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Influence of Microwave Cooking on Multi-Mycotoxin Levels in Plant-Based Meat Analogues

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1. General introduction

Mycotoxins are secondary metabolites produced by fungi mainly belonging to three genera: *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. (G. S. Shephard, 2008). Many other fungi (*Alternaria* spp., *Claviceps* spp.) can be mycotoxigenic (Liang et al., 2023). More than 300 compounds have been identified as mycotoxins. These are characterized by different toxicity and chemical proprieties (Alshannaq & Yu, 2017; G. S. Shephard, 2016; Stroka & Maragos, 2016; Yazdanpanah, 2011). The most studied mycotoxins are aflatoxins (AF), trichothecenes (TCT), fumonisins (FUM), zearalenone (ZEA), ochratoxins (OTA)(Amirahmadi et al., 2018; Ji et al., 2014; Sengling Cebin Coppa et al., 2019; Zaki et al., 2012). The infection of fungal species in crops or fields can coincide with a higher risk for human and animal health (Yazar & Omurtag, 2008). Fungi and plants can modify mycotoxins and change their structures. These mycotoxins are called “masked mycotoxins” because they are undetectable by standard analytical techniques (Berthiller et al., 2013; De Boevre et al., 2012). Masked mycotoxins are, in general, less toxic than the precursors, however, they can contribute to the total toxicity of the food (Gratz, 2017).

Mycotoxins have an enormous impact on human and animal health. The exposure can be direct by ingesting contaminated foods or indirectly through foods of animal origin that were exposed to mycotoxins (Sengling Cebin Coppa et al., 2019). The health effects of mycotoxin exposure can range from acute to chronic, depending on the duration and level of exposure. Acute symptoms can include nausea, vomiting, diarrhea, and abdominal pain, while chronic exposure has been linked to organ damage, neurotoxicity, reproductive issues, and increased susceptibility to infectious diseases (Bennett & Klich, 2003). Long-term exposure to mycotoxins has also been associated with an increased risk of developing cancers, particularly liver and esophageal cancer (Peers & Linsell, 1973; Xue et al., 2019). The disease caused by toxicological exposure to mycotoxins is mycotoxicosis, which has been responsible for some changes in human history (Peraica & Rašić, 2012).

There are different European Regulations about the legal maximum levels (MLs) of mycotoxins in food and feed:(Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs (Text with EEA Relevance), s.d.). The sum of mycotoxins is considered only for AFs (AFB_1, AFB_2, AFG_1, AFG_2) and FBs (FB_1, FB_2). This approach can underestimate the total toxicity of food and feed, leading to a higher risk for consumers (Ekwomadu et al., 2021).

The European Food Safety Authority (EFSA) made different risk assessments about the presence of mycotoxins in feed and food. More than nine groups of mycotoxins are regulated in more than 60 different food commodity groups (Montet et al., 2021). EFSA produced several scientific opinions about the presence of specific mycotoxins in food (es. OTA in grains), a modified form of some mycotoxins (masked FBs) or emerging mycotoxins like Enniatins (ENNs) (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014a, 2014b; Schrenk et al., 2020).

However, different products are not covered by European Regulations. For example, currently, soy products have not MLs set. These include vegetable burgers and ready-to-eat food for vegans and vegetarians with higher exposure to mycotoxins due to the higher consumption of cereal-based products (Penczynski et al., 2022).

Several analytical techniques have been developed to identify and quantify mycotoxins in different matrices, such as liquid chromatography (LC), gas chromatography (GC), mass spectrometry (MS), enzyme-linked immunosorbent assays (ELISA), and polymerase chain reaction (PCR). Each technique has advantages and limitations, but the most commonly used approach is LC-MS/MS due to its high sensitivity, selectivity, and accuracy (Turner et al., 2009).

One of the major challenges in mycotoxin analysis is the complexity and diversity of food matrices. Mycotoxins can be found in various agricultural commodities, including cereals, nuts, spices, and processed food products (Marín & Ramos, 2016; Martins et al., 2001; Yazar & Omurtag, 2008). These matrices often contain various interfering compounds, such as pigments,

lipids, sugars, and proteins, which can affect the analysis and identification of mycotoxins (Steiner et al., 2020). Therefore, sample preparation techniques, such as extraction and purification methods, are crucial for removing matrix interferences and concentrating the target analytes.

Another challenge is the lack of commercially available certified reference materials (CRMs) for mycotoxins. CRMs are essential for method validation, quality control, and ensuring accurate quantification of mycotoxins. However, due to the complexity and variability of mycotoxin structures, it is difficult to produce stable and homogeneous CRMs that represent the entire spectrum of mycotoxins (Köppen et al., 2013). Consequently, developing and producing CRMs that accurately mimic naturally occurring mycotoxin contamination in different matrices are ongoing research areas.

Furthermore, mycotoxins often occur in mixtures, where one sample can contain multiple mycotoxins simultaneously. The analysis of mycotoxin mixtures poses several challenges, including the need for selective and sensitive detection methods to analyze a wide range of mycotoxins with different physical and chemical properties.

The present thesis aims to develop an analytical method to analyze several mycotoxins in several food matrices and commodity groups with an LC-MS/MS method (Chapter I). Secondly, analyze mycotoxins in several food matrices that are alternative veggie-meal alternative (Chapter II) and try to investigate how the mycotoxins found can change depending on the cooking method (Chapter III).

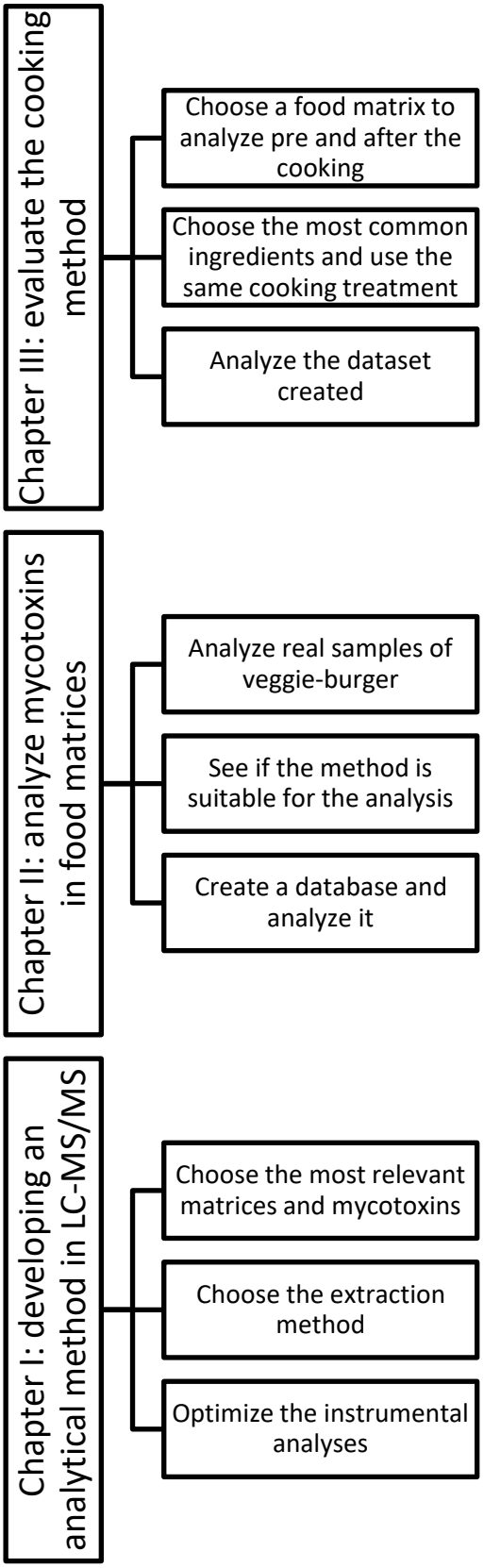

Fig. 1 Overview of the work done for the thesis

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1. Mycotoxins

The terms mycotoxins derived from the Greek μύκης (*mykes*, *mukos*) “fungus” and the Latin *toxicum* “poison”. The terms was used the first time in London (England) when in 1962 more than 100.000 turkey poults died mysteriously (Wannop, 1961). The mortality of this “X” disease was around 100%. Histopathological analysis (liver, kidney, spleen) found that the most significant alteration where in the liver, were the parenchyma was found in cirrhotic conditions. The meal based on groundnut were contaminated with *Aspergillus flavus,* which produce AFs (Pickova et al., 2021). Mycotoxin includes several compounds characterized by different toxicity and chemical proprieties (Hussein & Brasel, 2001; Richard, 2007). Not all fungi are responsible for the production of mycotoxins. The most important classes of fungi that can synthetize this secondary metabolites are *Alernaria*, *Aspergillus*, *Penicillum* and *Fusarium* (Greeff-Laubscher et al., 2020).

Therefore the classification can be different and can be done by their toxicity, chemical structures, syndrome that they can cause or their biosynthetic origins (Bennett & Klich, 2003; Zain, 2011). Different toxicities characterize these compounds. Even the same mycotoxin can be in different categories, AFs are characterized, for example, by mutagenic, teratogenic, and immunomodulatory effects (Y.-C. Lin et al., 2014; Mary et al., 2012; Wogan et al., 2012). Cereals represent the most common type of foods consumed and therefore represent the main route of exposure to mycotoxins in animals and humans (Janik et al., 2020). In a ten-year survey based on 74.821 feed and feed raw materials samples, 64% of samples were contaminated with at least two mycotoxins (Gruber-Dorninger et al., 2019). The detectable level of DON and ZEN in more than 500.000-grain samples was 60% and 80%, respectively (Eskola et al., 2020). We will discuss in the following paragraphs the class of compound analysed and the techniques used for the analyses.

1.1 Aflatoxins

The term “aflatoxins” refers to the fungus that mainly produces these mycotoxins, the Aspergillus genus. The most common fungus that can produce aflatoxins and infect foods is *A. flavus* (Ekwomadu et al., 2021; Rodríguez-Blanco et al., 2021). “Aflatoxins” is an abbreviation of ***As***pergillus ***fla***vus ***to****xins* (Schoental, 1967). Different spp. in the genus *Aspergillus* can produce aflatoxins such as *bombycis, nomius, rambellii, and toxicarius* (Smith et al., 2016). The most common aflatoxins found in food or feed are AFB_1, AFB_2, AFG_1, and AFG_2 (Jurišić et al., 2019; Kumar et al., 2017a). The acronyms “B” and “G” refer to the colour exhibited if exposed to ultraviolet light, that is “blue” and “green”, respectively (Bbosa et al., 2013; Benkerroum, 2020a).

AFs can be divided depending on the chemical structures in the difurocoumarocyclopentenone (AFB_1, AFB_2) and difurocoumarolactone (AFG_1, AFG_2) series. The main structure is shown in Figure 2; five rings characterize it. An aromatic ring (A), a six-membered lactone ring (D), a five (B aflatoxins) or six-member (G aflatoxins) lactone (E), and furofuran moiety (rings B, C) (Bräse et al., 2013a). These compounds are characterized by a bifuran ring fused to a coumarin nucleus with a pentanone (B aflatoxins) or a six-membered lactone (G aflatoxins) (Dhanasekaran et al., 2011). The AFM_1 and AFM_2 are the hydroxylated (hydroxylic group between the junction of the two furan rings) form of AFB_1 and AFB_2, respectively and are the results of the metabolism by lactating mammals when they eat contaminated feed (Campagnollo et al., 2016). They are less toxic than their precursors and can be found in meats and milk products of exposed animals (Becker-Algeri et al., 2016; Rushing & Selim, 2019).

AFs are soluble in organic polar solvents such as methanol, acetone and less soluble in apolar solvents and water (Janik et al., 2020). Strong conditions of pH can degrade AFs. Therefore, it needs to be in a stable pH range of 4-6 (Iram et al., 2015; Marchese et al., 2018).

AFB_1 is considered the most toxic among AFs and the most common food

contaminant (Janik et al., 2020; Marchese et al., 2018). It is classified as group 1 (cancerogenic to humans) by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 1999). It is toxic in all animal species tested at the date (Haschek & Voss, 2013).

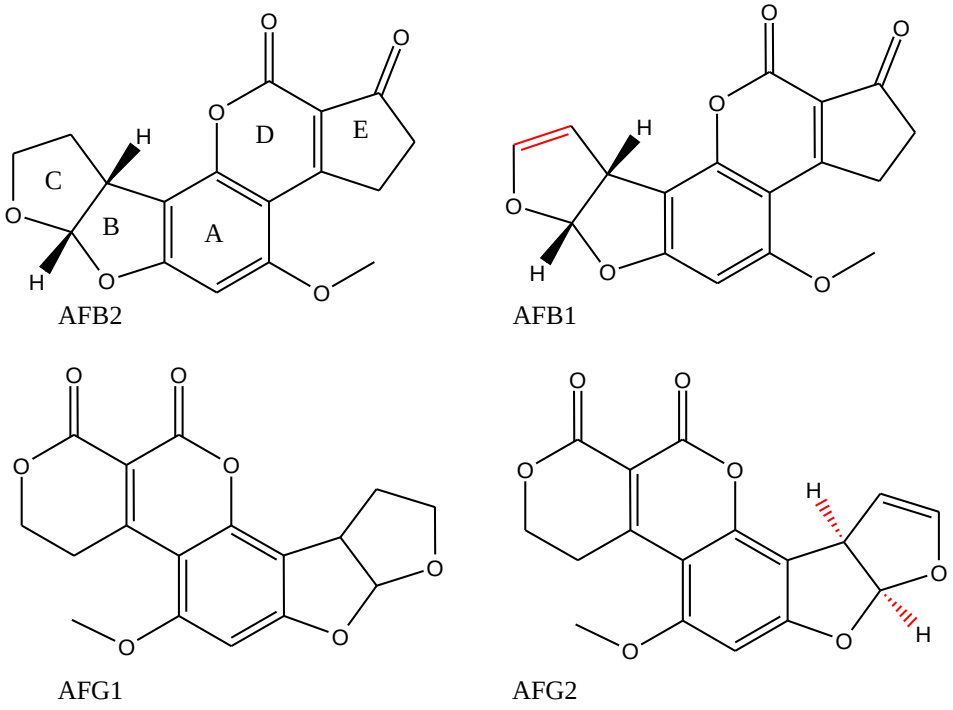


Fig. 2 Chemical structures of aflatoxins.

1.1.1 Occurrence

AFs are known to contaminate cereals such as corn, wheat, rice, and others. The presence of AFs in cereals has been reported in all continents, especially in the humid environment of tropical regions (Al-Zoreky & Saleh, 2019; Ruadrew et al., 2013). This results in high exposure to AFs, especially because cereal-based products are the most consumed in developing countries (Grace et al., 2015; Temba et al., 2017). Precise occurrence data is hard to obtain because it depends on many factors, such as the analytical method used, where data are obtained and sampling procedures. Estimations report that AFs contaminate at least 35% of cereals analysed (Grace et al., 2015). Maize plants are the most sensitive to *Aspergillus* contamination among cereals, especially *A. flavus*, *A. parasiticus A. flavi*

(FAO, 2018; Taniwaki et al., 2018). Regarding rice, presence of AFB_1 have been reported in Africa, Brazil, Europe, China (Frisvad et al., 2019; X. D. Sun et al., 2017).

Spices and dried fruits are prone to AFs contamination due to the condition of production (Martinez-Miranda et al., 2019). Storage and processing conditions can increase the AFs contents (Elshafie et al., 2002). The incidence of the presence of AFs in food, and in general of mycotoxins, will increase due to climate change (Decastelli et al., 2007). In fact, the seasons that are becoming hotter and drier increase the population of *A. Flavi* in Europe and AFs possible contaminations (Moretti et al., 2019).

1.1.2 Toxicity

The toxicity of AFs is well known. There were several outbreaks of aflatoxicosis in different countries, such as Kenya (2005), and Tanzania (2016), due to consuming of contaminated corn that led to the death of 225 individuals (Kamala et al., 2018; Probst et al., 2007). They are activated in less toxic but reactive electrophilic species in the phase I metabolism by P450 (CYP) CYP1A2, CYP3A5, and CYP3A4 (Deng et al., 2018; Rushing & Selim, 2019). The phase II metabolism leads to a conjugation with glutathione, and the conjugate B_1-glutathione is extracted with the bile into the intestinal tract. The susceptibility of the different species is inversely proportional to the activity of glutathione-S-transferase (GST) (Kensler et al., 2011). AFs are hepatotoxic, mutagenic and genotoxic. AFs can create adducts with the guanine in deoxyribonucleic acid (DNA) (Ferreira et al., 2019). The mutation of the DNA is associated with different genes that can increase the calcium in mitochondria, interfere with the p-53 codon, induce conversion from G:C to A:T and, in general, increase apoptosis (Y.-C. Lin et al., 2014; Park et al., 2019; Paul et al., 2015; Soman & Wogan, 1993). The acute aflatoxicosis is associated with histopathological damage in the liver (Benkerroum, 2020a). Other organs, such as the kidney, pancreas, bone, and nervous system, are subjected to AG-induced genotoxicity (Benkerroum, 2020b). AFs are also an immunosuppressor and reduce the activity of immune cells (Y. Sun et al., 2023).

