and precision values [136]. The difficulty of this step is the considerable heterogeneity of samples in which mycotoxins can appear, from raw to processed foodstuffs, and also the heterogeneity of mycotoxin distribution in the matrices. Inappropriate preserving conditions can cause the emergence of localised moulds with elevated concentrations of mycotoxins, also known as “hot spots”, which are arbitrarily distributed [137]. This can lead to an underestimation of mycotoxin contamination or, on the contrary, to an overestimation if contaminated particles are casually selected. The presence of these hot spots can hinder the sampling procedure of some mycotoxins such as AFs, since existing data suggest that AFs are more heterogeneously distributed than OTA and DON [137]. For these reasons, it is important to ensure a good homogenisation process, especially with AFs, before starting sampling in order to obtain consistent samples, and prevent sampling above or below the real value of the whole sample.

Making mistakes during sample preparation is associated with a large percentage of error in the whole mycotoxin analytical procedure. In particular, the steps that accumulate a large percentage of error are grinding and subsampling [136]. For instance, Hallier *et al.* [138] studied the variability in the quantification of DON in wheat grain samples. The results showed that grain sampling was the most critical step, contributing about 46% of the total variability. In order to reduce this variability in the mycotoxin determination, some general criteria need to be set. For this reason, the EU has established the main methods for sampling and analysing some mycotoxins, as has already been described in regulation (EC) No 401/2006 in section 1.1.5. [124]. According to this regulation, the heterogeneous distribution of the mycotoxins means that the samples must be prepared and ground with extreme care to ensure complete homogenisation. In general, the regulation establishes maximum concentration levels for the dry matter of each commodity, so samples must also be accurately dried before sampling. Then, the lot to be analysed should be divided into sublots, depending on weight, commodity and particle size. So, for heavy lots with large particles, the resulting

sublots will weigh more than the sublots from lighter lots with smaller particles. These sublots are then analysed and the results are extrapolated to the whole lot. If these methods are followed, the results will be closer to the real values [124].

The most common steps in treating samples that are to be used for mycotoxin determination are shown in Figure 7. First of all, a statistically valid sample is taken from the whole lot and must be conserved to prevent further mould growth. Then, this sample is lyophilised in order to work with dry weight, which is more accurate and also prevents further mycotoxin growth. Then, it is ground to reduce the particle size and improve the extraction, and sifted, normally using a 500 µm sieve. However, a sieve of 100 µm is often used when PLE is the extraction technique, and the particles smaller than 100 µm are discarded to avoid problems with the equipment. Then, a homogenising step is done and various subsamples are selected for individual analysis. All these pre-treatments are indispensable for guaranteeing the representativeness of the samples collected and the reliability of the results.

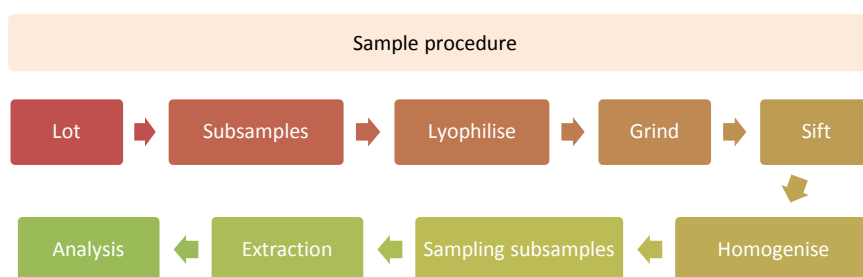


Figure 7. Schematic diagram of the sample procedure.

Once sampling has been completed, the next step is the extraction before analysis. The choice of extraction technique depends not only on the matrix and the target analytes, but also on the purpose of the extraction. If the extraction is performed to monitor the presence of high concentrations of mycotoxins, simple extraction techniques are enough. On the other hand, if low detection and quantification limits are required to determine the presence of these mycotoxins, more exhaustive extraction techniques are needed. For instance, for exposure assessment studies which focus on the determination of the presence of mycotoxins to control their ingestion, the methods selected should be able to extract the target mycotoxins at very low concentrations and prevent interferences which may affect the final determination. In this case, a more selective extraction technique followed by a further clean-up step would be a good option.

Numerous extraction techniques have been reported in the literature. The use of one or another depends on the type of matrix (for example, processed or unprocessed, solid or liquid, etc.), the physical and chemical properties of the mycotoxins to be analysed and the subsequent separation and detection techniques. The sections below give a brief review of the extraction techniques that the present doctoral thesis focuses on, and which are commonly used for mycotoxin determination.

1.2.1.1. Solvent extraction

Solvent extraction techniques have been used for a long time, and are probably the oldest techniques used for extracting mycotoxins from cereal samples. They are still used nowadays because they are simple and do not require expensive equipment. SLE is applied in solid matrices and LLE liquid matrices. Depending on the mycotoxins to be extracted and the matrix to be treated, two main factors need to be taken into account: the extraction solvent and the time of the extraction.

Solvents need to be able to extract the target mycotoxins and remove, as much as possible, the interfering compounds. When the solvent is used for the extraction of multiple mycotoxins, a compromise between all the mycotoxins is required. Polar organic solvents are the most used solvents since nearly all mycotoxins are insoluble in apolar solvents, and are soluble in polar and slightly polar solvents. Often, apolar solvents such as hexane or cyclohexane are used as a clean-up step to remove interferences such as lipids. Hence, the most used solvents are organic solvents, such as methanol (MeOH) [139], acetonitrile (ACN) [140], acetone [141], chloroform, toluene, dichloromethane and ethyl acetate [142], mixed with water or small quantities of acids [143,144]. For instance, Warth *et al.* [145] (2012) used a mixture of ACN/water/acetic acid (CH₃COOH) (79:20:1) as the extraction solvent to extract 63 mycotoxins in cereals and feed. Similarly, Beltrán *et al.* [146,147] used a mixture of ACN/water (80:20, v/v) with the addition of 0.1% formic acid (HCOOH) to extract 11 mycotoxins from cereal and cereal derivatives. Recoveries were between 70% and 120%. Water is often added for the extraction because it helps the solvent mixture penetrate the matrix and increases the extraction efficiencies [143,144]. And it is also useful for such mycotoxins as DON and FBs [143,148] that often present recovery values that are under the recommended 70%. Furthermore, using water as the extraction solvent has some advantages since its environmental impact is nearly negligible so it is a green solvent extraction technique. On the other hand, this efficient low cost method is not suitable for other compounds. For instance, water cannot be used to extract AFs, which have hydrophobic characteristics [148]. Acetone is also a common organic solvent used for the extraction of mycotoxins from cereals. Capriotti *et al.* [141] used a mixture of acetone/water/CH₃COOH (80:19:1, v/v/v) with good results and

moderate signal suppression, and complied with the current guidelines on mycotoxin control. Recently, chlorinated chemicals have stopped being used because of their ecological hazards [149], although they have good extraction properties and have been widely used for mycotoxin extraction [150,151]. The addition of small quantities of acids, such as HCOOH or CH₃COOH, to the extraction solvent commonly increases extraction efficacies because they interrupt the interactions that may occur between mycotoxins and compounds from the matrix like proteins [143,144].

The type of matrix, the length of the extraction and the extraction solvent are important factors when extracting mycotoxins that usually present low recoveries. It has been reported that extractions from processed maize are difficult [151] and, in most cases, longer extraction times enhance the extraction [152]. For instance, the presence of interferences found by Beltrán *et al.* [146] was significant, especially for the FBs which presented signal enhancement after an extraction of 90 minutes. Temperature is not usually taken into account when extracting mycotoxins by solvent extraction techniques, and room temperature (24 °C) is the most common.

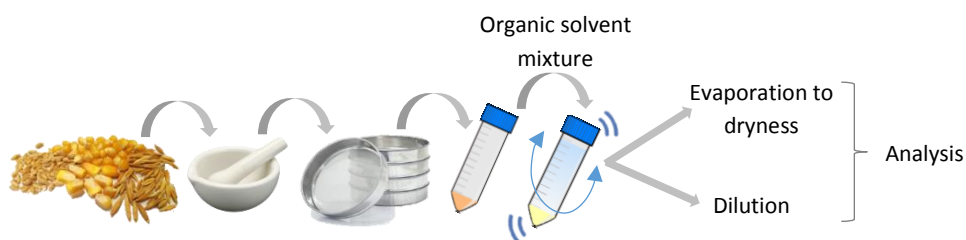


Figure 8. Illustration of the sample procedure steps for SLE in cereal samples.

Figure 8 shows the usual SLE procedure in which several authors use only one extraction step followed by a dilution to reduce the presence of interferences, or followed by a drying and reconstitution step to increase the detection and quantification limits. For instance, Juan *et al.* [153] and Klötzel *et al.* [140] used a mixture of ACN/water (84:16, v/v) for the extraction of *Fusarium* mycotoxins from cereals and cereal products, followed by evaporation and reconstitution. On the other hand, instead of evaporating the supernatant, Hickert *et al.* [154] and Nathanail *et al.* [155] diluted the supernatant before the injection. Different dilutions were tested to determine whether more diluted samples reduce the presence of the matrix effect, which was reduced in all mycotoxins and in all the matrices tested [147]. Conversely, more sensitive instruments are needed to detect the lower mycotoxin concentration levels. In fact, extracts are often diluted when

isotopically-labelled standards for each compound cannot be used because of their high cost.

LLE has also been used for the extraction of mycotoxins from food samples such as bovine milk [139], and also from biological samples such as blood, urine and saliva [156]. For the extraction of OTA from milk, the same volume of sample is mixed with MeOH, and recovery values are more than 90% [139]. Conversely, for the extraction of 28 mycotoxins from biological samples, 5 mL of acetonitrile/water/CH₃COOH (80:19:1, v/v/v) was mixed with 200 µL of sample. Recoveries were between 70% and 102%, except for FBs [156].

SLE and LLE with a single extraction step in some cases enabled a high number of mycotoxins to be extracted in a single analysis with suitable recoveries. Ediage *et al.* [142] validated a method for the extraction of 25 mycotoxins from peanut cake, maize and cassava flour. Sulyok *et al.* [157,158] validated two methods for the extraction of 87 and 39 mycotoxins from cereal matrices, and Zachariasova *et al.* [159] validated a methodology for 32 mycotoxins from beer samples. On the other hand, solvent extractions often have the disadvantage that they use large amounts of solvent. It must be taken into account that for multiple mycotoxin extractions, organic solvents are used instead of water because of their overall better extraction results. For instance, Erisken *et al.* [160,161] extracted several trichothecenes twice with 41 mL ethylacetate from urine, plasma, faeces and ileum digesta samples.

These one-step extractions are easy, fast and economic when low amounts of solvent are used. As a consequence, SLE and LLE are good tools for the rapid monitoring of mycotoxins since most validated methodologies comply with the requirements of the legislation. Taking into account all the solvent extraction combinations described above, recoveries were good with these techniques. However, matrix effects were considerable and limits of detection (LOD) and limits of quantification (LOQ) were high as a consequence of the simplicity of these extraction techniques. Often, the presence of interferences prevents the mycotoxins from being correctly determined. For this reason, more efficient and selective extraction techniques are generally used.

1.2.1.2. Pressurised liquid extraction

PLE is a fully automated methodology that combines high pressures with high temperatures. The homogenised sample is poured into a stainless steel extraction cell, together with a dispersive agent, such as diatomaceous earth (DE). The extraction cell is heated for a few minutes and then the analytes are extracted from the matrix using the

selected extraction solvent at high temperatures and pressures. Thus, the collection vessels contain a mixture of the extraction solvent and the target analytes.

High temperatures increase the solvent's ability to wet the sample and improve the contact of the analytes with the solvent. They also help to break down the bonds between the matrix and the mycotoxins. Thus, high temperatures contribute to the solubilisation of the analyte. At the same time, high pressures help to maintain the solvent in the liquid state at high temperatures and, consequently, extractions are fast and effective. As a general rule, higher temperatures increase extraction efficiency, but at the same time matrix interferences are extracted, which decreases selectivity. Nonetheless, automation and high efficiency are two of this technique's most important advantages [162], so a compromise must be struck between efficiency and selectivity. Despite these advantages, PLE is not an extraction technique that is frequently used to extract mycotoxins. However, when PLE has been used to extract several mycotoxins from cereal and cereal derivatives, results have always been satisfactory.

Accordingly, various extraction parameters can be optimised with PLE: extraction solvent, temperature, pressure, extraction time, number of cycles and cell size. Moreover, several cleaning parameters can also be used: for example, dispersing agents (in-cell clean-up) or previous on-cell clean-up. Generally, the optimisation of the PLE methodology starts by selecting the best extraction solvent or the best solvent extraction mixture. MeOH, ACN and a mixture of ACN/water are the three most used solvents for extracting mycotoxins, and whether one or the other is used depends largely on the matrix and the analytes of interest. For instance, D'Arco *et al.* [163] and González *et al.* [164] used MeOH as the solvent to extract FBs from corn-based baby foods and OTA from rice and rice products, respectively. Recoveries were between 68% and 83% in the case of FBs, and over 90% in the case of OTA. In contrast, Campone *et al.* [162] selected ACN as the solvent to extract AFs from nuts, since the extracts obtained were those with the lowest lipid content. Recoveries were between 77% and 93%. Some aqueous mixtures of ACN have also been selected by several authors [156,165,166], usually with the addition of CH₃COOH [165,166]. Again results were good. In the case of Zinedine *et al.* [165], a method for extracting OTA from breakfast and infant cereals was validated with a recovery value of 82%, whereas Cao *et al.* [156] validated a method for 28 different mycotoxins from biological samples, with recoveries between 71% and 100% with the exception of FBs, for which recoveries were lower. Acidified water as the extraction solvent has not been used to extract mycotoxins to the best of our knowledge, until section 3.1.2 of this doctoral thesis. Conversely, this extraction technique, also called subcritical water extraction (SWE) and pressurised hot water extraction (PHWE), has been

widely used to extract organic contaminants from foodstuff such as antibiotics, insecticides, pesticides and herbicides [167].

The efficiency of PLE also depends on the selected temperature, whereas pressure commonly has less effect [162]. Flush volume and purge time are also optimising parameters, but they have no significant effect on the extraction efficiency. Authors have used different temperatures between 40 °C and 140 °C. However, Desmarchelier *et al.* [166] achieved extraction at room temperature, which is probably why they needed 3 cycles, whereas other authors extracted in a single cycle. Different pressures have also been used to extract mycotoxins, from 500 psi to 2,000 psi, although 1,500 psi is the most common pressure [156,162,164,168,169].

Several authors extracted mycotoxins using PLE with no further clean-up step and with successful results [156,162,165]. However, when the PLE method is used for the extraction of mycotoxins from food and feedstuffs, the presence of interferences is often quite high and further clean-up steps are necessary after the extraction. DE is widely used as a dispersing agent. Moreover, several authors have used dispersing agents as in-cell clean-up sorbents to reduce the presence of interferences when extracting analytes other than mycotoxins. Sorbents such as activated magnesium silicate (Florisil®), alumina or silica have been tested with suitable results for the extraction of musk fragrances from food samples [170,171]. The same dispersive sorbents, and others, have also been tested for the extraction of FBs from cereal samples, but none of them produced a significant reduction in the presence of interferences [163]. Furthermore, a previous on-cell clean-up using a solvent with complementary properties is also a good clean-up strategy because it removes lipids from the sample before the extraction, which decreases the percentage of matrix effect [172]. This methodology consists of a first extraction, for instance with hexane as the extraction solvent, followed by the extraction with the extraction solvent of interest. The extract obtained from the first extraction is discarded and the extract from the second, which will contain fewer lipid compounds, is analysed.

1.2.1.3. QuEChERS

Another extraction technique that is widely used for the determination of mycotoxins is QuEChERS. This technique consists of a first extraction with solvent followed by the addition of salts, which promotes the phase separation. Nowadays, there are three commonly used QuEChERS methods. Method 15662 from the European Committee for Standardization (CEN) [173] uses 4 g of anhydrous magnesium sulphate (MgSO₄), 1 g of NaCl, 1 g of trisodium citrate dehydrate and 0.5 g of disodium hydrogencitratasesquihydrate. The AOAC Official Method 2007.01 [174] uses 6 g of

MgSO₄ and 1.5 g of sodium acetate. And the original QuEChERS method by Anastassiades *et al.* [175] uses 4 g of MgSO₄ and 1 g of NaCl. These chemical reagents, together with 10 mL of water and 10 mL of ACN, are part of the initial single-phase extraction of the whole QuEChERS procedure. Depending on the target mycotoxins, an acid can be added. This mixture is shaken vigorously, then centrifuged and, as a result, three layers are formed. The bottom layer is water, the middle layer contains the chemical reagents and the upper layer is organic and contains the mycotoxins. Then, a purification step is usually carried out on the upper layer with the dSPE sorbents. In the original QuEChERS method, Anastassiades *et al.* [175] mixed 150 mg of anhydrous MgSO₄ and 25 mg of PSA sorbent with 1 mL of the ACN layer, although many of the sorbents mentioned above could be used, such as primary secondary amine (PSA), graphitised carbon black (GCB), Florisil® and octadecyl silica (C₁₈) [176,177].

The QuEChERS technique has been applied to extract analytes that are physically and chemically very different from a wide variety of samples (for example, pesticides, biopesticides and mycotoxins from organic wheat, cucumber and red wine products) [178]. Many studies have been published on mycotoxin extraction using the QuEChERS method [176,177,179], although the mycotoxins and the matrices used have often led to modifications being made to the technique [69,180]. As well as being flexible, this technique has other advantages: no equipment is required and the amounts of solvent consumed are small. It also provides good recoveries and matrix effects when extracting mycotoxins. For instance, Veprikova *et al.* [181] developed an extraction technique for 57 mycotoxins from plant-based dietary supplements. Of the 57 mycotoxins tested, AFs, DON, DON3G, 3AcDON, 15AcDON, FBs, OTA, ZEA, HT-2 and T-2 toxins were evaluated. Recoveries ranged between 71% and 122% for all mycotoxins, except for DON3G for which recoveries were between 40% and 65% depending on the matrix. The mycotoxins DON, OTA, AFs, FBs, ZEA HT-2 and T-2 were also evaluated by Arroyo-Manzanares *et al.* [180] and by Rubert *et al.* [69], who obtained recoveries up to 70% and matrix effects up to 31% in most cases. Furthermore, they carried out the QuEChERS extraction with no further dSPE clean-up step. Zhou *et al.* [182] obtained recoveries between 70% and 114% for all the 10 mycotoxins they tested from wheat flour, and matrix effects between 15% and -15% with the addition of the dSPE step. These results demonstrate that, depending on the target mycotoxins and the matrices, the clean-up step is an option if the presence of interferences needs to be reduced.

1.2.1.4. Solid-phase extraction

SPE is a sorptive-based extraction technique in which the target analytes interact with a sorbent surface. The steps involved in this technique are represented in Figure 9. First,

the sorbent is conditioned with a suitable solvent solution, the composition of which is similar to that of the matrix. Then, the sample is passed through the sorbent to extract the analytes from the matrix, and the cartridge is washed to remove as many impurities as possible without losing the analytes of interest. Finally, the target analytes are eluted using a suitable organic solvent.

SPE cartridges are commercially available in a variety of formats and with different sorptive materials. Common sorbents are Florisil®, aluminium oxide (alumina), C₁₈ and silica gel. Mixtures of these sorbents and mixtures with other sorbents are also commercially available and have been commonly applied as a clean-up step in the determination of mycotoxins [156,168,169]. The most well-known and widely used commercial cartridge for extracting mycotoxins is OASIS HLB (provided by Waters) [156,168]. In this cartridge, polar groups are introduced into the polymer structure to enhance its polarity. This is important considering the polar characteristics of mycotoxins. This cartridge has been applied in the literature for different kinds of mycotoxins and matrices. The application is direct in the case of liquid samples, but a first extraction is needed in the case of solid samples. As an example, Cao *et al.* [156] developed an extraction assay for 28 mycotoxins from various biological samples, using an OASIS HLB after PLE.

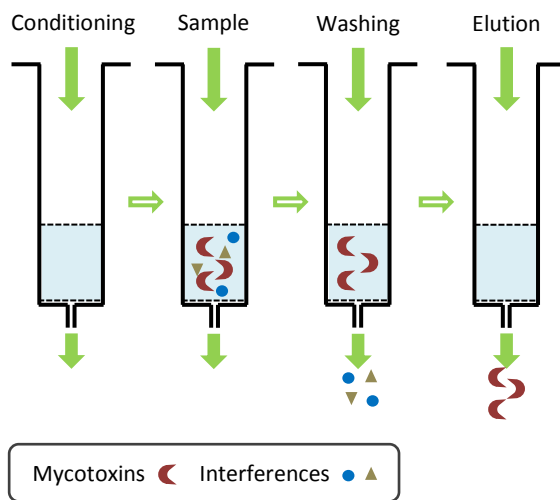


Figure 9. Schematic protocol of SPE procedure.

The IAC is another cartridge format that uses specific antibodies to the analytes of interest rather than sorbent compounds. Target analytes are retained by immunoaffinity

not by adsorption as in classic SPE. The IAC technique is one of the most commonly used SPE strategies for extracting mycotoxins, since these columns are highly specific and selective for target mycotoxins and remove matrix interferences. The disadvantages of this methodology are the cost of IACs and that they are commonly designed for one type of analyte, and so have a limited capacity of multi-mycotoxin determination [183]. However, there are several commercially available IACs that can be used for more than one mycotoxin. Two examples are Myco6in1+™, which is suitable for the simultaneous determination of AFs, OTA and *Fusarium* toxins from cereals and cereal products [184], and aflaochra HPLC™, which is used to determine aflatoxins and OTA [183], as its name suggests.

In the determination of mycotoxins, SPE is commonly used as a clean-up step after the extraction strategy. SPE is also common as a pre-concentration step and even two successive SPE cartridges are used. As an example, Schollenberger *et al.* [185] used two consecutive SPE cartridges, a Florisil® and a cation exchange cartridge, as the clean-up step for the extraction of several *Fusarium* mycotoxins.

Another format commonly used as a clean-up strategy is dSPE, in which the sorbent is dispersed and in contact with the sample during the extraction procedure. The most widely used sorbents in dSPE are MgSO₄, PSA, GCB, Florisil®, alumina and silica gel. Several authors have evaluated the effectiveness of using sorbents for mycotoxin extraction [176,182,186], although one study reports that these sorbents do not make any significant improvement [177]. GCB is useful for removing pigments and sterols and PSA, Florisil® and C₁₈ are useful for removing fatty acids and sugars. As each sorbent is useful for different purposes, results are better when different mixtures of these sorbents are used. For example, Sharmili *et al.* [176] concluded that a ratio of 3:1 of C₁₈:GCB was optimal for the multi-mycotoxin extraction from vegetable oils, and Zhou *et al.* [182] concluded that a ratio of 1:1 of PSA:C₁₈ was optimal for multi-mycotoxin extraction from wheat flour. Depending on the target mycotoxins and the matrices, different combinations are suitable as the clean-up step.

Nowadays, numerous extraction techniques have been reported in the scientific literature, especially for the mycotoxins dealt with in this doctoral thesis. As an overview, some examples of the extraction techniques listed above, applied to different matrices and different groups of mycotoxins, are provided in Table 6.

Table 6. Common extraction techniques used for the determination of mycotoxins.

Mycotoxins	Matrix	Extraction technique	Clean-up	Extraction solvent	%ER	Ref.
AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA	Maize kernels, dry pasta and multicereal babyfood	SLE	-	ACN/water 80:20	58 - 120	[146]
6 <i>Fusarium</i> mycotoxins	Organic and conventional pasta	SLE	-	ACN	85 - 110	[87]
T-2 and HT-2	Oat-based media	SLE	-	MeOH/water 80:20	98 - 128	[187]
AFB ₁ , AFB ₂ , AFG ₁ , DON, ZEA, OTA	Rice	SLE	-	ACN/water 3:1	69 - 99	[188]
DON-3-glucoside, DON-15-glucoside	Wheat	SLE	SPE	ACN/water 84:16	60 - 80	[189]
17 <i>Fusarium</i> mycotoxins	Maize, sorghum, millet and <i>ogi</i>	SLE + SPE	Multifunctional column + glass microfilter	ACN/water/HCOOH 79:20:1	75 - 110	[79]
23 mycotoxins	Sorghum varieties	SLE + hexane defatting	SPE	MeOH/ethyl acetate/water 70:20:10	0.2 - 11 Expressed as bias	[190]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA	Maize-based breakfast cereals, barley and peanuts	SLE	IAC	ACN/water/CH ₃ COOH 79.5:20:0.5	63 - 112	[191]

Table 6. (Cont.).

Mycotoxins	Matrix	Extraction technique	Clean-up	Extraction solvent	%ER	Ref.
31 different <i>Fusarium</i> , <i>Aspergillus</i> , <i>Penicillium</i> , and <i>Claviceps</i> metabolites	Wheat, barley, and oat grains	PLE	-	ACN/water 90:10	51 - 122 With some exceptions	[192]
17 mycotoxins	Cereal-based commodities	PLE	QuEChERS	ACN/water/ CH ₃ COOH 80:19:0.5	70 - 120 With some exceptions	[166]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂	Nuts	PLE	LLE	ACN	77 - 93	[162]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂ , OTA, HT-2, T-2, among other pesticides and biopesticides	Wheat, cucumber, red wine	QuEChERS	-	ACN 1% HCOOH	71 - 110 With some exceptions	[178]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA, NIV, among others	Pseudocereals, spelt and rice	QuEChERS	-	ACN 5% HCOOH	60 - 103	[180]
56 mycotoxins and mycotoxin metabolites	Complex feeding matrices	QuEChERS	dSPE	Water 0.1% HCOOH + ACN	62 - 118	[193]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂ , OTA, DON, ZEA	Vegetable oil	QuEChERS	dSPE	ACN	88 - 106	[176]

1.2.2. Separation and detection techniques

After the extraction step, an effective chromatographic separation is needed because of the similar physicochemical properties of some mycotoxins, and to reduce as much as possible the ME since interferences are reduced by separating the target mycotoxins and unwanted co-eluting matrix components. Nowadays, liquid chromatography (LC) and gas chromatography (GC) are the predominant techniques for identifying mycotoxins and their modified forms. The choice of one technique or the other depends on the physicochemical properties of the target analytes (for example, their polarity, volatility and thermal stability).

As mentioned above, GC has been used for the identification and separation of mycotoxins. However, GC could involve more than one derivatisation reaction because of the polarity of the mycotoxins. Furthermore, a wide variety of mycotoxins usually co-occur. Consequently, GC is not the preferred separation technique for determining mycotoxins. In spite of this, several authors have recently developed multi-analyte methods based on GC-MS/MS for the determination of mycotoxins in biological samples [194,195]. For instance, Mahmoud *et al.* [194] developed a GC-MS/MS method for the determination of trichothecenes from chicken liver extracted with a modified QuEChERS technique. Another example is the methodology developed by Rodríguez-Carrasco *et al.* [195] for the determination of multiple mycotoxins in human urine samples. They proposed a GC method as an alternative to LC for determining mycotoxins in human urine. As well as these examples, GC has also been used for the determination of mycotoxins in cereals and cereal derivatives [144,196–198].

In spite of this, LC has become the preferred separation technique for analysing mycotoxins in feed and foodstuffs, mainly because of the polarity of mycotoxins [199]. With LC, good chromatographic signals and high levels of sensitivity can be achieved. Furthermore, LC-based methods make it possible to efficiently separate multiple mycotoxins. Thus, a large number of mycotoxins can be included in a single LC method.

As far as detection techniques are concerned, MS/MS with triple-quadrupole (QqQ) as analyser is the most frequently used because it is highly sensitive and selective to mycotoxins in a wide variety of food, feed and biological samples. For instance, concentration levels in biological samples are much lower than those found in grain samples. MS/MS-based methods enable mycotoxins to be determined at these low concentration levels, which is useful, for example, when monitoring dietary exposure. In contrast, in routine analysis, simpler detection approaches are used, such as single MS,

which can determine mycotoxins at sufficiently low concentrations in food and feed samples.

The sections below discuss the main features of LC coupled to MS/MS since the methods developed in this doctoral thesis for mycotoxin determination were based on these techniques.

1.2.2.1. Liquid chromatography

As mentioned, LC is the most commonly used technique for separating mycotoxins in extracts from food, feed and biological samples, because of their polar and non-volatile characteristics. Of the factors that influence mycotoxin separation, the stationary phase and the mobile phase selection are the most important.

Selecting the appropriate stationary phase is perhaps the most important part of LC separation. Several stationary phases can be used for the chromatographic separation, depending on the physical and chemical structure of the target mycotoxins. Because of the polarity of the main mycotoxins, reversed-phase columns are used (particularly C₁₈). However, other reversed-phase stationary phases such as C₈ [81,200] and pentafluorophenyl [169] can also be used. Less apolar stationary phases would slow down mycotoxin elution. Therefore, despite the wide variety of common mycotoxins, most studies use the C₁₈ stationary phase, as described in Table 7. For instance, Romero-González *et al.* [178] used a C₁₈ column for the simultaneous determination of more than 90 compounds including mycotoxins, pesticides and biopesticides, with a total chromatographic run-time of 13 minutes.

Short narrow columns filled with small particles produce ultra-high pressures of up to 15,000 psi. Consequently, retention times (RT) of the analytes are reduced which makes most apolar mycotoxins, such as NIV and DON, elute within the first two minutes [147]. In this way, the analysis time is shortened without compromising the peak resolution. Thus, ultra-high performance LC (UHPLC) is actually the most used technique for separating mycotoxins, as is detailed in Table 7 in which most of the examples use this technique. These columns are usually filled with small particles between 1.7 µm and 1.8 µm, which can give good performances in shorter times. These columns also frequently have small diameters, commonly of 2.1 mm, as described in Table 7. As a consequence, reduced flow rates between 0.2 and 0.5 mL min⁻¹ can be applied without compromising the analysis time and peak performance. If columns with wider diameters and larger particles are used, flow rates might need to be faster, such as 1 mL min⁻¹, like some of the examples described in Table 7. Continuing with the same example as above, Romero-

González *et al.* [178] applied a flow rate of 0.45 mL min^{-1} (100 mm x 2.1 mm, $1.7 \mu\text{m}$), while Berthiller *et al.* [189] applied a flow rate of 0.8 mL min^{-1} (100 mm x 4.6 mm, $3 \mu\text{m}$).

At ultra-high pressures, multiple mycotoxins can be included in a single method, which is important when mycotoxins are determined. The multi-mycotoxin LC methods are an important tool for increasing food safety, since globalisation and the increasing presence of processed products mean that all sorts of mycotoxins can be found in all sorts of matrices. In this way, in recent years, several UHPLC-MS/MS based multi-mycotoxin methods have been developed. For instance, Veprikova *et al.* [181] developed a LC-MS/MS method for the simultaneous separation and determination of 57 mycotoxins and Sulyok *et al.* [157] developed another LC-MS/MS method for the determination of 87 mycotoxins and some of their metabolites. In the second of these articles, target mycotoxins were eluted within two chromatographic runs of 21 minutes, under different ionisation conditions. As was expected, co-elution was not avoided, because of the large quantity of target analytes, although most compounds were easily distinguished in the MRM mode.

The specific composition of the mobile phases depends on the target mycotoxins and the polarity mode used during the analysis. The two most common solvents for the organic phase are ACN and MeOH. Following the examples described in Table 7, Dors *et al.* [188] used ACN as the organic mobile phase after testing several mobile phases consisting of mixtures of MeOH, ACN and water, for the determination of AFs, DON, OTA and ZEA by LC-DAD. On the other hand, Vaclavikova *et al.* [191] used MeOH for the determination of a similar group of mycotoxins by LC-QTrap. The aqueous phase is usually mainly water although, on some occasions, a small percentage of organic solvent is also added [79].

At the same time, both mobile phases are usually mixed with small percentages of acids and small concentrations of salts in order to improve the analyte ionisation. HCOOH and CH_3COOH are the most used acids and ammonium acetate (NH_4Ac) and ammonium formate (NH_4HCO_2) are the most used salts, because they are compatible with MS ionisation. On some occasions, the mobile phase additives are only added to one phase. However, to obtain reproducible results and ensure the same concentration during the whole analysis, additives can be included in both mobile phases. For instance, Romero-González *et al.* [178] used water 5 mM NH_4HCO_2 as the aqueous mobile phase and MeOH as the organic one, while Schwartz-Zimmermann *et al.* [61] added 0.1% of HCOOH in both phases, which were water and MeOH. Another example is the study made by Zachariasova *et al.* [201], in which 5 mM NH_4HCO_2 was added to the methanol organic

phase, but the mobile phase had to be subsequently acidified by HCOOH, since FBs were not detectable under the previous conditions.

Furthermore, in order to enhance the reproducibility of the methods, the analytical column is generally thermostatted in an oven in a temperature range between 30 °C and 40 °C [146,178,180].

1.2.2.2. Mass spectrometry detection

After the chromatographic separation, the detection and quantification techniques also need to be coupled. Over the last two decades, LC coupled to MS has become the main methodology used in mycotoxin analysis. However, initially, the most common detectors were fluorescence (FLD) [162], ultraviolet (UV) [202] and diode array (DAD) [188] detectors. These determination techniques have fewer properties than others such as MS, but the limits obtained are adequate for routine and service laboratories. These laboratories determine the presence of regulated mycotoxins to see whether the concentration levels comply with current legislation. Consequently, these equipment are frequently used to determine mycotoxins in food and feed samples. One disadvantage is that these analytical methods are commonly limited to a single group of structurally-related mycotoxins for each analysis. As can be observed in the examples given in Table 7, Dors *et al.* [188] developed a method for determining 6 mycotoxins with LC-DAD and Campone *et al.* [162] developed a method for 4 AFs with LC-FLD.

Hence, as mentioned above, LC-MS methods have more applications in mycotoxin analysis. In the last two decades, LC-MS methods have been extended to include multi-mycotoxin methods, which analyse a wide range of structurally diverse mycotoxins in a single analysis, and increase sensitivity at the same time. Mass spectrometer detectors have become indispensable tools for mycotoxin studies, since as is discussed above, several mycotoxins commonly co-occur at low concentration levels.

In addition, because mycotoxins are present in a wide range of complex matrices at very low concentration levels, MS/MS is the most used technique for the qualitative and quantitative determination of mycotoxins combining different analysers such as QqQ, quadrupole - Time of Flight (Q-ToF) and quadrupole ion trap (QTrap). In recent years, the tandem approach that has most been used to determine mycotoxins is QqQ since most of the studies focus on the determination and quantification of target mycotoxins in food and feed samples at low concentrations and the QqQ analyser provides high sensitivity in multiple reaction monitoring (MRM) mode. When these modes are used, three transitions are generally selected for each mycotoxin: one quantifies the transition, which

is the most intensive, and two qualify the transitions in order to confirm the target mycotoxin. As a result of the high sensitivity, detection and quantification limits can be low. Table 7 shows that these limits may depend on the mycotoxin of interest, on the matrix and on the extraction and determination techniques. Complex matrices with a considerable presence of interferences make it difficult to achieve low limits. For instance, AFs, and more specifically AFG₁, are usually acquired at low quantification limits, whereas DON is usually acquired at higher quantification limits. By way of example, Table 7 shows that Arroyo-Manzanares *et al.* [180] determined a complex group of 15 mycotoxins using a QuEChERS extraction followed by LC-QqQ from several cereal samples, one of which was white rice. In this matrix, the method quantification limit (MQL) for AFG₁ was 0.23 µg kg⁻¹ whereas for DON it was 18.2 µg kg⁻¹, which is a difference of almost two orders of magnitude. Continuing with the white rice example, it should also be pointed out that the MQL obtained for NIV was 233 µg kg⁻¹. Beltrán *et al.* [146] obtained similar results when they determined the presence of AFG₁ and DON in maize kernels, dry pasta and multicereal baby food samples, using SLE followed by LC-QqQ. MQLs for AFG₁ in dry pasta and in multicereal baby food were 0.3 µg kg⁻¹ and 0.5 µg kg⁻¹, respectively, and for DON they were 80 µg kg⁻¹ for both. In this study, the differences between matrices can also be observed, since the MQL obtained for DON in maize (150 µg kg⁻¹) was almost double that obtained in the other matrices detailed above. As these two examples show, the differences between mycotoxins is notable but so are the differences between matrices. Consequently, special attention must be paid to the mycotoxins and matrices of interest.

The choice of the most suitable tandem approach depends on the purpose of the research. The Q-ToF analyser provides qualitative information that confirms the identification of the analytes in the samples. Consequently, Q-ToF is commonly used for non-targeted analysis, and generally for the evaluation of mycotoxin metabolites. It can be used to identify and confirm the presence of mycotoxins although it is generally less sensitive than other hybrid approaches, like QqQ. Of the two examples of Q-ToF analysis in Table 7, Nathanail *et al.* [155] used this device for the stable isotopic labelling of HT-2 and T-2 and for the identification of numerous derivative products from planta biotransformation. Likewise, Schwartz-Zimmermann *et al.* [61,62] used Q-ToF to determine several compounds with the same precursor ions, such as DON-sulfonates and DON-glucuronides in biological samples. On the other hand, Kostelanska *et al.* [203] used Q-ToF to quantify DON and some of its derivatives from beer and beer subproducts. They found a minimum presence of interferences and low detection limits (1 µg L⁻¹ for all analytes), similar to the results obtained with QqQ analyser.

As mentioned above, another tandem approach used for determining mycotoxins is QTrap. This analyser improves performance and enhances sensitivity in full scan and product ion scan modes, and provides accurate quantification and structural information at the same time [204]. Hence, QTrap analyser gives qualitative and quantitative information. As exemplified in Table 7, several papers have used QTrap to determine mycotoxins and modified mycotoxins. For instance, Berthiller *et al.* [189] used LC-(APCI) QTrap to determine DON glucosides, and Schwartz-Zimmermann *et al.* [61] used LC-QTrap to determine DON glucuronides.

Of the ionisation modes, electrospray ionisation (ESI) is the most used, mainly for polar and mid-polar compounds in environmental and food samples. It is followed by atmospheric pressure chemical ionisation (APCI), mainly for mid-polar and non-polar compounds. ESI and APCI are the most used sources because of their robustness, high sensitivity, accuracy, selectivity and compatibility with practically the whole assortment of analyte polarities [205]. They are both atmospheric pressure ionisation interfaces, which are soft ionisation methods since the molecules are not excessively fragmented. Most published studies use ESI as the ionisation mode because it is more sensitive thanks to the higher APCI source fragmentation (see Table 7). Additionally, ESI is more suitable than APCI for polar mycotoxin metabolites, especially for charged metabolites, because APCI cannot transfer charged ions into the gas phase [60]. Furthermore, it has also been reported that ESI seems to be more robust than APCI [205]. On the other hand, as explained above, APCI is more suitable for mid-polar and non-polar compounds, and it is frequently used for the ionisation of several *Fusarium* mycotoxins, like DON and DON glucosides [60]. Consequently, depending on the target mycotoxins, a comparative assessment of the efficiency of the two sources of ionisation, ESI and APCI, is frequently made [159]. By way of example, Berthiller *et al.* [189] developed a method to determine the natural occurrence of DON glucosides in wheat and maize, using APCI as the ionisation source (Table 7). These interfaces can undergo ionisation difficulties, due to the possible competitiveness in the ionisation process between the target analytes and the presence of matrix compounds. These difficulties can enhance or suppress signals, leading to the overestimation or underestimation of analyte concentrations. In these cases, clean-up steps for reducing the presence of interferences, matrix-matched calibration curves, and/or the use of isotopically labelled reference standards can be used to solve these ionisation difficulties and quantify the analytes more accurately.

Selecting a suitable polarity mode – positive or negative – is also important. However, when multiple mycotoxins are determined with a single LC method, different polarity modes are often necessary to increase the analyte sensitivity. Some authors have

combined two ionisation modes in the same chromatographic run whereas other authors have used two consecutive chromatographic runs with different polarity modes. For instance, Beltrán *et al.* [147] determined 11 mycotoxins in a single chromatographic run in both positive and negative ionisation modes. Sulyok *et al.* [157] determined 87 mycotoxins in two consecutive chromatographic runs, one in positive mode and one in negative mode. And to improve the sensitivity of 31 target mycotoxins, Kokkonen *et al.* [192] used two consecutive chromatographic runs in both positive and negative polarities.

From all the information given in section 1.2, it can be concluded that the accuracy of mycotoxin analysis will be determined by the extraction and determination techniques selected, the chemical characteristics of the target compounds and the composition of the matrix or matrices of interest.

Table 7. Common techniques used for the determination of mycotoxins.

Mycotoxins	Column & flow rate	Mobile phases	Determination technique	LOQ ($\mu\text{g kg}^{-1}$)	Ref.
AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , OTA, HT-2, T-2, among other pesticides and biopesticides	C ₁₈ , 100 x 2.1 mm, 1.7 μm 0.45 mL min ⁻¹	A: MeOH B: Water 5 mM NH ₄ HCO ₂	LC-(ESI)QqQ	10	[178]
17 Fusarium mycotoxins	C ₁₈ , 150 x 2.1 mm, 5 μm 0.3 mL min ⁻¹	A: 94:5:1 B: 2:97:1 Water/MeOH/ CH ₃ COOH both with 5 mM NH ₄ Ac	LC-(ESI)QqQ	0.64 – 30.6 Except for NIV and Fusarenon-X (41.2-175)	[79]
AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA	C ₁₈ , 50 x 2.1 mm, 1.7 μm 0.3 mL min ⁻¹	A: Water B: MeOH Both with 0.5 mM NH ₄ Ac, 0.1% HCOOH	LC-(ESI)QqQ	0.3 – 3.5 Except for DON (80-150) and HT-2 (6.5-15)	[146]
AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA, NIV, among others	C ₁₈ , 50 x 2.1 mm, 1.8 μm 0.4 mL min ⁻¹	A: Water B: MeOH Both with 5 mM NH ₄ HCO ₂ , 0.3% HCOOH	LC-(ESI)QqQ	0.2 – 3.4 (white rice) Except for DON (18.2)	[180]
AFB ₁ , AFB ₂ , AFG ₁ , AFG ₂ , DON, OTA, HT-2, T-2, FB ₁ , FB ₂ , ZEA	C ₁₈ , 100 x 2.1 mm, 1.7 μm 0.4 mL min ⁻¹	A: Water B: MeOH Both with 5 mM NH ₄ Ac	LC-(ESI)QTrap	0.1 – 10	[191]
DON-3-glucoside, DON-15-glucoside, DON, 3AcDON, 15AcDON	C ₁₈ , 100 x 4.6 mm, 3 μm 0.8 mL min ⁻¹	MeOH/water 15:85	LC-(APCI)QTrap	LODs: 6 – 20	[189]

Table 7. (Cont.).

Mycotoxins	Column	Mobile phases	Determination technique	LOQ ($\mu\text{g kg}^{-1}$)	Ref.
31 different <i>Fusarium</i> , <i>Aspergillus</i> , <i>Penicillium</i> , and <i>Claviceps</i> metabolites	Inertsil phase 150 x 2.1 mm, 5 μm 0.1-0.2 mL min ⁻¹	A: water with 0.2% HCOOH B: ACN with 0.2% HCOOH C: 1 mM NH ₄ Ac D: ACN	LC-(ESI)QqQ	1 – 620 (wheat)	[192]
6 <i>Fusarium</i> mycotoxins	C ₁₈ , 150 x 2 mm, 3 μm 0.2 mL min ⁻¹	A: ACN B: MeOH 20 Mm NH ₄ HCO ₂	LC-(ESI)QqQ	0.05 – 0.5	[87]
iso-DON glucuronides iso-deepoxy-DON glucuronides	C ₁₈ , 150 x 2.1 mm, 2.6 μm 0.25 mL min ⁻¹	A: Water B: MeOH Both with 0.1% HCOOH	LC-(ESI)QToF LC-(ESI)QTrap	Qualitative assay	[61]
HT-2, T-2	C ₁₈ , 150 x 2.1 mm, 1.8 μm 0.25 mL min ⁻¹	A: Water B: MeOH Both with 0.1% HCOOH	LC-(ESI)QToF	Qualitative assay	[155]
T-2 and HT-2	C ₁₈ , 150 x 4.6 mm, 3 μm 1 mL min ⁻¹	A: Water B: ACN	LC-DAD	T-2: 254.1 HT-2: 229.4	[187]
AFB ₁ , AFB ₂ , AFG ₁ , DON, ZEA, OTA	C ₁₈ , 250 x 4.6 mm, 4 μm 1 mL min ⁻¹	A: Water B: ACN	LC-DAD	0.18 – 1.5	[188]
ABF ₁ , AFB ₂ , AFG ₁ , AFG ₂	C ₁₈ , 250 x 4.6 mm, 5 μm 1 mL min ⁻¹	MeOH/ACN/water 20:20:60	LC-FLD	0.04 – 0.2	[162]

1.3. References

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CHAPTER 2. OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI

ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

The main objective of the research discussed in this doctoral thesis is the development and improvement of different analytical methods to determine mycotoxins and their derivatives in different types of matrices. For that, different extraction strategies are evaluated focusing on both increasing the preconcentration factor to lower the LODs and cleaning-up the matrix. All these strategies are coupled to liquid chromatography with tandem mass spectrometry. Then, the analytical methods are applied to different varieties of matrices, in either solid or liquid state, of either food or biological origin.

Another objective is to provide new information regarding the metabolism of mycotoxins once they are consumed by animals. The first part includes the identification and analysis of possible microbial changes due to intestinal detoxification processes, while the second part identifies and evaluates the modified forms excreted by animals.

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CHAPTER 3. EXPERIMENTAL, RESULTS AND DISCUSSION

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As discussed in the introduction, the determination of the presence of mycotoxins in food and feedstuff is necessary to ensure food security. Effective routine analysis must be carried out to do so. Moreover, it is difficult to effectively determine them because of the diverse modifications that can occur in their chemical structure, producing derivatives.

Because mycotoxins are often found at very low concentration levels either in cereal or in biological samples, further research is needed to develop more sensitive analytical methods that more accurately detect and quantify these mycotoxins and their derivatives, including metabolites and detoxification products, in plants and animals.

Taking this into account and the objectives specified in the previous section, related studies have been carried out over the course of this doctoral thesis. This chapter presents the experimental part, the results and the discussion. Obtained results have already been published, or are in the process of being published, in peer-reviewed scientific journals. These publications are presented below in article format in three sections with the exception of the last study, which is still in its preliminary stage and it would not been yet presented in article form. However, obtained results from this last research are also discussed in its corresponding section. At the beginning of each section, there is a brief introduction to establish the context of the research, and the most notable results are discussed at the end. Lastly, the more relevant conclusions drawn in these sections are also presented. The list of the articles published as a result of the research conducted within the framework of this doctoral thesis is included in Appendix II.

This is the research group's first contact of with mycotoxins, although it has determined other common environmental contaminants in food samples. This new research line related mycotoxins was started with a period of time to acquire knowledge. Therefore, and as a first contact, the research was started with the development of an analytical method for the determination of mycotoxins in cereal-based beverage samples obtained from different local supermarkets. This research was followed by the targeted determination of mycotoxins and modified mycotoxins from cereal matrices through PLE using water as the extraction solvent, also followed by LC-MS/MS determination. Cereal samples also were obtained from different supermarkets in Tarragona (Catalonia). The target mycotoxins were selected based on their prevalence, together with the selection of the matrices. Thus, the methodologies developed in this first section provide a useful tool for the determination of common mycotoxins on common matrices. For the first time, these strategies were applied to these kind of matrices.

After this first contact with mycotoxins, the research was followed along the second section, where the results from two different studies are presented and discussed. This section starts with the investigation of how the microbiota reacts to the consumption of the DON mycotoxin. To do so, a metagenomic assay was carried out on rat gut samples after two months of DON consumption. At the same time, the presence of DON and DOM-1 in faeces was monitored in order to observe changes in the mycotoxins excretion during the treatment. Then, the extraction strategy used previously for *Fusarium* mycotoxins in complex matrices, such as faecal samples, was improved with the objective of apply it for further *Fusarium* derivatives.

All the experimental part explained so far was developed in collaboration with two centres: the Centre for Omic Sciences (COS), Joint Unit University Rovira i Virgili – EURECAT Technology Centre of Catalonia in Reus and the Group of Chromatography, Environmental Applications of the University Rovira i Virgili.

The research developed in the third section was focused on the investigation for the first time of the metabolism of the trichothecenes nivalenol and nivalenol-3-glucoside in rats. The obtained results from this last study are not presented in article form, as previously mentioned. However, a brief introduction of the state of the art, and an explanation of the experimental part and results obtained are described. This study was carried out in Christian Doppler Laboratory for Mycotoxin Metabolism, Center for Analytical Chemistry (Department of Agrobiotechnology, IFA-Tulln) of the University of Natural Resources and Life Sciences (BOKU) in Vienna, during an European placement that took place during the course of the thesis.

3.1. Determination of mycotoxins in liquid and solid cereal samples

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As mentioned in the introduction, several toxicological effects can be produced after the mycotoxins consumption from food refusal and emesis [1] to carcinogenic effects [2]. For this reason, and because of their prevalence, the presence of mycotoxins in foodstuffs has been identified as a critical food security issue. Even though there are several processing methods to reduce mycotoxin levels, it is well established that most mycotoxins are not efficiently removed by conventional treatments or by food processing [3,4]. Numerous studies have reported the presence of mycotoxins in a multitude of samples, although they are mainly present in cereal and cereal derivatives samples [5,6]. Their prevention and control depend mainly on the commodity and the producer fungi. The most common commodities are cereals, but cereals can be consumed in different forms, either in raw format or as a derivative product. Consequently, depending on the commodity, the presence of mycotoxins may vary substantially. Thus, it is important to control the mycotoxin concentration levels present in any particular food and feed samples, especially for those products intended for the direct consumption, as commonly happens in feedstuffs.

For the mycotoxins determination, several methods based on LC-MS/MS have been developed. Furthermore, most of them are multi-mycotoxin analytical methods, which allow simultaneous determination of structurally different types of mycotoxins. Moreover until now, numerous sample preparation techniques have been applied for the extraction of mycotoxins from the above detailed matrices [7,8], mainly in cereal raw grains. Various techniques commonly used and previously described in the introduction are SLE, QuEChERS and SPE. From these techniques, one of the most common is the SLE due its simplicity. However, this technique is commonly related with high presence of interferences and, consequently, with elevated dilution factors. For this reason, more selective extraction methods or the addition of a clean-up step in the extraction process are required. Considering this premise, QuEChERS (Section 3.1.1.) and PLE (Section 3.1.2.) along with different clean-up strategies have been evaluated in this present section as extraction techniques for liquid and solid matrices, respectively.

In the first study presented in this doctoral thesis, the included mycotoxins are a group comprising *Aspergillus*, *Penicillium* and *Fusarium* mycotoxins. These particular mycotoxins were chosen because of their toxicity and because they have been widely present in food and feed samples. Reason why they can be expected in several cereal derivatives, such as plant-based beverages. During the last five years the consumption of plant-based beverages, such as oat, rice and soy beverages, has increased considerably and for this reason these matrices were selected. Moreover, from the best of our knowledge, plant-based beverages have not been yet studied to evaluate the mycotoxin presence. Thus, the main objective of this first study is focalised into the

sample treatment for the simultaneous determination of 11 mycotoxins in a no-studied matrix, the plant-based beverages using LC-(ESI)MS/MS. Considering the sample treatment, parameters such as recovery, ME and limits of the method were taken into account, for the two extraction techniques tested: a simple LLE and QuEChERS, an extraction widely applied for mycotoxins determination in a wide range of samples [9].

The second study presented in this section is focused on the determination of *Fusarium* mycotoxins, including modified mycotoxins, in cereal grains. Most modified mycotoxins are not regulated and consequently, they are not included in routine analysis. However, as it was detailed in the introduction, these mycotoxins can suffer chemical modifications releasing the parent mycotoxin becoming as dangerous as regulated mycotoxins. The extraction technique applied was PLE followed by SPE, and several extraction solvents were tested. As described in the introduction, there are few articles in literature about mycotoxins extracted using PLE, and all of them using organic solvents as the extraction solvent. However, acidified water as the extraction solvent was also tested in this research.

The results obtained from these two studies have been published in *Food Chemistry* 229 (2017) 366-372 and in *Food Analytical Methods* 4 (2018) 1113-1121, respectively, and they are presented below.

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*3.1.1. Determination of mycotoxins in plant-based beverages using QuEChERS
and liquid chromatography-tandem mass spectrometry*

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DETERMINATION OF MYCOTOXINS IN PLANT-BASED BEVERAGES USING QUECHERS AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A method was developed for the simultaneous determination of 11 mycotoxins in plant-based beverage matrices, using a QuEChERS extraction followed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry detection (UHPLC-(ESI)MS/MS). This multi-mycotoxin method was applied to analyse plant-based beverages such as soy, oat and rice.

QuEChERS extraction was applied obtaining suitable extraction recoveries between 80 and 91%, and good repeatability and reproducibility values. Method Quantification Limits were between 0.05 $\mu\text{g L}^{-1}$ (for aflatoxin G₁ and aflatoxin B₁) and 15 $\mu\text{g L}^{-1}$ (for deoxynivalenol and fumonisin B₂). This is the first time that plant-based beverages have been analysed, and certain mycotoxins, such as deoxynivalenol, aflatoxin B₁, aflatoxin B₂, aflatoxin G₁, aflatoxin G₂, ochratoxin A, T-2 toxin and zearalenone, were found in the analysed samples, and some of them quantified between 0.1 $\mu\text{g L}^{-1}$ and 19 $\mu\text{g L}^{-1}$.

Keywords: *Mycotoxin; Plant-based beverages; QuEChERS; UHPLC-(ESI)MS/MS*

1. Introduction

Mycotoxins are natural secondary metabolites produced by some species of filamentous fungi of the *Aspergillus*, *Penicillium* and *Fusarium* genera (Richard, 2007). Over 400 types of mycotoxins are reported, classified by their structure, their biological source or the moment of production from preharvest on the plant culture to storage, transport or processing stages (Bhat, Rai, & Karim, 2010). Modern techniques and good practices of handling and preserving food and feed reduce the presence of mycotoxins. Nevertheless, these species also grow in cereals, fruit and milk (Bhat et al., 2010). Of all mycotoxins, aflatoxin B₁ (AFB₁) is the most potent carcinogen, but all mycotoxins are harmful in different ways, displaying acute and chronic toxicity, such as genotoxicity, carcinogenic toxicity, immunotoxicity (immunostimulatory or immunosuppressive), mutagenicity, nephrotoxicity and teratogenicity attributes (EFSA, 2007, 2014).

The main foods affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and several types of fruit, particularly apples, or sub-products produced from contaminated raw materials, such as wine and beer (EFSA, 2013). Mycotoxins are a serious health risk present throughout the entire food

chain as they display stability at high temperatures and withstand cooking processes (Bullerman & Bianchini, 2007). People can be intoxicated if they eat either contaminated food or products, such as eggs, meat and milk from animals that previously consumed these toxins. In order to reduce the effects of mycotoxin ingestion, the European Union Commission Regulation establishes the maximum levels allowed in certain kinds of food for the major mycotoxins, such as aflatoxins (AFG₁, AFG₂, AFB₁, AFB₂), fumonisins (FB₁, FB₂), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) (EFSA, 2007), and recommends the maximum levels for the sum of T-2 toxin (T-2) and HT-2 toxin (HT-2) (EC, 2013). For example, the maximum level allowed in the case of AFB₁ in all cereals and all derivatives is 2.0 µg kg⁻¹. Consequently, this might be the maximum level permitted for oat- and rice-based products. However, this regulation does not consider the mycotoxin levels that may exist in legumes, such as soybeans. Soybeans are not a product that favours the production of certain mycotoxins. However, there is still a risk as the presence of the main fungi contributor to aflatoxin production has been reported in this type of legume (Nesheim & Wood, 1995).

Over the last few years, the consumption of beverages of plant

origin has increased for medical reasons (e.g. due to intolerances and allergies), or as part of an alternative lifestyle (Lawrence, Lopetcharat, & Drake, 2016; Mårtensson, Öste, & Holst, 2000). If the raw material contains mycotoxins, the resulting beverage will also probably contain these toxins. To analyse these mycotoxins during beverage production, it is important to note that, depending on the raw plant material composition, the beverage might be very different (Mäkinen, Uniacke-Lowe, O'Mahony, & Arendt, 2015), which results in different interferences between matrices when determining the analytes of interest. Considering these differences, finding a common method to determine different mycotoxins for all of the different types of beverages is challenging.

There are different extraction techniques suitable for mycotoxin isolation, such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE) for liquid samples, and pressurized liquid extraction (PLE) and solid-liquid extraction (SLE) for solid samples, among others (Köppen et al., 2010; Capriotti et al., 2012). The method selection depends on the nature of the matrix, its characteristics and complexity. However, some of these methods are expensive, complex, and/or involve considerable consumption in terms of

time and solvent. In order to minimize the sample treatment but prevent exposure to matrix effects, a Quick, Easy, Cheap, Effective, Rugged and Safe method (QuEChERS) is a suitable alternative. The QuEChERS method has been used for mycotoxin extraction from food, both in solid samples, such as dried fruit (Azaiez, Giusti, Sagratini, Mañes, & Fernández-Franzón, 2014), pseudocereals, spelt and rice (Arroyo-Manzanares, Huertas-Pérez, García-Campaña, & Gámiz-Gracia, 2014), and in liquid samples, such as wine (Pizzutti et al., 2014) and beer (Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). However, plant-based beverages have not previously been analysed and QuEChERS extraction could be a proper choice.

The aim of this study is to develop a method for the simultaneous determination of 11 mycotoxins in soy, oat and rice plant-based beverages, using QuEChERS extraction followed by UHPLC- (ESI)MS/MS.

2. Materials and methods

2.1. Reagents and chemicals

The target mycotoxins, which are restricted or subject to recommendations by the European legislation (EC, 2007, 2013), were four aflatoxins (AFB₁, AFB₂, AFG₁, and AFG₂), OTA and six *Fusarium* toxins (DON, ZEA,

T-2, HT-2, FB₁ and FB₂). They were purchased (> 99% purity) from Trilogy Analytical Laboratory (Washington, WA, USA). AFB₁, AFB₂, AFG₁ and AFG₂ were in acetonitrile (ACN) at 25 mg L⁻¹; ZEA, DON and OTA were in methanol (MeOH) at 25 mg L⁻¹, 100 mg L⁻¹ and 10 mg L⁻¹, respectively; T-2 and HT-2 were in ACN at 100 mg L⁻¹; and a mixture of FB₁ and FB₂ was in ACN/water (50:50, v/v) at 100 mg L⁻¹ and 30 mg L⁻¹, respectively. A mixed solution of all of the analytes was prepared at 1 mg L⁻¹ for all of the analytes, except in the case of FB₂ at 0.3 mg L⁻¹, in MeOH/H₂O (1:1, v/v). Mixed solutions were stored at 4 °C for six months.