1.1.3 Regulatory limits in food

Due to the toxicity, the presence of AFs has been regulated in almost all countries (Van Egmond et al., 2007). In European Countries, the allowed maximum levels (MLs) have been regulated by the Commission Regulation (EC) No 2023/915, repealing Regulation (EC) No 1881/2006. Annex I of the Regulation is shown in Table 1. Children are considered "vulnerable populations"; therefore, the MLs are lower for each matrix. The lower MLs is 0.10 ppb of AFB_1 in food for special medical purposes for infants and young children. The food listed in Annex I cannot be used as raw materials or ingredients in foods if contaminated in higher quantities allowed in Annex I.

Table 1 Annex I of the regulation (EU) 2023/915 regarding the maximum levels for certain contaminants in food (Commission Regulation (EU) 2023/915, 2023).

1	**Mycotoxins**				
1.1	**Aflatoxins**	**Maximum level (µg/kg)**			**Remarks**
		B_1	**Sum of B_1, B_2, G_1 and G_2**	**M_1**	For the sum of aflatoxins, maximum levels refer to lower bound concentrations, which are calculated on the assumption that all the values below the limit of quantification are zero.
1.1.1	Dried fruits to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food except products listed in 1.1.3	5,0	10,0	-	
1.1.2	Dried fruits used as only ingredient or processed products from dried fruits, placed on the market for the final consumer or use as an ingredient in food except products listed in 1.1.3	2,0	4,0	-	In the case of food consisting of dried fruits used as only ingredient or in the case of processed products consisting at least of 80 % from the dried fruits concerned, the maximum levels as established for the corresponding dried fruits apply also to those products.
1.1.3	Dried figs	6,0	10,0	-	In the case of food consisting of dried figs used as only ingredient or in the case of processed products consisting at least of 80 % from dried figs, the maximum levels as established for dried figs apply also to those products.
1.1.4	Groundnuts (peanuts)	8,0	15,0	-	Except groundnuts (peanuts) and other oilseeds for

	and other oilseeds, to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food				crushing for refined vegetable oil production. If groundnuts (peanuts) and other oilseeds with inedible shell are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part.
1.1.5	Groundnuts (peanuts) and other oilseeds used as only ingredient or processed products from groundnuts (peanuts) and other oilseeds, placed on the market for the final consumer or use as an ingredient in food	2,0	4,0	-	Except crude vegetable oils destined for refining and refined vegetable oils. If groundnuts (peanuts) and other oilseeds with inedible shell are analysed, it is assumed when calculating the aflatoxin content that all the contamination is on the edible part. In the case of food consisting of groundnuts (peanuts) and other oilseeds used as only ingredient or in the case of processed products consisting at least of 80 % from the groundnuts (peanuts) and other oilseeds concerned, the maximum levels as established for the corresponding groundnuts (peanuts) and other oilseeds apply also to those products.
1.1.6	Tree nuts to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food except products listed in 1.1.8 and 1.1.10	5,0	10,0	-	If tree nuts ‘in shell’ are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part.
1.1.7	Tree nuts used as only ingredient or processed products from tree nuts, placed on the market for the final consumer or use as an ingredient in food except products listed in 1.1.9 and 1.1.11	2,0	4,0	-	If tree nuts ‘in shell’ are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part. In the case of food consisting of tree nuts used as only ingredient or in the case of processed products consisting at least of 80 % from the tree nuts concerned, the maximum levels as established for tree nuts apply also to those products.
1.1.8	Almonds, pistachios and apricot kernels to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food	12,0	15,0	-	If tree nuts ‘in shell’ are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part.
1.1.9	Almonds, pistachios and apricot kernels, placed on the market for the final consumer or use as an ingredient in food	8,0	10,0	-	If tree nuts ‘in shell’ are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part. In the case of food consisting of almonds, pistachios and apricot kernels used as only ingredient or in the case of processed products consisting at least of 80 % from the tree nuts concerned, the maximum levels as established for the corresponding tree nuts apply

					also to those products.
1.1.10	Hazelnuts and Brazil nuts, to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food	8,0	15,0	-	If hazelnuts 'in shell' are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part.
1.1.11	Hazelnuts and Brazil nuts, placed on the market for the final consumer or use as an ingredient in food	5,0	10,0	-	If hazelnuts 'in shell' are analysed, it is assumed, when calculating the aflatoxin content, that all the contamination is on the edible part. In the case of food consisting of hazelnuts and Brazil nuts used as only ingredient or in the case of processed products consisting at least of 80 % from the tree nuts concerned, the maximum levels as established for the corresponding tree nuts apply also to those products.
1.1.12	Cereals and products derived from cereals except products listed in 1.1.13, 1.1.18 and 1.1.19	2,0	4,0	-	Including processed cereal products. Products derived from cereals relate to products containing at least 80 % cereal products.
1.1.13	Maize and rice to be subjected to sorting or other physical treatment before placing on the market for the final consumer or use as an ingredient in food	5,0	10,0	-	
1.1.14	Dried spices: *Capsicum spp.* Pepper Nutmeg (*Myristica fragrans*) Turmeric (*Curcuma longa*)	5,0	10,0	-	For *Capsicum spp.* (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne or paprika) For Pepper (fruits of *Piper* spp, including white and black pepper) The Regulations is applied for mixtures of dried spieces containing at least one of the species regulated.
1.1.15	Ginger (*Zingiber officinale*) (dried)	5,0	10,0	-	
1.1.16	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	-	-	0,050	
1.1.17	Infant formulae, follow-on formulae and young-child formulae	-	-	0,025	The maximum level applies to the products ready to use (placed on the market as such or after reconstitution as instructed by the manufacturer).
1.1.18	Baby food and processed cereal-based food for infants and young children	0,10	-	-	The maximum level applies to the dry matter of the product as placed on the market.
1.1.19	Food for special medical purposes intended for infants and young children	0,10	-	0,025	The maximum level applies in the case of milk, milk products and similar products to the products ready to use and in the case of products other than milk, milk products and similar products to the dry matter.

AFs are the mycotoxins with the lowest MLs due to their toxicity. The Codex Alimentarius Commission (CAC) regulate the MLs for mycotoxin contamination in food and feed internationally (J.-G. Lee et al., 2021). Creating and developing a food safety regulation needs scientific and economic factors. In fact, despite CAC, each country can have more stringent limits based on scientific and logical factors (Roberts & Unnevehr, 2005). United States (US) and Kenya established the MLs in cereals as 20 and 10 ppb for the sum of AFB1-AFB2 and AFG1-AFG2, respectively, while in Europe, the MLs are at 2 and 4 ppb (Mutegi et al., 2018). Mercosur (Argentina, Brazil, Uruguay, Paraguay, and Venezuela), ASIA, and Members of the Gulf Cooperation Council (the United Arab Emirates, Saudi Arabia, Qatar, Oman, Kuwait, Bahrain) have their regulations for aflatoxin (Al-Jaal et al., 2019; Martínez Miranda et al., 2013).

1.2 Ochratoxins

Ochratoxins are a group of toxic secondary metabolites produced by certain moulds, particularly fungi from the genera *Aspergillus* and *Penicillium* (el Khoury & Atoui, 2010). These compounds are characterized by isocumarin structures coupled with β-phenylalanine. The most important compound of ochratoxins is ochratoxin A (OTA). The dechloro analogue of OTA is ochratoxin B (OTB), the ethyl ester of OTA is called ochratoxin C (OTC), and the isocumaric derivate of OTA is called ochratoxin α (Otα). It's dechloro analogue ochratoxin β (OTβ). Molecular structures are shown in Figure 3. The pH influences the solubility of OTA. OTA is soluble in polar organic solvents (MeOH, ACN) in acid and neutral pH. In contrast, higher pH tends to increase the solubility in water-alkaline solutions (el Khoury & Atoui, 2010). This molecule is heat resistant, and contaminated foodstuffs can contain OTA even after high-pressure steam sterilization at 121°C for three h (Trivedi et al., 1992). Studies have pointed out that OTA heat resistance depends on the water contents present in contaminated foodstuffs (Pleadin et al., 2014).

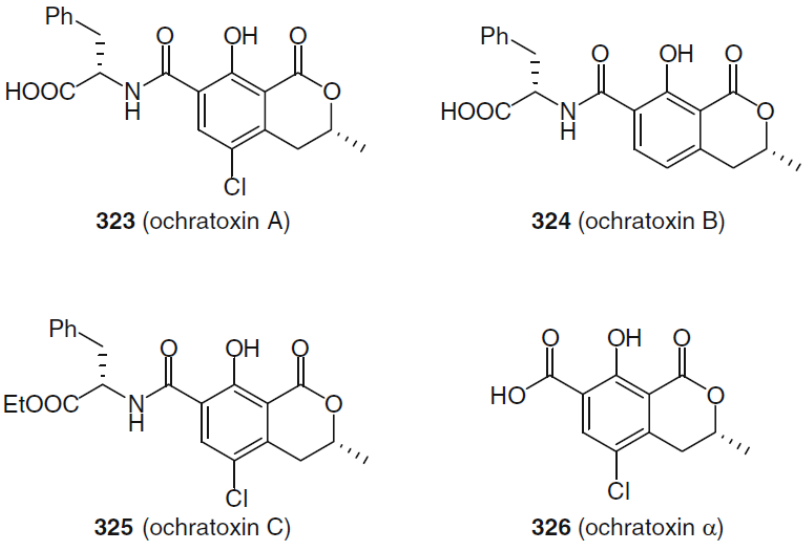


Fig. 3 Chemical structures of some ochratoxins (Bräse et al., 2013b).

1.2.1 Occurrence

The occurrence of OTA in foodstuffs can vary widely across different products and geographical regions. Cereals are considered the most contaminated foodstuffs regarding OTA contamination (Vega et al., 2009); they can become contaminated during growth, harvesting, and storage, especially if the conditions are conducive to fungal growth (X. Li et al., 2021). A ten-year global survey, with a total of 78.821 samples and more than 100 nations, revealed that more than 15% of feed samples were contaminated with OTA, and a higher incidence of positive samples was found in Asia (Gruber-Dorninger et al., 2019). A similar percentage was revealed in a survey in the United States, where OTA was detected in 12.2% of cereals (wheat, barley, durum) samples (n=252) (Kuruc et al., 2015). Consequently, animal feed can also be contaminated, leading to the indirect exposure of OTA to animals and potential carryover into animal-derived products (Duarte et al., 2012). Therefore, OTA can be found in salami, beer, cheese, and meat (Altafini et al., 2017; Lhotská et al., 2016; Pattono et al., 2011).

Wines represent the second most important source of OTA in Europe (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014a). Grapes can be infected by OTA-producing moulds, which can contaminate

wine (Battilani & Pietri, 2002). Additionally, OTA can be introduced during winemaking processes, leading to its presence in the final product (Dachery et al., 2015). Several studies have been conducted on the presence of OTA in wines. A survey of 100 samples of wine produced in Portugal, Spain, and Italy collected from 1984 to 2017 found four positive samples with a detectable amount of OTA (Silva et al., 2019). The general quality of wine in Italy is high. Three different surveys were done in Italy, and no wines were over the MLs of the European Regulation of 2 µg/L (Di Stefano et al., 2015, 2015; Gentile et al., 2016; Vella et al., 2019).

Coffee beans, both during growth on the plant and during processing and storage, can be contaminated by OTA-producing moulds (Barcelo & Barcelo, 2018). The toxin can persist through roasting, brewing, and consumption (Napolitano et al., 2007). It represents the third-largest source of OTA intake in Europe (X. Li et al., 2021). OTA is found in roasted coffee, soluble coffees, and decaffeinated coffee worldwide (Jonatova et al., 2020; Tozlovanu & Pfohl-Leszkowicz, 2010; Vecchio et al., 2012).

Other foodstuffs that can be contaminated with OTA are dried fruits and spices (Pantano et al., 2021). In both cases, the drying process can create conditions favourable for mould growth and mycotoxin production (Iqbal et al., 2013, 2021; Jalili & Jinap, 2012).

1.2.2 Toxicity

Ochratoxins are characterized by different toxicity. *In vivo* and *in vitro* studies showed that OTB is much less toxic than OTA (Doster et al., 1974; Heussner & Bingle, 2015; Peckham et al., 1971). Once ingested, OTA binds to plasma albumin in a percentage that depends on the species (Duarte et al., 2012). The half-life ($t_{1/2}$) ranges from 8h in rabbits to over 500 h in monkeys (Joint FAO/WHO Expert Committee on Food Additives et al., 2001). The toxicity depends also on gender and age (Coronel et al., 2010; Vettorazzi et al., 2014). Due to its binding proprieties, OTA's main toxicity target is the kidney, which can damage renal proximal and distal tubules (Abid et al., 2003; Hmaissia Khlifa et al., 2012). OTA possesses a phenyl-alanine

structure and competes with phenylalanine for the binding sites. OTA's toxicity arises from its ability to interfere with various cellular processes.

One of its primary mechanisms of action is the inhibition of protein synthesis by targeting the ribosomes within cells (Mally et al., 2005; Wiger & Størnier, 1990). This effect is caused by the inhibition of phenylalanine tRNA synthetase by ocratoxins. Other processes involved in the toxicity mechanisms are the inhibition of mitochondrial respiration and oxidative damage of macromolecules (Kőszegi & Poór, 2016). Prolonged exposure to ochratoxin A has been associated with chronic kidney disease, and it's considered a potential risk factor for Balkan Endemic Nephropathy, a kidney disease prevalent in certain regions (Castegnaro, Canadas, et al., 2006). OTA has been classified as possibly carcinogenic to humans by the IARC («IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man», 1976). This classification is mainly due to its observed carcinogenic effects in animal studies, particularly in relation to kidney tumours (Maaroufi et al., 1994). OTA has been associated with reproductive and developmental toxicities. It induces craniofacial malformations and alters embryo growth (Heussner & Bingle, 2015).

1.2.3 Regulatory limits in food

Given these health concerns, regulatory agencies and international organizations have established guidelines and maximum permissible limits for OTA levels in various food products. The European Union has established some of the most comprehensive regulations regarding ochratoxin A. Maximum levels for OTA are specified for various food products, including cereals, dried fruits, coffee, wine, and grape juice. The levels are set to minimize exposure to ochratoxin A and protect public health. The range is between 0,5 and 80 µg/kg. The lowest limit is in baby food, while the highest is in liquorice extracts. An extract of the MLs in Europe is presented in Table 2. China has European's similar European MLs for their products: 2 µg/kg in wine and must, 5 µg/kg in grains and cereals and 10 µg/kg in instant coffee (Heussner & Bingle, 2015). To date, other ochratoxins are not regulated

worldwide.

Table 2 Annex I of the regulation (EU) 2023/915 regarding the maximum levels for certain contaminants in food. MLs for Ochratoxin A (Commission Regulation (EU) 2023/915, 2023).

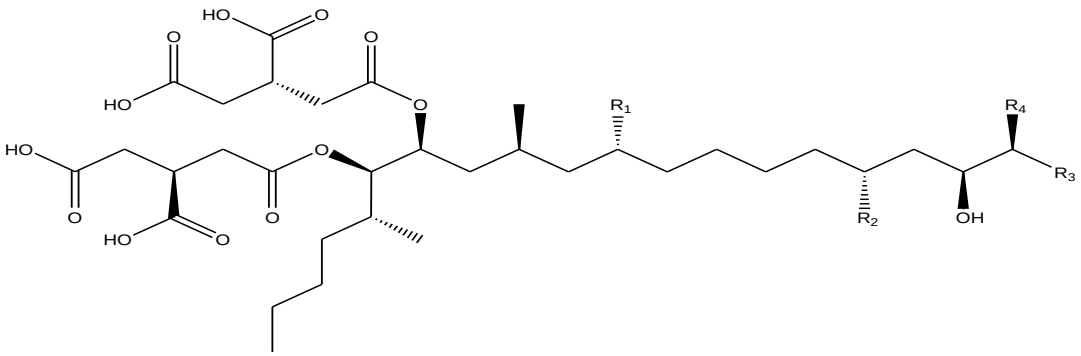
1.2	**Ochratoxin A**	**MLs (µg/kg)**
1.2.1	Dried fruits	
1.2.1.1	Dried vine fruits and dried figs	8,0
1.2.1.2	Other dried fruits	2,0
1.2.2	Date syrup	15
1.2.3	Pistachios to be subjected to sorting or other physical treatment before placing on the market for final consumer or use as an ingredient in food	10,0
1.2.4	Pistachios placed on the market for final consumer or use as ingredient in foods	5,0
1.2.5	Dried herbs	10,0
1.2.6	Ginger roots (dried) for use in herbal infusions	15
1.2.7	Marshmallow roots (dried), dandelion roots (dried) and orange blossoms (dried) for use in herbal infusions or in coffee substitutes	20
1.2.8	Sunflower seeds, pumpkin seeds, (water) melon seeds, hempseeds, soybeans	5,0
1.2.9	Unprocessed cereal grains	5,0
1.2.10	Products derived from unprocessed cereal grains and cereals placed on the market for the final consumer except products listed in 1.2.11, 1.2.12, 1.2.13, 1.2.23 and 1.2.24	3,0
1.2.11	Bakery wares, cereal snacks and breakfast cereals	
1.2.11.1	products not containing oilseeds, nuts or dried fruits	2,0
1.2.11.2	products containing at least 20 % dried vine fruits and/or dried figs	4,0
1.2.11.3	other products containing oilseeds, nuts and/or dried fruits	3,0
1.2.12	Non-alcoholic malt beverages	3,0
1.2.13	Wheat gluten not placed on the market for the final consumer	8,0
1.2.14	Roasted coffee beans and ground roasted coffee except products listed in 1.2.15	3,0
1.2.15	Soluble coffee (instant coffee)	5,0
1.2.16	Cocoa powder	3,0
1.2.17	Dried spices except products listed in 1.2.18	15
1.2.18	*Capsicum spp.* (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne or paprika)	20
1.2.19	Liquorice (*Glycyrrhiza glabra*, *Glycyrrhiza inflata* and other species)	
1.2.19.1	Liquorice root (dried), including as an ingredient in herbal infusions	20
1.2.19.2	Liquorice extract for use in food in particular beverages and confectionary	80
1.2.19.3	Liquorice confectionery containing ≥ 97 % liquorice extract on dry basis	50
1.2.19.4	Other liquorice confectionery	10,0
1.2.20	Wine and fruit wine	2,0
1.2.21	Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2,0
1.2.22	Grape juice, grape juice from concentrate, concentrated grape juice, grape nectar, grape must and concentrated grape must, placed on the market for the final consumer	2,0
1.2.23	Baby food and processed cereal-based food for infants and young children	0,50

1.2.24	Food for special medical purposes intended for infants and young children	0,50

1.3 Fumonisins

Fumonisins' term is derived from the *genre* of fungus that produces them, *Fusarium*. Some species that produce fumonisins are *F. verticillioides*, *F. proliferatum*, *F. anthophilum* (Blacutt et al., 2018; Jurado et al., 2010). Four different groups of fumonisins have been characterized (A, B, C, P). The structures of some fumonisins are shown in Table 3. The “A” series has an N-acetate group instead of an amino group. In contrast, the “C” series doesn’t have the methyl group in R_3. The “P” group have an N-linked 3-hydroxy pyridinium group instead of the amino group. Fumonisins of “B” category are the most studied and toxic (Rheeder et al., 2002).