MeOH and ACN, both for LC-MS, were purchased from Panreac (Barcelona, Spain). Ultrapure-grade water was obtained from a MilliQ water purification system (Millipore, Darmstadt, Germany). Formic acid (HCOOH) ~ 98% and 10 M ammonium formate (NH₄HCOO) aqueous solution were purchased from Fluka (St. Louis, MO, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. QuEChERS extraction packets (4 g MgSO₄, 1 g NaCl) were obtained from Agilent Technologies (Waldbronn, Germany).

Real samples were soy, oat and rice plant-based beverages obtained from local supermarkets. Three different commercial brands were selected for each cereal.

It is important to take certain security measures when handling mycotoxins, such as wearing double gloves (latex underneath and nitrile on top) and cleaning all laboratory materials that have been in contact with mycotoxins, including old solutions, with 20% commercial sodium hypochlorite (NaClO).

2.2. Liquid chromatography-mass spectrometry

Chromatographic analyses were performed in an Agilent 1290 Infinity LC Series coupled to a 6495 iFunnel Triple Quadrupole MS/MS with an electrospray ionisation (ESI) interface, all from Agilent Technologies, operating in positive ion mode. Chromatographic separation was performed using a Cortecs UHPLC C₁₈ column (100 mm x 2.1 mm, 1.6 μm) from Waters (Wexford, Ireland).

The chromatographic separation was performed by gradient elution using a binary mobile phase constituted of water (solvent A) and MeOH (solvent B), both with 5 mM NH₄HCOO and 0.1% HCOOH. The elution started at 10% of B and increased up to 50% in 4.5 min, then to 95% in 7.5 min, remaining in isocratic mode for 2.5 min. The injection volume was 10 μL, the flow rate was fixed at 0.45 mL min⁻¹ and the column temperature was held at 40 °C.

Samples were kept in the autosampler at 4 °C until analysis.

The source parameters were a capillary voltage of 4,000 V for aflatoxins and 3,500 V for the rest of compounds, desolvation gas flow and temperature of 18 L min⁻¹ and 160 °C, nebulizer pressure of 35 psi, nozzle voltage of 500 V, fragmentor voltage of 380 V, cell acceleration voltage of 5 V, and sheath gas flow and temperature of 11 L min⁻¹ and 350 °C. The high and low pressure funnel parameters were, respectively, 180 and 150 V for aflatoxins and 150 and 90 V for the rest of compounds. The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity. For each analyte, three characteristic MRM transitions were monitored, in accordance with the European Commission guidelines (SANTE, 2015). Four different time segments were also established in order to improve sensitivity. All these parameters are specified in Table 1.

2.3. Sample preparation

For the extraction of soy, oat and rice plant-based beverages, the original QuEChERS extraction method (Anastassiades, Lehotay, Štajnbaher, & Schenk, 2003) was used just with the addition of formic acid in the extraction buffer. Briefly, 10 mL of sample was added to a 50 mL centrifuge tube with

10 mL ACN with 1% HCOOH and shaken for 3 min. Then, 4 g of MgSO₄ and 1 g of NaCl were added to the solution, and shaken vigorously for 3 min. Afterwards, the tubes were centrifuged at 10,000 rpm at 20 °C for 5 min. Finally, 1 mL aliquot of the supernatant phase organic layer) was diluted 1:1 (v/v) with solvent A of the mobile phase, and filtered with a 0.2 µm nylon filter (GVS Filter Technology, Indianapolis, IN, USA). The extracts were stored at 4 °C until analysis in order to preserve their stability.

3. Results and discussion

3.1. Instrumental optimisation

With the aim of identifying the optimal conditions for the ESI of mycotoxins, different concentrations of HCOOH (0-0.3%) and NH₄HCOO (0-10 mM) on mobile phase were tested, since the addition of buffers to the mobile phase allows a reduction in sodium adducts, improving analyte ionisation (Campone et al., 2015). The addition of HCOOH is important, especially in the case of fumonisins (FB₁ and FB₂), because it increases their sensitivity and improves their peak shape (Zollner & Mayer-Helm, 2006). However, higher buffer concentrations cause ion suppression (Beltrán, Ibáñez, Sancho, & Hernández, 2009). After testing the different mobile phase compositions, the best one was 0.1%

HCOOH and 5 mM NH₄HCOO (pH 3.1), which allows the highest level of ionisation for all of the analytes in a suitable chromatographic separation under the gradient applied. Under these conditions, all of the mycotoxins are better ionised in positive mode, presenting an abundance of [M+H]⁺ ion, except for the T-2 and HT-2 toxins, which were ionised as ammonium adducts [M+NH₄]⁺ in a more abundant form. All these adducts are well study and reported by previous works (Arroyo- Manzanares et al., 2014; Azaiez et al., 2014; Beltrán et al., 2009; Jackson, Kudupoje, & Yiannikouris, 2012; Lattanzio, Ciasca, Powers, & Visconti, 2014).

Once the precursor ions were selected, different collision energies were applied to obtain three product ions for each mycotoxin and thus three MRM transitions, which are specified in Table 1. These three selected transitions enable the correct identification of every toxin as recommended by the EU directive (SANTE, 2015) and most of them have previously been reported in the literature (Arroyo-Manzanares et al., 2014; Beltrán et al., 2009; Jackson et al., 2012; Lattanzio et al., 2014).

After studying the instrumental linearity (with $r^2 \geq 0.992$), the detection limits (LOD) and quantification limits (LOQ) were determined by adopting

the criteria of a signal-to-noise ratio (S/N) equivalent to 3 and 10, respectively.

Obtained LODs were 0.001 µg L⁻¹ (for AFG₂, AFG₁, AFB₂ and AFB₁), 0.04 µg L⁻¹ (for FB₁, FB₂ and ZEA), 0.01 µg L⁻¹ (for OTA and T-2), 0.1 µg L⁻¹ (for DON) and finally 0.25 µg L⁻¹ (for HT-2). Regarding to obtained LOQs they were 0.003 µg L⁻¹ (for AFG₂, AFG₁, AFB₂ and AFB₁), 0.2 µg L⁻¹ (for FB₁, FB₂ and ZEA), 0.03 µg L⁻¹ (for OTA and T-2), 0.3 µg L⁻¹ (for DON) and finally 0.9 µg L⁻¹ (for HT-2). Linear range was from LOQ to 100 µg L⁻¹ (for AFG₂, AFG₁, AFB₂, AFB₁ and OTA), to 500 µg L⁻¹ (for DON, FB₂ and T-2) and to 1000 µg L⁻¹ (for FB₁, HT-2 and ZEA).

3.2. QuEChERS extraction optimisation

Initially, a simple solid-liquid extraction method successfully applied by Beltrán et al. (Beltrán et al., 2013) for solid matrices was adapted for these liquid matrices. To specify, the method involved mixing 250 µL of plant-based beverage with 1 mL of ACN 0.1% HCOOH, which was then shaken for 20 min, and centrifuged at 4,000 rpm for 10 min, before adding a supernatant aliquot diluted with aqueous solvent of the mobile phase (1:4, v/v). However, the content of the extracts caused a loss in the reproducibility of the results obtained. Thus, to solve this problem a

Table 1. LC-MS/MS parameters for mycotoxin determination.

Start time (min)	Mycotoxin	Retention Time (min)	Precursor ion (m/z)	Dwell time (ms)	Product ion (m/z)*	CE (eV)	
0	DON	2.2	297.1	[DON + H] ⁺	170	249.1	10
						231.1 (57)	10
						203.1 (61)	12
3	AFG ₂	4.9	331.1	[AFG ₂ + H] ⁺	40	313.1	25
						245.1 (40)	25
						257.0 (65)	35
	AFG ₁	5.2	329.1	[AFG ₁ + H] ⁺		243.0	30
						200.0 (60)	45
						283.0 (35)	25
AFB ₂	5.5	315.1	[AFB ₂ + H] ⁺		287.0	30	
					259.0 (93)	30	
					243.0 (43)	45	
AFB ₁	5.8	313.1	[AFB ₁ + H] ⁺		241.0	42	
					285.1 (99)	25	
					213.0 (60)	50	
6.1	HT-2	6.3	442.2	[HT-2 + NH ₄] ⁺	55	215.0	12
						263.0 (89)	15
						197.0 (48)	20
	FB ₁	6.5	722.4	[FB ₁ + H] ⁺		334.1	42
						352.1 (77)	35
						703.9 (78)	35
T-2	6.8	484.2	[T-2 + NH ₄] ⁺		215.1	15	
					185.1 (82)	15	
					305.2 (95)	10	
7	FB ₂	7.0	706.4	[FB ₂ + H] ⁺	55	336.1	45
						318.1 (65)	40
						74.1 (29)	35
	OTA	7.1	404.1	[OTA + H] ⁺		239.0	30
						220.8 (41)	45
						193.0 (34)	45
ZEA	7.2	319.2	[ZEA + H] ⁺		283.1	15	
					187.1 (59)	20	
					203.0 (39)	20	

pretreatment with QuEChERS was applied.

With respect to the different QuEChERS methods (the European Committee for Standardization (CEN) Method 15662, the AOAC Official Method 2007.01 and the original QuEChERS method (Anastassiades et al., 2003)), different studies (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014; Martínez-Domínguez, Romero-González, & Garrido Frenich, 2016; Rubert et al., 2014) have shown that there are no significant differences between them. Thus, considering the simplicity of the original QuEChERS method, it was selected for the present study with the extraction buffer with formic acid.

Prior to recovery studies, the samples (oat, soy and rice plant-based beverages) were analysed in order to subtract the possible signal of analytes present. Then, analytes were added to fortify samples at two different group concentrations to calculate extraction recoveries. One concentration group was near to the highest concentration range and was at 50 $\mu\text{g L}^{-1}$ (for AFG₂, AFG₁, AFB₂, AFB₁ and OTA), at 250 $\mu\text{g L}^{-1}$ (for DON, FB₂ and T-2) and at 500 $\mu\text{g L}^{-1}$ (for FB₁, HT-2 and ZEA). The other concentration group was lower than the previous but analytes concentrations were according to their sensitivity in UHPLC-(ESI)MS/MS, with

the aim of obtaining similar analyte response values. To do so, samples were spiked to concentrations of 10 $\mu\text{g L}^{-1}$ of AFB₁, AFB₂, AFG₁, AFG₂, ZEA, OTA, FB₁ and T-2, 3 $\mu\text{g L}^{-1}$ of FB₂, 50 $\mu\text{g L}^{-1}$ of DON and 100 $\mu\text{g L}^{-1}$ of HT-2. Obtained results were similar at both concentrations and finally only lower concentrations were used to calculate extraction recoveries because there were near to the real concentrations that usually appear in real samples.

Extraction recoveries (ER) were calculated by comparing the analyte concentration when the sample was spiked before and after extraction. Matrix effects (ME) were calculated by comparing the concentration when the sample was spiked after extraction with the calibration standard response, as well as taking into account the analyte concentration in non-spiked samples. ER and ME percentages were calculated according to following equations:

$$\%ER = \frac{C \text{ before} - C \text{ non spiked}}{C \text{ after} - C \text{ non spiked}} \times 100$$

$$\%ME = \left(\frac{C \text{ after} - C \text{ non spiked}}{C \text{ calibration curve}} \times 100 \right) - 100$$

The results, which are detailed in Table 2, show excellent extraction recoveries in all matrices, with values between 80% and 91%. The recoveries obtained were in accordance with previously

reported recoveries in liquid matrices, such as wine (Pizzutti et al., 2014) and beer (Rodríguez-Carrasco et al., 2015). With respect to the ME, values among the three types of matrices were similar but differed depending on the mycotoxin, as can be observed in Table 2. As can be seen, all of the ME values were acceptable with values up to 45%, with the exception of DON, FB₁ and FB₂. DON underwent ion suppression in all of the matrices, and the high values obtained might be attributed to the polar nature of the analyte (Sobrova et al., 2010; Wang & Li, 2015). In contrast, FB₁ and FB₂ displayed significant ion enhancement, especially in the case of FB₂. This fumonisin enhancement was also previously observed in cereal grains (Jackson et al., 2012) and in liquid and powder milk (Wang & Li, 2015), where these mycotoxins showed strong ion enhancement. In view of these ME values, different attempts to reduce them were tested. However, none of these attempts were successful for the other mycotoxins studied. Thus, this ME was assumed in the rest of the study.

3.3. Method validation

The method validation was performed before its application to sample analysis, for the 11 selected mycotoxins in three different liquid matrices: oat soy and rice beverages.

For the method validation, linear range, limits of detection (MDL) and limits of quantification (MQL), accuracy, repeatability and reproducibility were studied. All of the above parameters were calculated when 10 mL of sample were analysed following the procedure described above. In order to compensate for the ME, the matrix-matched calibration approach was studied for each matrix. The linear range was between the MQLs and 200 $\mu\text{g L}^{-1}$ for AFB₁, AFB₂, AFG₁, AFG₂, OTA and ZEA, at 600 $\mu\text{g L}^{-1}$ for FB₂, and at 2,000 $\mu\text{g L}^{-1}$ for DON, HT-2, FB₁ and T-2. The linearity of the method was good with $r^2 \geq 0.993$ in all matrices.

MDL and MQL were estimated in the same way than instrumental limits detailed previously. Taking into account current guidelines (SANTE, 2015) obtained limits afford suitable precision, accuracy and recovery results making them acceptable. The MQLs are all shown in Table 3, which are in line with the response provided in the instrumental UHPLC-MS/MS. The MDLs in the present study were between 0.02 $\mu\text{g L}^{-1}$ and 0.4 $\mu\text{g L}^{-1}$ for AFG₂, AFG₁, AFB₂, AFB₁, FB₁, T-2, OTA and ZEA, and, for the rest of compounds, they were between 2 $\mu\text{g L}^{-1}$ and 5 $\mu\text{g L}^{-1}$. The maximum mycotoxin limits established for certain food commodities by the European Union Commission Regulation (EC, 2006) were used as

reference values for the studied samples, because of the lack of regulation. If these regulated levels are taken as a reference, the MQLs obtained are between 10 and 100 times lower.

The method repeatability (intra-day, n=5) and reproducibility (inter-day, n=5), expressed as relative standard deviation (%RSD), were tested at concentration levels that correspond to ten times the MQLs of each compound. Good repeatability and reproducibility results were obtained, all below 9% and 19%, respectively, in accordance with the guidelines.

With respect to accuracy, the obtained results were excellent for all three matrices and all of the analytes. The accuracy values for the oat-based beverage were between 82% and 110%, while the values for soy were between 91% and 112%, and, in the case of rice, the values were between 91% and 110%. As can be observed, there were no significant differences between the matrices.

Prior to analysis of different samples, a comparison was performed between matrix-matched calibration curves obtained for three matrices in order to identify whether there were

Table 2. Extraction recoveries (%) and matrix effects (%) obtained for the three samples studied spiked with the analyte mixture. See the text for information about concentrations.

Mycotoxin	Oat		Soy		Rice	
	Extraction recoveries	Matrix effects	Extraction recoveries	Matrix effects	Extraction recoveries	Matrix effects
DON	87	-52	84	-56	87	-56
AFG ₂	88	5	87	-10	86	-12
AFG ₁	89	35	87	34	87	12
AFB ₂	88	0	86	-7	85	-11
AFB ₁	86	43	88	38	86	19
HT-2	90	-2	88	-6	88	-6
FB ₁	80	76	82	63	85	75
T-2	86	13	89	-8	86	6
FB ₂	89	>100	80	>100	83	>100
OTA	91	23	89	21	87	1
ZEA	88	16	90	3	87	-5

significant differences between them. Firstly, slope standard deviations (S_b) of each matrix were compared using the *F*-Fisher test, and then the *T*-student test for the slope (*b*) comparison was applied, with $\alpha = 0.05$. The results showed that all of the slopes were comparable. In consequence, a single matrix-matched curve could be used for studying all of the different plant beverage matrices. The matrix-matched curve selected in the present study was the obtained from rice.

3.4. Application to beverage samples

The developed methodology was applied for the analysis in triplicate of three types of plant-based beverages (soy, oat and rice) from three different commercial brands obtained from local supermarkets. Some of the studied mycotoxins were detected and/or quantified (at very low concentrations) in the samples, and the results are shown in Table 3.

Table 3. Mycotoxin concentration ($\mu\text{g L}^{-1}$) found in the analysed beverage samples.

Mycotoxin	MQL* ($\mu\text{g L}^{-1}$)	Oat			Soy			Rice		
		A	B	C	A	B	C	A	B	C
DON	15	<MQL	<MQL	<MQL	-	-	-	-	19	15
AFG ₂	0.5	-	<MQL	<MQL	-	<MQL	<MQL	-	-	-
AFG ₁	0.05	-	0.1	-	-	<MQL	<MQL	-	-	-
AFB ₂	0.1	-	0.4	0.4	-	-	-	-	-	-
AFB ₁	0.05	-	0.3	0.2	-	<MQL	-	-	<MQL	-
HT-2	10	<MQL	-	-	-	-	-	-	-	-
FB ₁	2	-	-	-	-	-	-	-	-	-
T-2	0.5	1.3	1.2	0.4	-	-	-	-	-	-
FB ₂	15	-	-	-	-	-	-	-	-	-
OTA	0.1	-	0.2	0.2	-	<MQL	0.1	-	<MQL	-
ZEA	2	-	<MQL	<MQL	-	<MQL	-	-	<MQL	<MQL

(-) Not detected

*MQL average between the studied beverages

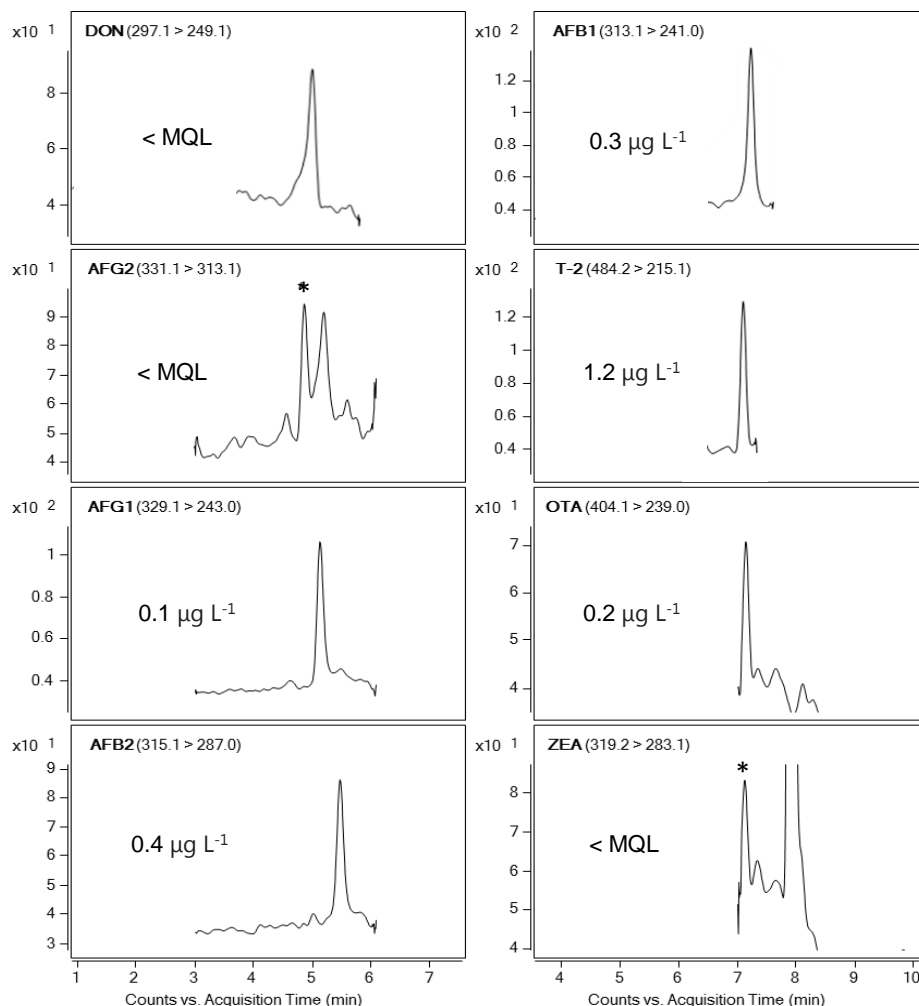


Figure 1. MRM chromatograms of quantitative transitions for detected mycotoxins in an oat sample. “*” denotes analyte’s peak.

In the case of oat beverages, DON, AFG₂, AFG₁, AFB₂, AFB₁, HT-2, T-2, OTA and ZEA were found in some of the analysed samples. One interesting feature is that the concentrations found for the analytes in samples B and

C were very similar. This is explained by the fact that, although they are from different brands, they were found to come from the same source. With respect to DON, it is also widely detected in solid oat samples (Jestoi et

al., 2004; Juan, Ritieni, & Mañes, 2013). HT-2 was found in one sample and T-2 was quantified in all of them, in line with the literature, which confirms that HT-2 and T-2 are predominantly detected in oat and oat-based products (Köppen et al., 2015). As an example, Figure 1 shows the MRM chromatograms for one of the oat samples analysed. In this figure, AFG₂ and ZEA showed poor resolution, although peak separations were tried to improve without success. Nevertheless, it should be taken into consideration that the concentration of these compounds is below MQL. In any case, analytes identifications were always performed with all the obtained product ions.

With respect to soy beverages, AFG₂, AFG₁ and AFB₁ were detected in one or two of the soy samples studied, in agreement with the previous literature, which found these aflatoxins in soybean samples and soy derivatives (Xie et al., 2014). Furthermore, OTA and ZEA were found in some of the analysed samples.

Finally, with regard to rice beverages, DON, AFB₁, OTA and ZEA were found in some of the studied samples. These results agree with those obtained by some authors, who found these toxins in different types of grain rice samples (Arroyo-Manzanares et al., 2014; Serrano, Font, Ruiz, & Ferrer,

2012). With respect to AFG₂, AFG₁ and AFB₂, they were not detected in rice beverage samples, which is also in agreement with other studies that analyse rice samples (Serrano et al., 2012).

4. Conclusions

This is the first study in which plant-based beverages have been analysed to determine the presence of several mycotoxins. A sensitive, reliable and multi-analyte method were developed for the quantification of eleven mycotoxins using QuEChERS extraction followed by UHPLC-(ESI)MS/MS.

The applied QuEChERS approach was suitable for the extraction of the target mycotoxins from this kind of matrices, as shown by the extraction recovery values obtained above 80%, and with ME values comparable to other studies that determine mycotoxins in other matrices.

The method was applied to the analysis of different plant-based beverages and some of the mycotoxins were found at low $\mu\text{g L}^{-1}$ levels.

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

3.1.2. Determination of trichothecenes in cereal matrices using subcritical water extraction followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

DETERMINATION OF TRICHOHECENES IN CEREAL MATRICES USING SUBCRITICAL WATER EXTRACTION FOLLOWED BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

Subcritical water extraction followed by solid-phase extraction and ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection is reported for the first time for the determination of 6 trichothecenes (deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, HT-2 toxin, and T-2 toxin) from different cereals. Water with 1% formic acid was used as the extraction solvent followed by a solid-phase extraction clean-up, achieving good performance with acceptable extraction recoveries, method detection limits between 0.05 $\mu\text{g kg}^{-1}$ and 4.0 $\mu\text{g kg}^{-1}$, and method quantification limits between 0.4 $\mu\text{g kg}^{-1}$ and 20 $\mu\text{g kg}^{-1}$. The use of water as the extraction solvent allowed a selective extraction affording low matrix effect levels and the detection and quantification of natural target trichothecenes at very low concentration levels. This extraction method was applied to different cereals, a pseudocereal and an oilseed sample, of which maize, millet, and oat were contaminated by at least one trichothecene.

Keywords: *Trichothecenes; Cereals; Subcritical water extraction; Solid-phase extraction; UHPLC-(ESI)MS/MS.*

1. Introduction

Cereals are the basis of human nutrition together with the consumption of fruits and vegetables. During recent years, some cereals, pseudocereals, and oilseeds have gained much more relevance than they formerly had, due to an increase in human interest with respect to having healthier nutrition, as well as an increase in food intolerances. Some examples are sorghum, millet, rye, buckwheat, quinoa, sesame seeds, oat, and spelt, among others (Arendt and Dal Bello 2008; Ačanski et al. 2015). The growth in cereal consumption also leads to an increase in the potential ingestion of mycotoxins. Although there are ways to try to reduce mycotoxin concentration, such as milling and cleaning the cereal grains, avoiding their growth is practically impossible (Kostelanska et al. 2011). For this reason, it is necessary to determine their presence in the human diet.

Among all of the reported types of mycotoxins, there is a family of cyclic sesquiterpenoids with low molecular weight (~ 200 – 500 Da) called trichothecenes, which appear predominantly in cereals and cereal derivatives, mainly wheat, barley, and corn (Pereira et al. 2014). These mycotoxins are divided into four groups (from type A to D), with types A and B

being the most common (Krska et al. 2007). The compounds that generate the greatest interest in view of their toxicity and occurrence classified as type A trichothecenes are HT-2 and T-2 toxins, and those classified as type B are deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3AcDON), and 15-acetyl-deoxynivalenol (15AcDON). Although acetylated forms are DON derivatives produced by fungi, they are considered to be native mycotoxins, which are a classification of free and unmodified mycotoxins (Payros et al. 2016). DON can also be modified biologically by the plant microbiota, producing deoxynivalenol – 3 – glucoside (DON3G), or animal microbiota, producing de-epoxy DON (DOM-1), 3-epi-DON, and 3-keto-DON (Payros et al. 2016). Acetylated forms of DON, which display similar or lower toxicity than their precursor (Pestka 2008), commonly appear simultaneously but less frequently than DON (Berthiller et al. 2013; EFSA 2013a). With regard to the glycosylated form, no toxic effects have been demonstrated to date for DON3G in mammals (JECFA 2011), but several authors have reported that colonic microbiota in the large intestine can hydrolyze DON3G, 3AcDON, and 15AcDON, releasing DON, which can be absorbed in the gut (Maresca 2013; Nagl et al. 2014). European regulations have established a maximum permitted level for DON (EC 2007), which varies

from 500 to 1,750 $\mu\text{g kg}^{-1}$, depending on the matrices of adult foodstuffs, and recommend a maximum level for HT-2 and T-2 toxins, which varies from 25 to 1000 $\mu\text{g kg}^{-1}$ (EC 2013). Although European regulations are in the process of including DON derivatives within its guidelines (EFSA 2013b), at present, there is no regulation affecting them. With respect to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), a provisional maximum tolerable daily intake (PMTDI) of 1 $\mu\text{g kg}^{-1}$ body weight (bw) for 3AcDON and 15AcDON has been established because the organization considers that toxicity of these derivatives is the same as their precursor's (JECFA 2011). Meanwhile, there is insufficient information on DON3G toxicity to establish a PMTDI (JECFA 2011). Thus, suitable analytical instrumentation and extraction methods can help to establish a clear approach to trichothecene regulation, as it should be able to monitor such low levels.

Previous studies have shown suitable extraction techniques for mycotoxins from different kinds of solid matrices, such as solid-liquid extraction (SLE) (Rubert et al. 2013), QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe extraction) (JiaoJiao et al. 2016; Zhou et al. 2016), pressurized liquid extraction (PLE) (Kokkonen and Jestoi 2009; Campone et al. 2015), and

microwave-assisted extraction (MAE) (Pallaroni et al. 2002; Pallaroni and Von Holst 2003). However, SLE and QuEChERS have certain disadvantages in comparison with PLE and MAE, such as they are less automated. The development of extraction methods using water is a sustainable alternative to these classical procedures. PLE and MAE are effective options because they provide effective extractions and they can be used with alternative and less contaminating solvents (Pallaroni and Von Holst 2003; Armenta et al. 2015). Comparing PLE and MAE, PLE might be better as the extraction process can be more automated and it is well-accepted for routine analysis of environmental and food contaminants (Campone et al. 2015). This technique can be also more sustainable if water is used as the extraction solvent, in which case, it is known as subcritical water extraction (SWE) or pressurized hot water extraction (PHWE). Using hot water under pressure, in order to maintain it in liquid state, allows the isolation of valuable components. SWE has largely been used to extract several analytes, such as insecticides and phenolic compounds, from diverse matrices, such as plants and oils, according to related reviews (Teo et al. 2010; Herrero et al. 2013). However, to the best of our knowledge, SWE has never been used to extract mycotoxins from cereal matrices.

Another advantage of the use of water as the solvent in PLE is that it allows the subsequent selective cleaning of the obtained extracts, using solid-phase extraction (SPE) without any previous solvent exchange, thereby reducing the analysis time. In this respect, the inclusion of a cleaning step reduces or even prevents matrix effects (ME) which can lead to significant overestimation or underestimation of mycotoxin concentration. An effective clean-up prevents or reduces these interferences, enabling sensitive, selective, and robust liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. Furthermore, the use of water allows milder extraction conditions and at the same time, more selective extraction.

The aim of the present research is to develop a method based on SWE followed by SPE clean-up and ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection (UHPLC-MS/MS), for the simultaneous determination of the six most abundant trichothecenes (DON and its derivatives DON3G, 3AcDON, and 15AcDON; HT-2; and T-2), from different types of cereals, a pseudocereal and an oilseed widely present in the human diet.

2. Materials and Methods

2.1. Reagents and Chemicals

The target mycotoxins were six: DON, T-2, HT-2, DON3G, 3AcDON, and 15AcDON (> 99% purity). DON, T-2, and HT-2 were purchased from Trilogy Analytical Laboratory (Washington, MO, USA) and DON3G, 3AcDON, and 15AcDON were purchased from Romer Labs (Union, MO, USA). DON was sold in methanol (MeOH) at 25 mg L⁻¹, T-2 and HT-2 in acetonitrile (ACN) at 100 mg L⁻¹, and DON3G in ACN at 50.9 mg L⁻¹. 3AcDON and 15AcDON were obtained in powder form. A mix solution of all of the mycotoxins at different concentrations was prepared, taking into account their response in (ESI)MS/MS, obtaining similar mycotoxin response values. HT-2 and DON3G were prepared at 1 mg L⁻¹; DON, 3AcDON, and 15AcDON at 0.5 mg L⁻¹; and T-2 at 0.1 mg L⁻¹. This mix solution was prepared in water/MeOH (80:20, v/v) and stored at -20 °C.

Ultra-pure-grade water was obtained by a Milli-Q water purification system (Millipore, Darmstadt, Germany). MeOH and ACN (both LC-MS grade) were obtained from Panreac (Barcelona, Spain), and acetone was obtained from VWR International

(Fontenay-sous-Bois, France). Formic acid (HCOOH) ~ 98% was purchased from Fluka (St. Louis, MO, USA). Ammonium formate (NH₄HCOO) aqueous solution 10 M was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diatomaceous earth (DE) was acquired from Thermo Scientific (Sunnyvale, CA, USA). The SPE cartridges were 150-mg OASIS HLB from Waters (Wexford, Ireland) and 200-mg ISOLUTE ENV+ from International Sorbent Technology LTD (Mid Glamorgan, UK).

Working with mycotoxins implies taking various security measures, such as using double gloves (made of latex and nitrile) and cleaning all the materials that have been in contact with mycotoxins with 20% commercial sodium hypochlorite (NaClO).

2.2. *Liquid Chromatography-Mass Spectrometry*

An Agilent 1290 Infinity LC Series coupled with a 6495 iFunnel Triple Quadrupole MS/MS with electrospray ionization (ESI) interface was used for chromatographic analysis, both from Agilent Technologies (Waldbronn, Germany). Chromatographic separation was achieved using a Cortecs UHPLC C₁₈ column (100 × 2.1 mm, 1.6 μm) from Waters. A binary mobile phase was used for the chromatographic separation, comprised of water (solvent

A) and MeOH (solvent B), both with 5-mM NH₄HCOO and 0.1% HCOOH. The gradient elution started at 10% B and maintained this percentage for 2 min. Over the next 5.5 min, the gradient increased to 20% and was held again under isocratic conditions for 3.5 min. It was then increased to 95% in 5 min and held under isocratic conditions for 2 min. Finally, it was returned to the initial conditions in 1 min and maintained for 2 min to equilibrate the column. The injection volume was 10 μL, flow rate was fixed at 0.45 mL min⁻¹, and the separation was performed at 40 °C. The autosampler was kept at 4 °C.

The optimized source parameters were capillary voltage of 4000 V for DON3G and 3500 V for the rest of compounds; desolvation gas flow and temperature of 18 L min⁻¹ and 160 °C, respectively; nebulizer pressure of 35 psi; nozzle voltage of 2000 V for DON3G and 500 V for the rest; fragmentor voltage of 380 V; cell acceleration voltage of 5 V; and sheath gas flow and temperature of 11 min⁻¹ and 350 °C, respectively. The high- and low-pressure funnel parameters were, respectively, 90 and 60 V for DON3G and 150 and 60 V for the rest of mycotoxins. Multiple reaction monitoring (MRM) experiments were carried out in positive polarity for all of the studied compounds with three representative MRM transitions for

each mycotoxin, in accordance with the European Commission guidelines (SANTE 2015). The collision energy was optimized for each product ion and they are detailed in Table 1, together with all MRM parameters obtained.

2.3. Sampling

Prior to the extraction and analysis, studied matrices were ground with the mill Taurus Aromatic (Taurus Group, Oliana, Spain), sifted twice in 500- and 100- μm sieves and homogenized. For spiked samples, 2 mL of acetone was added to 1 g of each sample in a 100-mL beaker, in order to spike the matrix homogeneously. Subsequently, 100 μL of the mix solution (see "Reagents and Chemicals" for concentrations) was added to the suspension and left overnight in a stirrer to let the mycotoxins come into contact with the sample and until the acetone was completely evaporated. Matrices were spiked at three different mycotoxin concentrations according to their sensitivity in UHPLC-(ESI)MS/MS, in order to obtain similar analytes response. The matrix used for method development and validation was maize, and the other matrices studied were three different cereals (spelt, millet, and oat), one pseudocereal (quinoa), and one oilseed (sesame seed), all

obtained from local markets.

2.4. Sample Extraction

For the SWE, a homogeneous mix of 1 g of sample and 1 g of DE was poured into an 11-mL stainless steel extraction cell, which was packed by inserting a layer of DE at the bottom and at the top (approximately 0.3 g for each layer) and a cellulose filter at the bottom, following the manufacturer's recommendations. Extractions were achieved on a Dionex ASE 350 accelerated solvent extractor (Dionex Corp., Sunnyvale, CA, USA). The SWE conditions were as follows: water with 1% of HCOOH as the extraction solvent, 80 °C with 5 min of cell preheating, 1500-psi extraction pressure, flush volume of 50%, purge time of 60 s, and a single extraction cycle of 5 min. The obtained extracts of volumes around 15 mL were cleaned up in OASIS HLB cartridges, previously conditioned with 10 mL of MeOH and 10 mL of water with 1% HCOOH (pH 2.0). The mycotoxins were eluted with 5 mL of MeOH and evaporated to dryness with a miVac vacuum concentrator (Genevac LTD, Ipswich, UK). The mycotoxins were resuspended with 2 mL of water/MeOH (80:20, v/v) and filtered with a 0.45- μm nylon filter (Phenomenex, Torrance, CA, USA) just prior to analysis.

Table 1. LC-MS/MS parameters for trichothecenes determination.

Start time (min)	Mycotoxin	Retention time (min)	Molecular weight (g mol ⁻¹)	Precursor ion (m/z)	Dwell time (ms)	Product ion (q/Q ratio)	CE (eV)
0	DON	2.9	296.3	297.1 [DON + H] ⁺	180	248.9	8
						231.1 (66)	10
						203.1 (59)	8
4.4	DON3G	3.6	458.4	297.1 [DON3G - 3G + H] ⁺	85	248.9	8
						231.1 (66)	10
						203.1 (59)	8
4.4	3AcDON	9.8	338.3	339.2 [3AcDON + H] ⁺	85	231.0	8
						203.0 (39)	24
						175.0 (23)	18
5.8	HT-2	14.7	424.4	442.2 [HT-2 + NH ₄] ⁺	85	339.1	16
						321.0 (42)	12
						136.9 (36)	4
5.8	T-2	15.1	466.5	484.2 [T-2 + NH ₄] ⁺	85	215.1	8
						262.9 (90)	8
						196.9 (31)	20
5.8	T-2	15.1	466.5	484.2 [T-2 + NH ₄] ⁺	85	214.9	16
						304.9 (80)	12
						185.0 (66)	10

3. Results and Discussion

3.1. Instrumental Optimization

Precursor ions were selected testing positive and negative modes with the mobile phase based on previous studies developed for similar mycotoxin groups (Zachariasova et al. 2010; Rubert et al. 2014; Veprikova et al. 2015; Miró-Abella et al. 2017). That is, the solvents tested were water/MeOH (50:50, v/v) with two acids (formic and acetic acid) at 0.1% (v/v) and two salts (ammonium formate and acetate) at 5 mM being added to both solvents, either alone or in combination, resulting in 6 different solutions. The mycotoxins were injected individually in order to select the ions from the target compounds by flow injection analysis (FIA) at a flow rate of 0.45 mL min⁻¹, at the following concentrations: 1 mg L⁻¹ for HT-2 and DON3G; 0.5 mg L⁻¹ for DON, 3AcDON, and 15AcDON; and 0.1 mg L⁻¹ for T-2. Taking into account adducts with the greater response in each mobile phase combination, the solution with ammonium formate and formic acid was the one that provided the highest response. In consequence, this was chosen as the mobile phase for the chromatographic separation. With this mobile phase, precursor ions appeared in greater abundance in positive mode. DON was ionized as [DON + H]⁺ in the more abundant form, and DON3G gave the same transition than DON by losing

the glucoside fragment. Therefore, DON and DON3G had the same precursor ion. With respect to both acetylated DON derivatives, their most abundant ion was the protonated form [M + H]⁺. However, [15AcDON + NH₄]⁺ was selected as the ion for 15AcDON, whereas the protonated adduct [3AcDON + H]⁺ was selected for 3AcDON, not only to avoid possible interferences, but also to enhance analyte selectivity and sensitivity. Finally, the ammonium adducts [M + NH₄]⁺ of HT-2 and T-2 toxins were selected, as they are the most abundant forms.

After the selection of the correspondent precursor ions and the mobile phase, different product ions were selected for each mycotoxin by applying different collision energies, in order to obtain the three most abundant MRM transitions that will facilitate the correct mycotoxin identification, as recommended by the EU Directive (SANTE 2015), and these are detailed in Table 1. Further source parameters were also optimized and are detailed in "Liquid Chromatography-Mass Spectrometry".

With regard to the chromatographic gradient, it was mainly focused on the separation of DON and DON3G which were well-resolved and it was possible to select the same precursor ion for both.

Once MS values were optimized and chromatographic separation was achieved, instrumental linearity and limits of detection (LOD) and quantification (LOQ) were established. LODs and LOQs were calculated as the lowest mycotoxin concentration that the quantifier and qualifier transitions displayed a signal-to-noise ratio (S/N) \geq to 3 and 10, respectively. The LODs obtained were from 0.01 to 0.2 $\mu\text{g L}^{-1}$ for all compounds, except for DON3G, for which was 0.7 $\mu\text{g L}^{-1}$. The LOQs ranged from 0.2 to 0.5 $\mu\text{g L}^{-1}$ for all compounds, except for DON3G, for which was 2.5 $\mu\text{g L}^{-1}$. The linearity was suitable (with $r^2 \geq 0.994$) and it ranged from LOQs used as the lowest concentration to 20 $\mu\text{g L}^{-1}$ for T-2, to 100 $\mu\text{g L}^{-1}$ for DON, to 500 $\mu\text{g L}^{-1}$ for acetylated forms, and to 1000 $\mu\text{g L}^{-1}$ for DON3G and HT-2.

3.2. Optimization of Extraction

Taking into consideration that in previous studies (Sánchez Maldonado et al. 2014; Plaza and Turner 2015), the SWE of several compounds in a wide range of matrices was achieved successfully, a SWE was tested to extract the target mycotoxins from the cereal matrices. Water was acidified with 1% of HCOOH (pH 2.0) in order to improve the extraction, as in the aforementioned studies. Using acidified water as the extraction solvent, it is not necessary to do any change of the

solvent for a clean-up process using a SPE cartridge.

Prior to SWE, the SPE process was optimized. Two different cartridges were tested: an OASIS HLB and an ISOLUTE ENV+. A total volume of 25 mL of water solution with target mycotoxins at 25 $\mu\text{g L}^{-1}$ for T-2; 125 $\mu\text{g L}^{-1}$ for DON, 3AcDON, and 15AcDON; and 500 $\mu\text{g L}^{-1}$ for HT-2 and DON3G, was loaded into the previously conditioned cartridge. The mycotoxins were then eluted with three sequential fractions of MeOH: a first fraction of 3 mL, a second fraction of 2 mL, and a third of 2 mL. Most of the mycotoxins eluted at the first 3 mL. The second fraction also contained some mycotoxins, with a recovery up to 10%. But in the third fraction, the mycotoxins' presence was insignificant. Consequently, a single elution of 5 mL of MeOH was selected. Table 2 details all the recovery results. Obtained recovery values (%Rec SPE std) were slightly higher for OASIS HLB, especially for the more polar compounds. However, both cartridges obtained good recovery values, all higher than 76%. Further tests were performed in order to discard interactions between the cartridge and the matrix. For that, instead of water solution, extracts from SWEs of non-spiked maize samples were used, which were spiked at the same concentration as above after SWE extraction. The obtained recoveries

(%Rec SPE matrix) were lower than in water solution, decreasing equally in both cartridges. However, recoveries were slightly higher for OASIS HLB (detailed in Table 2).

Then, SWE optimization was performed taking into consideration the parameters with the greatest influence, namely temperature and number of cycles, as well as the extraction solvent, and maintaining the other parameters as described in "Sample Extraction". To do so, 1 g of homogenized maize sample was poured into a stainless steel extraction cell with DE, as explained in "Sample Extraction", and two different SWE temperatures were examined: 80 °C and 100 °C. Both temperatures provided suitable results in a similar order of magnitude, so a temperature of 80 °C was selected. Moreover, the number of SWE cycles was tested. The second cycle obtained an insignificant signal response, and a single extraction cycle was finally selected.

Once SWE parameters were optimized, the SWE extract was loaded into both SPE cartridges, and the mycotoxins were eluted; the extract was evaporated and re-suspended with the same solvent conditions as the initial mobile phase: 1 mL of water/MeOH (80:20, v/v), in order to obtain their recovery of the whole extraction. Two different groups of

concentrate ions were tested to calculate the recoveries of the entire method. These two groups were chosen in order to obtain similar response values of all compounds and taking into account their linear ranges. One group was at 1 $\mu\text{g kg}^{-1}$ (for T-2), at 5 $\mu\text{g kg}^{-1}$ (for DON, 3AcDON, and 15AcDON), and at 20 $\mu\text{g kg}^{-1}$ (for HT-2 and DON3G). The other concentration group was at 15 $\mu\text{g kg}^{-1}$ (for T-2), at 75 $\mu\text{g kg}^{-1}$ (for DON, 3AcDON, and 15AcDON), and at 200 $\mu\text{g kg}^{-1}$ (for HT-2 and DON3G). The %Rec SWE + SPE was calculated by comparing the concentration obtained from samples spiked before the extraction process with the concentration obtained from samples spiked after the extraction process. The obtained recovery values were similar at both tested groups, and just values when the sample was spiked at the lower concentration are shown in Table 2. As can be seen, the recovery values (%Rec SWE + SPE) obtained when OASIS HLB was used in the SPE are slightly higher than those achieved with ISOLUTE ENV+. Thus, OASIS HLB was selected for further experiments. In addition, from the %Rec SWE + SPE values, we can confirm that the SWE parameters as well as the use of water as solvent are a suitable option to extract these mycotoxins from cereals.

In addition, ME were evaluated and the values were obtained by comparing the concentration obtained when the

Table 2. Trichothecene recoveries from SPE (%Rec SPE) and extraction recoveries from SWE and SPE (%Rec SWE+SPE) of both tested cartridges. Matrix effects from OASIS HLB (%ME) are also detailed, from a dilution of 1 and 2 mL. Matrix was the maize sample spiked with the mycotoxins mixture, which concentrations are specified in the text.

	ISOLUTE ENV+			OASIS HLB				
	%Rec SPE std	%Rec SPE matrix	%Rec SWE+SPE	%Rec SPE std	%Rec SPE matrix	%Rec SWE+SPE	%ME 1 mL	%ME 2 mL
DON	76	70	69	92	73	77	-48	-24
DON3G	84	69	49	101	76	63	-35	-21
3AcDON	105	87	51	94	93	69	13	10
15AcDON	106	98	46	110	103	59	-8	3
HT-2	94	76	45	94	78	56	-39	-25
T-2	88	37	41	82	41	53	-39	-24

n=3, %RSD lower than 3% in both %Rec SPE; lower than 12% in both %Rec SWE+SPE. Results obtained from a final resuspension of 2 mL.

samples were spiked after the whole extraction process with the concentration obtained with the pure standard, and considering $ME = 0$ (no matrix effect), $ME > 0$ (ion enhancement), and $ME < 0$ (ion suppression). The obtained ME values are shown in Table 2, and it can be observed that all of the mycotoxins, except the acetylated forms of DON, are highly affected by ion suppression due to the complexity and composition of the maize samples. In order to reduce these ME values, an option could be the use of isotopically labeled standards for each compound, but it could not be afforded because of their elevated cost. For that, the mycotoxins were diluted in a resuspension of 2 mL of water/MeOH (80:20, v/v) solution instead of 1 mL. The results improved slightly as can also be observed in Table 2, with the percentage of ME reduced in all cases. Even in the case of some mycotoxins, such as DON, HT-2 and T-2, the ME reduced by nearly half.

Once the recovery and ME results for maize were obtained, and in order to evaluate the applicability of the developed method to other samples, three different cereals (spelt, millet, and oat), one pseudocereal (quinoa), and one oilseed (sesame seed) were spiked with the target mycotoxins, in the same way and concentrations as the maize samples. Different extraction

recoveries and ME were obtained from each matrix after a dilution of 2 mL, as detailed in Table 3. The obtained results were similar to those obtained in maize samples, especially in the case of spelt and quinoa samples. Oat, millet, and sesame displayed slightly lower recoveries. In the case of 3AcDON, in sesame matrices, the recovery was not calculated since there was an interference which masked the mycotoxin and it was not possible to quantify it; thereby, they are not collected in Table 3. With regard to ME for all matrices, they were considerably low. A previous extraction research was based on the use of PLE with organic solvents (Kokkonen and Jestoi 2009), and the ME obtained were higher for the same analytes due to the use of a less selective extraction solvent. Thus, using water as extraction solvent could be a suitable alternative because it extracts the mycotoxins and at the same time, does not extract many interferences as can be observed with the lower percentage of ME obtained from the extracts diluted with 2 mL. The reported method is adequate to quantify trichothecenes which appear naturally in complex matrices and at low concentrations. In addition, the present procedure allows a more effective and selective extraction, with lower ME, and it is more sustainable than classical PLE.

Table 3. Trichothecene extraction recoveries (%Rec SWE + SPE) and matrix effects (%ME) of the studied samples. Spiking concentrations are specified in the text.

	Spelt		Millet		Oat		Quinoa		Sesame	
	%Rec SWE+ SPE	%ME	%Rec SWE+ SPE	%ME	%Rec SWE+S PE	%ME	%Rec SWE+S PE	%ME	%Rec SWE+S PE	%ME
DON	60	-18	48	-17	53	-34	45	-8	72	-45
DON3G	49	-3	39	-6	35	-15	42	-6	48	-5
3AcDON	40	13	28	15	41	-18	35	28	-	-
15AcDON	46	8	34	9	27	2	33	9	50	14
HT-2	52	-7	34	4	25	1	39	7	44	6
T-2	47	-17	39	-14	30	-15	37	-12	33	-9

n = 3, RSD lower than 20%

3.3. Method Validation

Method validation parameters, such as linear range, LOD, LOQ, repeatability, and reproducibility, were evaluated using 1 g of maize samples spiked with the target trichothecenes. First of all, the presence of natural contamination was evaluated and taken into account by substrating the signal from contaminated samples. Then, the linear range was assessed from LOQs to 40 $\mu\text{g kg}^{-1}$ for T-2, to 200 $\mu\text{g kg}^{-1}$ for DON and its acetylated forms, and to 400 $\mu\text{g kg}^{-1}$ for DON3G and HT-2. The linearity was acceptable with r^2 higher than 0.990. LODs and LOQs were obtained in the same way as in the case of instrumental limits described above in "Instrumental Optimization". The LODs obtained were 0.05 $\mu\text{g kg}^{-1}$ for T-2; between 0.5 and 1.0 $\mu\text{g kg}^{-1}$ for DON, 3AcDON, 15AcDON, and HT-2; and 4.0 $\mu\text{g kg}^{-1}$ for DON3G. With respect to LOQs, they ranged between 0.4 and 1.0 $\mu\text{g kg}^{-1}$ for DON, 3AcDON, 15AcDON, and T-2; 4.0 $\mu\text{g kg}^{-1}$ for HT-2; and 20 $\mu\text{g kg}^{-1}$ for DON3G. The regulation for maize samples permits a maximum level for DON of 1750 $\mu\text{g kg}^{-1}$ (EC 2007), recommends a maximum level for the sum of T-2 and HT-2 of 100 $\mu\text{g kg}^{-1}$ (EC 2013), and recommends a maximum level for 3AcDON and 15AcDON of 1 $\mu\text{g kg}^{-1}$ (JECFA 2011). Taking into account these regulated levels and using them

as reference values, the obtained LOQs are acceptable because they are below them. In some mycotoxins such as DON, HT-2, and T-2, LOQ values are more than 100 times lower than the regulation values, denoting that it could be a good method to detect possible food and feed trichothecene natural contamination. There is in the literature previous researches which analyze diverse mycotoxins, by PLE with organic solvents and LC-MS/MS (Kokkonen and Jestoi 2009; Desmarchelier et al. 2010). In these researches, target mycotoxins also were extracted, among others, obtaining LOD and LOQ values higher than those obtained in the present research, denoting that SWE could be a good tool to extract type A and type B trichothecenes.

Method repeatability (intra-day, $n = 5$) and reproducibility (inter-day, $n = 5$) were obtained from different trichothecene concentration tests: T-2 at 1 $\mu\text{g kg}^{-1}$; DON, 3AcDON, and 15AcDON at 5 $\mu\text{g kg}^{-1}$; and HT-2 and DON3G at 10 $\mu\text{g kg}^{-1}$. Repeatability and reproducibility were expressed as relative standard deviation percentage (%RSD), and they were acceptable in accordance with current guidelines (SANTE 2015). The obtained results were between 6 and 9% for the repeatability and between 16 and 18% for the reproducibility.

3.4. Application to Different Samples

Once the method was successfully applied to maize samples, the natural presence of trichothecenes was studied using three different commercial brands of each cereal, pseudocereal, and oilseed ($n = 18$). Considering that the extraction recoveries were satisfactory and the repeatability of the method too, quantification of mycotoxins in the cereal samples was proposed using external calibration curve and applying the total recovery values (recovery explained in "Optimization of Extraction"). This was further proved by quantifying the mycotoxins present in maize sample by using the two approaches: matrix - matched calibration curve and external calibration curve plus total recovery percentage. The accuracy of both approaches was from 76 to 112%.

At least one mycotoxin was detected in all of the six samples studied, and they could be quantified in three cases: maize, millet, and oat. Different interval concentrations were found in the three different brands, and they are detailed in Table 4. DON was found in all the samples at low level, except in sesame samples. DON was detected in spelt and quinoa samples and quantified in maize in values up to $17.8 \mu\text{g kg}^{-1}$, in oat up to $64.5 \mu\text{g kg}^{-1}$, and in millet up to $8.1 \mu\text{g kg}^{-1}$. This

mycotoxin displayed the greatest trichothecene incidence ratio. Previous studies have also reported the presence of this trichothecene in the samples indicated (Jestoi et al. 2004; Schollenberger et al. 2005; Krysińska-Traczyk et al. 2007; Juan et al. 2013). Furthermore, 15AcDON also was quantified in maize up to $16.7 \mu\text{g kg}^{-1}$ and in oat up to $10.6 \mu\text{g kg}^{-1}$. With regard to the oat matrix, mycotoxin co-exposure is common, as identified in the previous studies (Schollenberger et al. 2005). As such, three more mycotoxins were quantified in oat: DON3G up to $8.7 \mu\text{g kg}^{-1}$, HT-2 up to $35.2 \mu\text{g kg}^{-1}$, and T-2 up to $4.5 \mu\text{g kg}^{-1}$. The concentration found in these samples is similar to those described in a previous study (Gottschalk et al. 2007).

From all the studied samples, there were some maize samples which were visually contaminated by fungi. The results obtained showed the presence of DON at $164.3 \mu\text{g kg}^{-1}$, DON3G at $91.0 \mu\text{g kg}^{-1}$, 3AcDON at $3.7 \mu\text{g kg}^{-1}$, and 15AcDON at $5.3 \mu\text{g kg}^{-1}$, the quantitative transition MRM chromatograms of which are shown in Fig. 1. These values are not detailed in Table 4, since this sample was singular. If these concentrations are compared with those quantified in the maize samples without visual contamination, it can be observed that, for example, DON concentration was more than 5-

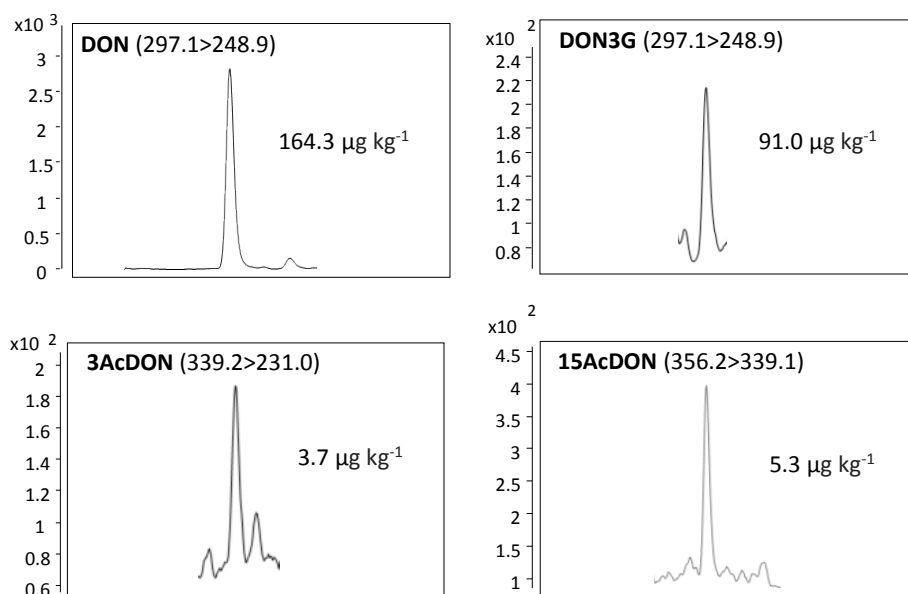


Fig. 1 Quantitative transition MRM chromatograms of detected trichothecenes in aged maize

fold. Therefore, it has been shown how visual contamination can anticipate the presence of mycotoxins.

From all these obtained results, one of the most important facts is that it was possible to extract these six different trichothecenes without using organic solvents at very low concentrations and with low ME.

4. Conclusions

For the first time, a method has been developed for the determination of six trichothecenes using SWE followed by an SPE clean-up and UHPLC-(ESI)MS/MS. The improved

alternative extraction used acidified water as solvent followed by a straightforward clean-up step. Although better recoveries would be obtained using an organic extraction solvent, water allowed better selectivity by obtaining lower ME levels. This decrease in ME levels involved the quantification of the target mycotoxins at very low concentrations and a selective detection of the natural presence of trichothecenes in the studied samples. The performance of the method may indicate a benefit of using alternative solvents, such as water, able to obtain results as sensitive and reliable as those provided by organic solvents.

Further research should be focused on the improvement of the purification step, by using less organic solvents and becoming more alternative, apart from broadening the applicability of the method by including more mycotoxins in different type of samples.

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3.1.3. Discussion of results

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Even though the results of the experimental research included in this section have been discussed individually in the previous papers, this section summarizes the most important outcomes of them. The research presented in this section presents original and new results. On one hand, information about mycotoxin contamination in matrices that could be considered new in to the market is given. On the other hand, results about an environmental friendly extraction, not commonly used to date, are achieved.

Two separate methods were developed for the analysis of different mycotoxins from diverse samples. Two extraction techniques were used for each type of sample, one of them adding a clean-up step. LC-(ESI)MS/MS was successfully employed and as expected, after analyzing various cereal and cereal derivative samples, the presence of mycotoxins was identified and determined.

On one hand, QuEChERS extraction was adapted from Anastassiades *et al.* [1] to determine *Aspergillus*, *Fusarium* and *Penicillium* mycotoxins from liquid samples, after ruling out a simple LLE technique. This extraction strategy was applied to soy, oat and rice plant-based beverage samples. QuEChERS was more suitable than LLE since the high sugar content of target samples produced losses in the reproducibility of the results obtained with LLE. Furthermore, there are numerous studies available in the literature applying QuEChERS for the extraction of mycotoxins from very diverse liquid samples, other than cereal-based beverages. Some of the more recently published articles were focused on samples such as wine [2], goat and cow kefir [3], cow milk [4,5], edible oils [6], fruit juices [7,8] and biological matrices [9], among others. Furthermore, QuEChERS extraction is useful when extracting a high number of compounds, apart from pesticides that were the original target compounds for this methodology. As demonstrated by Perez-Ortega *et al.* [11], over 600 different contaminants can be extracted using QuEChERS as the extraction technique. However and to the best of our knowledge, this was the first time that QuEChERS has been applied for the extraction of contaminants in plant-based beverages, and it was also the first time that mycotoxins were determined in plant-based beverages. After this study, another study determined AFs in soy-beverage samples, but using different extraction and detection strategies than those reported in the present doctoral thesis [10].

Suitable recovery results were obtained in this first article with the validated QuEChERS technique, between 80% and 91% for all the target compounds. However, ME results differ substantially between compounds, comprising ion suppression in some mycotoxins and ion enhancement in others. For instance, DON and HT-2 presented ion suppression in all the matrices, and FBs presented an important ion enhancement especially for FB₂. These results obtained for FBs are in agreement with the common

problems reported in the introduction related with FBs. Since the research followed after the first section of the present doctoral thesis was focused only with mycotoxins from the trichothecenes group, and considering the difficulties reported in the literature regarding FBs, results obtained for FBs in this study were accepted.