Table 3 Structures of fumonisins.



Name	R_1	R_2	R_3	R_4
FA_1	OH	OH	CH_3	NHAc
FA_2	H	OH	CH_3	NHAc
FA_3	OH	H	CH_3	NHAc
FA_4	H	H	CH_3	NHAc
FB_1	OH	OH	CH_3	NH_2
FB_2	H	OH	CH_3	NH_2
FB_3	OH	H	CH_3	NH_2
FB_4	H	H	CH_3	NH_2
FC_1	OH	OH	H	NH_2
FC_2	H	OH	H	NH_2
FC_3	OH	H	H	NH_2
FC_4	H	H	H	NH_2
FP_1	OH	OH	CH_3	3-hydroxypyridinium
FP_2	H	OH	CH_3	3-hydroxypyridinium
FP_3	OH	H	CH_3	3-hydroxypyridinium

FP_4	H	H	CH_3	3-hydroxypyridinium

Fumonisins are soluble in polar solvents such as water, acetonitrile, and methanol (Pietri & Bertuzzi, 2012). They are thermally stable and can resist in foodstuffs even at 250°C (Bryła et al., 2017).

1.3.1 Occurrence

Fumonisins are mycotoxins that can be found in different agricultural commodities depending on the geographical region and the processing methods. Geographical location, climate, agricultural practices, and storage conditions can influence the prevalence and levels of fumonisin contamination (Yli-Mattila & Sundheim, 2022). Corn is the main source of fumonisin exposure for humans (Braun & Wink, 2018). They tend to accumulate in corn kernels, mainly if the corn is grown under conditions favourable to fungal growth (D'Ovidio et al., 2007a). Consequently, corn-based products such as cornmeal, corn flour, corn chips, and tortillas can also be contaminated if they are made from fumonisin-contaminated corn (Zimmer et al., 2008). Corn in Southern Europe and the USA is more subject to fumonisin contamination (Braun & Wink, 2018). Corn contamination also includes the presence of high levels of fumonisins in gluten-free products made up of corn flour (Cendoya et al., 2018; Lo Magro et al., 2011). Processed foods containing corn ingredients, such as snacks, breakfast cereals, and baked goods, could potentially contaminate fumonisin if the raw materials used in their production were contaminated (Yoshinari et al., 2020; Zentai et al., 2019). Besides corn, other cereal grains like wheat, barley, and rice can also be contaminated with fumonisins. Contamination levels in these grains might be lower than in corn (Cendoya et al., 2018). However, it's still a concern, especially in areas where multiple crops are grown together (Farhadi et al., 2021). Fumonisins can enter the food chain through contaminated animal feed (Scott, 2012). When livestock consume feed contaminated with fumonisins, the mycotoxins can accumulate in animal tissues and products such as meat, milk, and eggs (Gazzotti et al., 2009; Y. Wang et al., 2021).FBs can also be detected in alcoholic beverages that use raw materials in fermentation that are contaminated with mycotoxins

(Kłosowski & Mikulski, 2010; Peters et al., 2017). Contaminated raw materials can influence the presence of fumonisins in oil obtained by oilseed (M. Yang et al., 2023). Spices are subjected to fumonisins contamination due to fungal growth during the drying and processing of these products (Al Ayoubi et al., 2021).

1.3.2 Toxicity

Fumonisin B1 is the most toxic fumonisin and is classified as a 2b carcinogen by IARC (International Agency for Research on Cancer, 1993). Fumonisins have different harmful effects on different species that are used as food sources, such as cattle, swine, sheep, and poultry (Gupta, 2018). The ingestion of fumonisins in horses causes species-specific syndromes called equine leukoencephalomalacia (ELEM) and porcine pulmonary oedema (PPE) (Marasas, 2001). FB1 in rodents showed carcinogenic activity, and a higher incidence of liver and kidney tumours was observed after FB1 oral administration (Qian et al., 2016; Xue et al., 2018). Fumonisins can damage other organs, such as the lungs, heart, and intestine (Jennings et al., 2020; Missmer et al., 2006; Norred et al., 1992; Terciolo et al., 2019; Voss et al., 2007). However, the correlation between cancer and fumonisins consumption is insufficient because other mycotoxins or factors can often occur (Braun & Wink, 2018; Come et al., 2019). The toxicity is caused by different mechanisms that include interference with the metabolism of sphingolipids, alteration of DNA methylation, and oxidative stress (X. Liu et al., 2019). Fumonisins have a chemical structure similar to sphinganine and sphingosine; therefore, they can interfere with their synthesis (Merrill et al., 2001). They can inhibit ceramide synthase and compete with fatty acyl-CoA, leading to cell signalling disruption and disease (Riley & Merrill, 2019). Research has shown that exposure to fumonisins during pregnancy can adversely affect neural tube development in the developing fetus (Voss & Riley, 2013). Neural tube defects (NTDs) are congenital severe malformations that result from the improper closure of the neural tube during early embryonic development; they can lead to severe neurological disabilities or even be fatal (Greene & Copp, 2014). Fumonisins can inhibit

dihydrofolate reductase (DHFR), which is essential for folic acid metabolism, and the folate receptor for the uptake of folic acid (Wolf, 1998). A positive association between fumonisins intake and growth impairment was found in different epidemiological studies where the consumption of food contaminated with FB1 is high (C. Chen et al., 2018; J.-G. Chen et al., 2021).

1.3.3 Regulatory limits in food

Fumonisins MLs are set in Europe by the EC Regulation 2023/915 (Table 3). Only FB1 and FB2 are considered and expressed as a sum among all fumonisins. The lowest limit is in baby food containing maize, while the highest is in unprocessed maize grains. The range is between 200 and 4000 µg/kg. The Food and Drug Administration (FDA) also includes FB3 in the sum of fumonisins, and the range is from 2 to 4 ppm, depending on the final consumers (Nutrition, 2023).

Table 4 MLs for fumonisins in Annex I of the regulation (EU) 2023/915 (Commission Regulation (EU) 2023/915, 2023).

1.6	**Fumonisins**	**Maximum level (µg/kg)**	**Remarks**
		Sum of B_1 and B_2	For the fumonisins, maximum levels refer to lower bound concentrations, which are calculated on the assumption that all the values below the limit of quantification are zero.
1.6.1	Unprocessed maize grains	4 000	Except unprocessed maize grains for which it is evident e.g. through labelling, and destination, that it is intended for use in a wet milling process only (starch production). The maximum level applies to unprocessed maize grains placed on the market before first-stage processing.
1.6.2	Maize placed on the market for the final consumer, milling products of maize placed on the market for the final consumer, maize-based food placed on the market for the final consumer except products listed in 1.6.3 and 1.6.5	1 000	
1.6.3	Maize-based breakfast cereals and	800	

	maize-based snacks		
1.6.4.1	Maize flour not placed on the market for the final consumer	2000	At least 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 µm.
1.6.4.2	Other milling products of maize not placed on the market for the final consumer	1400	Less than 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 µm.
1.6.5	Baby food containing maize and processed maize-based food for infants and young children	200	The maximum level applies to the dry matter of the product as placed on the market.

1.4 Trichothecenes

Trichothecenes are a group of mycotoxins that include more than 200 different compounds (Janik, Niemcewicz, Podogrocki, Ceremuga, Stela, et al., 2021; McCormick et al., 2011). They are produced by different fungi genera such as *Trichothecium, Trichoderma, Fusarium, and Myrothecium* (McCormick et al., 2011). A sesquiterpenoid structure from the precursor 12,13-epoxytrichothec-9-ene characterizes them. Trichothecenes have been classified into four types depending on the chemical structures: type A, B, C, and D (McCormick et al., 2011). The classification is based on the substituent in C-8. Type A trichothecenes can have a R-OH group, R-O-R, or no functional group at C-8. Type B trichothecenes have a carbonyl group at C-8, type C trichothecenes have an epoxide in C-7/C-8, while type D trichothecenes have a ring that links C4 and C15 (Ueno, 1984). The same fungi can produce different types of trichothecenes (Schollenberger et al., 2007). However, this classification has limitations because different chemical structures and functional groups can exist among the same type of trichothecenes. They are characterized by a small structure with amphipathic properties, two properties that increase their oral absorption and toxicity (Karlovsky et al., 2016). The most toxic and studied compounds among trichothecenes are T2, HT2 and deoxynivalenol (DON or vomitoxin) (Janik, Niemcewicz, Podogrocki, Ceremuga, Stela, et al., 2021). T2 and HT2 are trichothecenes of type A, while DON is a trichothecene of type B. Other compounds included in this class of trichothecenes are nivalenol, diacetoxyscirpenol, 3-acetyl-DON and 15-Ac-DON.

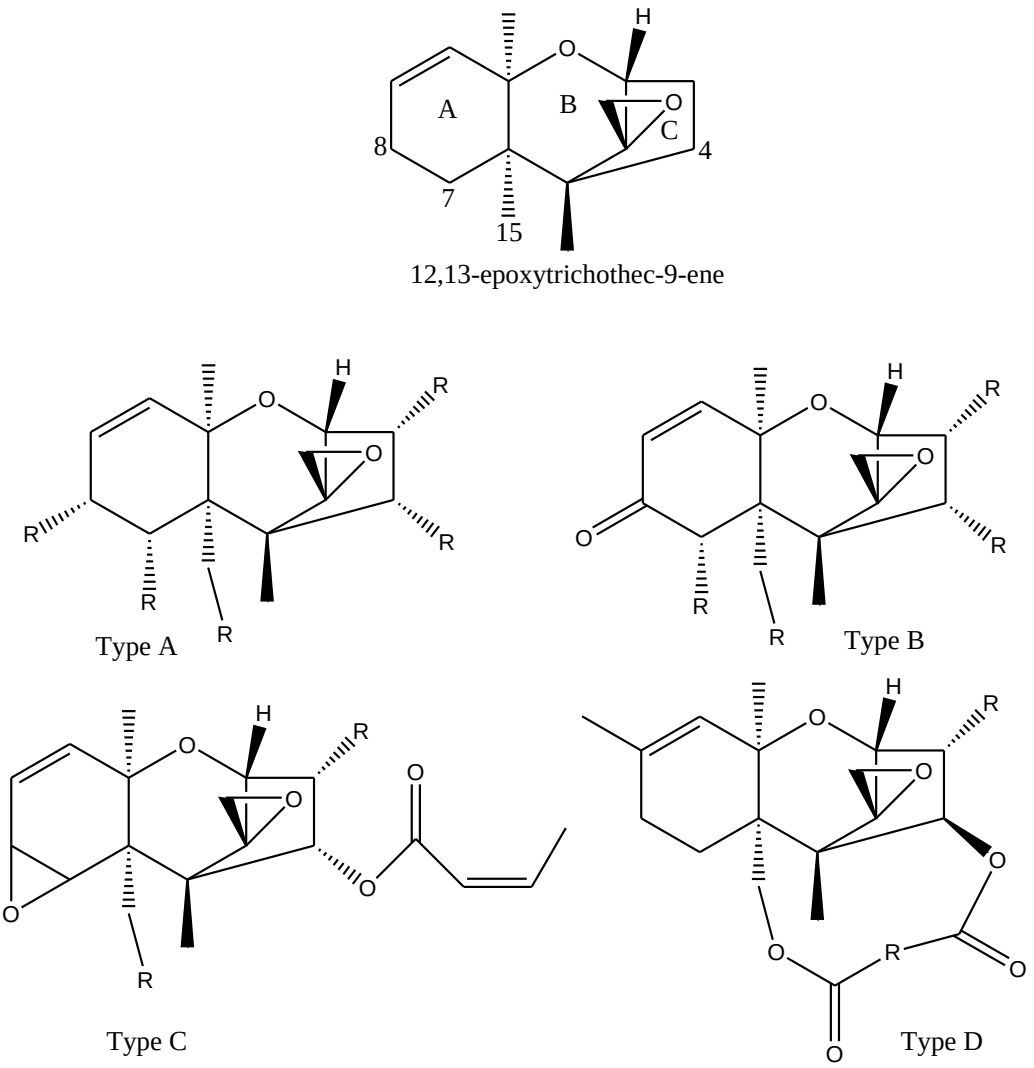


Fig. 4 Chemical structures of 12,13-epoxytrichothec-9-ene (Bräse et al., 2013b).

1.4.1 Occurrence

The contamination of trichothecenes is variable and depends on numerous factors. The climatic conditions such as humidity, temperature, and rain events significantly impact the growth of toxigenic species and, therefore, the presence of trichothecenes (Adhikari et al., 2017; Fink-Gremmels & van der Merwe, 2018; Toregeani-Mendes et al., 2011). T2, HT2, and DON toxins can be found in several food commodities such as cereals (wheat, maize, bearley, oats), animal feed, nuts (peanuts, pistachios, hazelnuts), oilseeds (sunflower seeds, soybeans, rapeseed), coffee, and spices (paprika, chilli peppers) (Authority (EFSA) et al., 2017; Meneely et al., 2023; Zinedine et al.,

2006). Cereals represent the most common foodstuffs contaminated with T2 and HT2; a study in Morocco found the presence of HT2 and T2 in 30% of the wheat and 20% of spices analyzed (Zinedine et al., 2006). The most common contaminated food with the presence of DON is wheat, and the presence of DON in cereals has been reported worldwide (Mishra et al., 2020). A survey conducted by EFSA in 2017 in Europe on the presence of T2 and HT2 in foodstuffs found that the highest mean concentration was found in “Grains for human consumption” and in oat products (Authority (EFSA) et al., 2017). This is dangerous because T2 and HT2 are compounds that can resist even heat treatment, such as baking and cooking (Van Der Fels-Klerx & Stratakou, 2010). Heat treatment such as baking, boiling, and frying can decrease the presence of DON in cereal-based products (Q. Wu, Kuča, et al., 2017). Other cereals, such as rye and maize, can be contaminated with T2 and HT2 (Kosicki et al., 2020; Maragos, 2023). Therefore, various processed foods containing cereal grains, such as pasta, snacks, and baked goods, may carry T2 and HT2 mycotoxins (Cano-Sancho et al., 2011). Animal feed can contain T2 and HT2; subsequently, these trichothecenes can contaminate products derived from animals, such as meat and eggs (Emmanuel K et al., 2020; Pleadin et al., 2021). Other products that can contain trichothecenes are spices and alcoholic beverages (beer, whiskey) (Negedu et al., 2018; Ostry et al., 2020).

1.4.2 Toxicity

All trichothecenes interfere with the synthesis of protein, with RNA and DNA replication, with mitochondrial respiration and increase the oxidative status of the organisms (Arunachalam & Doohan, 2013; Cai et al., 2020; Karacaoğlu & Selmanoğlu, 2017; J. Li et al., 2022). The trichothecenes, due to their chemical structure, can interfere with the translational mechanism of the 60S subunit (Mostrom, 2011). The presence of the different functional groups in C-3 and C-4 changes the phase of the protein synthesis that they can inhibit (W. Wang et al., 2021). Trichothecenes can increase the activation of the mitogen-activated protein kinases (MAPKs) by inducing apoptosis and pro-inflammatory responses (Arunachalam & Doohan, 2013). Immunomodulation activities characterize trichothecenes; they can increase or have

immunosuppression properties (Q. Wu, Wang, et al., 2017). This effect seems correlated with the time of exposure and dose. Low doses over a long period can have immunostimulation properties, while high doses in low time induce leukocyte apoptosis and, therefore, an immunosuppressive effect (Bondy & Pestka, 2000; Clarke et al., 2015; Q. Wu, Wang, et al., 2017). The ingestion of trichothecenes is responsible for weight growth suppression (Wan et al., 2015). This is due to several mechanisms: an increase of neurotransmitters that increase emesis (5-H5), the activation of the nucleus tractus solitarus (NTS) with anorexigenic activities, induction of proinflammatory cytokines that cause anorexia, and activation of vagal afferent axons (Pestka, 2010; Terciolo et al., 2018; W. Wu et al., 2013, 2016). Trichothecenes, therefore, can cause hematologic and immunologic alterations in animals (McCormick et al., 2011; Munkvold, 2017; Parent-Massin, 2004). DON can cause high economic loss, especially in swine industries, due to emesis after feed-contaminated ingestion and weight growth stops (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2018; Pestka, 2007). DON can cause alteration in the reproductive cycle of animals and interfere with the hormonal cycle in both ovarian and testicular development and function (Pinto et al., 2022). Regarding human health, DON has been associated with different mycotoxicosis in Asia. The consumption of DON-contaminated wheat caused gastrointestinal and neurological symptoms (C. Chen et al., 2019). In humans, T2 and HT2 are responsible for alimentary toxic aleukia (ATA) (Meneely et al., 2023). This syndrome is characterized by different symptoms that include anaemia, convulsions, and even death (Edwards, 2009). Epidemiological studies associate the presence of the Kashin-Beck disease with ingesting T-2 toxin (D. Li et al., 2016). This syndrome is characterised by movement impairments, fatigue, and cartilage damage (Ning et al., 2023). A chemical agent called “yellow rain” contains T2 toxin and has been used in Afghanistan, Laos and Vietnam as a biological warfare agent; T2 toxin can cause skin injury at lower doses than sulfur mustard (Etemad et al., 2023). The mortality rate of the person exposed was over 10%, and death occurred before a few weeks (Tucker, 2001).

1.4.3 Regulatory limits in food

Due to trichothecene toxicities, their presence has been regulated in food worldwide. There is often a co-occurrence of T2 and HT2 toxins. Therefore, the regulation considers the sum of these together. The EU Commission (2013/165/EU) on the presence of T-2 and HT-2 toxins in cereals and cereal products established the indicative levels for grains and cereals products intended as the sum of T2 and HT2. The indicative levels can be seen in Table 4. However, these limits may change as they are still discussed. Maximum Limits that may be established in the future can be seen in Table 4 (Meneely et al., 2023).

Table 5 Annex I of the Commission Recommendation (2013/165/EU) on the presence of T-2 and HT-2 toxin in cereals and cereal products (Commission Regulation (EU) 2023/915, 2023).

1.	**Unprocessed cereals**	**Indicative levels (µg/kg)**	**Maximum Limits under Discussion**
1.1	barley (including malting barley) and maize	200	100
1.2	oats (with husk)	1000	500
1.3	wheat, rye and other cereals	100	50
2.	**Cereal grains for direct human consumption**		
2.1	Oats	200	50
2.2	Maize	100	50
2.3	Other cereals	50	20
3.	**Cereal products for human consumption**		
3.1	Oat bran and flaked oats	200	50
3.2	cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100	50
3.3	other cereal milling products	50	20
3.4	breakfast cereals including formed cereal flakes	75	20
3.5	bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25	10
3.6	cereal-based foods for infants and young children	15	10
	Cereal products for feed and compound feed		
4.1	Oat milling products (husks)	2000	
4.2	Other cereal products	500	
4.3	Compound feed, with the exception of feed for cats	250	

Regarding DON, it is regulated by the Commission Regulation (EU) 2023/915. The maximum levels allowed in foodstuffs are listed in Table 5. The MLs in 1.41, 1.4.2, and 1.4.3 refer to “first-stage processing” that does not include cereals under physical or thermal treatment other than drying, cleaning, and sorting.

Table 6 Annex I of the Commission Regulation (2023/915/EU) on maximum levels for DON in food (Commission Regulation (EU) 2023/915, 2023).

1.4	**Deoxynivalenol**	**ML (µg/kg)**	**Remarks**
1.4.1	Unprocessed cereal grains except products listed in 1.4.2 and 1.4.3	1 250	Except unprocessed maize grains intended to be processed by wet milling and except rice. The maximum level applies to unprocessed cereal grains placed on the market before first-stage processing (6).
1.4.2	Unprocessed durum wheat grains and oat grains	1 750	The maximum level applies to unprocessed cereal grains placed on the market before first-stage processing (6).
1.4.3	Unprocessed maize grains	1 750	Except unprocessed maize grains for which it is evident e.g. through labelling or destination, that they are intended for use in a wet milling process only (starch production). The maximum level applies to unprocessed maize grains placed on the market before first-stage processing (6).
1.4.4	Cereals placed on the market for the final consumer, cereal flour, semolina, bran and germ as final product placed on the market for the final consumer except products listed in 1.4.7 and 1.4.8	750	Except rice and rice products.
1.4.5	Pasta	750	Pasta means pasta (dry) with a water content of approximately 12 %.
1.4.6	Bread, pastries, biscuits, cereal snacks and breakfast cereals	500	Except rice products. Including small bakery wares.
1.4.7	Milling products of maize not placed on the market for the final consumer		
1.4.7.1	Maize flour not placed on the market for the final consumer	1 250	At least 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 µm.
1.4.7.2	Other milling products of maize not placed on the market for the final consumer	750	Less than 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 µm.
1.4.8	Baby food and processed cereal-based food for infants and young children (3)	200	Except rice products. The maximum level applies to the dry matter (5) of the product as placed on the market.

1.5 Zearalenone

Zearalenone, often abbreviated as ZEA, is a mycotoxin belonging to the group of compounds known as fusariotoxins; the name refers to the mould species that produce ZEA that belong to the *Fusarium* genus (Guerre, 2015). ZEA is particularly interesting due to its potential health risks and occurrence

as a contaminant in various food and feed commodities. It is a mycotoxin among the group of resorcylic acid lactones (RALs). A resorcinol ring system with a lactone ring and a phenolic hydroxyl group characterizes it (Galaverna & Dall'Asta, 2012). The resorcylic acid core includes two benzene rings connected by a diene structure. It represents the core of its biological activity (Patocka et al., 2013). The lactone ring imparts stability to the molecule, and it is one of the main reasons why ZEA is characterised by estrogenic activity and multiple hydroxyl groups (-OH) (Balló et al., 2023). ZEA has a chemical structure resembling estrogen, a hormone in humans and animals. This structural similarity allows ZEA to interact with estrogen receptors, classifying it as an endocrine-disrupting compound (Kowalska et al., 2016).

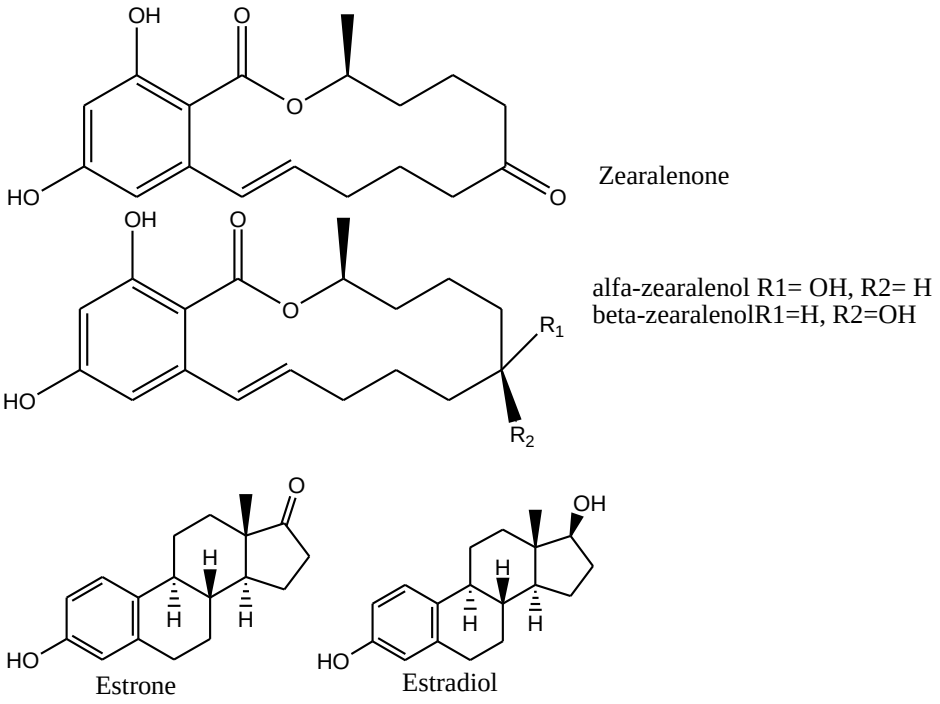


Fig. 5 Chemical structures of ZEA and its reduced form. Their estrogen activity is correlated with the chemical structures similar to estrogen.

1.5.1 Occurrence

The occurrence of Zearalenone in cereals is a widespread issue that has significant implications for both human health and agricultural productivity. ZEA can contaminate cereal crops worldwide, including wheat, maize, barley, oats, and sorghum (Yu et al., 2022a). The percentage of positive samples

varies across different regions. In Slovenia the 24% of the produced wheat was contaminated with ZEA, with an enormous economic loss (Kirinčič et al., 2015). A survey of 10 years found that the 46% of raw cereals are contaminated with ZEA (H. J. Lee & Ryu, 2017), with higher incidences in Africa and Europe, followed by America e Asia. These variations in positive percentages can be attributed to the different agricultural practices, storage methods, and climatic conditions in those regions (Leslie et al., 2021; Topan et al., 2023). Environmental factors play a crucial in the occurrence of ZEA in cereals. High humidity and elevated temperatures during cereal crops' flowering and grain development stages create favorable conditions for *Fusarium* infection and subsequent mycotoxin production (Mahato et al., 2021). Furthermore, the improper storage of cereals can also contribute to the occurrence of ZEA contamination. When cereals are not stored in optimal conditions, such as in humid environments or with inadequate temperature control, it can create an ideal environment for the growth of *Fusarium* fungi and the production of ZEA (Topan et al., 2023). Feed samples are the most common cereal-based products contaminated by ZEA. A study in China found the presence of ZEA in the 96.9% of the feed samples analyzed, including 3,499 samples with mean concentrations ranging from 48.1 to 326.8 µg/kg (Zhao et al., 2021, pp. 2018–2020). This leads to its presence in meat and dairy products, as animals consuming contaminated feed can metabolize and transfer the toxin into their tissues and milk (Falkauskas et al., 2022). ZEA contamination has been reported in barley used for brewing, which can lead to the presence of ZEA in beer where the brewing process does not eliminate mycotoxins like ZEA entirely, in pasta and noodles made with wheat or other cereals that contain ZEA (Mizutani et al., 2011; Ok et al., 2014).

1.5.2 Toxicity

The mechanisms of toxicity of ZEA are multifaceted and can vary depending on the specific cell types involved. ZEA is a mycotoxin that exhibits estrogen-like activity. Studies have shown that Zearalenone can bind to estrogen

receptors in cells, leading to disruption of hormonal regulation (Hueza et al., 2014). This disruption can affect reproductive processes, as well as other physiological and biochemical functions regulated by estrogen (Y. Li et al., 2012). Numerous studies have shown that ZEA has the ability to directly bind to estrogen receptors in cells, leading to disruption of hormonal regulation and an imbalance in the estrogenic signaling pathway. This disruption can have various detrimental effects on the body, including reproductive disorders, such as abnormal estrus cycles and reduced fertility in animals (Minervini & Dell'Aquila, 2008; Zheng et al., 2019). For these reasons, ZEA and its metabolites (α-zearalenol, α-zearalanol, β-zearalanol, β-zearalenol) are considered endocrine-disrupting chemicals or mycoestrogens (Anne & Raphael, 2000). In recent years, research has been conducted to understand the molecular mechanism of toxicity of ZEA and to assess its effects on human health (Kowalska et al., 2020). In addition to its estrogenic activity, ZEA has also been found to exhibit carcinogenic properties (Han et al., 2022). The IARC classified ZEA as the first Class 3 carcinogen, and studies in vivo indicate that ZEA can induce apoptosis and show genotoxicity by DNA fragmentation (International Agency for Research on Cancer, 1993). It may induce cancer and proliferation of breast cancer (Ahamed et al., 2001; Cai et al., 2019). Additionally, ZEA has been linked to the development of certain types of cancer and has been shown to have immunotoxic effects, compromising the body's immune system (Lecomte et al., 2019). In fact, it inhibits the T cell-mediated immune responses and induces the apoptosis of T lymphocytes (Cai et al., 2020, 2020; Hueza et al., 2014). ZEA has been identified as genotoxic, which indicates its ability to cause damage to DNA and potentially result in genetic mutations. Its toxic effects include the induction of chromosomal aberrations, creating DNA adducts, inducing lipid peroxidation and cellular apoptosis and inhibiting DNA synthesis (Han et al., 2022)

1.5.3 Regulatory limits in food

The maximum permissible levels of ZEA in foodstuffs to guarantee consumer safety have been set by European Regulations (EC) 2023/915. In the European Union, the maximum permitted levels for ZEA in various food

products range from 20 (in food destined for infants and children) to 350 µg/kg (in unprocessed maize grains). The limits are applicable in raw materials before the first stage of processing, which means any physical or thermal treatment, other than drying, of or on the grain.

The maximum limits change in various countries. In Brazil, wheat products have a maximum limit of 100 µg/kg, while in Ukraine and Russia, the limit is 1 mg/kg (Yu et al., 2022a). To date (October 2023), there are no specific legal regulations regarding zearalenone in the United States.

Table 7 Annex I of the Commission Recommendation (2013/165/EU) on the presence of ZEA toxin in cereals and cereal products (Commission Regulation (EU) 2023/915, 2023).

1.5	**Zearalenone**	**Maximum level (µg/kg)**	**Remarks**
1.5.1	Unprocessed cereal grains except products listed in 1.5.2	100	Except unprocessed maize grains intended to be processed by wet milling and except rice. The maximum level applies to unprocessed cereal grains placed on the market before first-stage processing.
1.5.2	Unprocessed maize grains	350	Except unprocessed maize grains for which it is evident e.g. through labelling, destination, that it is intended for use in a wet milling process only (starch production). The maximum level applies to unprocessed maize grains placed on the market before first-stage processing.
1.5.3	Cereals placed on the market for the final consumer, cereal flour, semolina, bran and germ as final product placed on the market for the final consumer except products listed in 1.5.5, 1.5.6 and 1.5.8	75	Except rice and rice products.
1.5.4	Bread, pastries, biscuits, cereal snacks and breakfast cereals except products listed	50	Except rice products.

	in 1.5.5		Includes small bakery wares.
1.5.5	Maize placed on the market for the final consumer Maize-based snacks and maize-based breakfast cereals	100	
1.5.6	Milling products of maize not placed on the market for the final consumer		
1.5.6.1	Maize flour not placed on the market for the final consumer	300	At least 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 μm.
1.5.6.2	Other milling products of maize not placed on the market for the final consumer	200	Less than 90 %, measured by weight, of the particles in the milling product have a size ≤ 500 μm.
1.5.7	Refined maize oil	400	
1.5.8	Baby food and processed cereal-based food for infants and young children	20	Except rice products. The maximum level applies to the dry matter of the product as placed on the market.

2. Determining mycotoxins in food

2.1 Analytical techniques

Analytical techniques play a crucial role in analyzing mycotoxins in foodstuffs and feed. The presence of mycotoxins in food can pose a significant health risk to humans, making it imperative to accurately detect and quantify these toxins (Alshannaq & Yu, 2017). Several analytical methods have been developed for the determination of mycotoxins in food, animal feed, dairy products, and other contaminated materials (Janik, Niemcewicz, Podogrocki, Ceremuga, Gorniak, et al., 2021). These methods include TLC (thin-layer chromatography) (Betina, 1985), competitive ELISA (enzyme-linked immunosorbent assay) (Maggira et al., 2022), capillary GC/MS (gas chromatography/mass spectrometry) (Colombo & Papetti, 2020; Rodríguez-Carrasco, Moltó, Mañes, et al., 2014), CE/MS (capillary electrophoresis/mass spectrometry), and HPLC (high-performance liquid chromatography) with fluorescence detection (Irakli et al., 2017a; Kecskeméti et al., 2020).

One important advancement in the field of mycotoxin analysis is the development and utilization of liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques. LC-MS/MS has emerged as a sensitive, rapid, and highly automated system for quantifying and identifying mycotoxins in food samples (Herebian et al., 2009). The use of LC-MS/MS in mycotoxin analysis has become increasingly popular due to its high sensitivity and ability to detect multiple mycotoxins in a single analysis (Martos et al., 2010; Pantano et al., 2021). LC-MS/MS allows for the simultaneous determination and accurate quantification of multiple mycotoxins in complex matrices such as foodstuffs and feeds (Kongkapan et al., 2016). It eliminates the need for separate analyses for each mycotoxin, saving time and resources, and provides a reliable assessment of mycotoxin contamination in foodstuffs and feed by allowing for the detection of mycotoxins at low levels. However, it has some drawbacks. A proper pretreatment for the extraction and clean-up of mycotoxins is required to ensure the accuracy and sensitivity of the assay, especially for detecting trace amounts of analyte in a complex matrix (Xiong et al., 2015).