On the other hand, PLE followed by SPE was applied to determine trichothecenes, including modified mycotoxins such as AcDON, in cereal grains. There are few articles in literature extracting mycotoxins using PLE [12–14], one of them also extracting trichothecenes and modified trichothecenes [14]. Nevertheless, there are no publications related with mycotoxin extractions using water as the extraction solvent. SWE has been useful in environmental analysis, in pharmaceutical industry and in food analysis among others [15], because it is an efficient, cheap and safe extraction technique. Water with 1% HCOOH (pH 2.0) was used as the extraction solvent in the second article of this section, and some parameters such as temperature and number of cycles were evaluated. However, at the beginning of the research some extractions using organic solvents as the extraction solvents were also tested. Obtained recovery results were suitable, but interferences were also extracted making difficult the extracts filtration while obtaining high percentages of ME. Considering the selectivity obtained when using acidified water as the extraction solvent, SWE was selected. Then, two different cartridges were also evaluated for the straight-forward clean-up step, with the final selection of OASIS HLB cartridge. This step is important, especially considering that target compounds are in an aqueous sample, avoiding a solvent exchange after the SWE. Furthermore, the fact of using water as extraction solvent allows more selective extractions, obtaining lower ME levels. Obtained recovery results were also suitable, allowing the extraction and subsequent quantification of the natural presence of trichothecenes. After all, SWE has been applied for the extraction of mycotoxins for the first time.

ME results obtained after SWE followed by SPE clean-up were between -18 and 15, with some exceptions. The fact of obtaining these low ME levels were due the selectivity achieved using acidified water as the extraction solvent. When using organic solvents for the extraction of target compounds, interferences are also commonly extracted. For instance, if results obtained in both articles are compared, ER values of the QuEChERS extraction are considerably higher than those obtained in the second article where SWE + SPE was used. However, ME values obtained when extracting with water are lower than those obtained when extracting with QuEChERS. Thus, SWE followed by SPE is a good option when more selective extractions are required and QuEChERS is more useful when higher recovery values are needed. Therefore, SWE + SPE might be a suitable

alternative for the extraction of mycotoxins from solid samples. And, it would be further applied to extract a broad group of mycotoxins as listed in the first study.

The developed methods were applied to different varieties of cereals and cereal derivatives obtained from local establishments in Tarragona. As detailed above, the validated QuEChERS extraction was applied to plant-based beverages, while SWE was applied to cereal grains, which were maize, spelt, millet, oat, quinoa and sesame. A brief summary of all the target mycotoxins detected in all the studied samples is given in Table 1. Overall, oat-based beverage was the matrix with more number of detected mycotoxins. However, the matrix with the largest concentration level was oat in grain, where DON was quantified at $64.5 \mu\text{g kg}^{-1}$. Concentration levels of DON were also high in rice-based beverage and maize, quantified up to $19 \mu\text{g kg}^{-1}$. Moreover, FBs were the only two mycotoxins that were not detected in any sample. In spite of these results, all the concentration levels quantified in the studied samples were well below the maximum permitted levels by the current European regulation, described in the introduction.

Table 1. Mycotoxins found in cereal and cereal derivative samples from Tarragona.

	Matrix	Mycotoxins
	Oat	DON, AFG ₂ , AFG ₁ , AFB ₂ , AFB ₁ , HT-2, T-2, OTA, ZEA
	Soy	AFG ₂ , AFG ₁ , AFB ₁ , OTA, ZEA
	Rice	DON, AFB ₁ , OTA, ZEA
	Maize	DON, DON3G, 3AcDON, 15AcDON, HT-2, T-2
	Spelt	DON, 15AcDON
	Millet	DON, 15AcDON, T-2
	Oat	DON, DON3G, 15AcDON, HT-2, T-2
	Quinoa	DON, 3AcDON, HT-2
	Sesame	-

In this way, both extraction methods are able to determine target mycotoxins in different matrices at low concentration levels. However, there are few advantages and disadvantages for using each strategy. On one hand, QuEChERS extraction is simple and effective, as obtained ME and recovery results demonstrate. However, this method is not a good option when extracting a large number of samples because it is not automated. In this way, SWE is a more automated strategy than QuEChERS facilitating the extraction of multiple samples with less hand work. On the other hand, extraction

recovery results obtained using SWE + SPE were not those that would be expected and are considerably lower than those obtained with QuEChERS.

Thus, considering the necessary equipment for the SWE, the possible related technical problems with the equipment, the extraction recovery results obtained with both strategies and the simplicity of the QuEChERS method in comparison with the SWE + SPE method, it is concluded that the QuEChERS extraction would be more suitable when determining mycotoxins in cereal and cereal derivative samples.

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3.2. Evaluation of trichothecenes in rats and their determination in excretion samples

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In the previous section, there is the first contact with mycotoxins and also with modified mycotoxins, with the application of two different extraction techniques for cereal and cereal derivative samples. The main concept of this second section is related with the transformation of a group of mycotoxins (trichothecenes) after its consumption.

As already explained in the introduction, once mycotoxins are consumed by animals, their chemical structure can be modified resulting in numerous modified mycotoxins [1] depending on each animal, such as sulfonates [2], glucuronides [3] and sulfates [4]. Then, these metabolites are often excreted by urine and faeces [5,6]. It is described that these biological transformations frequently respond to the capability of the organism to decrease the toxic effects of parent mycotoxins [1,7]. For instance, the mycotoxin DOM-1 is reported to be less toxic than its parent DON mycotoxin [7,8]. As a result, it is considered that microbiota composition can be modified in order to better adapt to these detoxification processes [9,10].

Toxicological effects produced by the consumption of high concentration levels of mycotoxins are widely described for most parent mycotoxins [11–13]. However, mycotoxins are commonly found in food and feed samples at low concentration levels. For instance, in the first section of this doctoral thesis, the highest mycotoxin concentration that was quantified was $64.5 \mu\text{g kg}^{-1}$ of DON in oat grains. Consequently, chronic effects produced by the consumption of low concentration levels during large periods of time, such as bacterial changes, are hardly described. In 2009 it was the first time that it was demonstrated that DON is capable to modify the bacterial composition in pigs [14]. However, to date research in this field has not progressed considerably.

In order to know how low mycotoxin doses can affect to the microbiota composition of animals, a metagenomics assay was achieved in the first study of this second section. This research was accomplished after the administration to rats of low concentration levels of DON during 7 weeks. As mentioned above, part of the mycotoxins excretion is produced by faeces and accordingly, the presence of the mycotoxins DON and DOM-1 was monitored daily by UHPLC-(ESI)MS/MS. The aim of this monitoring was to observe an increment of DOM-1 excretion, explained by an increment of DON detoxification capability. As soon as treatment with DON had finished, gut biodiversity was explored through 16s rRNA high throughput sequencing.

Once the presence of modified mycotoxins in different parts of the organism has been reported, the development of high sensitive determination methods has become an important issue. As described in the first section, there are numerous developed

methodologies for the determination of modified mycotoxins mostly in cereal and cereal derivative samples. However, animal excretions are more complex samples and methods obtaining low detection and quantification limits and low presence of interferences must be applied. As a result, different extraction methods comprising SLE, QuEChERS and PLE were tested, and a clean-up strategy was optimised for the determination of trichothecenes in rat faecal samples. The addition of a clean-up step is important in order to reduce the presence of interferences and obtain lower limits, taking into account the complexity of the excretion samples and the low concentration levels that these compounds are commonly found. Obtained results are detailed in the second article reported in the present section.

Two papers discussing the results obtained from these studies have been reported and are presented below. The first paper has been published in *Food and Chemical Toxicology* 121 (2018) 124–130, and the second one has been submitted for publication in *Journal of Chromatography B*.

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3.2.1. Monitoring and evaluation of the interaction between deoxynivalenol and gut microbiota in Wistar rats by mass spectrometry-based metabolomics and next-generation sequencing

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MONITORING AND EVALUATION OF THE INTERACTION BETWEEN DEOXYNIVALENOL AND GUT MICROBIOTA IN WISTAR RATS BY MASS SPECTROMETRY-BASED METABOLOMICS AND NEXT-GENERATION SEQUENCING

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Abstract

Published evidence has demonstrated the several toxic characteristics of mycotoxins and their considerable risk to human and animal health. One of the most common uncertainties regards whether if very low concentrations of the mycotoxin deoxynivalenol (DON), easily consumed within the Mediterranean Diet, can cause metabolic alterations; some of them produced by the interaction between DON and gut microbiota. Accordingly, faecal samples were collected from Wistar rats that had consumed the mycotoxin DON at low levels (60 and 120 $\mu\text{g kg}^{-1}$ body weight of DON per day), and were analysed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection, in order to monitor the mycotoxin DON and its metabolite de-epoxy deoxynivalenol (DOM-1). The obtained results showed an evolution in DON excretion and the metabolite DOM-1 which has less toxic properties, over the course of the days of the study. To elucidate whether intestinal microbiota had a role in the observed detoxification process, the changes in microbial gut biodiversity were explored through 16s rRNA high throughput sequencing. No main changes were detected but significant increase in Coprococcus genus relative abundance was found. Further studies are needed to confirm if intestinal microbiota composition and function are affected by low concentrations of mycotoxins.

Keywords: *Mycotoxins; Rats; Faeces; Microbiota; Metagenomics; UHPLC-(ESI)MS/MS.*

1. Introduction

The mycotoxin deoxynivalenol (DON) is a secondary metabolite produced by several *Fusarium* moulds and it is considered the most widespread mycotoxin in food and feed matrices [1], especially in cereals and cereal-based products. The consumption of DON may have numerous hazardous effects, ranging from vomiting, diarrhoea, gastroenteritis, growth impairment and immune dysfunction [2,3], to leukopenia, haemorrhage, endotoxemia and shock-like death [3]. The toxicology of DON is dose-dependent and its susceptibility is determined by differences between animal species in terms of DON metabolism, absorption, distribution and secretion. Thus, pigs are the most sensitive animals, while rats have medium sensitivity and ruminants are the least sensitive [2]. There are certain toxicological parameters for examining the degree of toxicity of hazardous compounds, such as the median lethal dose (LD50), the minimum emetic dose (MED) and the no-observed adverse effect level (NOAEL). These parameters also vary between species and the type of administration. In the case of orally administered DON in rats, the estimated NOAEL was found between $150 \mu\text{g kg}^{-1}$ body weight (b.w.)/day [2–4] and $500 \mu\text{g kg}^{-1}$ b.w./day [3,4], which means that, at this dose, no adverse

effects should be observed, but does not mean that intestinal microbiota modifications may not occur. European regulations have set maximum levels for DON in different kind of matrices, especially in cereal and cereal derivatives [5], varying from $500 \mu\text{g kg}^{-1}$ to $1,750 \mu\text{g kg}^{-1}$ in adult foodstuffs, to $200 \mu\text{g kg}^{-1}$ in foods for infants and young children.

Once DON is consumed, it is mostly absorbed and rapidly distributed to all tissues, with blood, the gastrointestinal tract, the lymphatic system and the immune system being the main targets. Then, it is eventually eliminated, mostly in urine and faeces [1], without bioaccumulation, which means that trace concentrations found in food of animal origin are not a public health problem [2]. Of the entire DON structure, the 12,13-epoxy-trichothec-9-ene skeleton can be modified by intestinal bacteria in order to detoxify the mycotoxin. It is known that the 12,13-epoxide group is responsible for the elevated toxicity of the molecule and, specifically, this group is removed by intestinal microbiota for DON detoxification, generating the toxin de-epoxy deoxynivalenol (DOM-1), which is less toxic than its precursor [1]. This modification of DON into DOM-1 has been observed *in vitro* [6,7] and also *in vivo* [8] experiments, mostly by anaerobic bacteria from the gut lumen [9]. Nevertheless, not all bacteria can

generate DOM-1 through DON detoxification [10]. Some researchers suggest that chronic consumption of certain mycotoxins may change the intestinal microbiota in order to try to acquire or increase the detoxification capability [9,11]. Thus, it is reported that the presence of ochratoxin A and DON can modify animals' intestinal functions [9] and intestinal microbiota [12,13]. In 2009, it was demonstrated for the first time that DON modifies intestinal microflora in pigs [14]. However, as yet, there has been no identification of the taxonomic categories responsible of these changes in the gut microbiota, which may differ between animal species.

The main objective of the present research is to evaluate gut microbiota to find the bacteria population responsible of the DON metabolism through its administration at NOAEL. Over the course of the present work, the concentration of DON and its derivative DOM-1 in rat faeces were determined by ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection (UHPLC-MS/MS), to monitor and evaluate the possible changes in mycotoxin metabolism. Since the dose of DON administered to rats is low, the presence of this mycotoxin together with their metabolites deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3AcDON) and 15-

acetyl-deoxynivalenol (15AcDON) easily found in cereal samples [15], was also controlled. These derivatives are easily metabolised to DON by rat gut intestinal microflora [15] and their presence might interfere the results of the present research.

2. Results

2.1. Mycotoxin monitoring with UHPLC-MS/MS

2.1.1. UHPLC-MS/MS parameters optimisation

The instrumental optimisation was done for DON and its metabolite DOM-1, but also for DON3G, 3AcDON and 15AcDON, in order to control their possible presence in feed samples. The mobile phase and the chromatographic gradient was selected in accordance with previous research studies in which DON and DON derivatives were separated, except for DOM-1 [16]. Under these conditions, DOM-1 was injected individually by flow injection analysis (FIA) and its precursor and product ions were selected, with its optimised collision energy. The precursor and product ions for DON, DON3G, 3AcDON and 15AcDON were the same as in the previous research [16], ionised in positive mode. DOM-1 also displayed greater abundance in positive mode, with the $[M+H]^+$ ion. Three MRM transitions were selected

for every target compound, as detailed in Table 1, so as to enable the correct identification of the mycotoxins in accordance with the recommendations of the EU Directive [17].

With respect to instrumental optimisation, the detection limits (LOD) and the quantification limits (LOQ) were established by assuming the criteria of a signal-to-noise ratio (S/N) equivalent to 3 for LOD and equivalent to 10 for LOQ. Thus, LODs ranged from 0.01 $\mu\text{g L}^{-1}$ to 0.25 $\mu\text{g L}^{-1}$, and LOQs from 0.02 $\mu\text{g L}^{-1}$ to 0.5 $\mu\text{g L}^{-1}$. Suitable linearity was obtained (with $r^2 \geq 0.994$) of the linear range which was from LOQ

to 200 $\mu\text{g L}^{-1}$ for DON, 3AcDON and 15AcDON, to 100 $\mu\text{g L}^{-1}$ for DON3G, and to 40 $\mu\text{g L}^{-1}$ for DOM-1.

2.1.2. Faecal extraction optimisation

Since only DON and DOM-1 are expected to be quantified in the faecal samples, the method optimisation was developed only for these two mycotoxins. The selection of the solid liquid extraction technique took into account previous studies that extracted mycotoxins from faecal samples [18–20]. The selected method was adapted from the literature [18–20], but using a small quantity of faeces, less organic

Table 1. Parameters of LC-MS/MS for mycotoxin determination in feed and faecal samples.

Start time (min)	Mycotoxin	Retention time (min)	Precursor ion (m/z)	Dwell Time (ms)	Product ion (m/z) and q/Q relation	CE (eV)
0	DON	2.9	297.1	[DON + H] ⁺	248.9 (100)	8
					231.1 (55)	10
					203.1 (60)	8
4.4	DON3G	3.6	297.1	[DON3G – 3G + H] ⁺	248.9 (100)	8
					231.1 (60)	10
					203.1 (50)	8
7.5	DOM-1	5.8	281.0	[DOM-1 + H] ⁺	233.2 (100)	8
					109.1 (96)	14
					215.1 (83)	10
7.5	3AcDON	9.8	339.2	[3AcDON + H] ⁺	231.0 (100)	8
					203.0 (44)	24
					175.0 (30)	18
7.5	15AcDON	10.1	356.2	[15AcDON + NH ₄] ⁺	339.1 (100)	16
					321.0 (38)	12
					136.9 (35)	4

solvents and avoiding a final dilution step if the matrix effects (ME) were sufficiently low to quantify at lower concentration levels.

First of all, faecal samples were analysed as to detect and quantify the possible natural presence of the target mycotoxins, DON and DOM-1, and no presence of these mycotoxins was observed. For the method optimisation, faecal samples were spiked and were blended with 1 mL of two different solvents in order to ascertain which is better for extraction: MeOH and MeOH 1% of HCOOH. These samples were sonicated for 15 minutes, and centrifuged at 15,000 rpm for 10 min. A volume of 700 µL of supernatant which is the maximum quantity of solvent possible to subtract, was evaporated to dryness under nitrogen flow. The extraction was performed twice with the purpose of assessing whether a second extraction was necessary in order to completely extract the mycotoxins. To do so, 1 mL of solvent was added to the wet faecal samples repeating the same procedure as before, but 1 mL of supernatant was evaporated to dryness, instead than 700 µL. Samples were re-dissolved in 1 mL of MeOH/water (20:80, v/v), filtered and analysed by UHPLC-MS/MS.

To obtain quantifiable results, extraction recovery (ER) and the ME were considered. The presence of ME

can interfere and cause a systematic error in the determination of the analyte of interest, distorting the signal by enhancing or suppressing it. Thus, the determination of the ME as well as the identification of the %ER, are useful tools for the method validation, which were calculated as follows:

$$(1) \%ER = \frac{(C \text{ before} - C \text{ non-spiked})}{(C \text{ after} - C \text{ non-spiked})} \times 100$$

$$(2) \%ME = \left[\frac{(C \text{ after} - C \text{ non-spiked})}{(C \text{ calibration curve})} \times 100 \right] - 100$$

C indicates concentration, ME=0 indicates no ME, ME>0 indicates ion enhancement and ME<0 indicates ion suppression. The obtained %ER from both types of extraction solvents were similar but slightly higher in the case of MeOH alone (detailed in Table 2) than in the case of MeOH with HCOOH (which were 50% and 70% for DON and DOM-1, respectively). The extraction results obtained from the second extraction were below than 10% and, consequently, one extraction was considered sufficient. Regarding the ME in the tested faecal samples, there was ion suppression due the elevated presence of interferences since the obtained percentage results were -67% for DON and -62% for DOM-1. These results were similar to those obtained from faeces extractions in previous studies [18,19]. In order to reduce these %ME, samples were diluted 1:1

Table 2. Method validation parameters for DON and DOM-1 in faecal samples.

Mycotoxin	%ER	%ME	MDL ($\mu\text{g kg}^{-1}$)	MQL ($\mu\text{g kg}^{-1}$)	Linearity ($\mu\text{g kg}^{-1}$)
DON	59	-42	0.7	1.5	MQL to 150
DOM-1	68	-39	0.3	0.7	MQL to 30

n=3, RSD \leq 4

with MeOH/water (20:80, v/v) before injection into the UHPLC-MS/MS. As a result, MEs were significantly decreased, as detailed in Table 2. Considering these results, faecal sample extractions were finally extracted once with MeOH and re-suspended after supernatant evaporation with 2 mL of MeOH/water (20:80, v/v), since more diluted extracts obtained better results.

Obtained method limits for DON and DOM-1 were appropriate for their detection and quantification in faeces and are detailed in Table 2, together with the linearity of the method which was acceptable for both mycotoxins with the r^2 higher than 0.996.

2.1.4. Faecal mycotoxin monitoring

First of all, the feed given to the rats was analysed in order to quantify the possible presence of natural mycotoxins which could contribute to consumption. For that, the presence of DON, DON3G, DOM-1, 3AcDON and

15AcDON was determined. DON was quantified in the tested samples at $3.8 \mu\text{g kg}^{-1}$, and the rest of the mycotoxins were not detected. Taking into account the weekly feed controls –each rat consumed daily an average of 24 g of feed– and the daily DON dose administered, the consumption of DON through the feed was below 0.5% of the dose. Thus, the amount of DON consumed through the feed is insignificant and it was discarded.

The target mycotoxins DON and DOM-1, which are those involved in the de-epoxidation process, were monitored and quantified in each faecal sample (35 samples per subject) in order to assess possible concentration variations on all the days of the study. Faecal samples were monitored due to the main excretion of DON metabolites by faeces in rats [21].

The quantity of DON and DOM-1 excreted in faeces ranged from $20 \mu\text{g kg}^{-1}$ to $230 \mu\text{g kg}^{-1}$ and from $5 \mu\text{g kg}^{-1}$ to $50 \mu\text{g kg}^{-1}$, respectively. These values

are very low in comparison to the DON consumed, and it may be due to the excretion of DON sulfonates and DOM sulfonates as major DON metabolites in faeces from rats treated with DON [20]. From all the faecal concentrations found during the present study, different comparisons were performed between groups using the Student's t-test statistic. As a result, significant differences were found between the control group and the group treated with 60 $\mu\text{g kg}^{-1}$ b.w. of DON per day (P60) and between the control group and the group treated with 120 $\mu\text{g kg}^{-1}$ b.w. of DON per day (P120) in the case of both mycotoxins, DON and DOM-1. At all time points, the concentration of DON found between P60 and P120 was higher than those found in the control faeces. Moreover, comparing both

groups of rats that consumed DON at different concentrations (P60 and P120 groups), there were no significant differences in the DON concentration levels present in faeces.

Regarding DOM-1, its concentration is higher in P120 than in P60, in almost all the weeks of study as it is detailed in Figure 1. The presence of DOM-1 in faecal samples also increased over the seven weeks of study, especially after the fifth week of DON consumption (Figure 1). The gradual increase in the DOM-1 concentration level was as expected, although the great increase produced in the fifth week surprised the authors. This increase is more prominent in the faeces from rats that consumed 120 $\mu\text{g kg}^{-1}$ per dose, than in faeces from rats that consumed 60 μg

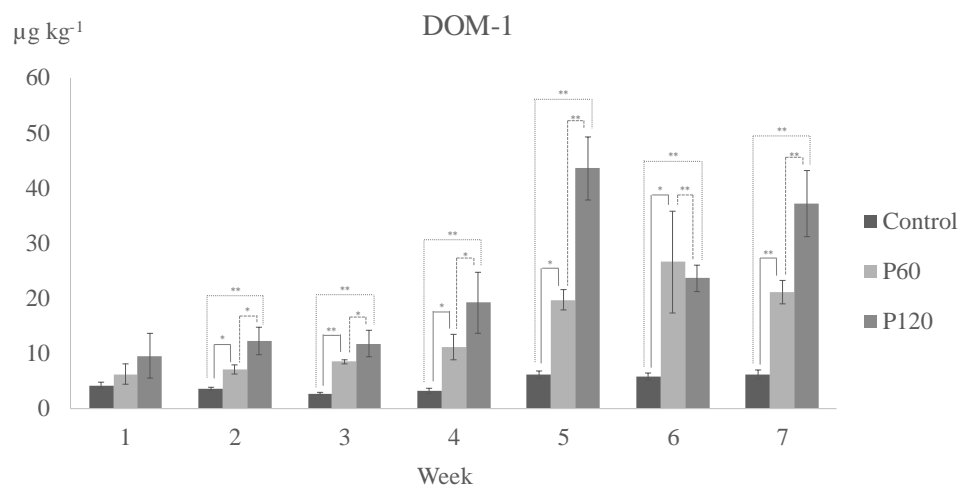


Figure 1. Concentration of DOM-1 quantified in faecal samples over the 7 weeks of study. Statistic significant differences are shown (one-way ANOVA with Bonferroni post-hoc correction), * $p < 0.05$; ** $p < 0.01$.

kg⁻¹ per dose, in which the growth is more progressive. Additional unexpected results were those obtained during the sixth week of the research, where the concentration excreted of DOM-1 decreased instead than increase, as it was expected. Although one should take also into account the great variability of P60 results at sixth week.

2.2. Microbial diversity analysis by high-throughput sequencing

In order to determine and quantify differences between the intestinal microbiota compositions of the three groups in the present research, metagenomic analysis was performed at the end point. The sequencing run produced a total of 6,697,663 paired-end reads that were reduced to

4,117,943 readings after quality filtering. The criteria for quality filtering considered that reads shorter than 50 bp were removed as well as reads with Phred score under 20. These readings were then analysed with QIIME and SPSS. The two phyla Bacteroidetes and Firmicutes dominated the community in all samples (Figure 2) with varying relative abundance (16.7-45.5% and 52.2-82.2%, respectively), which is consistent with previously published mammalian gut microbiome descriptions [22,23].

When the microbial relative abundance between groups was compared, no difference was found at any taxonomic level, except at genus level. The one-way ANOVA test revealed that the relative abundance of the Coprococcus genus was slightly

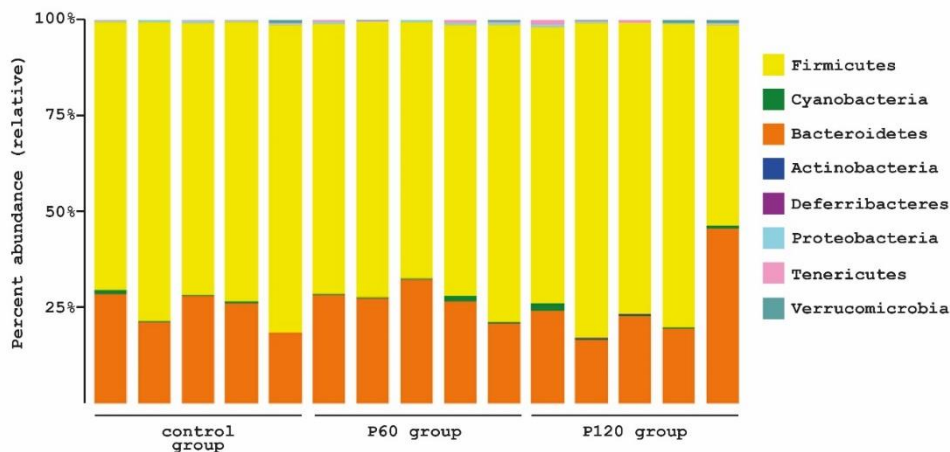


Figure 2. Phylum-level taxonomic distribution. Bars represent the relative abundance (percentage) of each phylum detected per sample.

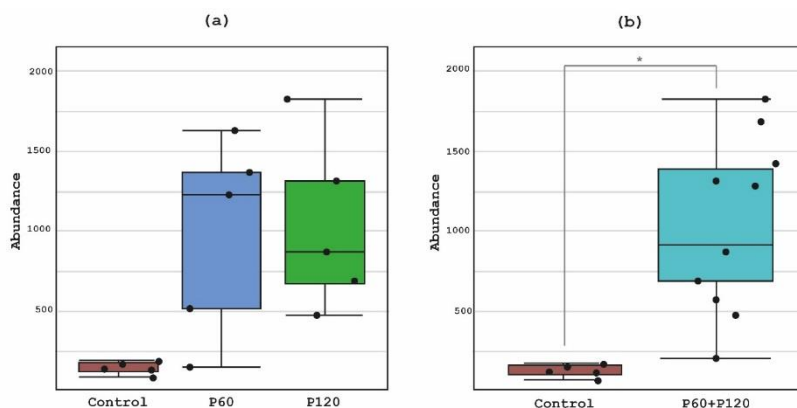


Figure 3. Graphics from Kurskall-Wallis tests comparing the abundance of the Coprococcus genus from the tested groups (applying false discovery rate correction). Abundance refers to the number of readings for the Coprococcus genus. (a) Comparison between the group control, P60

higher in the P60 group compared to the control group (0.24% vs 1.6%, $p=0.039$) and also higher in the P120 group than in the control group (0.24% vs 1.7%, $p=0.030$), data not shown. However, when a Kurskall-Wallis test was done with multiple test correction using false discovery rate (q), only significant differences were found in Coprococcus genus abundance when treated rats (independently of the dose) and control group were compared (Figure 3b, $q=0.031$). Those differences were not found when both treated groups were separately compared with control group (Figure 3a, $q=0.34$).

3. Discussion

As has already been hypothesised [9,11], the observed rise of the faecal

presence of DOM-1 must presumably be caused by gut microbiota increasing detoxification capability. Probably, intestinal microbiota from rats increased the detoxification ability during the treatment. These results can be compared with a study of faecal samples from pigs [11] in which the de-epoxidation ability was found only in animals fed with contaminated feed from the second week of exposure onwards. Additionally, the intestinal microbiota composition was different between animals that had increased the ability to de-epoxidate mycotoxins in comparison to animals that were not exposed to mycotoxins.

Regarding the microbial diversity analysis, the gut abundance of Coprococcus has changed due the treatment with the mycotoxin DON.

However, Coprococcus belongs to a group of anaerobic cocci that are known to produce butyrate, which is an essential metabolite in the human colon. Butyrate is the preferred energy source for the colon epithelial cells. It contributes to the maintenance of the gut barrier functions, and has immunomodulatory and anti-inflammatory properties [24]. Evidence indicating that disruption of the intestinal epithelial barrier due to DON exposure is well established [25]. Further research is needed to know if the Coprococcus genus can play a role in the detoxification of DON, which may explain the increased amount of DOM-1 in faeces, described in the UHPLC-MS/MS results section.