2.2 Chromatographic separation techniques for determination of mycotoxins in food: High-Performance Liquid Chromatography

One key analytical technique used for the analysis of mycotoxins in foodstuffs and feed is liquid chromatography. It is a popularly used technique for the separation and analysis of mycotoxins in food and feed samples (Alshannaq & Yu, 2017). It is a powerful analytical technique that utilizes the differential migration of components in a liquid mobile phase through a stationary phase to separate and quantify mycotoxins. This mobile phase is liquid, and it is typically a mixture of solvents, which carries the analytes through a stationary phase. The stationary phase, usually a solid or liquid-coated material, serves as a barrier that interacts with the analytes based on their chemical properties, allowing for their separation (Bakalyar, 1981).

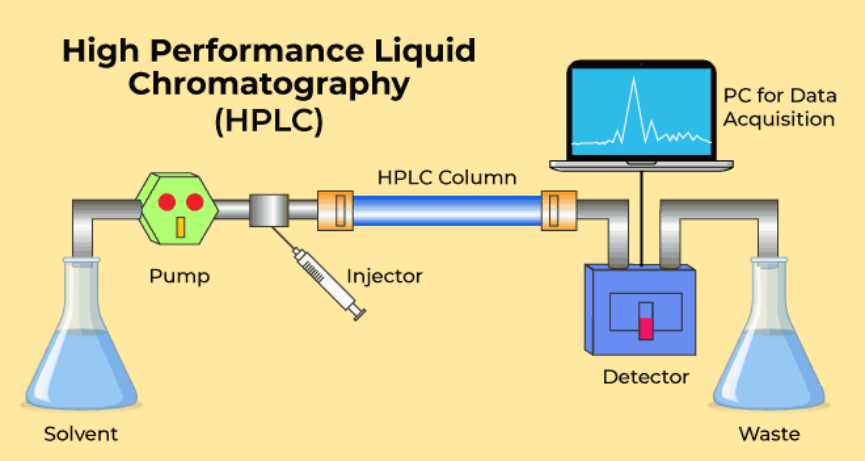


Fig. 6 General equipment for HPLC. Credits: **(«Chromatography», 2021)**

Normally, the term “normal-phase chromatography” refers to the combination of a polar stationary phase and a nonpolar mobile phase, while in “reversed-phase” chromatography, the stationary phase is characterized by a nonpolar affinity while the mobile phase is polar. The separation of the analytes in the stationary phase can occur with different electrostatic charges, sizes, and affinity (Rusli et al., 2022). The most common for analyzing mycotoxins is the differences in the affinity to the compounds between the stationary and mobile phases (Khayoon et al., 2010; Lattanzio et al., 2007a).

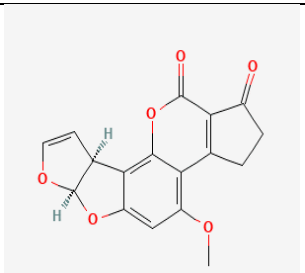
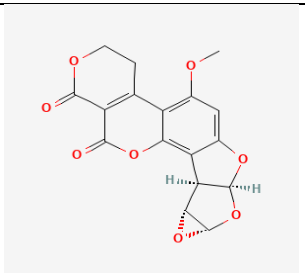
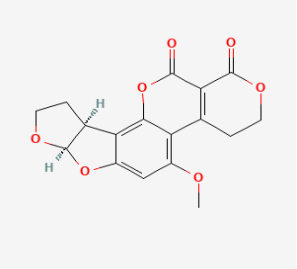
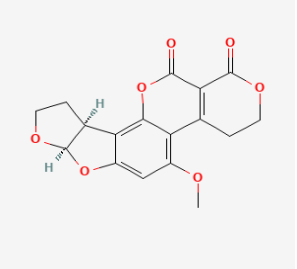
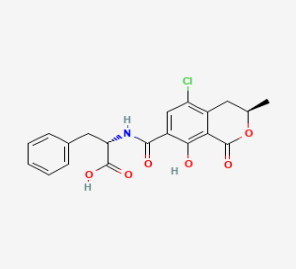
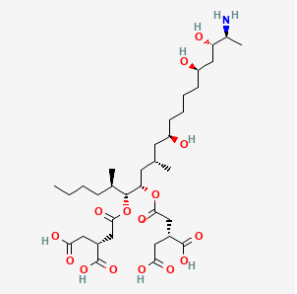
The standard column used for the mycotoxin analysis contains silica particles

functionalized with octadecyl (C18). This phase is ideal for separating aflatoxins because it provides good retention, separation, and selectivity for these compounds (Bi et al., 2022; Guo et al., 2023). The HPLC system, coupled with different revelators, allows the analysis of different mycotoxins together, and the introduction of multi-mycotoxin analytical methods is useful for analyzing and separating mycotoxins with different chemical structures (Hickert et al., 2015; Sarkar et al., 2022; Spanjer et al., 2008).

The mobile phase's composition is chosen during the development of the method. Generally, a common mobile phase is a mixture of hydrophilic and organic mobile phases that are water, acetonitrile, or methanol (Nakhjavan et al., 2020). In mycotoxin analysis, a gradient elution is often used, where the composition of the mobile phase changes during the chromatographic run. This is particularly beneficial for separating mycotoxins that are characterized by different polarities, as shown in **Table 5**. The gradient typically starts with a higher proportion of water. It gradually transitions to a higher percentage of the organic solvent (Keskin & Eyupoglu, 2023; Kiseleva et al., 2020). The binary system used in HPLC methods, such as a mixture of water and acetonitrile, ensures efficient elution and separation of mycotoxins (Salim et al., 2021). Acetonitrile acts as an organic solvent that helps dissolve the mycotoxins, while water serves as a polar component to facilitate the interaction of the analytes with the stationary phase (G. Ren et al., 2018). Other variations may use a ternary solvent system, which includes an additional modifier or buffer to adjust the pH and improve the separation of mycotoxins (Pernica et al., 2019).

In the field of mycotoxin analysis, a range of detectors are employed in high-performance liquid chromatography, including UV-VIS detectors (HPLC-UV), fluorescence detectors (HPLC-FLD), and mass spectrometry detectors (HPLC-MS) (Keskin & Eyupoglu, 2023; Salim et al., 2021). UV-VIS detectors rely on the absorption of ultraviolet or visible light by mycotoxins, while fluorescence detectors measure the emission of fluorescent light following excitation at a specific wavelength (Irakli et al., 2017a; Mochamad & Hermanto, 2017).

Aflatoxins and other mycotoxins possess natural fluorescence, a characteristic that can be used for analytical determination (Singh & Mehta, 2020).

Analyte	Chemical structure	pKa and Log P at pH 3/pH 7	Analyte	Chemical structure	pKa and Log P at pH 3/pH 7
AFB1	 $C_{17}H_{12}O_6$	pKa: 17.79/-4.4 Log P: 0.45	AFG1	 $C_{17}H_{12}O_8$	pKA: /-4.4 LogP: 1.81/1.37
AFB2	 $C_{17}H_{14}O_7$	pKa: 17.79 ± 0.1 /-4.1 log P: 1.63 / 1.57	AFG2	 $C_{17}H_{14}O_7$	pKA: /-1.6 LogP: 3.71/2.68
OTA	 $C_{20}H_{18}ClNO_6$	pKa: 3.29 ± 0.1 /-2.20 ± 0.4 log P: 4.41 / 1.10	FB1	 $C_{34}H_{59}NO_{15}$	pKa: 3.64 ± 0.23 / 9.24 ± 0.39 Log P: - 0.61/-3.23

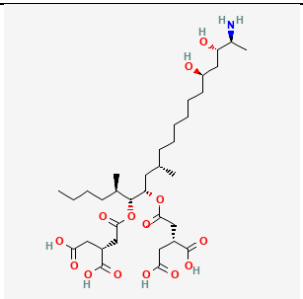
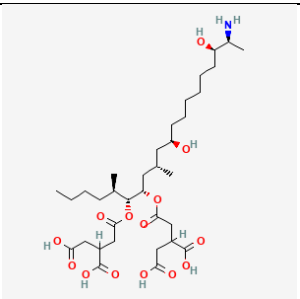
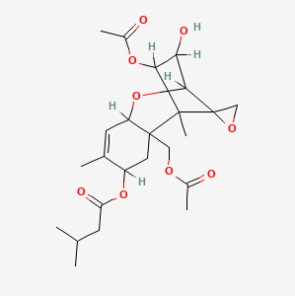
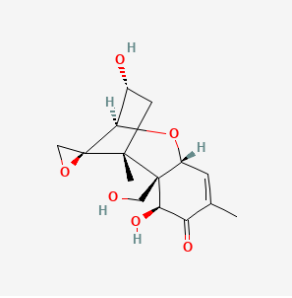
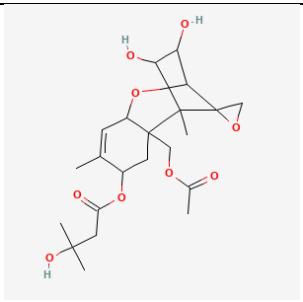
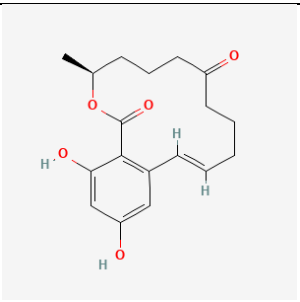
FB2	 $C_{34}H_{59}NO_{14}$	pKa: 3.64 ± 0.23 / 9.25 ± 0.39 Log P: 1.58 / -1.04	FB3	 $C_{34}H_{59}NO_{14}$	//
T2	 $C_{24}H_{34}O_9$	pKa: 13.23 ± 0.7 Log P: 2.25 / 2.25	DON	 $C_{15}H_{20}O_6$	pKa: 11.91 ± 0.7 Log P: -1.41 / -1.41
HT2	 $C_{22}H_{32}O_9$	pKa: 13.26 ± 0.7 Log P: 2.27 / 2.27	ZEA	 $C_{18}H_{22}O_5$	pKa: 7.41 ± 0.4 Log P: 3.83 / 3.72

Table 8 Chemical structure and pKA-values of mycotoxins analysed. From: **(Lauwers et al., 2019)**

2.3 Mass spectrometry (MS): triple quadrupole

Mycotoxin analysis has experienced significant advancements with the integration of mass spectrometry (MS). This analytical technique plays a crucial role in identifying and quantifying mycotoxins in complex matrices. By detecting and measuring the mass-to-charge (m/z) ratio of ionized mycotoxin molecules, MS provides accurate results for researchers. There are different mass analyzers, single quadrupole (Q) or triple quadrupoles (QqQ), ion traps, and time of flights (TOF). All of them are used in mycotoxins analysis (Arroyo-Manzanares et al., 2021; Mavungu et al., 2009).

The first use of mass spectrometry in mycotoxin analysis dates back to 1971 when thin-layer chromatography (TLC) was coupled with a mass spectrometer for determining mycotoxins (Haddon et al., 1971). The first multi-method methods for analyzing mycotoxin in samples were reported thirteen years in 1984 published a multi mycotoxins method for the analyses of aflatoxins (B_1, B_2, G_1, G_2, M_1 and M_2) in corn (Plattner et al., 1984). Since then, mass spectrometry has become increasingly important in the field of mycotoxin analysis (Sforza et al., 2006; G. Shephard et al., 2011). The introduction of liquid chromatography (LC) as an analytical technique for separating compounds allowed a high throughput of sample analysis with different analytes with a single run. Furthermore, mass spectrometry coupled with liquid chromatography has the advantage of creating a multi-mycotoxins method to discover new mycotoxins in new matrices, such as fumonisins in figs and fumonisins in coffee beans (Moretti et al., 2005; Noonim et al., 2009). Moreover, high-resolution mass spectrometry (HRMS) can be used for retrospective analysis and to discover if new mycotoxins were present in samples already analysed (Castaldo et al., 2019).

One commonly used type of mass analyzer is the quadrupole, which consists of four parallel rods with circular cross-sections. These rods are arranged in a specific configuration, and varying voltages are applied to them to create an electric field (Mellon, 2003). Quadrupole analyzers operate based on the principle that ions with a specific mass-to-charge ratio can pass through the analyzer while others are filtered out.

This arrangement allows for the manipulation of ions based on their mass-to-charge ratios as they pass through the quadrupole analyzer. The quadrupole analyzer operates by applying a radio frequency (RF) and direct current (DC) voltage to the rods, creating a quadrupolar electric field. This electric field selectively filters ions, allowing only those with specific mass-to-charge ratios to pass through and reach the detector. Different ions can be selectively filtered by varying the RF and DC voltages, providing a method for accurate mass analysis (Hoffmann et al., 2011).

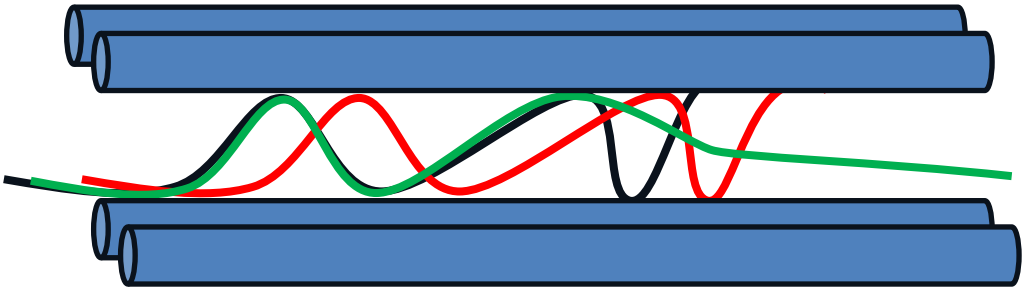


Fig. 7 Semplifed scheme of a quadrupole. Only the ions on the green trajectory will be detected by the detectors because they do not collide with the rods.

The direction of the ion entering the space between the rods depends on the charge. A positive ion will take the direction towards a negative rod; if it changes the charge during this trajectory, it will take another path. Applying a particular DC e RF voltage, the ions with the specific m/z will pass through the quadrupole without colliding with the rods (Punetha & Kotiya, 2023).

A triple quadrupole is a type of mass spectrometer composed of three sets of quadrupole mass filters, hence the name "triple quadrupole". Each set of quadrupole filters consists of four rods that create an electric field, allowing only desiderated ions of a specific mass-to-charge ratio (m/z) to pass through (Arnott, 2001).

The entire process of a triple quadrupole mass spectrometer involves three main steps: ionization, mass analysis, and detection. First, the sample is ionized, typically through techniques such as electron impact ionization or electrospray ionization. Molecules are converted into ions and are introduced into the mass spectrometer. In the mass analysis step, the first quadrupole filter acts as a mass filter, selecting ions of a specific m/z ratio from the ionized

sample. These selected ions are then directed towards a collision cell, where they undergo collision-induced dissociation (CID) or other fragmentation techniques. This fragmentation step breaks the ions into smaller fragments, allowing for further analysis and identification. The second quadrupole filter serves as a mass filter again, selectively transmitting only the fragments of interest. This step enables the isolation and analysis of specific fragments for further investigation. Finally, the third quadrupole filter acts as a mass filter or a detector, selectively detecting ions that match the desired m/z ratio (Hopfgartner et al., 2004).

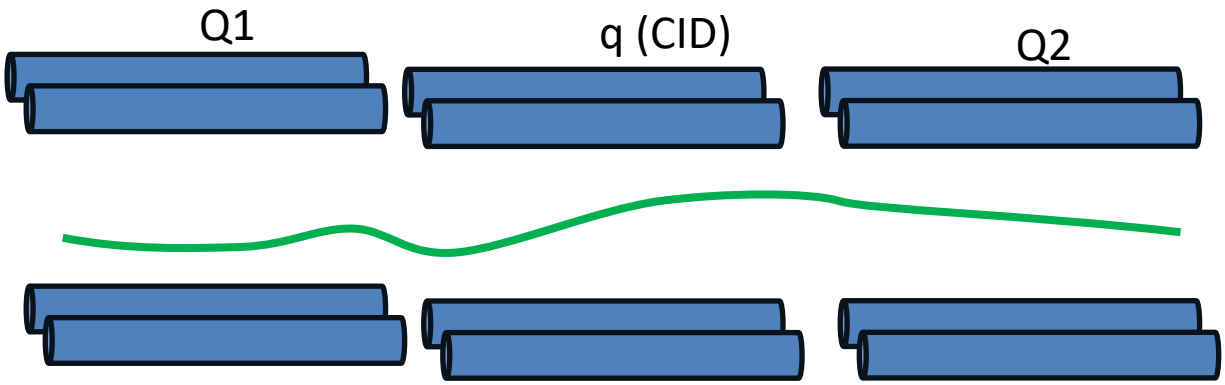


Fig. 8 Simplified scheme of a triple quadrupole. Q1 acts as a mass filter, q as a cell where molecules can be fragmented, and Q2 as a mass filter or as a detector.