Nevertheless, the results reported here do not agree with already published *in vivo* studies. For example, Saint-Cyr and colleagues [13] used quantitative PCR to determine an increment of Bacteroides and Prevotella genera, as well as a decrease in *Escherichia coli* in stool samples from rats after the administration of 100 µg kg⁻¹ b.w. for four weeks. These findings were not corroborated, probably because the microbiota of these rats was of human origin and transplanted. Another study by Waché and colleagues [14] reported that DON had a moderate effect on cultivable bacteria and on capillary

electrophoresis single strand conformation polymorphism patterns corresponding to the Eubacteria genus in the pig intestine. They found that aerobic mesophilic bacteria increased while anaerobic sulphite-reducing bacteria remained unchanged. These outcomes were not replicated in our tests nor in a later study conducted also on a pig model [26], in which, moreover, no change in microbiota composition in response to DON administration was found. However, our study is the first to apply a metagenomic approach sequencing regions of 16s RNA gene, providing a general overview of microbiota composition, and all the previous publications mentioned only looked for specific bacteria. Therefore a variation in the Coprococcus genus may have gone unnoticed.

Additionally, some *in vitro* studies have demonstrated the ability of certain bacterial species to promote DON metabolism, by binding or detoxification [7,27–29], but none of the genus where these species belong to were found to be significantly increased in our treated groups. An explanation for this may be that these species do not react to mycotoxin exposure *in vivo* or perhaps they respond by increasing gene expression of proteins related to DON detoxification rather than by increasing their cellular abundance.

Overall, technical issues coupled with the dose and the extent and duration of treatment, as well as the animal model, could explain the discordances between the results of the *in vitro* studies published and our *in vivo* study.

4. Conclusions

Mycotoxins are fungal secondary metabolites, potentially hazardous to human and animal health following direct consumption through contaminated food or feed. The impact of mycotoxin consumption in microbial gut composition has already been demonstrated and our results support the hypothesis that microbiota composition slightly changes in response to mycotoxin consumption. In this regard, several *in vitro* and *in vivo* studies have identified a modulation of certain groups of intestinal bacteria due to mycotoxin exposure but, to date, this is the first metagenomic study that assesses the response of gut microbial composition to DON administration. An increase in DOM-1 was found in faecal samples due to mycotoxin consumption and a correlation of this rise with a significant increase in the relative abundance of the Coprococcus genus.

Long-term exposure to mycotoxins may produce significant changes in microbiota composition and their

metabolic activity, and these issues require further experimentation to elucidate the mechanism of action in order to promote them and find a new way of preventing or treating the effects of mycotoxin. Thus, further *in vivo* and *in vitro* studies are needed to shed some light on the response of microbiota to mycotoxins. According to present knowledge, the identification of specific bacterial genus or species that have detoxification capability opens the possibility of their use as feed additives [30]. For example, *Eubacterium* strains reduce the epoxy group from mycotoxins and their effect has already been reported in chicken models [31]. Thus, the administration of this species as feed additives in high-probably exposed populations could prevent, palliate or even restore the chronic damage caused by DON and other mycotoxins.

However, metagenomic analysis is restricted to the identification of microbial diversity, while the molecular functionality of this community remains ignored. As most biological mechanisms involve more than one type of biomolecule, further studies should combine multiple omic strategies (i.e. metagenomics and metaproteomics) to achieve a comprehensive, structured and interactive overview of the mycotoxin-microbiota interplay.

5. Materials and Methods

5.1. Experimental design

Male Wistar rats (7-weeks-old, 200-240 g b.w.) were purchased from Charles River Laboratories (Barcelona, Spain). Water and radiated pelleted feed from Teklad Global Diets (Madison, WI, USA), free of fungi that can generate mycotoxins, were provided ad libitum. The eighteen rats were housed individually in polycarbonate cages under controlled conditions in terms of temperature (22 ± 2 °C), humidity (50-60% relative humidity) and a light/dark cycle (12h). The animals were acclimatised for five days and were distributed fairly into three groups taking into account their b.w. and their contents in fat, lean tissue, free water and total water, obtained through Nuclear Magnetic Resonance (NMR) measurements. The study groups (N=6) were depending on the mycotoxin administration: P60 group was treated with $60 \mu\text{g kg}^{-1}$ b.w. per day of DON and P120 group was treated with $120 \mu\text{g kg}^{-1}$ b.w. per day of DON, using in all cases 600 μL of diluted condensed milk, five days per week for eight weeks. Following the same administration protocol, 600 μL of diluted condensed milk were used as the vehicle for the control group. The individual dose of toxin was revised weekly according to their b.w. Faecal samples were individually collected five

days per week for seven weeks and stored at -80 °C until analysis. Water, feed and b.w. controls were performed weekly. At the end of the eighth week of study, the rats were anaesthetised using pentobarbital sodium and then decapitated. The kidneys, liver, brain, muscle, the caecum and the heart were removed and weighed as a control parameter, and caecum was frozen with liquid nitrogen and stored at -80 °C until metagenomic analysis.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures.

5.2. Standard mycotoxin solutions

Methanol (MeOH, for LC-MS grade) and ethanol (EtOH) were purchased from Panreac (Barcelona, Spain), and formic acid (HCOOH) ~ 98% was acquired from Fluka (St. Louis, MO, USA). Acetone, 10 M ammonium formate (NH_4HCOO) aqueous solution and pentobarbital sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure-grade water was obtained by a Milli-Q water purification system (Millipore, Darmstadt, Germany).

The target mycotoxins were DON and DOM-1, both obtained from Bioser (Barcelona, Spain), and DON3G, 3AcDON and 15AcDON were purchased from Romer Labs (Union, MO, USA).

DON, 3AcDON and 15AcDON were obtained in powder form and DOM-1 and DON3G were purchased in acetonitrile (ACN) solution at 50 mg L⁻¹ and at 50.9 mg L⁻¹, respectively. DON was dissolved at 1.8·10³ mg L⁻¹ and 3AcDON and 15AcDON were dissolved at 104 mg L⁻¹, all in water/EtOH (80:20, v/v) and stored at 4 °C during the experiment. A mixed stock solution of all mycotoxins was prepared at different concentrations depending on their response in (ESI)MS/MS, in order to obtain similar response values. Thus, DON, DON3G, 3AcDON and 15AcDON were at 0.5 mg L⁻¹ and DOM-1 was at 0.1 mg L⁻¹, in water/MeOH (80:20, v/v), and this mix solution was stored at -20 °C for six months.

5.3. Faecal mycotoxin monitoring

5.3.1. Mycotoxin faecal sample extraction and method validation

Aliquots of 100 mg of freeze-dried and homogenised faecal samples were blended with 1 mL MeOH and sonicated for 15 minutes. Samples were centrifuged at 15,000 rpm for 10 min, and 700 µL aliquots of supernatant were evaporated to dryness under nitrogen air and re-suspended in 2 mL of MeOH/water (20:80, v/v). Before analysis by UHPLC-(ESI)MS/MS, the extracts were filtered with a 0.45 µm nylon filter (Phenomenex, Torrance, CA, USA) and stored at 4 °C.

For the method optimisation, faecal samples without the presence of mycotoxins, or the least possible presence, were used to obtain the ER and ME values. To do so, 100 mg of homogenised freeze-dried faecal samples were spiked with the mycotoxins at two different concentrations: DON was at 50 µg kg⁻¹ and DOM-1 was at 10 µg kg⁻¹. Samples were spiked with 200 µL of mycotoxin solution in acetone in order to distribute it homogeneously throughout the matrix, and left overnight. Then, samples were treated as detailed above.

The method validation was done for the two faecal target mycotoxins DON and DOM-1. It was performed by studying the method detection limits (MDL) and method quantification limits (MQL), linear range, accuracy, repeatability and reproducibility. To do so, 100 mg of faecal sample was analysed following the extraction method described above. MDLs and MQLs were estimated by the criteria of a signal-to-noise ratio (S/N) equivalent to 3 and 10, respectively.

The repeatability (n=5, intra-day) and the reproducibility (n=5, inter-day) of the method were below 4% and 12%, respectively, expressed as the relative standard deviation (%RSD). These results were obtained by spiking the samples at a concentration near to

the MQL and at a concentration ten times higher than the MQL, for each compound. Thus, repeatability and reproducibility were acceptable in accordance with current guidelines [17].

5.3.2. UHPLC-MS/MS analysis

DON and DON derivatives were analysed chromatographically on an Agilent 1290 Infinity LC Series UHPLC system (Agilent Technologies, Waldbronn, Germany), and separated using a Cortecs UHPLC C₁₈ column (100 mm x 2.1 mm, 1.6 µm) from Waters (Wexford, Ireland). The column was kept at 40 °C and mycotoxins were eluted at a flow rate of 0.45 mL min⁻¹ using a binary mobile phase constituted of water (eluent A) and MeOH (eluent B), both with 5 mM of NH₄HCOO and 0.1% of HCOOH. The gradient elution was the same as the previous research [16], the injection volume was 10 µL and the autosampler was kept at 4 °C.

Then, the LC eluent was directed into a 6495 iFunnel Triple Quadrupole MS/MS with an electrospray ionization (ESI) interface from Agilent Technologies. Source parameters were taken from previous research [16] for DON, DON3G, 3AcDON and 15AcDON, and they were also applied for DOM-1 as follows: capillary voltage of 3,500 V,

desolvation gas flow of 18 L min⁻¹, desolvation gas temperature of 160 °C, nebuliser pressure of 35 psi, nozzle voltage of 500 V, fragmentor voltage of 380 V, cell acceleration voltage of 5 V, sheath gas flow of 11 L min⁻¹ and sheath gas temperature of 350 °C. The high and low pressure funnel parameters were 150 and 60 V, respectively. All compounds were acquired by Multiple Reaction Monitoring (MRM) mode in positive polarity, in which three characteristic MRM transitions were monitored for each mycotoxin, in accordance with the European Commission guidelines [17]. Suitable collision energies were tested for each transition, and different time segments were optimised in order to improve analyte sensitivity. All these parameters mentioned above are detailed in Table 1.

5.4. 16s rRNA-metagenomics

5.4.1. DNA extraction

To obtain DNA from caecum samples, the QIAmp DNA Stool Kit (Qiagen, Venlo, the Netherlands) was used replacing the 70 °C lysis incubation recommended by the protocol, by a 95 °C lysis. DNA purity and integrity were assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA).

5.4.2. Partial 16S rRNA gene amplification and purification

Sequences from the V3 and V4 regions of 16S rRNA gene were amplified from the extracted faecal DNA through two primer pairs: 341F-532R (5'-CCTACGGGRSGCAGCAG-3'; 5'-ATTACCGCGGCTGCT-3') for the V3 region, 515F-806R (5'-GTGCCAGCMGCCGCGGTAA-3'; 5'-GGACTACHVGGGTWTCTAAT-3'), for the V4 region. These primers comprise, at their 5' end, one of the two adaptor sequences used in the Ion Torrent sequencing library preparation protocol linking a unique Tag barcode of 10 bases to identify different samples. To perform the V3 region amplification, 1 μL of extracted DNA (50 ng mL^{-1}), 7.5 μL of water, 12.5 μL of AmpliTaq Gold 360 (Applied Biosystems, California, USA), 2 μL of each primer Forward ($5 \mu\text{M}$) and 2 μL of the primer Reverse ($5 \mu\text{M}$) were mixed in this order. Meanwhile, to perform the V4 region amplification, 1 μL of extracted DNA (50 ng mL^{-1}), 8.5 μL of water, 12.5 μL of AmpliTaq Gold 360, 1.5 μL of each primer Forward ($5 \mu\text{M}$) and 1.5 μL of the primer Reverse ($5 \mu\text{M}$) were mixed, also in this order. Different Polymerase Chain Reaction (PCR) cycle parameters were used for the V3 and V4 regions. For the V3 region, the parameters were 5 min at $95 \text{ }^\circ\text{C}$, 35 cycles of 30 sec at $94 \text{ }^\circ\text{C}$, 30 sec at $55 \text{ }^\circ\text{C}$ and 1.3 min at $72 \text{ }^\circ\text{C}$, followed by 10 min at $72 \text{ }^\circ\text{C}$. For the V4

region, the parameters were 3 min at $94 \text{ }^\circ\text{C}$, 30 cycles of 30 sec at $94 \text{ }^\circ\text{C}$, 45 sec at $57 \text{ }^\circ\text{C}$ and 1 min at $72 \text{ }^\circ\text{C}$, followed by 2 min at $72 \text{ }^\circ\text{C}$. Reactions were carried out by using a Verity Thermocycler (Applied Biosystems, Waltham, MA, USA). In order to confirm the PCR products, a 2% agarose gel was used and the specific bands were excised and purified with the Nucleospin Gel and the PCR clean-up kit (Macherey-Nagel, Berlin, Germany). The concentration of the PCR amplicons was analysed by electrophoresis on a Bioanalyser (Agilent Technologies). Equimolar pools of each fragment and sample were combined to obtain a multiplexed pool.

5.4.3. Sequenced-based microbiome analysis and Statistics

The library pool was diluted to a DNA concentration of 60 pM prior to clonal amplification. The Ion 520 & Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, USA) was employed for template preparation and sequencing according to the manufacturer's instructions. Prepared samples were loaded on to a 530 chip and sequenced using the Ion S5 system of the Ion Torrent Platform (Life Technologies). Once sequencing was achieved, Ion Torrent Suit software removed low quality and polyclonal sequences and those readings were then analysed using QIIME (v1.9.1),

selecting only sequences with 150 to 200 bp and omitting homopolymers greater than 6 [32–36]. QIIME was used to summarise the relative abundance of microbial clades at different taxonomic levels, generating an OTU (operational taxonomic unit) table for each taxonomy level. Afterwards, SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used with data contained in each OTU table to determine significant differences between phylum, classes, orders, families and genus between the groups of interest. To perform statistical analysis, relative abundance of microbial clades lower than 0.01% were ignored. A one-way ANOVA test with a Bonferroni correction for post hoc analysis was performed between all the groups for each taxonomy level.

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

3.2.2. Optimised extraction methods for the determination of trichothecenes in rat faeces followed by liquid chromatography-tandem mass spectrometry

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

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OPTIMISED EXTRACTION METHODS FOR THE DETERMINATION OF TRICHOHECENES IN RAT FAECES FOLLOWED BY LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY

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Abstract

The mycotoxin deoxynivalenol (DON) and some of its derivatives, such as 3-acetyl deoxynivalenol (3AcDON), 15-acetyl-deoxynivalenol (15AcDON), deoxynivalenol-3-glucoside (DON3G) and de-epoxy deoxynivalenol (DOM-1), are commonly found in food and biological samples. However, literature does not present suitable methodologies for detecting and quantifying these mycotoxins at very low levels, which would be especially useful when they are present in biological samples. The main goal of the present paper was to evaluate different extraction techniques for the determination of these mycotoxins in rat faecal samples, in order to reduce the interferences present in the matrix and be able to quantify the mycotoxins at low concentration levels. Using diverse extraction methodologies such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and pressurised liquid extraction (PLE), the clean-up strategy was optimised. QuEChERS extraction followed by a dispersive solid phase extraction (dSPE) clean-up step with activated carbon was the method with the best extraction recovery results, ranging between 78% and 83% (except for DON3G). The matrix effect values were from 32 -2% to -20% which supposed a reduction in comparison with the other tested strategies. These results enabled low quantification limits to be achieved, from 0.2 µg kg⁻¹ to 3.4 µg kg⁻¹. In view of the results, it was possible to quantify the natural presence of DON and DOM-1 from the tested faecal samples, at low concentration levels.

Keywords: *Trichothecenes; Rats; Faeces; QuEChERS, dispersive solid-phase extraction; LC-(ESI)MS/MS.*

1. Introduction

Mycotoxins are widely present in food chains of animals and humans. In view of the toxicological effects that these toxins can have, it is important to control their presence. Mycotoxins, especially trichothecenes, can be found in a wide range of feed samples, predominantly based on cereals [1], where the mycotoxins deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyl-deoxynivalenol (15AcDON) are commonly found [1, 2]. In addition, there are other DON derivatives that may be present in food samples, such as deoxynivalenol-3-glucoside (DON3G) [3], or also in biological samples, such as de-epoxy deoxynivalenol (DOM-1) [4].

However, in fact, DON, also known as vomitoxin, is the most common trichothecene found in food samples [1], and it is related to numerous toxic effects, such as food refusal, emesis and immunotoxicity, among others [5, 6]. All DON derivatives produce toxicity that is lower than or similar to than DON [7] and, for this reason, the presence of these mycotoxins has been widely monitored in biological samples, in order to better understand them in terms of exposure and metabolism. DON metabolism differs between animal species, but in spite of this, the most common biological samples tested are urine [8–10] and faecal

samples [11–13]. In the case of rats, DON is mainly present in faecal samples [14]. The main DON metabolites in rats that are DON sulfonates, are also mainly excreted by faeces in comparison to urine [12]. Moreover, it was demonstrated that the elimination of DON in form of DOM-1 in urine is less relevant than in faeces [8].

Sometimes the extraction techniques used in the analytical methods for determining mycotoxins in biological samples are complicated due to the complexity of the matrices. In spite of this, the most habitual sample treatment applied to faecal samples are solid-liquid extractions (SLE) [11, 12, 15, 16]. Nevertheless, SLE presents some drawbacks such as the huge amounts of organic solvents [10, 17], it must be repeated more than once thereby increasing the extraction time [11, 13], and the use of high dilution factors [8] which requires more sensitive equipment. Moreover, a low quantity of sample, such as 100 mg, is often used to avoid interferences but as a result, higher limits of detection and quantification can be achieved in comparison with those that can be obtained with higher amounts, for example with 1 g of sample.

Subsequently, the main objective of the present study was to test different extraction techniques in order to obtain the best extraction results possible for

the mycotoxins DON, 3AcDON, 15AcDON, DON3G and DOM-1 from rat faecal samples. Performance of various extraction methods was evaluated, including SLE, pressurised liquid extraction (PLE), either using organic solvents or water as the extraction solvent, and Quick, Easy, Cheap, Effective, Rugged and Safe method (QuEChERS), as well as some clean-up procedures, such as solid-phase extraction (SPE), dispersive SPE (dSPE) or in-cell clean-up for PLE, as these procedures are commonly found in the literature for the extraction of mycotoxins from food and biological samples [12, 13, 18–24]. Often, in the studies in the literature suitable recovery results were achieved but, at the same time, the high level of interferences present promoted the matrix effect, which causes diminution in method detection and quantification limits and/or inaccurate quantification. For instance, some of the methods detailed above quantified DON and DOM-1 between $3 \mu\text{g kg}^{-1}$ and $202 \mu\text{g kg}^{-1}$, and between $3 \mu\text{g kg}^{-1}$ and $476 \mu\text{g kg}^{-1}$, respectively [8, 25]. Taking into account these studies from the literature, depending on the purpose of the extraction methodology simple methods obtaining high limits would be enough. In the present research, a more efficient extraction would be necessary. The present paper focuses on exploring extraction and clean-up techniques in order to obtain the

highest pre-concentration factors, as well as cleaner extracts, thus obtaining lower detection and quantification limits.

2. Materials and Methods

2.1. Chemical reagents and standard solutions

Methanol (MeOH) and acetonitrile (ACN), both LC-MS grade, were purchased from J.T. Baker (Deventer, The Netherlands). Acetone for pesticide residue analysis was purchased from VWR International (Fontenay-sous-Bois, France). HPLC-grade ethanol (EtOH) absolute was supplied by Scharlab (Barcelona, Spain), and ammonium formate (NH_4HCOO) was sourced from Fluka (Buchs, Switzerland). Formic acid (HCOOH) $\geq 95\%$ was purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure-grade water was obtained by an ultrapure water purification system from Veolia Water (Sant Cugat del Vallès, Barcelona, Spain), and diatomaceous earth (DE) was sourced from Thermo Scientific (Sunnyvale, CA, USA).

QuEChERS extraction tubs (4 g magnesium sulphate, 1 g sodium chloride) and 150-mg OASIS HLB cartridges were obtained from Waters (Wexford, Ireland). For dSPE, the tested sorbents were: activated carbon of $150 \mu\text{m}$ purchased from J.T. Baker, C₁₈

sorbent of 40-60 μm from Scharlab (Barcelona, Spain), silica gel of 40-63 μm from Merck (Darmstadt, Germany), aluminium oxide $\geq 98\%$ of 45 μm and Florisil® PR of 149-250 μm both from Sigma - Aldrich, activated coconut charcoal of 420-841 μm and Supel™ QuE Z-Sep⁺ Tubes of 500 mg and 50 μm , both from Supelco (Sigma-Aldrich).

All mycotoxin standards were supplied individually by Bioser (Barcelona, Spain). Standard solutions of DOM-1 and DON3G were in acetonitrile (ACN) solution at 50 mg L⁻¹ and at 50.2 mg L⁻¹, respectively. DON, 3AcDON and 15AcDON were obtained in powder form and were dissolved in water/EtOH (80:20, v/v), at 1.8·10³ mg L⁻¹ in the case of DON, and at 104 mg L⁻¹ in the case of the acetylated forms. A mix stock solution of all mycotoxins was prepared at 2 mg L⁻¹ for all compounds in water/MeOH (80:20, v/v), and it was stored at -20 °C for six months.

2.2. Sample preparation

Faecal rat samples were obtained from rats treated with DON, from a previous study [26]. For the method validation, the faecal samples used were from rats without DON treatment. For the determination of mycotoxins and modified mycotoxins, the samples used were from different rats fed with DON. Samples for the

validation and for the determination of mycotoxins were from different individuals in order to obtain more representative results, taking into account that the composition of faecal matrix may be very variable between rats. After individual collection, the samples were immediately preserved in a freezer, and later lyophilised (miVac SpeedTrap™ Genevac, Ipswich, UK) and grounded and homogenised with the Moulinex mill (Barcelona, Spain). For spiked samples, 1 g of each homogenous faecal sample was weighed into a 100 mL beaker and 2 mL of acetone was added in order to spike the matrix homogeneously. Then, samples were spiked with the mixed stock solution, with final concentrations of 10 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ for all the target mycotoxins. The suspension was left overnight to allow the complete evaporation of the acetone and to enable the mycotoxins to come into contact with the matrix.

2.3. Extraction procedure

The different extraction methods tested in the present research were SLE, PLE with two different extraction solvents, and QuEChERS. These methods were accurately examined with the application, in some cases, of several clean-up steps, such as dSPE and in-cell clean-up. All the details for the development of these methods are explained in the following sections.

2.3.1. Solid-liquid extraction

A portion of 1 g of freeze-dried and homogenised faecal sample was mixed with 10 mL of MeOH, sonicated for 15 min and centrifuged at 15,000 rpm for 10 min. An aliquot of 7 mL of supernatant was evaporated to dryness under nitrogen flow, re-suspended in 2 mL of MeOH/water (20:80, v/v), filtered with a 0.45 µm nylon filter (Membrane Solutions, Kent, WA, USA) and stored at 4 °C until analysis.

2.3.2. Pressurised liquid extraction

PLE was carried out on a Dionex ASE 350 accelerated solvent extraction system (Dionex Corp., Sunnyvale, CA, USA) with stainless steel extraction cells of 11 mL. Firstly, a cellulose filter was placed at the bottom of the extraction cell, in accordance with the manufacturer's recommendations, followed by a DE layer of about 0.3 g. Then, 1 g of faecal sample and 1 g of DE were mixed and poured into the extraction cell, followed by another DE layer on the top. The PLE conditions were as tested previously by the authors [20]: temperature at 80 °C, a single extraction cycle of 5 min, 5 min of cell preheating, 1,500 psi extraction pressure, flush volume of 50% and a purge time of 60 s.

The two tested extraction solvents were MeOH and ultrapure water with

1% HCOOH. When using MeOH as the extraction solvent, different clean-up steps were tested. Firstly, an on-cell clean-up was examined before the MeOH extraction, but using hexane as the extraction solvent, applying the same PLE conditions as previously described. The obtained extracts were discarded and subsequently, an extraction with MeOH was performed. The obtained extract (around 15 mL) was evaporated to dryness through a miVac vacuum concentrator (Genevac). Extracts were re-suspended with 2 mL of MeOH/water (20:80, v/v), filtered with a 0.45 µm nylon filter and stored. Different in-cell clean-up steps were also tested using silica gel, C₁₈, Florisil® PR and aluminium oxide as sorbents and MeOH as the extraction solvent. A portion of 1 g of each sorbent was added either to the mixture of the DE and the sample or separately as a layer. Similarly, the DE layer and the cellulose filter were added to the bottom of the extraction cells.

In the case of the extraction using ultrapure water with 1% HCOOH as the extraction solvent, the obtained extract (around 15 mL) was passed through an OASIS HLB cartridge, previously conditioned with 10 mL of MeOH and 10 mL of the PLE extraction solvent. Then, elution was carried out with 5 mL of MeOH and the samples were evaporated to dryness, re-suspended, filtered and stored.

2.3.3. Quick, easy, cheap, effective, rugged and safe (QuEChERS)

For the QuEChERS extraction, 1 g of dried and homogenised faecal sample was weighed into a 50 mL centrifuge tub and blended with 10 mL of ultrapure water and 10 mL of ACN, and the tube was shaken vigorously for 3 min. Then, the sample was mixed with the content of the extraction salt tube and was shaken vigorously for 3 more minutes, and was centrifuged at 9,000 rpm at 20 °C for 10 min. After this, 9 mL of supernatant, which belongs to the acetonitrile layer, was evaporated to dryness under nitrogen flow and re-suspended in 2 mL of MeOH/water (20:80, v/v). The extract was filtered with a 0.45 µm nylon filter and stored at 4 °C before injection.

Then, different clean-up sorbents for the dSPE were tested: silica gel, C₁₈, Florisil® PR, activated coconut charcoal, activated carbon and Supel™ QuE Z-Sep⁺. The 9 mL of supernatant was transferred to a 15 mL centrifuge tub containing 200 mg of a dispersive sorbent, instead of evaporate it. This mixture was vortexed for 1 min and centrifuged at 9,000 rpm at 20 °C for 10 min. Finally, 8 mL of supernatant was evaporated and treated as above.

2.4. Chromatographic analysis

The LC-(ESI)MS/MS analyses were performed on an Agilent 1290 Infinity LC Series (Agilent Technologies, Waldbronn, Germany) coupled with a 6495 iFunnel Triple Quadrupole MS/MS with electrospray ionisation (ESI) interface, also from Agilent Technologies. The target mycotoxins were separated using a Cortecs HPLC C₁₈ column (100 mm x 2.1 mm, 1.6 µm) from Waters (Wexford, Ireland) at 40 °C, and through a gradient elution from a binary mobile phase. This was constituted of water (eluent A) and MeOH (eluent B), both with 0.1% HCOOH and 5 mM NH₄HCOO. The main chromatographic and source parameters to optimise were the same as the authors' previous research [17], as the target mycotoxins were the same. Accordingly, the flow rate was set at 0.45 mL min⁻¹ and the injection volume was 10 µL. The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity and, in accordance with the European Commission guidelines [23], three characteristic MRM transitions were monitored for each mycotoxin, with their most suitable collision energies. All these parameters are detailed in Table 1.

Table 1. Instrumental parameters for trichothecene determination and quantification.

Mycotoxin	Retention Time (min)	Precursor ion (m/z)	Dwell time (ms)	Product ion (m/z) and q/Q ratio	CE (eV)
DON	2.9	297.1	[DON + H] ⁺	249.1 (100)	8
				230.9 (65)	10
				202.8 (60)	8
DON3G	3.5	297.1	[DON3G - 3G + H] ⁺	249.1 (100)	8
				230.9 (65)	10
				202.8 (60)	8
DOM-1	5.7	281.2	[DOM1 + H] ⁺	233.2 (100)	8
				109.1 (95)	14
				215.1 (80)	10
3AcDON	9.7	339.2	[3AcDON + H] ⁺	231.1 (100)	8
				202.9 (40)	24
				175.0 (25)	18
15AcDON	10.0	356.2	[15AcDON + NH ₄] ⁺	339.1 (100)	16
				321.1 (40)	12
				137.0 (35)	4

3. Results and discussion

3.1. Instrumental optimisation

As a result of the optimised instrumental method, all the target mycotoxins were precisely identified and separated, with the exception of the acetylated compounds. The mycotoxins 3AcDON and 15AcDON were not completely separated, but they could be identified and quantified separately due to the differences in their molecular and fragment ions. The rest of the compounds were separated in the chromatographic run time of 18 minutes, with suitable peak shapes.

With all the optimised parameters, instrumental linearity (with $r^2 \geq 0.997$) and limits of detection (LOD) and quantification (LOQ) were determined. LOD and LOQ were calculated assuming the criteria of a signal-to-noise ratio (S/N) equivalent to 3 and 10, respectively. The LODs obtained were 0.005 $\mu\text{g L}^{-1}$ for DOM⁻¹, 0.01 $\mu\text{g L}^{-1}$ for DON, 3AcDON and 15AcDON, and 0.1 $\mu\text{g L}^{-1}$ for DON3G. Meanwhile, the LOQs obtained were 0.02 $\mu\text{g L}^{-1}$ for DOM-1, 0.05 $\mu\text{g L}^{-1}$ for DON, 3AcDON and 15AcDON, and 0.5 $\mu\text{g L}^{-1}$ for DON3G. The linear range went from LOQ as the lowest concentration to 300 $\mu\text{g L}^{-1}$ for all compounds.

3.2. Extraction method optimisation

In order to evaluate and compare the different extraction methods, the percentages of extraction recovery (%ER) and matrix effects (%ME) were considered and calculated according to the following equations. To obtain the %ER values, the analyte concentration from the sample spiked before the extraction was compared with that obtained when spiked after the extraction. To calculate the %ME values, the analyte concentration from the sample spiked after the extraction was compared with the concentration of the standard, and interpreted as ME<0 (ion suppression), ME=0 (no ME) and ME>0 (ion enhancement).

$$(1) \%ER = \frac{(C \text{ before} - C \text{ non-spiked})}{(C \text{ after} - C \text{ non-spiked})} \times 100$$

$$(2) \%ME = \frac{[(C \text{ after} - C \text{ non-spiked}) / (C \text{ standard})] \times 100}{100}$$

For both values, the analyte concentration present in non-spiked samples from no treated rats was taken into account. To do so, the faecal samples used to optimise the method were first analysed to determine the presence of any target mycotoxin. The results showed peaks of DON in the chromatogram and so its peak area average was subtracted from the corresponding peak areas of the spiked samples.

Then, the target mycotoxins were added to fortify 1 g of faecal samples at two different concentrations to calculate the ER and ME values. Spiking concentrations were 10 $\mu\text{g kg}^{-1}$ and 100 $\mu\text{g kg}^{-1}$ for all the target compounds. The obtained results were similar at both tested concentrations and so only values obtained at the lower concentrations are detailed in this paper, since these values were closer to those expected to be found in real samples.

Regardless of the extraction methodology, samples were filtered previously to their injection to the LC-MS/MS. The possible losses of the compounds were evaluated but it was not taken into account since the difference was not greater than 10%.

3.2.1. Solid liquid extraction

Since the objective of the present research was to evaluate and improve mycotoxin extraction from faecal samples, the SLE method from the authors' previous research [26] was used as the initial method with one modification: a portion of 1 g of sample was used instead of 100 mg. Suitable ER results were obtained but, at the same time, there was a high level of interferences present, with %ME in form of suppression of around -60% in most cases. To reduce the ME one strategy is the use of isotopically

Table 2. Percentages of extraction recoveries (%ER) and matrix effects (%ME) from the different tested methodologies.

	Solid liquid extraction		PLE		PLE (MeOH) + silica layer		PLE hexane + PLE MeOH		PLE water + OASIS HLB		QuEChERS ACN 100%		QuEChERS 1% HCOOH	
	%Ext Rec	%ME	%Ext Rec	%ME	%Ext Rec	%ME	%Ext Rec	%ME	%Ext Rec	%ME	%Ext Rec	%ME	%Ext Rec	%ME
DON	57	-77	81	-54	79	-57	62	-58	60	-25	137	-47	136	-49
DON3G	54	-65	76	-22	77	-12	67	-34	58	-20	68	-38	86	-45
DOM-1	72	-68	78	-38	75	-38	65	-40	57	-9	137	-47	138	-46
3AcDON	73	-50	69	-44	70	-41	61	-58	23	-47	94	-47	90	-57
15AcDON	75	-60	69	-63	70	-60	64	-65	25	-51	97	-55	94	-70

n=3
 RSD ≤ 15 (if %ER > 50%)

labelled standards for each compound, which supposes an elevated cost. Other extraction methods were assayed before affording this cost. The results are detailed in Table 2 and improving them was the main purpose of this research.

3.2.2. Pressurised liquid extraction

In order to improve the SLE results, PLE using MeOH as the extraction solvent was then tested, in view of the high extraction capacity of MeOH. The results in terms of %ER and %ME improved considerably, especially with regard to DON, DON3G and DOM-1. However, the %ME still remained very high and an extraction method using a more selective extraction solvent was tested. For this purpose, acidified water was selected as the extraction solvent, as it was expected to prevent the extraction of interferences. In this case, the same PLE conditions described above were used, using water with 1% HCOOH as the extraction solvent instead of MeOH. The fact that water is used in the PLE enables the subsequent selective cleaning of the obtained extracts using SPE, without requiring a solvent exchange. Therefore, in all the PLE experiments using water, a clean-up with OASIS HLB was also used, conditioned and eluted as detailed in Section 2.2.2., as optimised previously [20]. The results detailed in Table 2 exhibited a reduction in the %ME in the

case of DON, DON3G and DOM-1 as compared to the values obtained with SLE, but there was no difference in the case of the acetylated forms, which, at the same time, underwent a significant reduction in terms of their %ER. Taking all these results into account, the PLE method with acidified water as the extraction solvent was discarded.

The next step, using MeOH as solvent in PLE, was to test whether various clean-up methods were useful in terms of reducing the ME, but maintaining the high ER values obtained when using MeOH as the extraction solvent. To the best of our knowledge, in-cell clean-up sorbents have not been used to date in the extraction of DON and its derivatives, but have been used for fumonisins [24] and other food contaminants [25]. Silica gel, C₁₈, Florisil® PR and aluminium oxide were the four sorbents examined, of which the first three have previously been used as cleaning sorbents in dSPE for mycotoxin extraction [23, 24]. A portion of 1 g of each sorbent was located, mixed with the DE and the matrix and performing the PLE using the same conditions as above. The obtained percentage values of ER and ME showed no significant differences between the tested sorbents. The following step was to check whether locating the sorbents as a layer at the bottom of the extraction cell had any influence. Once again,

there was no difference between applying the clean-up step or not, and there was no difference between the tested sorbents. For this reason, only the results obtained from the extraction using silica in the layer mode are shown in Table 2. In the case of the other sorbents, which are not included in Table 2, %ER ranged from 59% to 70% (except for DON3G), and %ME ranged from -22% to -55%. Therefore, the in-cell clean-up was ruled out.

Another clean-up step was tested with an on-cell clean-up before the extraction. The PLE conditions were the same as the in the case of the extraction with MeOH but using hexane. This apolar solvent might remove the possible presence of lipids in the tested samples, as it is used in the literature [26]. The hexane extraction was followed by an extraction with MeOH as the extraction solvent, again under the same PLE conditions. At the end, the on-cell clean-up with hexane had no effect on the %ME, but also resulted in a reduction in the ER of about 10%, as can be observed in Table 2. For these reasons, this strategy was also discarded.

3.2.3. QuEChERS

As the results obtained with PLE were not promising, the next step was the selection of another extraction

method. The original QuEChERS extraction method [30] was tested with some variations. To do so, two extraction buffers were tested: ACN or ACN acidified with 1% of HCOOH. The obtained results (Table 2) from both assessments showed an increase in the %ER of all mycotoxins in comparison with the results obtained from all the previously examined extraction methods. An unexpected result were the high values of %ER obtained with respect to DON and DOM-1 in both cases, probably due to the presence of interferences. However, as a result of this extraction, there was a reduction in %ME in comparison with those obtained from the SLE in both cases, with HCOOH and without it, as shown in Table 2. As can be also observed in Table 2, there were practically no differences between both tested buffers. Thus, taking into account all the obtained results, the QuEChERS method with only ACN as the extraction buffer was selected. Moreover, in order to further reduce the %ME, different dSPE sorbents were tested such as silica gel, C₁₈, Florisil® PR, activated coconut charcoal, activated carbon and Supel™ QuE Z-Sep⁺. As suggested in the manufacturer's recommendations, 500 mg of the Supel™ QuE Z-Sep⁺ sorbent was used, while 200 mg was weighed in the rest of the cases. As shown in Table 3, the results obtained from all the tested sorbents, clearly showed a reduction in the %ME in comparison

Table 3. Percentages of extraction recoveries (%ER) and matrix effects (%ME) from the different sorbents tested as a clean-up step after QuEChERS extraction using ACN as solvent.

	Silica		C ₁₈		Florisil®		Activated coconut charcoal		Active carbon		Z-Sep+	
	%ER	%ME	%ER	%ME	%ER	%ME	%ER	%ME	%ER	%ME	%ER	%ME
DON	86	-28	79	-34	84	-22	73	-20	83	-18	74	-18
DON3G	60	-13	64	-17	49	-8	52	-4	43	-2	35	0
DOM-1	86	-13	82	-19	82	-9	76	-8	78	-8	71	-6
3AcDON	84	-34	86	-37	81	-27	81	-35	82	-20	78	-25
15AcDON	87	-44	85	-46	82	-35	81	-40	82	-18	80	-32

with those obtained without any clean-up step (Table 2). More concretely, Silica, C₁₈, Florisil® and activated coconut charcoal reduced considerably the %ME, especially for DON, DON3G and DOM-1. In the case of active carbon and Z-Sep⁺, the %ME reduced by more than half, compared to the QuEChERS extraction without clean-up. These results were in accordance with the supernatant colouring form of the dSPE when transferred to the evaporation tubes. The colour of the supernatants from both carbon sorbents was transparent while the other supernatants had a yellowish colouring and accordingly, the two carbons, together with Z-Sep⁺, obtained the lowest percentage of ME. With respect to %ER, all the results obtained from all the tested dispersive solvents were higher than those obtained with the SLE method, with the exception of DON3G results. From the great majority of the tested extractions, the %ER values of DON3G were low in comparison with those from the rest of mycotoxins, especially when using QuEChERS. On the other hand, the obtained values of %ME obtained from the QuEChERS extraction with the subsequent clean-up are under -20%, concluding that the matrix effect in form of signal suppression might be accepted.

As a consequence, the QuEChERS method followed by a dSPE was selected for the extraction of the target trichothecenes from faecal samples. From all the tested sorbents and taking into account a compromise between the percentages of ER and ME, the activated carbon and Z-Sep⁺ were the sorbents with better results. Finally, the activated carbon was selected because provides the best %ME results in spite of using less amount of sorbent, because of its extraction simplicity and due the higher value of %ER of the analyte DON3G.

3.3. Method validation

Once the extraction method had been optimised, it was also validated. Parameters such as method detection limits (MDL), method quantification limits (MQL), linear range, accuracy, and intra-day and inter-day repeatability were evaluated for the target mycotoxins using 1 g of faecal samples, and following the method described above. The faecal samples from no treated rats used to validate the method, were also first analysed to determine the natural presence of any target mycotoxins, and were considered by subtracting the signal from contaminated samples.

The linear range was then evaluated by matrix-matched calibration by spiking faecal samples at in triplicate. Six concentration levels were used for all compounds, which were within the +/- 20% as required by the EC guidelines [31]. The linear range was between the lowest concentration, which was the MQL of each mycotoxin, and the maximum concentration that was 300 $\mu\text{g kg}^{-1}$ for all compounds. The resultant linearity was suitable, with correlation coefficients ≥ 0.994 .

MDL and MQL were estimated in the same way as the instrumental limits, detailed in Section 2.3. The MQLs obtained were 0.2 $\mu\text{g kg}^{-1}$ for DOM-1, 0.5 $\mu\text{g kg}^{-1}$ for DON, 3AcDON and 15AcDON, and 5 $\mu\text{g kg}^{-1}$ for DON3G, while the obtained MDLs were 0.05 $\mu\text{g kg}^{-1}$ for DOM-1, 0.1 $\mu\text{g kg}^{-1}$ for DON, 3AcDON and 15AcDON, and 1 $\mu\text{g kg}^{-1}$ for DON3G. These limits were lower than some limits found in the literature. For instance, MDL found by Saint-Cyr *et al.* [25] for DON and DOM-1 were 3 $\mu\text{g kg}^{-1}$, which are limits between 30 and 60 times higher than those found with the present method.

The precision was evaluated using the repeatability (intra-day precision, five replicated samples measured during the same day, $n=5$) and the reproducibility (inter-day precision, five

replicated samples analysed for three consecutive days, $n=15$), expressed as % relative standard deviation (%RSD). The method repeatability and reproducibility were tested at two different concentration levels: at 5 $\mu\text{g kg}^{-1}$ and 50 $\mu\text{g kg}^{-1}$, which correspond to 10 and 100 times, respectively, the calculated MQLs. At both tested concentrations, the obtained results were between 5 and 8% for the repeatability, and between 13 and 19% for the reproducibility.

The obtained limits for the developed method are part of the goal of this research, as well as improving the values of the %ER and %ME, as they were considerably reduced in comparison to the previous method, which used SLE as the extraction technique [26].

3.4. Analysis of samples

As soon as the method was successfully validated and to evaluate its applicability, the natural presence of mycotoxins was studied using the faecal samples from rats treated with DON from the previous research [26]. A pool of different individual faecal samples from treated rats was analysed. Three samples from the pool were analysed using the optimised method. The target mycotoxins were determined and quantified using the

matrix-matched calibration curve, as faecal samples of an equal composition were considered.

With the current method, it was possible to quantify DON and DOM-1, at the levels of $235 \mu\text{g kg}^{-1}$ and $55 \mu\text{g kg}^{-1}$, respectively. However, the mycotoxin DON3G was not detected in any of the analysed samples, similar to 3AcDON and 15AcDON. Figure 1 shows the quantitative and one qualitative MRM chromatograms for DON and DOM-1 found in one of the analysed

faecal samples from the pool of rats treated with DON.

From all the spiked mycotoxins, the natural presence of acetylated mycotoxins was not expected in the analysed faecal samples, since the main acetylated forms produced by fungi are hydrolysed once they are consumed. However, it is known that there are other modified mycotoxins which can appear to the faecal samples like sulfonates, but there are no commercial standards available for

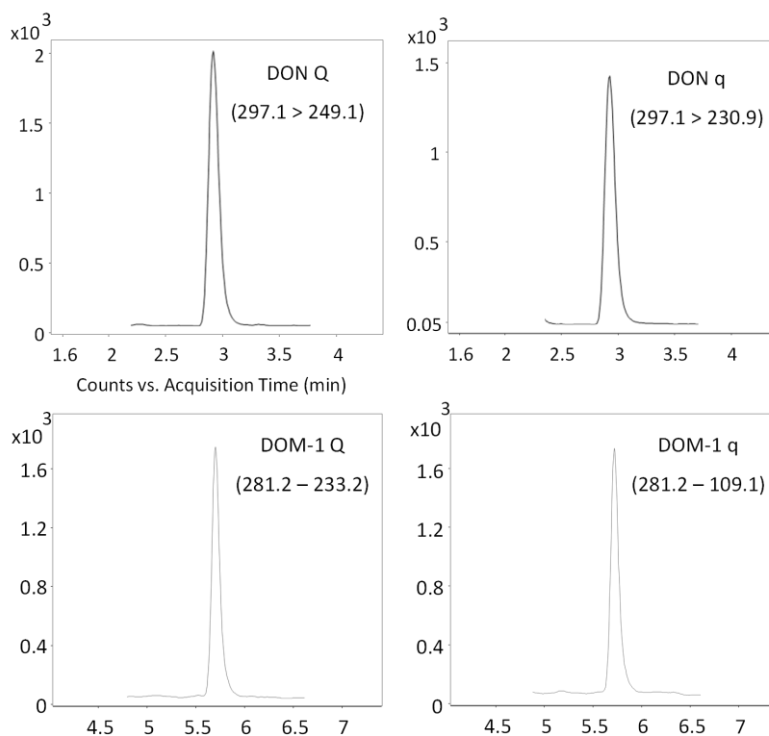


Fig. 1 Quantitative and qualitative MRM chromatograms for DON and DOM-1 of one non spiked faecal sample, where the analytes were quantified at the levels of $235 \mu\text{g kg}^{-1}$ and $55 \mu\text{g kg}^{-1}$, respectively.

them. On the contrary, there are standards commercially available for acetylated DON derivatives. Taking into account the obtained results for the acetylated mycotoxins and the nature of the other derivatives, the methodology can be slightly modified. For instance, it is known that the presence of acid improves the DON sulfonates extraction. Thus, the addition of acid to the extraction solvent can be considered for these compounds. Summing-up with the present developed methodology, the high ER values together with the low ME values make that is a good option when determining mycotoxins from faecal samples.

Thus the reduction of the method limits enabled the possibility of quantifying mycotoxins at levels that were not possible with the previous simple method [26]. This fact allows the possibility to apply the developed methodology for the determination of further modified mycotoxins.

4. Conclusions

From all the tested approaches, the highest ER values and the lowest matrix effect were obtained with the QuEChERS method with the subsequent clean-up using activated carbon in dSPE. And, the developed method can be successfully applied for the routine determination of

mycotoxins in rat faecal samples due the suitable results achieved, with the possibility of detection and quantification at very low concentrations.

Even though faecal matrices are complex samples, the low percentage of ME indicates the reliability of the clean-up step performed in the presented method that effectively reduces the presence of interferences and, thus, the ME, which allows the quantification of the natural presence of the mycotoxins DON and DOM-1 in the tested faecal rat samples at low concentration levels.

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

3.2.3. Discussion of results

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

After mycotoxins consumption, there are two main consequences that can be derived: biological modifications due toxicological effects or production of modified mycotoxins. Considering these effects, the possible gut microbiota alterations were studied in the first study of this second section. Since efficient extraction methodologies are needed for the determination of mycotoxins in biological excretion samples, the optimisation of the extraction methods was evaluated and presented in the second article of this section.

The main objective of the first paper was to examine if low concentration levels can produce bacterial modifications. For that, DON was administered to Wistar rats during almost two months at two different concentration levels: $60 \mu\text{g kg}^{-1}$ bw and $120 \mu\text{g kg}^{-1}$ bw. After the gut biodiversity exploration a difference between the relative abundance of Coprococcus genus was found between rats treated with DON at $120 \mu\text{g kg}^{-1}$ bw and rats without treatment. No other alterations were showed meaning that low concentration levels, such as $60 \mu\text{g kg}^{-1}$ bw of the DON mycotoxin, have no significant effects on rats gut microbiota.

However, our results are not correlated with results found by Saint-Cyr *et al.* [1] that also have evaluated the exposure of low concentration dose of DON in rats. In their research, rats were initially germ free and were inoculated with human faecal flora. These rats were treated with DON during 4 weeks with $100 \mu\text{g kg}^{-1}$ bw. Their results showed significant fluctuations of microbiota groups after DON consumption, different than Coprococcus, such as *Bacteroides / Prevotella* group and *Escherichia coli*. Taking into account the results presented in this doctoral thesis and results presented by Saint-Cyr *et al.*, it is demonstrated that DON at low concentration levels could induce gut microbiota alterations. However, the research in this field is still in its initial phase, and more studies are needed to conclude which bacteria is involved on DON's detoxification process. The achievement of the bacteria responsible of this detoxification will move forward on the probably understanding of the biological detoxification processes and the formation of modified mycotoxins.

Furthermore, during this research, a monitoring of the presence of DON and DOM-1 was achieved. On one hand, the concentration levels of DON quantified daily during the 7 weeks of study did not showed significant differences, concluding that the excretion of DON did not vary during the treatment. On the other hand, the excretion of DOM-1 varied, especially during the last 2-3 weeks. One explanation for this fact could be the detoxification capability acquired by rats along the treatment. However, there are poor evidences to correlate this evolution of the excretion of DOM-1 and the alteration of the Coprococcus genus, previously described. For this reason and as detailed before, more

research regarding the metabolism of mycotoxins such as DON is needed, especially considering the results obtained in our research.

For the determination of the presence of DON and DOM-1 in the rats' faecal samples on a daily basis, a simple extraction technique was developed. However, some authors indicate that modified mycotoxins can be partially lost when analysing if the extraction procedure is not the appropriated [2]. Therefore, our research was followed by improving the extraction method used previously when monitoring DON and DOM-1 in rat faecal samples. For that, several extraction techniques were tested, with the application of some clean-up procedures. Some of the extraction techniques tested presented complications due the complexity of the faecal matrices. For instance, the first extraction strategy applied was PLE using MeOH as the extraction solvent. The combination of high temperature, high pressure and the use of the organic solvent produced the extraction of numerous matrix interferences. Furthermore, the following filtration presented the difficulty that the filter was easily saturated, interfering in the appropriate filtration. Taking into account these difficulties in the extraction procedure, different clean-up steps were applied, without obtaining significant differences.

When QuEChERS extraction was finally selected due to the better recovery results obtained, the addition of a final clean-up step was also considered. Six different sorbents were tested, with the final selection of the activated carbon clean-up sorbent as dSPE. The extracts obtained after the extraction and the clean-up with three of the six tested sorbents are presented in Figure 1, in duplicate.

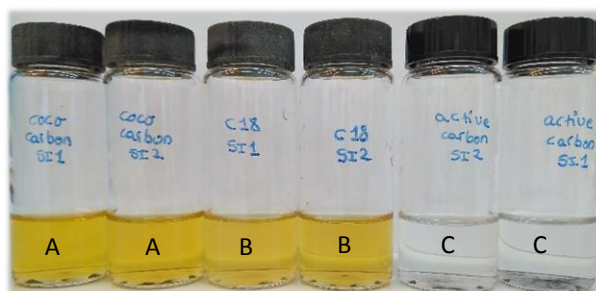


Figure 1. Extracts obtained from three different clean-up sorbents tested after QuEChERS extraction. A: coconut carbon, B: C₁₈, C: activated carbon.

The first two vials are after the clean-up with coco carbon, the two following vials are after C₁₈ sorbent and the last two vials are after the dispersive SPE with activated carbon. As can be observed, the sorbents coco carbon and C₁₈ conserve the yellowship coloration, while activated carbon is able to obtain completely transparent extracts. Thus, activated carbon was the sorbent that visually eliminated more interferences. Extraction recovery and matrix effect results are according to this fact, since activated carbon was the sorbent able to obtain higher recovery results and lower presence of interferences. The final results obtained for all the validated trichothecenes open new insights to the extraction of other modified trichothecenes easily found in faecal samples, mostly at low concentration levels, described in the following third section of the present doctoral thesis.

Therefore, with the final optimised extraction strategy it would be interesting to repeat the extraction of the rat faecal samples collected during the treatment with the DON. Considering the possible presence of other modified mycotoxins than those selected for this research, as it is considered in the following third section, this proposed investigation would be useful for the better understanding of mycotoxins metabolism.

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

3.3. Preliminary research in metabolism of nivalenol and nivalenol-3-glucoside in rats

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ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

During the development of the present doctoral thesis, our research group has been progressively introduced in the area of mycotoxins. At the beginning, we started testing and optimising different extraction strategies with different kinds of mycotoxins and matrices. Then, the research continued investigating modified mycotoxins, either with the optimisation of extraction strategies or with the investigation of their metabolism. The synthesis and the determination of non-targeted compounds such as mycotoxin metabolites, was an unknown field for our research group. To learn about mycotoxin metabolites, a research stay was accomplished at Christian Doppler Laboratory for Mycotoxin Metabolism, in IFA-Tulln, BOKU under the supervision of Dr. Franz Berthiller and Dr. Heidi Schwartz-Zimmermann. This research group has largely investigated mycotoxins and mycotoxin metabolism by plants, microbes and animals. Therefore, the research stay was very fruitful because of working with non-targeted compounds and because their experience enriched the knowledge of this thesis regarding non-targeted mycotoxins.

The research developed during the stay is presented in this third section. The main objective was to investigate the absorption, distribution, metabolism and excretion of NIV and NIV3G in rats. An introduction about the state of the art, followed by the developed experimental part and the obtained results to date are presented below. This study is not presented in article form considering its preliminary state, and more experimental work is still needed to present all the results.

METABOLISM OF NIVALENOL AND NIVALENOL-3-GLUCOSIDE IN RATS

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The present study addresses a recent claim of the European Food Safety Authority [1] to determine the toxicokinetics of modified mycotoxins. Modified mycotoxins are formed in various crop plants as a detoxification mechanism. By conjugating mycotoxins with polar compounds like glucose and storing the conjugates in the vacuole, infected plants are able to - at least temporarily - inactivate mycotoxins [2]. Modified mycotoxins occur in a variety of plant based food and feed, and also nivalenol-3-glucoside (NIV3G) has been identified in wheat [3].

Currently there are no European guidance values for NIV and NIV3G in food and feed. Based on a long-term feeding study with mice [4] the Scientific Committee on Food adopted 0.7 mg NIV per kg of bw per day as the lowest dose with an observed toxic effect (= LOAEL) and set a temporary tolerable daily intake (= t-TDI) of 0.7 $\mu\text{g kg}^{-1}$ bw per day for humans. The use of a safety factor of 1000 is common practice and allows for a potentially different metabolism of toxins by animals and humans. Specifically, it was recently shown that DON is converted to a variety of metabolites in rats that are, to the best of our knowledge, not formed in humans. The novel metabolites included DON sulfonates 1, 2 and 3, DOM sulfonates 2 and 3 [5], iso-DON-3-glucuronide, DOM-3-glucuronide, and iso-DOM-3-glucuronide [6]. DON- and DOM sulfonates excreted into faeces made up almost 50% of the total DON administered [5].

Administration of DON3G to rats resulted in formation of the same metabolites plus DON3G sulfonate 2. As detailed in Figure 1, NIV is structurally similar to DON equal than NIV3G and DON3G, formation of NIV sulfonates, NIV3G sulfonates, NIV glucuronides, deepoxy-NIV (DNIV), DNIV sulfonates and DNIV glucuronides is likely.

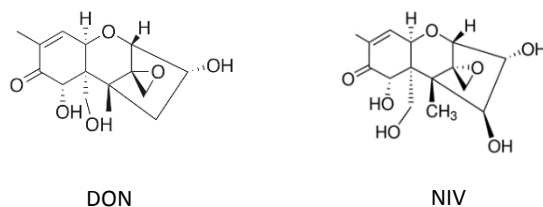


Figure 1. Chemical structures of the mycotoxins DON and DOM-1.

The risk of ingesting food or feed containing modified mycotoxins is that mycotoxin conjugates could be hydrolysed back to the toxic parent mycotoxins in the digestive tract, as has been shown for DON3G [7,8] and zearalenone-14- and -16-glucoside [9]. Likewise, NIV3G might be cleaved to NIV in the gastro-intestinal tract. As a consequence, the released NIV could be absorbed, thus increasing the total toxin burden of an individual. Indeed, an *in vitro* study was recently published showing the partial cleavage of NIV3G after incubation with human faeces [10]. This study reinforces the importance of an *in vivo* study to investigate the fate and toxic effects of NIV3G in animals.

The overall aim of this study was to investigate the absorption, distribution, metabolism and excretion (ADME) of NIV and NIV3G in rats for the first time. The first step was to administer NIV and NIV3G to rats and to collect urine and faeces samples. The second step was to screen urine and faeces samples for the expected sulfonate and glucuronide compounds. As a third step, the formed metabolites as reference standards should be produced for identification and quantification. Subsequently, analytical methods for the quantitative determination of NIV, NIV3G and their metabolites in rat faeces and urine should be developed and validated. Overall, the results of this study will extend the current knowledge about the *in vivo* metabolism of NIV and NIV3G, thus contributing significantly to the further risk assessment of these compounds.

The chemicals and standards used for the development of the research were MeOH and ACN (both LC gradient grade), purchased from VWR International GmbH (Vienna, Austria). Glacial acetic acid (LC-MS grade), formic acid and EtOH were obtained from Sigma-Aldrich (Vienna, Austria), Merck KGaA (Darmstadt, Germany) and from Carl Roth GmbH and Co. KG (Karlsruhe, Germany), respectively. Water was purified with an ultrapure water system (Sartorius arium pro, Göttingen, Germany).

Solid NIV was purchased from Romer Labs GmbH (Tulln, Austria) and NIV3G was produced as described in [11]. Toxin solutions for the animal trial were prepared in water and contained 70 mg L⁻¹ of NIV and 106 mg L⁻¹ of NIV3GAc. Stock solutions of NIV (1000 mg L⁻¹) and NIV3G (5340 mg L⁻¹) for use as reference standards and for preparation of NIV- and NIV3G metabolites were prepared by dissolving the solid compounds in water (NIV) and MeOH (NIV3G) and stored at -20 °C.

The animal experiment was approved by the institutional ethics committee and the national authority (BMFW-66.016/0002-WF/V/3b/2017, decision of 11 September 2017) according to § 26 of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 2012. Six male Hsd:Sprague Dawley rats (6 weeks old, 146.1 ± 2.3 g) were delivered by Envigo (Casatenovo, Italy). Animals were allowed to acclimatise for seven days before the start of the experiment. During the animal experiment which lasted for 31 days in total, the rats had *ad libitum* access to water and feed. The diet was analysed for its concentration of NIV and NIV3G as described in [12].

Using a 5 x 6 design, the rats (n = 6) received water (negative control), NIV (350 µg kg⁻¹ bw; positive control) and the equimolar dose of NIV3G (532 µg kg⁻¹ bw) per gavage (orogastric application using stainless steel feeding tubes (Part No. FTSS-20S-38, 20 ga (0.9 mm OD x 0.6 mm ID) x 38 mm, Instech, Solomon, Plymouth Meeting, PA USA)) on day 1, 8, 15, 22 and 29 of the experiment (see Table 1). On average, rats weighed 197 g on the first treatment day and 314 g on day 29. Hence, on average, 69 µg of NIV and 105 µg of NIV3G (0.98 mL of the respective aqueous stock solutions containing 70 mg L⁻¹ of NIV and 106 mg L⁻¹ of NIV3G) were administered on day 1, and 111 µg of NIV and 169 µg of NIV3G (1.59 mL of the individual stock solutions) were given on day 29. After each application, the rats were kept separately in metabolic cages (Tecniplast 3700M-071, floor area 320 cm², cage height 14 cm) for 48 hours, whereas they were kept in pairs in Makrolon type III cages (Fa. Ehret, PB1230) between sampling period and novel toxin application. Urine and feces were quantitatively collected for the periods 0-24 h and 24-48 h after dosing. Urine was measured volumetrically on site and stored at -20 °C until analysis. Faecal samples were stored at -20 °C until lyophilisation and weighed after

freeze-drying. The general condition of the animals was observed and registered daily during the experiment.

Table 1. Administration of water, NIV and NIV3G to six rats using a 5 x 6 design.