The ability to perform targeted analysis of specific ions or fragments enhances the accuracy and precision of measurements. Additionally, the triple quadrupole can be used for structural analysis, as the fragmentation patterns obtained during CID can provide valuable information about the molecular structure of the analyzed compounds (Mary et al., 2012).

There are different ways of using a tandem mass spectrometer. However, the most common scan techniques are:

1. Reaction Monitoring (RM): RM is a technique used for targeted analysis and quantification of specific analytes in complex samples. It involves selecting a specific precursor ion in the first quadrupole filter, fragmenting it in the collision cell, and then monitoring one or more specific product ions in the third quadrupole filter. This technique provides high selectivity and sensitivity for the targeted analytes. It allows for the precise quantification of

trace-level compounds in complex matrices. When only a precursor is selected, it is called single reaction monitoring (SRM); when multiple precursors are selected, it is called multiple reaction monitoring (MRM) (Zainudin et al., 2022).

2. Precursor ion scan (PIS): to perform a precursor ion scan, the first quadrupole of the mass spectrometer is set to transmit a specific range of precursor ions, while the second quadrupole acts as a collision cell. The third quadrupole is set to transmit a specific range of product ions that result from the fragmentation of the selected precursor ions (Josephs, 1996).
3. Selected Ion Monitoring (SIM): SIM is another technique used in mass spectrometry for targeted analysis. It involves monitoring specific ions of interest without performing fragmentation. In SIM, the first quadrupole filter is set to transmit a narrow mass range, allowing the detection of specific ions in that range without fragmentation. SIM is commonly used for screening or profiling applications, where the focus is on detecting and identifying specific compounds rather than quantification. It provides high sensitivity and can be useful for analyzing low-abundance compounds or identifying unknown substances (Zöllner & Mayer-Helm, 2006).

All MS analyses are prone to the “matrix effect”. This phenomenon refers to the interference or influence of components present in a sample matrix on the ionization and detection of analytes during mass spectrometry analysis (Steiner et al., 2020). In the context of mycotoxin analyses using an MS, the presence of the sample matrix, which includes components like carbohydrates, lipids, proteins, and other co-extracted compounds, can affect the accuracy and reliability of the results (Kovač et al., 2022). The matrix effect in mycotoxin analysis can manifest in different ways. Some components of the sample matrix can lead to ion suppression or enhancement, affecting the ionization efficiency of mycotoxins. This can result in decreased sensitivity or inaccurate quantification if not properly accounted for during analysis (Malachová et al., 2014). The matrix components may cause shifts in the retention time and impact the identification and quantification of mycotoxins; they can contribute to the background noise or interfere with the detection of ions. The choice of a good extraction procedure can mitigate these effects.

2.4 Extraction process

2.4.1 Sample preparation

Sample preparation is a crucial step in mycotoxin analysis as it aims to ensure representative subsampling, minimize heterogeneity, and facilitate efficient extraction of mycotoxins from the sample matrix (Razzazi-Fazeli & Reiter, 2011). The first step in sample preparation is to ensure that the sample taken for analysis is representative of the entire lot or population being studied. In Europe, the sampling procedure is regulated by the Commission Regulation (EC) No 401/2006, which “laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs”. It does provide general guidelines for sampling and analysis methods (Commission Regulation (EC) No 401/2006, 2014; Commission Regulation (EC) n. 519/2014, 2014). For example, for cereals with lots between 300 and 1500 tons, the sampling plan shall be used with 100 incremental samples depending on the weight, resulting in an aggregate sample of 10 kg. Homogenization is essential to ensure that the subsample taken for analysis is uniform and representative. It involves thorough mixing of the sample to achieve a consistent distribution of mycotoxins throughout (Janik, Niemcewicz, Podogrocki, Ceremuga, Gorniak, et al., 2021; Spanjer et al., 2006). The homogenization technique used depends on the sample type and its physical properties. For solid samples, grinding or milling can be employed to reduce particle size and enhance homogeneity (Cheli et al., 2013). The particle size should be around 500 mm and mixed after the homogenization (Commission Regulation (EC) No 401/2006, 2014). For liquid or semi-solid samples, gentle mixing or shaking is typically sufficient (Spanjer et al., 2006).

2.4.2 Extraction

The choice of solvent in the extraction of mycotoxins is a critical factor that can significantly impact the efficiency and selectivity of the extraction process. The selection of an appropriate solvent depends on several factors, including the polarity of the mycotoxin(s) of interest, the sample matrix, the desired extraction efficiency, and the analytical method employed for

subsequent analysis. The extraction solvent is often a mixture of polar and not polar compounds such as methanol, acetonitrile, and water (Perestrelo et al., 2019; Salvi et al., 2017; Y. Yang et al., 2020). Mycotoxins can vary in their polarity, ranging from highly polar to non-polar compounds (see Table 7), and solvents with appropriate polarity are selected to extract the target mycotoxins from the sample matrix efficiently. For example, polar mycotoxins like DON are often extracted using polar solvents like aqueous solutions or organic solvents with high water content (Kappenberg & Juraschek, 2021), while less polar mycotoxins like aflatoxins may require more nonpolar organic solvents (Hutchins & Hagler, 1983). The mix of polar and organic solvents is also compatible with the analytical method. Some analytical techniques have specific requirements for the solvent used to extract mycotoxins. For example, high-performance liquid chromatography (HPLC) often requires the use of organic solvents like acetonitrile or methanol, while immunoaffinity column-based methods may require specific buffer solutions (Castaldo et al., 2019; Pantano et al., 2021; Rubert et al., 2011).

Several extraction techniques are employed for the extraction of mycotoxins from food and feed samples. These techniques aim to efficiently extract mycotoxins while minimizing interference from the sample matrix and achieving high extraction yields. The liquid-liquid extraction (LLE) or pressurized liquid extraction (PLE) with accelerated solvent extraction (ASE) use the different miscibility of mycotoxins in the aqueous and organic phases (Andrade et al., 2013; Miklós et al., 2020; Rubert et al., 2012). The most common extraction technique is QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) (Frenich et al., 2011; González-Curbelo et al., 2015; González-Jartín et al., 2019; Juan et al., 2017; Pereira et al., 2015; Perestrelo et al., 2019; Y. Yang et al., 2020). QuEChERS is a widely used extraction method for mycotoxin analysis in food and feed samples. It is known for its simplicity, cost-effectiveness, and ability to extract a wide range of mycotoxins efficiently. QuEChERS combines extraction and cleanup steps in a single process, providing a streamlined approach to sample preparation (Janik, Niemcewicz, Podogrocki, Ceremuga, Gorniak, et al., 2021). The sample is first homogenized, and then an extraction solvent, such as

acetonitrile or a mixture of acetonitrile and water, is added. Salt is added to induce phase separation, and the extract is centrifuged. The resulting supernatant is analysed after further cleanup with dispersive solid-phase extraction (dSPE) using sorbents like magnesium sulfate and primary-secondary amine (Pantano et al., 2021).

2.5 Purification process

In the extraction process, interferences from matrices are co-extracted due to the polar and non-polar characteristics of mycotoxins. Therefore, a purification process is needed to improve and enhance the results' recovery, accuracy, and reliability. Solid Phase Extraction (SPE) is a widely used technique for sample purification in mycotoxin analysis. It involves using solid sorbents, such as silica, C18, or immunoaffinity columns, to retain mycotoxins while unwanted matrix components are washed away selectively (Alshannaq & Yu, 2017; Razzazi-Fazeli & Reiter, 2011). The sample extract is passed through the SPE column, and the mycotoxins are retained based on their affinity to the sorbent. After washing away the interfering substances, the mycotoxins are eluted using an appropriate solvent for subsequent analysis. IAC (Immunoaffinity Chromatography) is a highly effective and specific purification technique widely used in mycotoxin analysis. It utilizes antibodies specifically designed to recognize and bind to target mycotoxins of interest. IAC offers excellent selectivity, enabling the removal of interfering substances from complex matrices, such as food and feed samples, while retaining the mycotoxins for subsequent analysis. IAC offers several advantages in mycotoxin purification, including high specificity, excellent cleanup of complex matrices, and minimal interference from co-extracted compounds (X. Liu et al., 2018). It allows for the selective extraction and enrichment of target mycotoxins, enabling sensitive and accurate quantification. However, IAC purification can be materially affected by organic solvents that can ruin the antibodies and have high operating costs (Castegnaro, Tozlovanu, et al., 2006; X. Liu et al., 2018).

2.6 Validation and analytical performance characteristics

The validation of an analytical method refers to the process of systematically evaluating and verifying the performance characteristics and reliability of the method for its intended purpose. It is critical to ensure that the method consistently produces accurate and reliable results. Validation provides scientific evidence that the method is fit for its intended use, whether for routine analysis, research, regulatory compliance, or quality control.

The term “validation” is explained in ISO/IEC 17025, ISO 15189 and ISO 15195, and it refers to “*the confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled”* (ISO 15195: 2018, 2018; ISO, 2005; Schneider et al., 2017). (Schneider et al., 2017).

In order to comprehensively evaluate the performance characteristics of an analytical method for mycotoxin analysis, several factors must be considered. These factors include specificity, accuracy, precision, linearity, limit of detection (LOD), and limit of quantitation (LOQ). Each of these parameters plays a crucial role in assessing the method's reliability and suitability for its intended purpose.

The Commission Regulation (EC) No 401/2006 provides guidelines for the validation of analytical methods used in mycotoxin analysis. These guidelines outline the recommended procedures, acceptance criteria, and documentation requirements for method validation, ensuring harmonized and standardized approaches in mycotoxin analysis.

- **Specificity** (or selectivity): this fundamental characteristic ensures the method's ability to accurately measure the target mycotoxin(s) in the presence of potential interferences or matrix components. It involves determining the method's selectivity by evaluating its response to closely related compounds or potential interfering substances commonly found in the sample matrix. The specificity can be assessed by analyzing fortified matrix samples with the target mycotoxins.
- **Cut-Off**: In analytical methods, the term "cut-off" refers to a predetermined threshold or limit used to distinguish between positive and negative results

or to determine the presence or absence of a particular analyte or substance (Valcárcel et al., 2002). The cut-off value is typically established based on scientific and regulatory considerations. The purpose of setting a cut-off value is to define a clear boundary that separates samples or measurements into distinct categories. For example, in mycotoxin testing, a cut-off value is established to determine whether a sample contains a concentration of mycotoxin above a certain level, indicating the presence of the substance in the specimen (Lattanzio et al., 2019). This cut-off value helps differentiate between positive and negative results, aiding in identifying mycotoxin-positive samples. This parameter is used in screening analysis where a certain concentration is not determined. The logistic regression classifies the sample as “positive” or “negative”. The “cut-off” is the concentration with a 5% probability of a false positive. Generally, it is assessed with 20 fortified samples at a concentration of MLs and 20 blank samples.

- **Working range**: the working range in an analytical method refers to the concentration range over which the method exhibits acceptable accuracy, precision, linearity, and sensitivity. It represents the range of analyte concentrations within which the method can reliably and accurately quantify the mycotoxins of interest («Uses (Proper and Improper) of Correlation Coefficients», 1988). The working range is typically determined with a regression model between the instrumental response and the analyte concentration. In LC-MS/MS, the model is linear and made with five points.
- **Accuracy:** accuracy refers to the closeness of the measured values to the true or reference values. It is typically assessed by analyzing known standards or certified reference materials and comparing the obtained results against the reference or confirmatory method. Accuracy evaluation allows for the determination of any bias or systematic error associated with the method. An example of measuring accuracy is recovery which can be expressed as coefficient variation (CV).

- **Precision:** is another critical parameter that assesses the method's repeatability and intermediate precision. Repeatability evaluates the degree of agreement between repeated measurements of the same sample under defined conditions. At the same time, intermediate precision assesses the agreement between measurements performed in different laboratories or by different analysts. Precision evaluation provides insights into the method's reliability and reproducibility. In mycotoxins analyses, the intermediate precision is made with the analysis of fortified samples on five different days (Regolamento (UE) n. 519/2014, 2014).

- **Linearity**: examines the method's ability to generate results that are directly proportional to the concentration or amount of the analyte(s) within a defined range. This parameter is typically assessed by analyzing a series of standards with known concentrations and plotting the response against the concentration. The resulting calibration curve allows for the determination of linearity, which is crucial for accurate quantification of mycotoxins.

- **Ruggedness and robustness:** are additional aspects that should be considered in method validation. Ruggedness refers to the method's reliability and reproducibility across different laboratories, analysts, and equipment. Robustness, on the other hand, assesses the method's ability to remain unaffected by small, deliberate variations in parameters, such as changes in pH, temperature, or sample preparation conditions. Evaluating ruggedness and robustness demonstrates the method's reliability in practical applications and ensures consistent results across different settings.

In addition, especially for mycotoxin analysis, assessing the “matrix effects” is mandatory. It refers to sample components' influence on the analyte's detection and quantification. It is necessary to assess and mitigate matrix effects through appropriate sample preparation and calibration strategies.

2. Research goals

This thesis aims to develop and validate an analytical method for the accurate analysis of mycotoxins in various commodity groups as specified by the EU Commission Regulation (EC) No 401/2006.

The newly implemented method will be utilized for official control purposes at the Istituto Zooprofilattico Sperimentale della Sicilia (IZS-Palermo), where real samples will be analyzed. The collected data will encompass sample types and analytical results from different commodity groups, providing crucial insights into the occurrence of mycotoxins in European cereals and other matrices.

The next steps will be the analysis of “meat-alternative” products made up of soy proteins and understanding the incidence of mycotoxins in these products. A comparative analysis will be conducted to identify potential differences among these matrices.

Finally, the primary objective of this research was to conduct a detailed quantitative analysis of various mycotoxins present in different food matrices. This involved measuring the levels of mycotoxins and how they are depredated in the most common matrices that compose alternatives to meat products. The effects of microwave cooking conditions were investigated. This analysis provided insights into how different cooking durations and intensities influence mycotoxin levels in various food matrices. To understand the variations, the concentrations of mycotoxins across different food matrices were compared. This comparison was crucial in identifying which food items demonstrated higher persistence or effective degradation of mycotoxins.

Chapter I: Developing an Analytical Method in LC-MS/MS

This chapter is a slightly modified version of (Pantano et al., 2021) published in open access in International Journal of Environmental Research and Public Health, 18(7), 3774. The introduction section was not included because a wide background was already written in the first part of this thesis.

1. Material and methods

1.1 Chemical and standards

All the solvents (methanol, acetonitrile, and formic acid) were LC–MS-grades (>99.9%), and were purchased from Sigma-Aldrich (Amsterdam, Holland). Ultrapure water was obtained in the laboratory using a Milli-Q system (Millipore Burlington, MA, USA). OTA, ZEA, DON, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, FB_3, T2, and HT2 were purchased by Sigma-Aldrich.

1.2 Materials

The products Supel QuE Citrate (EN) Tube (55227-U) and Supel QuE PSA (primary, secondary amine, EN) Tube (55228-U) were purchased from Sigma-Aldrich (Amsterdam Holland). The composition of 55227-U is 4 g $MgSO_4$, 1 g NaCl, 0.5 g sodium citrate dibasic sesquihydrate, and 1 g sodium citrate tribasic dihydrate. 55228-U contains 0.9 g $MgSO_4$ and 150 mg of Supelclean PSA.

1.3 Working solutions

Standards were mixed to obtain the following working solution: OTA, AFLA (AFB_1, AFB_2, AFG_1, AFG_2), FUMO (FB_1, FB_2, FB_3), ZEA, DON, MIX T2 (T2, HT2). Acetonitrile was used as a solvent, except for SMix1 (low level) and Smix2 (high level), where methanol was used. A working solution was used to fortify the blank matrix sample, and low-level and high-level were used as calibration solutions. The list of how were prepared is shown in Table 8.

1.4 Sample Preparation

The method was applied to screen mycotoxins in Sicily (Southern Italy). About 10 kg of maize and 5 kg of black pepper were collected from 2 different local vendors in Palermo (Sicily) and used for validation procedures. Samples were grounded using a Mixer B-400 laboratory mill by BÜCHI (Cornaredo, Italy) at ambient temperature with knives' rotation speed of 9000 rpm. Samples grounded were stored at −10 °C until analysis.

1.5 Sample Extraction

About 5.0 ± 0.1 g of the sample was weighted in a falcon tube of 50 mL. A total of 150 µL of OTA-d5 with a concentration of 100 µg/L was added to all samples (3.0 µg/Kg, 7.5 µg/L). One sample for each matrix was fortified with the working solution as described above (Table 8). After 10 minutes, 10 mL of bidistilled water and 10 mL of an acetonitrile/formic acid solution (80:20 v/v) were added to the sample. The sample was vortexed for 15 min and left to rest for 15 minutes at −20 °C. A mixture of salt (4 g MgSO4, 1 g NaCl, 0.5 g sodium citrate dibasic sesquihydrate, 1 g sodium citrate tribasic dihydrate) was added, handle shacking occurred for about 1 minute, and the mixture was centrifugated for 10 min at 5000 rpm. The supernatant was transferred into a mix of salt, 900 mg MgSO4, and 150 mg Supelclean PSA. After 1 minute of handle shacking and 5 minutes of centrifugation at 5000 rpm, 3 mL of the supernatant was evaporated (40 °C) and dissolved in 600 µL of methanol/water (50/50 v/v). The sample was ready for the injection.

1.6 Instrumentation

The analysis was performed on a Thermo Fischer Ultra High Performance Liquid Chromatography(UHPLC) system (Thermo Fisher Scientific, California, CA, USA) consisting of an ACCELA 1250 quaternary pump and an ACCELA autosampler. A Thermo Scientific Hypersil Gold reversed-phase UHPLC column (50 mm, 2.1 mm ID, 1.9 µm) was used for mycotoxin analysis.

The mobile phase (Table 9) was a time-programmed gradient using water

(eluent A) and methanol (eluent B). Both contained 2.50 mM of ammonium formate and 0.1% formic acid. The chromatographic run started with 100% of A with a variation of 20% in 0.5 min. The conditions were maintained for 1 min, and then A decreased until a percentage of 40% in 0.1 min. Linear decrease of A occurred with a total percentage of B of 100% in 2.6 min. Conditions were maintained for 0.7 min, and the system returned to 100% A and 0% of B in 0.1 min for 1 min.

A triple quadrupole TSQ Vantage (Thermo Fisher Scientific, California, CA, USA) in positive electrospray ionization (ESI) mode was used as a spectrometer. The product ion scans were obtained by a direct infusion of each analyte dissolved in methanol/water (50/50 v/v).

The ESI parameters were set as follows: capillary temperature 310 °C, vaporizer temperature 300 °C, sheath gas pressure 40 psi, auxiliary gas pressure 30 psi, and capillary voltage 4.8 kV. Collision gas, peak resolution, scan time, and scan width parameters were set as described in (Cammilleri et al., 2019). Trace Finder version 4.1 from Thermo Fisher (Kandel, Germany) was used to record and elaborate data. Results obtained from the analyte's direct infusion (parent, product 1, product 2, CE) and chromatography runs can be seen in Table 10.

Table 9 Working solution for the mycotoxin analyses. Different levels of mycotoxin are correlated with different limits in EU regulation in matrices.

Working Solutions		**Conc.**	**Fortified Sample**		**SMix 1 (Low Level)**		**SMix 2 (High Level)**	
			Conc µg/Kg (µg/L)	**Vol [2] (µL)**	**Conc.**	**Vol [3] (µL)**	**µg/L**	**Vol [3] (µL)**
OTA	OTA	100	3.0 (7.5)	150	1.5	15	7.5	75
AFLA	AFB_1	100	1.6 (4.0)		0.8		4	
	AFG_1	100	1.6 (4.0)	80	0.8	8	4	40
	AFB_2	25	0.4 (1.0)		0.2		1	
	AFG_2	25	0.4 (1.0)		0.2		1	
ZEA	ZEA	1000	75 (187.5)	375	37.5	37.5	187.5	187.5
FUMO	FB_1	10,000	400 (1000)		200			
	FB_2	10,000	400 (1000)	200	200	20	1000	100

Working Solutions		Conc.	Fortified Sample		SMix 1 (Low Level)		SMix 2 (High Level)	
			Conc µg/Kg (µg/L)	Vol [2] (µL)	Conc.	Vol [3] (µL)	µg/L	Vol [3] (µL)
	FB_3	10,000	400 (1000)		200			
DON	DON	10,000	100 (250)	50	50	5	250	25
MIX T2	T2	1000	25 (62.5)		12.5			
				125		12.5	62.5	62.5
	HT2	1000	25 (62.5)		12.5			
OTA-d5 [1]	OTA D5	100	3.0 (7.5)	150	7.5	75	7.5	75

[1] Internal standard. [2] Volume of working solution to add to the fortified samples. [3] Volume of working solution added to obtain 1 mL of SMix 1 (low level) and 1 mL of SMix2 (high level).

Table 10 The mobile phase in the chromatography run.

Time (min)	A (%)	B (%)
0	100	0
0.5	80	20
1.5	80	20
1.6	40	60
4.2	0	100
4.9	0	100
5	100	0
6	100	0

Table 11 Retention time, the most abundant m/z ions and optimal collision energy (CE).

Mycotoxin	Rt	Parent	Product 1 (m/z)	CE (V)	Product 2 (m/z)	CE (V)
OTA	3.52	404.2 $[M + H]^+$	239.2	27	221.7	37
ZEA	3.43	319.1 $[M + H]^+$	283.2	20	187.0	22
AFB_1	2.96	313.1 $[M + H]^+$	285.1	25	241.0	38
AFB_2	2.91	315.1 $[M + H]^+$	287.1	27	259.0	30
AFG_1	2.87	329.1 $[M + H]^+$	243.0	27	311.0	25
AFG_2	2.82	331.1 $[M + H]^+$	245.0	30	313.0	30
DON	1.04	297.2 $[M + H]^+$	203	20	249.2	15
FB_1	3.28	722.2 $[M + H]^+$	334.2	40	252.2	30
FB_2	3.45	706.3 $[M + H]^+$	354.2	37	336.1	37
FB_3	3.55	706.3 $[M + H]^+$	354.2	37	336.1	37
T2	3.32	484.2 $[M + NH_4]^+$	305.0	15	215.0	15
HT2	3.17	442.2 $[M + NH_4]^+$	263.0	15	215.0	15

1.7 Validation procedure

The method was validated according to the EU Commission Decision 2002/657/EC, following the Council Directive 96/23/EC and Regulation (EC) no. 401/2006. Linearity, specificity, precision (repeatability and reproducibility within-laboratory), and ruggedness were determined. The specificity was determined by analyzing 20 blank and fortified samples for each matrix to assay the absence of interfering peaks. The linearity was tested with a standard curve of 5 points, including zero, as follows: AFB_2- AFG_2 (200–2000 µg/L), AFB_1-AFG_2 (800–8000 µg/L), DON (50,000–500,000 µg/L), FUMO (200,000–2,000,000 µg/L), T2-HT2 (125,00–125,000 µg/L), ZEA (37,500–375,000 µg/L), OTA (1500–15,000 µg/L). The linear coefficients for each calibration in curve were $r^2 > 0.99$. The precision was assessed by fortifying 20 samples at screening target concentration (STC) and by analyzing 4 of them each day for 5 days to calculate intermediate precision (RSD_{Ri}); then, the cut-off was calculated. As required from Commission Regulation (EC) no. 401/2006, STC must be under or equal with MLs report into (EC) Regulation 1881/2006. The ruggedness test was performed according to Youden (Karageorgou & Samanidou, 2014) by determination of the effect of changing conditions (speed and time of centrifugation, time and speed of stirring,

evaporation temperature). The identification of analytes was made by comparing the retention time in the sample (TR_s) and the spiked sample (TR_a) with a range of ±0.1 min. The semi-quantification of analytes was made by extrapolation of data obtained in the linear regression between low level and high level (Table 8), and the concentration in the sample was obtained with the following formula:

$$C_C = C_S * D \quad (1)$$

Where C_c is the concentration in the matrix (µg/Kg), C_s is solution (µg/L), and D is the dilution factor. Matrix effect (MEs) in maize and spice were calculated as described by Juan Sun et al (J. Sun et al., 2016):

$$ME_s = 100(1 - (\frac{A_{bm}}{A_s}) \quad (2)$$

A_{bm} is the area in the blank matrix and A_s is the area of mycotoxin standard in solvent (n = 3). Accuracy was evaluated with the extraction of 20 fortified samples for each matrix.

1.8 Real samples

After validation, the method was tested with real samples collected during an inspection in Palermo (Sicily). Maize (n = 25) and wheat (n = 25) were collected from the same local vendors; black pepper (n = 25) and coffee (n = 25) were from two different local supermarkets in Palermo (Sicily). All samples were transported daily in the lab and, if needed, grounded as described in Section 1.4 of Material and methods. The extraction was performed as described in Section 1.5 of Material and methods.

2. Results and Discussion

2.1 Method development

2.1.1 Extraction solvent and Cleanup

The extraction method developed allows for extracting 12 mycotoxins in cereals and 5 in black pepper with a cheap and fast procedure. The best performance was attained using water and a mixture of acetonitrile/formic acid (80:20 v/v) Other ratios (60:40, 40:60, 20:80, 50:50) were examined to reach this conclusion (Figure 10). Acetonitrile and formic acid enhance analytical performance, as previously reported in the literature (Beltrán et al., 2009). Acetonitrile/water extraction (in different percentages) is one of the most common mixtures used for mycotoxin analysis (Union, 2017). Acetonitrile can reduce the extraction of lipophilic materials such as fats and has a high capacity to extract molecules characterized by different polarities (González-Curbelo et al., 2015). All mycotoxins analyzed are soluble in acetonitrile, and a higher percentage of acetonitrile can improve analytes' extraction. OTA, AFLA, and ZEA are soluble in polar organic solvents such as methanol and acetonitrile (el Khoury & Atoui, 2010; Hidy et al., 1977; Moss, 2003). FUMO are hydrophilic mycotoxins (Figure 9) and are soluble in the same solvents and water (Visconti et al., 1994).

Regarding trichothecenes, T2, HT2, and DON are low soluble in water but highly soluble in ethanol and organic solvents (Knutsen et al., 2017). For these reasons, acetonitrile, methanol, and water were used for the extraction procedure. Furthermore, water is added in high starch or low water matrices to reduce the interaction between them and analytes (Stahnke et al., 2012).

Formic acid and citrate salts decrease pH and contrast PSA's effect of increasing pH in the second step (Koesukwiwat et al., 2014; Y. Liu et al., 2014). $MgSO_4$ and NaCl increase the recovery of polar analytes, and MSO_4 with PSA performs better and increases mycotoxins' recovery (González-Curbelo et al., 2015; Vaclavik et al., 2010).

The ratio between $MgSO_4$ and PSA in the sorbent d-SPE step is always greater than 1 in all the literature, even if the quantity can change (González-Curbelo et al., 2015). However, 900 $MgSO_4$ and 150 mg of SupelcleanTM PSA is already used in different analytical techniques that use QuEChERS methods for the extraction of analytes such as pesticides (Giaccone et al., 2018; Madureira et al., 2014), likewise for the 4:1 w/w ratio between $MgSO_4$ and NaCl as salt added in the extraction procedure (Cunha & Fernandes, 2010; Galluzzo et al., 2021; Koesukwiwat et al., 2014, 2014; Rodríguez-Carrasco, Moltó, Berrada, et al., 2014).

2.2 Matrix Effect in Mycotoxin Analysis

LC–MS/MS is susceptible to MEs, a common and unpredictable problem that can influence the validation process (Smeraglia et al., 2002). The ESI ionization is subject to ion suppression more than other atmospheric pressure ionization (API) techniques such as atmospheric pressure chemical ionization (APCI) (Cortese et al., 2020).

Isotope-labeled internal standards can be useful to handle with MEs and are considered the “gold standard” approach (De Nicolò et al., 2017). This approach is useful for predicting the strong MEs in the analytical signal (Fabregat-Cabello et al., 2016). However, isotope-labeled internal standards are very expensive and do not exist for all mycotoxins, and recovery is not required in the screening method (Boevre et al., 2012). For these reasons, the use of a fortified sample for each analyte session seems more reasonable. Nevertheless, OTA-d5 was used in the validation procedure and in real samples to evaluate the entire process. In the present work, a strong ME was observed for AFLA with a range of (+43.74, −20.18) in maize and (−26.17, −0.89) in black pepper (Table 12).

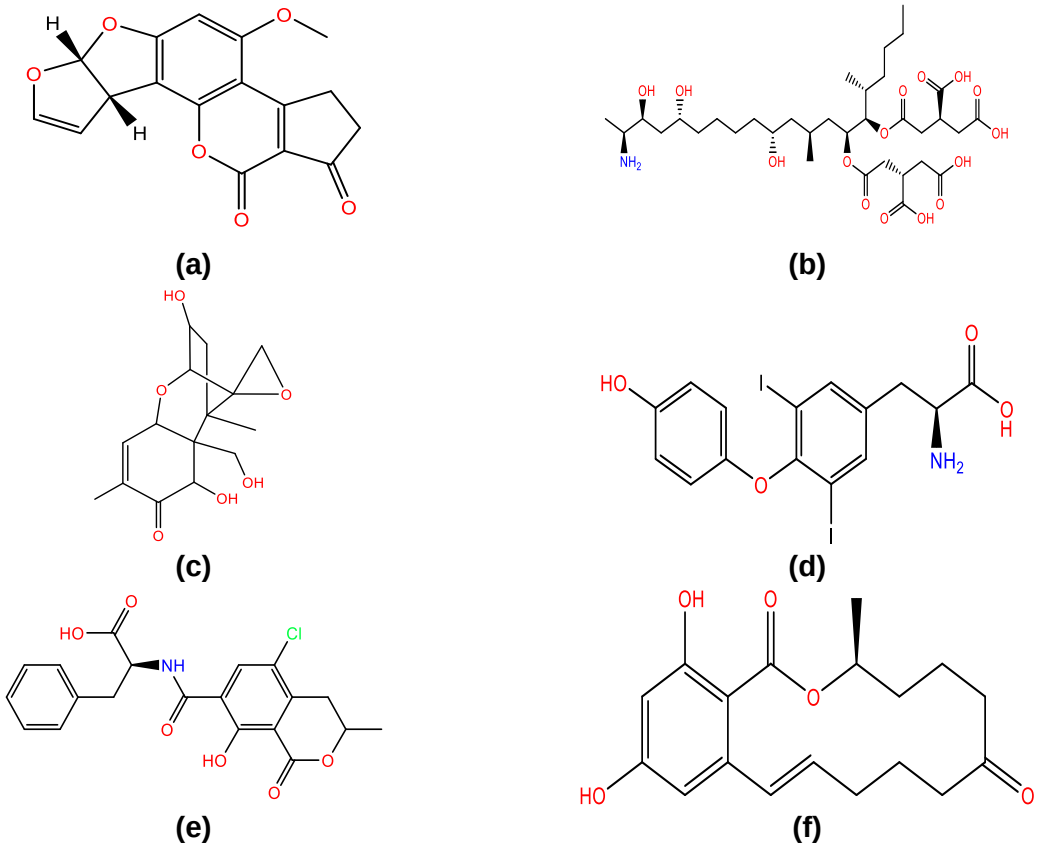
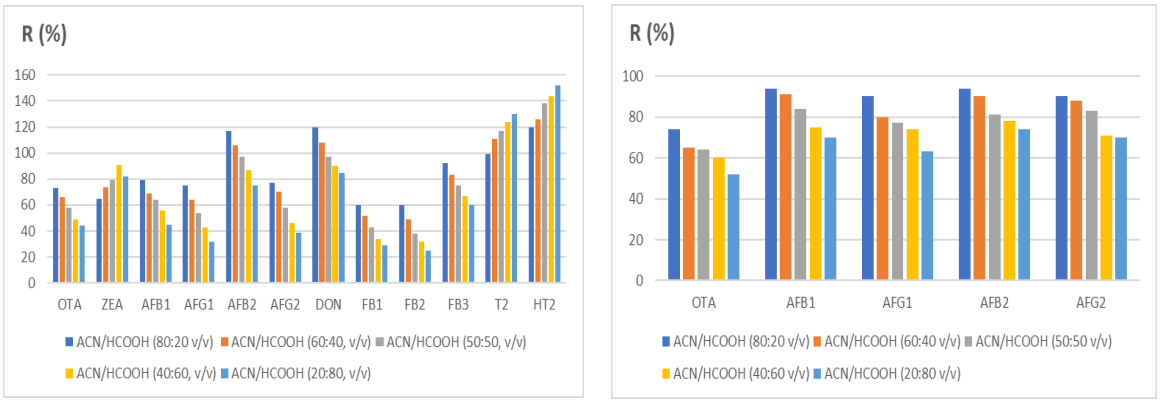


Fig. 9 Molecular structure of some mycotoxins: (a) AFB1; (b) FB1; (c) DON; (d) T2; (e) OTA; (f) ZEA.



(a) **(b)**

Fig. 10 Effect of solvents on the extraction recovery: (a) maize; (b) black pepper. ACN, acetonitrile, HCOOH, formic acid. The increase of ACN increase the recovery of all mycotoxins.

Table 12 Results of the validation procedure.