Day	Animal					
	1	2	3	4	5	6
1	water	-	-	NIV	NIV3G	water
8	NIV3G	water	-	-	NIV	NIV3G
15	-	NIV	NIV3G	water	-	-
22	NIV	NIV3G	water	-	-	NIV
29	-	-	NIV	NIV3G	water	-

NIV sulfonates 1, 2 and 3 were produced by incubating an aqueous stock solution containing 1000 mg L⁻¹ NIV with sodium sulfite (10% w/v, for NIVS 1 and 2) or sodium metabisulfite (15% w/v in phosphate buffer, for NIVS 3) as described for DON sulfonates 1, 2 and 3 in [13]. Similarly, NIV3G sulfonate 2 was produced by incubating an aqueous solution containing 1000 mg L⁻¹ NIV3G with sodium sulfite (10% w/v). Preparative isolation of the formed NIV- and NIV3G sulfonates was carried out on an Agilent 1100 Series preparative HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Sedex LT-ELSD Model 85LT low temperature evaporative light scattering detector (Sedere, Alfortville, France). Compounds were separated in gradient elution mode on a Gemini-NX C₁₈ column (150 mm × 21.2 mm i.d., 5 µm Phenomenex, Aschaffenburg, Germany) with a guard column of the same material at 25 °C. Mobile phase A consisted of water/HCOOH (99:1, v/v), mobile phase B of MeOH/HCOOH (99:1, v/v). Gradient elution started at 5% B for 0.5 min and continued with a linear increase to 35% B that was reached at 6.9 min. From 7.0-8.4 min, the column was flushed at 100% B. Column re-equilibration at 5% B was achieved between 8.5 and 10.5 min. The flow rate was 16 mL min⁻¹, and the injection volume was 400 µL. The column effluent was split 1:70, one part being directed into the evaporative light scattering detector (ELSD) and the main part being sent to the fraction collector. NIVS 1 was collected between 3.6 and 4.2 min, NIVS 2 between 4.4 and 4.8 min, NIVS 3 between 4.9 and 5.3 min and NIV3G sulfonate 2 was collected between 4.6 and 5 min.

DNIV was produced by incubation of 6.5 mg of NIV with the anaerobic bacterial strain BBSH 797 in 30 mL of oxygen free culture medium at 37 °C for 10 days as described earlier for production of DOM [5]. The diluted reaction mixture was analysed by LC-MS/MS as described in 2.6. In addition to DNIV, NIVS 1, 2 and DNIVS 2 were

detected. The formed DNIV and sulfonates were cleaned-up by solid phase extraction on 14 Strata C₁₈ T cartridges (200 mg, 3 mL, Phenomenex, Aschaffenburg, Germany). After conditioning with MeOH and MeOH/water/HCOOH (5:94.9:0.1, v/v/v), the supernatant of the centrifuged reaction solution was applied, the cartridges were washed with 1 mL of MeOH/water/HCOOH (5:94.9:0.1, v/v/v) and the compounds were eluted with 5 mL of MeOH. Both the washing solution and the eluate were analysed by LC-MS/MS. Subsequently, both solutions were evaporated to 4 mL and subjected to preparative chromatography using the same conditions as described for isolation of NIV sulfonates.

NIV glucuronides were produced by incubation of NIV with rat liver microsomes, UDP-glucuronic acid and several other reagents as previously described for production of DON glucuronides [6]. After incubation overnight, the reaction solutions were centrifuged, partly evaporated and cleaned up by solid phase extraction on Strata C₁₈ T cartridges (200 mg, 3 mL). After conditioning with MeOH/CH₃CHOOH (99.9:0.1, v/v) and water/CH₃CHOOH (99.9:0.1, v/v), one mL aliquots of the pooled supernatants of the reaction solution were applied, the cartridges were washed with 1 mL of water/CH₃CHOOH (99.9:0.1, v/v) and the compounds were eluted with 5 mL of MeOH. Preparative isolation of NIV-glucuronides was carried out on the same preparative HPLC system as described above. Mobile phase A was water/CH₃CHOOH (99.9:0.1, v/v), mobile phase B ACN/CH₃CHOOH (99.9:0.1, v/v). The following gradient was used: 0 min: 5% B, 0.5 min: 5% B, 10 min: 50% B, 12 min: 100% B, 14 min: 100% B, 14.1 min: 5% B, 16 min: 5% B. Fractions were collected from 2-14 min (5 fractions per minute) and analysed for NIV glucuronides by LC-MS/MS (see 2.6).

Liquid chromatography high resolution mass spectrometry (LC-HR-MS) on an X500R QTOF instrument from SCIEX (Darmstadt, Germany) was used to record product ion spectra of the novel metabolites. The structural formulas of the identified NIV metabolites are shown in Figure 2.

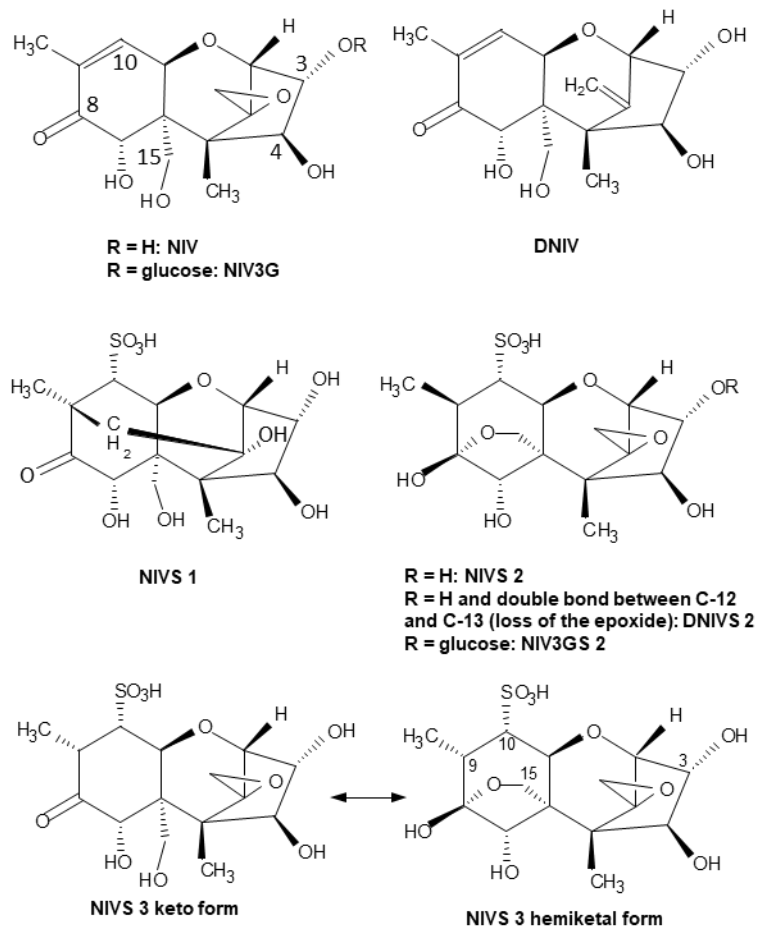


Figure 2. Chemical structures of NIV metabolites.

Urine samples were diluted 1:5000 with water and the creatinine content was determined by LC-MS/MS as described by [14]. For determination of NIV and its metabolites in rat urine, both a dilute and shoot approach and IAC clean-up were performed. In the dilute and shoot approach, urine samples were diluted to 0.5 mM creatinine with MeOH/water (50:50, v/v) and centrifuged at 14350 x g for 10 min. Prior to HPLC-MS/MS analysis, ^{13}C -labelled NIV was added as internal standard at a concentration of 30 ng mL⁻¹. For the IAC clean-up, DON/NIV WB columns (Vicam, Milford, MA, USA) were used. Urine samples containing higher creatinine

concentrations than 10 mM were diluted to 10 mM creatinine with water, urine samples with creatinine concentrations between 5 and 10 mM were diluted to 5 mM creatinine and urine samples containing between 2.5 and 5 mM creatinine were diluted to 2.5 mM creatinine. Prior to IAC, urine samples were centrifuged at 14350 x g for 10 min and 200/400/800 μ L of urine samples diluted to 10/5/2.5 mM creatinine were diluted with 3.8/3.6 and 3.2 mL 200 mM PBS buffer. After application of the diluted urine samples to DON/NIV WB columns, the columns were washed with 10 mL 200 mM PBS and 10 mL water. Finally, the analytes were eluted with 0.5 mL of MeOH and 1.5 mL of ACN, the combined eluates were evaporated and the dried residues were dissolved in 200 μ L MeOH/water (20:80, v/v).

For determination of NIV metabolites in faeces, 200 mg aliquots of freeze-dried and thoroughly homogenized feces samples were extracted three times (30/20/10 min) with 4, 3 and 3 mL of MeOH/water/HCOOH (49.5:49.5:1, v/v/v) on a GFL rotary shaker (type 3017, Burgwedel, Germany) by shaking in 15 mL polypropylene tubes (Sarstedt GmbH, Nümbrecht, Germany). Subsequently, a 0.5 mL aliquot of the pooled extracts was diluted 1+1 with water and centrifuged at 14350 x g for 10 min prior to LC-MS/MS analysis.

Analysis of urine and faecal samples was performed on a 1290 Infinity series UHPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a 6500+ QTrap mass spectrometer equipped with an IonDrive TurboV source (SCIEX, Foster City, CA, USA). Analyst software version 1.6.3 (SCIEX) was used for instrument control and data analysis. Chromatographic separation was carried out on a Kinetex EVO C₁₈ column (150 x 3 mm, 2.6 μ m, Phenomenex, Aschaffenburg, Germany). Eluent A consisted of water/HCOOH (99.9:0.1, v/v), eluent B was composed of ACN/HCOOH (99.9:0.1, v/v). After an initial period of 0.5 min at 5% B, the proportion of B was linearly increased to 13% at 5.5 min. At 6.5 min, 100% was reached which was held until 8.4 min. Afterwards, the column was re-equilibrated at 5% B for 2.5 min, resulting in a total run time of 11 min. The flow rate was 450 μ L min⁻¹, the column temperature was 30 °C and the injection volume was 3 μ L. Mass spectrometric detection was performed in negative electrospray ionisation mode and selected reaction monitoring (SRM) was applied as scan type. The source parameters were as follows: source temperature 400 °C, ion spray voltage -4500 V, curtain gas 35 psi, ion source gas 160 psi and ion source gas 240 psi. SRM transitions of compounds available as pure standards were optimised by syringe pump infusion of analyte solutions and software controlled parameter optimisation. For compounds not available as reference standard, theoretical SRM transitions were calculated. Optimised and calculated parameters are provided in Table 2.

Table 2. HPLC-MS/MS parameters achieved for the instrumental validation.

Analyte	Precursor ion (m/z)	Ion species	Product ions (quant/qual, m/z)	CE (quant/qual, eV)	Relative intensity
NIV	357.1	[M+HCO ₂] ⁻	45.0/281.1	-42/-22	0.18
13C-NIV	372.1	[M+HCO ₂] ⁻	45.0/295.1	-42/-22	0.18
DNIV	341.1	[M+HCO ₂] ⁻	45.0/265.1	-42/-22	0.32
NIVS 1	393.1	[M-H] ⁻	80.0/81.0	-98/-68	0.06
NIVS 2	393.1	[M-H] ⁻	81.0/80.0	-68/-98	0.18
NIVS 3	393.1	[M-H] ⁻	80.0/363.1	-98/-36	0.36
DNIVS 2	377.1	[M-H] ⁻	81.0/80.0	-68/-98	0.27
NIV-3-Glc	519.2	[M+HCO ₂] ⁻	263.1/443.1	-30/-28	1.05
NIV3GS 2	555.2	[M-H] ⁻	81.0/443.2	-78/-44	0.44

The LC-MS/MS based methods for determination of NIV metabolites in faeces and urine were validated with respect to apparent recovery (RA), extraction recovery (ER), mass spectrometric matrix effects (SSE), limits of detection (LODs), limits of quantification (LOQs), intra- and inter-day repeatability and linearity of calibration functions. The blank matrices required for spiking experiments were prepared by pooling individual urine and finely homogenized lyophilized faecal samples from rats treated solely with water.

For determination of RA, ER and SSE in rat faeces, 200 mg aliquots of pooled rat faeces were spiked in triplicate before extraction with 100 µL of standard mixtures containing NIV, DNIV, NIV3G, NIVS 1, 2 and 3, DNIVS 2 and NIV3GS 2 at six concentration levels between 0.18 and 54 mg L⁻¹, corresponding to 0.09 and 27 µg g⁻¹ in lyophilized faeces and to 1 to 300 ng mL⁻¹ in measurement solution at 100% RA. One hour after spiking, the spiked and two unspiked samples were worked-up as described in 2.5. In addition, pure solvent and matrix-matched calibration functions were prepared containing between 0.3 and 300 ng mL⁻¹ of all analytes. RAs, ERs and SSE were calculated by comparison of the slopes of standard addition, matrix matched and pure solvent calibration functions as outlined in [12].

Blank rat urine for validation of the dilute and shoot method was obtained by pooling equal volumes of individual blank urine samples diluted to 0.5 mM creatinine. SSE of NIV, DNIV and NIV-glucuronide in urine diluted to 0.5 mM creatinine were assessed by comparing the slopes of matrix matched and pure solvent calibration functions containing between 0.3 and 300 ng mL⁻¹ of analytes and 30 ng mL⁻¹ of 13C-NIV.

Matrix effects of the internal standard were determined by comparing the average peak areas of 13C-NIV in matrix matched and pure solvent calibration curves.

For validating the IAC clean-up procedure, three pools of blank urine containing 10, 5 and 2.5 mM creatinine were prepared. Each pool was used for establishing one standard addition curve in the range from 0.1 to 30 μg NIV and NIV-3-glucuronide (NIV-3-GlcAc) mmol^{-1} creatinine, corresponding to 1-300 ng mL^{-1} in 10 mM urine, 0.5-150 ng mL^{-1} in 5 mM urine and 0.25-75 ng mL^{-1} in 2.5 mM urine. Aliquots of 200/400/800 μL of 10/5/2.5 mM spiked urine were cleaned-up by IAC as described in 2.5. The final volume was 200 μL for all samples, corresponding to 1-300 ng mL^{-1} of NIV and NIV-3-GlcAc in measurement solution at 100% recovery. RAs were calculated by dividing the slopes of the standard addition curves by the slopes of pure solvent calibration functions prepared at the same concentration levels as the standard addition curves ($n = 6$ levels/curve) and multiplication by 100.

Faeces and urine samples were worked-up in duplicate as described in 2.5. When single values deviated by more than 20%, sample work-up and measurement was repeated. Analytes were quantified on the basis of pure solvent calibration functions (0.3-300 ng mL^{-1} , peak area versus analyte concentration) established in Analyst® software version 1.6.3 (SCIEX). Concentrations determined in faeces sample extracts were corrected by the RA and the dilution factor. For quantification of NIV in urine samples measured by the dilute and shoot method, each single sample was corrected by the SSE determined for 13C-NIV in the same sample and multiplied by the dilution factor. Concentrations of NIV and NIV-3-GlcAc obtained after IAC clean-up were corrected by the RA and the dilution factor.

Analyte concentrations between LOD and LOQ are referred to as traces and were included as half of the LOQ value for further calculations (see also [7]). NIV equivalent concentrations were calculated by dividing the analyte concentration by the molecular weight of the analyte and multiplying by the molecular weight of NIV. The total amounts of excreted analytes were obtained by multiplying the NIV equivalent concentrations in faeces and urine by the total amount of lyophilized faeces (0.4-6.5 g) or the total volume of urine (1.5-15.5 mL) excreted per day.

As soon as all the experimental part that is detailed before was carried out, some of the results expected at the beginning of the research were finally obtained. Considering the literature and information reported above, the formation of NIV sulfonates, NIV3G sulfonates, NIV glucuronides, deepoxy-NIV (DNIV), DNIV sulfonates and DNIV glucuronides was expected and investigated. For that, and as explained before, a unique

dose of $350 \mu\text{g kg}^{-1}$ bw of NIV and a unique dose of $532 \mu\text{g kg}^{-1}$ bw of NIV3G were administered to 6 rats per gavage. In order to avoid possible toxic effects in rats, the administered doses were considered taking into account the TDI proposed by the Scientific Committee on Food of $700 \mu\text{g kg}^{-1}$ bw for humans [15] and the research done by Takahashi et al. [16]. It was a 90-day study with rats where a significant reduction in the white blood cell count was observed at $0.4 \text{ mg NIV per kg of bw per day}$ (= LOAEL). For these reasons, $0.35 \text{ mg NIV per kg bw day}^{-1}$ (and the equimolar dose of NIV3G) was administered in the present study. During the whole experiment, no clinical symptoms were observed in any of the rats. In addition, there was no statistically significant difference in feed intake of rats treated with water, NIV or NIV3G. The average feed intake on treatment days was $13.0 \pm 1.8 \text{ g per rat}$.

Prior to production of NIV metabolites, selected rat urine and faeces samples were analysed by a generic LC-MS/MS based method employing the LC conditions as stated in 2.6 and calculated theoretical SRM transitions for the expected metabolites. Sample preparation was carried out as described in 2.5, using the dilute and shoot approach for urine samples. The tentatively identified NIV and NIV3G metabolites in faeces were NIV sulfonates 1, 2 and 3, DNIV, DNIV sulfonate 2, NIV3G sulfonate 2. Samples collected after NIV consumption showed NIV sulfonate 2 as the second major NIV metabolite. However, the first major NIV metabolite is still not be unequivocally identified. Samples collected after NIV3G consumption showed DNIV, NIV-3-glucoside sulfonate 2 and NIV sulfonate 2 as the major metabolites. A chromatogram from a rat faecal sample after the consumption of NIV3G is reported in Figure 3, where the main metabolites can be observed. Regarding urine samples, NIV-main-glucuronides have been identified in

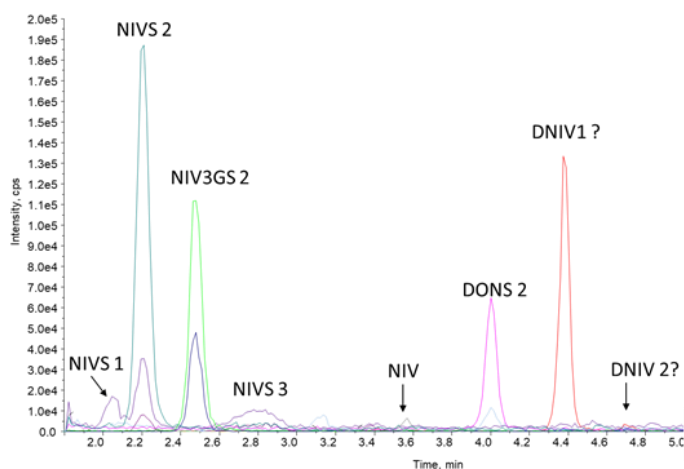


Figure 3. Chromatogram obtained from a faecal sample of a rat treated with NIV3G.

samples of the NIV group.

Then, the dilute and shoot approach for quantification of NIV metabolites in urine was validated by determining the matrix effects of NIV, DNIV and NIV-glucuronide in urine diluted to 0.5 mM creatinine. Regarding faecal samples, RAs obtained for NIV metabolites were between 90 and 105%, with the exception of NIV sulfonate 3 that obtained an RA of 122%, resulting from a signal enhancement.

The biological recoveries of NIV and NIV3G administered to rats is estimated close to 100%. However, there is still work to do to ensure this value. However, obtained ME results were not suitable and an IAC clean-up step was required after the dilute and shoot approach. Currently, further NIV-glucuronides are under investigation using the IAC clean-up procedure, together with further compounds verifications and identifications. New results are expected in order to obtain accurate conclusions.

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CHAPTER 4. CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI

ANALYTICAL TOOLS TO DETERMINE MYCOTOXINS AND MODIFIED MYCOTOXINS

Eugènia Miró Abella

The major conclusions that can be drawn from the studies presented in this doctoral thesis can be summarised as follows:

1. Different sample strategies were successfully optimised in this doctoral thesis for the extraction of mycotoxins and modified mycotoxins from different kinds of matrices. Techniques such as QuEChERS, pressurised liquid extraction (PLE), solid-phase extraction (SPE) and solid-liquid extraction (SLE) were effectively applied obtaining high recovery results while achieving low presence of matrix effects.
2. When these extraction techniques were combined with liquid chromatography tandem mass spectrometry to determine target mycotoxins, the achieved detection and quantification limits, at $\mu\text{g kg}^{-1}$ and $\mu\text{g L}^{-1}$ range, were suitable for their determination in different kinds of matrices. These limits are below the maximum allowed concentration levels allowed by European legislation and, consequently, the developed methodologies are suitable for the determination of the natural presence of numerous mycotoxins in food and feed samples.
3. The simple and useful QuEChERS strategy was successfully applied for the extraction of 11 mycotoxins from plant-based beverages, obtaining high recovery results between 80 and 91% and low presence of matrix effects, up to 45% with some exceptions. Consequently, the natural presence of mycotoxins was determined for the first time in rice, soy and oat plant-based beverages at $\mu\text{g L}^{-1}$ levels.
4. PLE with acidified water as the extraction solvent was applied for the first time for the selective extraction of trichothecenes from different complex cereal samples, which were spelt, millet, oat, quinoa and sesame. This technique allowed the subsequent addition of straight-forward clean-up step by SPE. The fact of using acidified water as the extraction solvent achieved extraction recovery results up to 73% for all compounds and matrices. However, with the selectivity provided by water it was possible to obtain low percentage of matrix effects from -18% to 15%, with few exceptions. The performance of the method may indicate a benefit of using alternative solvents, such as water, able to obtain results as reliable as those provided by organic solvents.

5. At least one mycotoxin was determined in practically all the food samples analysed during this doctoral thesis. In addition, in most cases more than one mycotoxin was determined in the same food sample, confirming the presence of multi-mycotoxin contaminations. DON, 15AcDON, OTA and ZEA were the most abundant compounds of the food analysed samples. All the concentration levels found in the analysed food samples were below the maximum allowed levels for the used cereals by current regulation.
6. For the first time a metagenomic study assessed the response of gut microbial composition to DON administration at two different concentration levels, simulating the low doses easily found in food and feed samples. Microbial gut biodiversity from rats was explored after the treatment that slightly changed in rats treated at $120 \mu\text{g DON kg}^{-1} \text{ body weight (bw) day}^{-1}$, since a significant increase in the relative abundance of the Coprococcus genus was observed. No significant changes were observed in rats that were treated with $60 \mu\text{g kg}^{-1} \text{ bw day}^{-1}$.
7. The presence of the concentration levels of DON and its metabolite DOM-1 was also monitored along the 7 weeks of DON's treatment. The presence of DOM-1 in faecal samples increased along the days after the consumption of DON, as a consequence of the increasing capability of the organism to detoxify DON. The increase of the excretion of DOM-1 could be related with the increase of the relative abundance of Coprococcus genus, but further research is needed to confirm this statement.
8. Different extraction techniques were compared for the determination of trichothecenes, including modified trichothecenes, from the rat faecal samples. The QuEChERS extraction technique followed by a dSPE clean-up step with activated carbon was shown to be the most suitable. Different clean-up strategies such as in-cell, on-cell, SPE and the several sorbents were tested without reducing considerably the percentage of matrix effect. The two different tested carbons, coco carbon and activated carbon, reduced successfully the presence of faecal interferences.

9. Analysed rat faecal samples presented some difficulties due the complexity of the matrix. However, using 1 g of sample, the optimised method allowed the achievement of low quantification limits from $0.2 \mu\text{g kg}^{-1}$ to $5 \mu\text{g kg}^{-1}$ and detection limits from $0.05 \mu\text{g kg}^{-1}$ to $1 \mu\text{g kg}^{-1}$ for the determination of DON and DON derivative compounds.
10. For the first time, an investigation of the absorption, distribution, metabolism and excretion of NIV and NIV3G in rats was achieved. Different NIV and NIV3G metabolites were identified in faecal and urine samples. In faeces, NIV sulfonates 1, 2 and 3, DNIV, DNIV sulfonate 2 and NIV3G sulfonate 2 were tentatively identified. In urine, traces of one glucuronide were detected.
11. The studies presented in this doctoral thesis have further demonstrated the natural presence of mycotoxins in food samples and the effects related with the consumption of DON at concentrations similar than those found in foodstuffs. However, the presence of modified mycotoxins is still not completely known. The identification of unknown modified mycotoxins present in food and feed samples and also in biological samples could avoid the consumption of non-controlled mycotoxins and the complete understanding of mycotoxin metabolism. Further research should be focused on the identification of these unknown compounds.

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Appendix I. List of abbreviations

AcDON	Acetylated deoxynivalenol
ACN	Acetonitrile
ADME	Absorption Distribution Metabolism Excretion
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
APCI	Atmospheric Pressure Chemical Ionisation
ASE	Accelerated Solvent Extractor
BW	Body Weight
C ₁₈	Octadecyl Silica
CH ₃ COOH	Acetic acid
CE	Collision Energy
CEN	European Committee for Standardization
DAD	Diode array
DE	Diatomaceous earth
DNA	Deoxyribonucleic acid
DNIV	Deepoxy-Nivalenol
DNIVS	Deepoxy-Nivalenol Sulfonate

DOM-1	Deepoxy-Deoxynivalenol
DON	Deoxynivalenol
DON3G	Deoxynivalenol-3-Glucoside
dSPE	dispersive Solid Phase Extraction
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Extraction Recovery
ESI	Electrospray Ionisation
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization
FBs	Fumonisin
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FIA	Flow Injection Analysis
FLD	Fluorescence Detector
GAP	Good Agricultural Practices
GC	Gas Chromatography
GCB	Graphitised Carbon Black
GMP	Good Manufacturing Practices
HCOOH	Formic acid
HPLC	High Performance Liquid Chromatography

HR	High Resolution
HT-2	HT-2 toxin
IAC	Immunoaffinity Columns
IARC	International Agency for Research on Cancer
JECFA	Joint Expert Committee on Food Additives
LC	Liquid Chromatography
LD ₅₀	Median Lethal Dose
LLE	Liquid-Liquid Extraction
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MAE	Microwave-Assisted Extraction
MDL	Method Detection Limit
ME	Matrix Effect
MED	Minimum Emetic Dose
MeOH	Methanol
MgSO ₄	Magnesium Sulphate
MLQ	Method Quantification Limit
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	mass-to-charge ratio

NaClO	Sodium hypochlorite
NIV	Nivalenol
NIV3G	Nivalenol-3-Glucoside
NIV3GS	Nivalenol-3-Glucoside Sulfonate
NIV-3-GlcAc	Nivalenol-3-Glucuronide
NIVS	Nivalenol Sulfonate
NMR	Nuclear Magnetic Resonance
NOAEL	No-Observed Adverse Effect Level
OJEU	Official Journal of the European Union
OTA	Ochratoxin A
PAT	Patulin
PCR	Polymerase Chain Reaction
PHWE	Pressurised Hot Water Extraction
PLE	Pressurised Liquid Extraction
PMTDI	Provisional Maximum Tolerable Daily Intake
PSA	Primary Secondary Amine
q	Qualifier transition
Q	Quantifier transition
QqQ	Triple Quadrupole
Qtrap	Quadrupole-ion trap
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe Extraction
R ²	Coefficient of determination

RA	Apparent Recovery
RNA	Ribonucleic acid
RSD	Relative Standard Deviation
RT	Retention Time
SCOOP	Scientific Co-operation on Question relating to Food
SLE	Solid-Liquid Extraction
S/N	Signal-to-Noise ratio
SPE	Solid-Phase Extraction
SRM	Selected Reaction Monitoring
SSE	Mass Spectrometric Matrix Effects
Std	Standard
SWE	Subcritical Water Extraction
T-2	T-2 toxin
TDI	Tolerable Daily Intake
t-TDI	temporary Tolerable Daily Intake
TLC	Thin layer chromatography
TOF	Time Of Flight
UHPLC	Ultra-High Performance Liquid Chromatography
UK	United Kingdom
UV	Ultraviolet
WHO	World Health Organization
ZEA	Zearalenone

ZEA-14-Glc	Zearalenone-14- <i>O</i> - β -Glucoside
ZEA-14-S	Zearalenone-14-Sulfate
ZEA-16-Glc	Zearalenone-16- <i>O</i> - β -Glucoside
3AcDON	3-Acetyl-Deoxynivalenol
15AcDON	15-Acetyl-Deoxynivalenol

Appendix II. List of publications

List of publications originated from the experimental part of the present doctoral thesis:

1. Miró-Abella E, Herrero P, Canela N, Arola L, Borrull F, Ras R, Fontanals N (2017) Determination of mycotoxins in plant-based beverages using QuEChERS and liquid chromatography-tandem mass spectrometry, *Food Chem* 229:366–372.
2. Miró-Abella E, Herrero P, Canela N, Arola L, Ras R, Fontanals N, Borrull F (2017) Determination of trichothecenes in cereal matrices using subcritical water extraction followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry, *Food Anal. Methods* 11:1113-1121.
3. Miró-Abella E, Torrell H, Herrero P, Canela N, Arola L, Borrull F, Ras R, Fontanals N (2018) Monitoring and evaluation of the interaction between Deoxynivalenol and gut microbiota in Wistar rats by mass spectrometry-based metabolomics and next-generation sequencing, *Food Chem. Toxicol* 121:124-130.
4. Miró-Abella E, Herrero P, Canela N, Arola L, Ras R, Borrull F, Fontanals N (2018) Optimised extraction methods for the determination of trichothecenes in rat faeces followed by liquid chromatography-tandem mass spectrometry, *J Chromatogr. B (Submitted)*.
5. Schwartz-Zimmermann HE, Binder SB, Miró-Abella E, Hametner C, Schwarz C, Michlmayr H, Reiterer N, Labudova S, Adam G, Berthiller F (2018) Metabolism of nivalenol and nivalenol-3-glucoside in rats (*In edition*).

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