Sample Type	Mycotoxin	Linearity (µg/L)	Matrix Effect (%)	STC µg/kg	Cut-Off µg/kg	Repeatability	Recovery (%)
Maize	OTA	1.5–15	−25.40	3.0	0.93	1.81	73
	ZEA	37.5–375	−2.85	75	43	7.83	65
	AFB_1	0.8–8	−12.18	1.6	0.95	0.95	79
	AFG_1	0.8–8	+43.74	1.6	1.06	0.19	75
	AFB_2	0.20–2	−20.18	0.4	0.37	0.14	117
	AFG_2	0.20–2	+6.69	0.4	0.27	0.057	77
	DON	50–500	+37.06	100	75	36.7	120
	FB_1	200–2000	+57.26	400	75	36.7	60
	FB_2	200–2000	+66.82	400	46	141	60
	FB_3	200–2000	+21.47	400	212	223	92
	T2	12.50–125	−13.35	25	23	1.99	99
	HT2	12.50–125	−2.21	25	26	6.02	120
Black pepper	OTA	1.5–15	−26.03	3.0	1.33	1.27	74
	AFB_1	0.8–8	−13.64	1.6	1.44	0.09	94
	AFG_1	0.8–8	−1.41	1.6	1.38	0.1	90
	AFB_2	0.2–2	−26.17	0.4	0.35	0.03	94
	AFG_2	0.2–2	−0.89	0.4	0.34	0.026	90

MEs have been reported several times in the literature, but with discordant data, enhancing or suppressing black pepper (Fabregat-Cabello et al., 2016; Yogendrarajah et al., 2013; L. Zhang et al., 2018) and maize (Fabregat-Cabello et al., 2016). FUMO was strongly enhanced with an overall increase of 48.51%, as already reported by Beltrán et al. (2009) (Beltrán et al., 2009). Curiously, OTA suppression in black pepper and maize was similar and had been reported before for maize (Lattanzio et al., 2007b). The strong MEs in the mycotoxin analysis was caused by the lipid/water/protein content of the matrices analyzed (Spanjer et al., 2008). For these reasons, the analysis of different matrices should be validated according to (EC) no. 401/2006 that divides food into commodity groups to validate screening methods (Commission Regulation (EC) No 401/2006, 2014). In this case, the research validated the commodity group of difficult or unique commodities (black pepper)

that include cocoa beans and products thereof; copra and products thereof; coffee, tea, and liquorice and the commodity group cereal grain and products thereof (maize), which include wheat, rye, barley, rice, oats, whole meal bread, white bread, crackers, breakfast cereals, and pasta. Confirmatory methods must be validated in each matrix, so screening methods increase the chance of discovering new incidents and protect the consumers from high mycotoxin exposure (Commission Regulation (EC) No 1881/2006, 2006).

2.3 Method Optimization

2.3.1 Validation Parameters

The analytical parameters of the methods used for mycotoxin analysis are regulated in Europe by the Commission Regulation (EC) no. 401/2006 which defines the criteria of analysis for the official control of the levels of mycotoxins in foodstuffs (Commission Regulation (EC) No 401/2006, 2014). The analysis was performed under RSD_{Ri}, and all cut-off levels were under STC. The linear correlation of the level tested in the range of linearity was acceptable (r_2 > 0.99). Regarding black pepper, maximum levels of OTA (15 µg/kg), AFB1 (5 µg/kg), and the sum of AFB1 + AFB2 + AFG1 + AFG2 (10 µg/kg) were established from current legislation (Commission Regulation (EC) No 1881/2006, 2006). More mycotoxins are regulated for maize and unprocessed cereals intended for direct human consumption: OTA (3 µg/kg), DON (750 µg/kg), ZEA (75 µg/kg), AFB1 (2 µg/kg), the sum of AFB1 + AFB2 + AFG1 + AFG2 (4 µg/kg), FB1 + FB2 (400 µg/kg), and the sum of T2-HT2 (3 µg/kg). All mycotoxins listed were validated according to (EC) no. 401/2006 regarding the screening method for mycotoxin analysis; the cut-off level must be equal or lower than the STC level, and the method developed was complied with. The validation data can be seen in Table 12.

2.3.2 Instrumental Methods

A cheap and simple screening method of 12 (cereal) and 5 (black pepper) mycotoxins was validated. Ammonium formate and formic acid were used to form ammonium adduct and protonated precursor ion, respectively. The

ammonium adduct was selected as precursor ion only for T2 and HT2 (Table 11). All mycotoxins were detected within 4 min. The adequate resolution was obtained between ions with the same m/z, which would be indistinguishable from the mass spectrometer if they coalesced. LC–MS/MS is used frequently to analyze molecules regulated by EU Legislation (Camilleri & Vulliet, 2015; He et al., 2018; Kmellár et al., 2010; Stachniuk et al., 2017), and it is possible to perform semi-quantitative analysis (Sulyok et al., 2010). For this reason, LC–MS/MS was preferred over other screening analytical techniques such as enzyme-linked immunosorbent assay (ELISA) (Folloni et al., 2011; Oplatowska-Stachowiak et al., 2018). Several parameters can influence the performance of mycotoxin analysis. Regarding instrument setting, the positive ion mode (ESI^+) was chosen because there is a better response for the overall of mycotoxin (Beltrán et al., 2009; Spanjer et al., 2008), especially AFLA that among mycotoxins are more regulated in the EU Legislation (Commission Regulation (EC) No 1881/2006, 2006). Water and methanol as mobile phases provided the best performance for peak resolution and run time for the chromatographic run. The same result was reported in the literature (Rubert et al., 2012; Spanjer et al., 2008; J. Sun et al., 2016). Ammonium acetate and formic acid addiction in the mobile phase increase the analytical performance (Beltrán et al., 2009; Y. Ren et al., 2007; D. Sun et al., 2019; Tamura et al., 2011). The best results were achieved with 0.1% of formic acid and 2.5 mM of NH_4COOH, as already reported by other authors (Boevre et al., 2012).

With a total chromatography run of 6 min and an extraction procedure that takes approximately 1 hour, the method developed is faster than other methods already reported in the literature (Abdallah & Abd-Allah, s.d.; Azaiez et al., 2014a; Njumbe Ediage et al., 2015; Oliveira et al., 2017; Tansakul et al., 2013) and is useful for quick screening. AFLA are not sensitive to heat treatment and can increase during the food storage period. A quick screening before storage can be useful to have some data on mycotoxins' presence (Magan et al., 2010; Milani & Maleki, 2014; Scott, 1984). Black pepper is a less studied matrix, and increased data on mycotoxins presence can help in the risk assessment of mycotoxins exposure. This is especially the case because there is a possible co-occurrence of mycotoxin due to the multiple fungal infections (Santos et al.,

2009)and because they are used as flavor-enhancers in convenience foods. However, this result has not been reached without compromises. Masked mycotoxins such as 3-acetyl deoxynivalenol (3-AcDON) and 15-acetyl-deoxynivalenol (15-AcDON) that present different toxicities (Gratz, 2017) are not currently regulated in EU Legislation and were not analyzed. Some peaks are moderately overlapped, such as for FB_2 and FB_3; however (EC) no. 1881/2006 requires only sum of FB_1 + FB_2 (Figure 11). Ergot sclerotia and ergot alkaloids required from (EC) no. 1881/2006 were not analyzed.

2.4 Ring Test and Application to Real samples

2.4.1 Ring Test

The laboratories' performance was assessed by proficiency tests (PTs) following ISO/IEC 17025:2018. The analytical quality of the validated method was assured by the participation in the interlaboratory study. The following PTs were purchased by Progetto Trieste (Test Veritas, Padova, Italy): MA2050 that consist of maize with an assigned values of AFB_1+, AFB_2+, AFG_1+, AFG_2 of 16.57 µg/kg expressed as sum; maize MA2051 with DON 702.14 µg/kg; ZEA 232.61 µg/kg; F2061 feed with 11.42 µg/kg AFB1; 2.42 µg/kg AFB_2; 7.65 µg/kg AFG_1; wheat WH2062 with DON 527.05 µg/kg and T2 18.31 µg/kg; dried figs DF2064 with AFB1 7.30 µg/kg; AFG1 5.35 µg/kg; and OTA 8.79 µg/kg; and GC + C2053 that consists of coffee with an assigned value of 7.53 µg/kg for OTA (Progetto Trieste, Test Veritas, Padova, Italy). The results of all tests were compliant with ISO/IEC 17025:2018. It is worth noting that according to (EC) no. 401/2006, dried figs are classified as "high sugar and low water content", which is a commodity group not validated, and despite this, the result was compliant with ISO/IEC 17025:2018.

2.4.2 Application of the Method to Real Samples

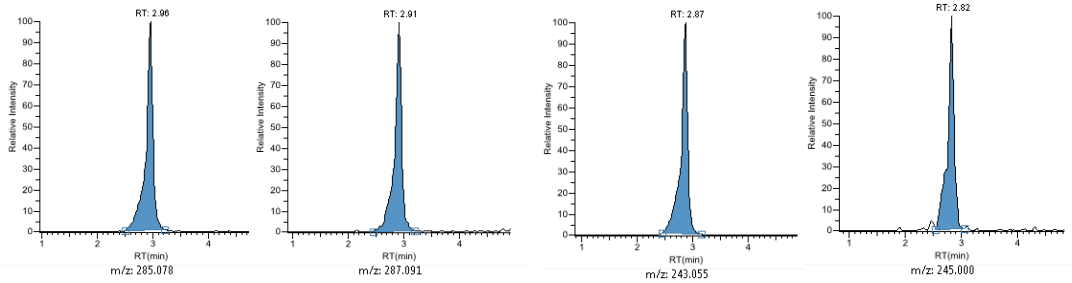
All samples were compliant and following (EC) no. 1881/2006. One sample of maize resulted with OTA at 2.53 µg/Kg, and one sample of black pepper resulted with 1.85 µg/Kg of OTA and the contemporary presence of 0.358 µg/Kg of AFB_2 (Table 13).

Table 13 Results of the analyses on real samples. A total of 25 samples were analyzed for each matrix. Dash indicates that all results were under the cut-off levels

Sample Commodity	Detected Mycotoxin	Number of Sample with a Detectable Amount of Mycotoxin	Amount
Maize	OTA	1	2.53 µg/kg
Wheat	-	0	-
Black pepper	OTA AFB_2	1 1	1.85 µg/kg 0.358 µg/kg
Coffee	-	0	-

3. Conclusion

A new method for detecting 12 mycotoxins in cereals and 5 mycotoxins in spices (black pepper) was developed and validated according to (EC) no. 401/2006. QuEChERS extraction was used effectively. The best performances were obtained with acetonitrile/formic acid (80:20 v/v) as extraction solvent. Strong MEs were observed in all the FUMO analyzed in maize, while AFLA had enhancing or suppressing effects. In black pepper, there was a suppression of signals for all mycotoxins analyzed. Six PTs were developed to evaluate the performance of the method. The method was applied to 100 real samples (25 maize, 25 wheat, 25 black pepper, and 25 coffee). Two samples had a detectable amount of mycotoxin, maize (OTA, 2.53 µg/Kg), and black pepper (OTA, 1.85 µg/Kg, and AFB_2, 0.358 µg/Kg). The method proposed is suitable for screening and routine analysis to monitor mycotoxins content in foodstuff following European Regulamentation. Further studies are needed to increase the number of mycotoxins analyzed and increase commodity groups analyzed with the same method.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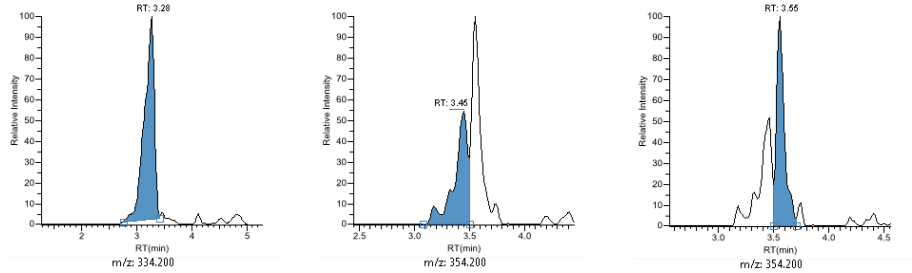
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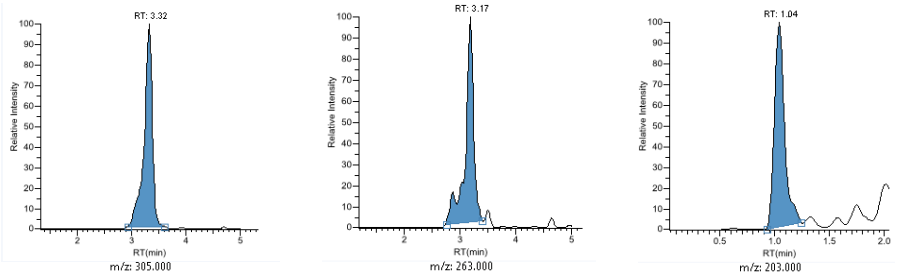
(**c**)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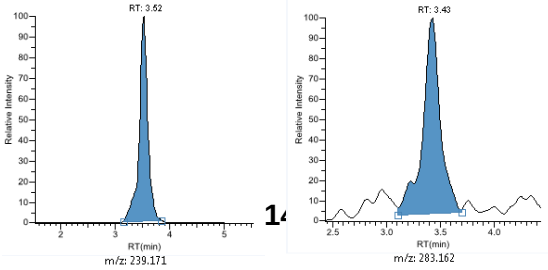
(**d**)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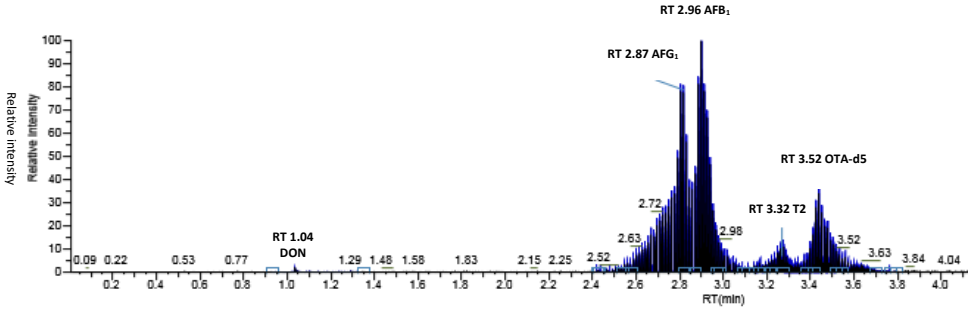
14

(e)



(f)

Fig. 11. UHPLC-MS/MS chromatograms obtained from a blank maize spiked with working solution (Table 1). Fumonisins were spiked at 100 mg/kg for a better visualization. From left to right: (a) AFB1, AFB2; (b) AFG1, AFG2; (c) FB1, FB2, FB3; (d)T2,HT2,DON;(e)OTA, ZEA; (f) total ion chromatogram (TIC).

Chapter II: Analyze mycotoxins in plant-based meat alternatives (PBMAs) products.

1. Introduction

1.1 Plant-based meat alternatives (PBMAs)

In recent years, there has been a remarkable surge in the popularity of plant-based meat alternatives, signaling a profound transformation in consumer dietary preferences and notable shifts in the trends of the food industry. These innovative products, meticulously crafted to emulate the flavor, texture, and nutritional profile of traditional meat while exclusively utilizing plant-derived ingredients, are collectively referred to as "plant-based meat alternatives" or, in acronym form, PBMAs (Messina et al., 2022). What was once considered a niche category of vegetarian products has now seamlessly transitioned into the mainstream of the food market. This transition can be attributed to the growing prominence of societal concerns surrounding critical issues such as climate change and animal welfare (Alcorta et al., 2021; Goldstein et al., 2017).

The flourishing market for PBMAs has witnessed substantial annual growth, with figures escalating from a valuation of USD 1.6 billion in 2019 to a projected USD 3.5 billion by 2026 (Alcorta et al., 2021). It is worth noting that PBMAs fall within the classification of ultra-processed foods according to the NOVA framework. This categorization stems from the fact that they are predominantly composed of substances derived from foods and supplemented with additives, with minimal to no presence of whole, unprocessed foods (Monteiro et al., 2018). In contrast to their animal-based counterparts, the ingredient lists for PBMAs tend to be significantly longer (Flint et al., 2023; Melville et al., 2023; Penna Franca et al., 2022).This notable expansion of ingredients raises concerns regarding the potential for mycotoxin contamination, particularly considering the susceptibility of cereals (such as grains, maize, and rice) and legumes (notably soybeans) to such contaminants (Bogueva & McClements, 2023; Sabuncuoğlu et al., 2020). In particular, soybeans have been identified as vulnerable to contamination by Aspergillus and Fusarium fungi, which can produce mycotoxins such as aflatoxins and fumonisins (Hassan et al., 2014). What further complicates matters is the resilience of mycotoxins to thermal

degradation; they remain stable within the range of processing temperatures employed by manufacturers, making their complete elimination during production challenging (Karlovsky et al., 2016; Romero et al., 2023; Solfrizzo et al., 2015).

As of the present date, November 2023, it is important to note that European regulations have yet to establish Maximum Levels (MLs) for mycotoxins in these matrices. Consequently, further research and data are deemed necessary to comprehensively address this issue.

2. Material and methods

2.1 Samples collection

A total of 20 samples of Plant-Based Meat Alternatives (PBMAs) were procured from local supermarkets in Palermo, Italy. The products under examination comprised soy burgers (n=10), sausages (n=5), and meatballs (n=5). Samples were homogenized separately using a Büchi B-400 vertical homogenizer (Flawil, Switzerland). Homogenized samples were stored at -20°C until the analyses.

2.2 Analyses of the samples

The chemical and instrumental analyses were done as described in Chapter I with the same chemicals and standards. The method was validated for cereals matrices that in the European Regulation can be referred to the commodity groups “high starch and/or protein content and low water and fat content” to the composition of this materials (Commission Regulation (EC) No 401/2006, 2014). For each batch, one portion of the samples were fortified as described in Table 9 to calculate the in-session recoveries. Specificity was assessed by analysing 5 blanks for each matrices and no interferences were found. Tweny samples were analysed for hamburger. LODs (limits of detection) and LOQs (limits of quantification) were determined by identifying the lowest analyte concentrations at which the signal-to-noise ratios equalled 3 and 10, respectively. The sample preparation and the sample extraction were as described in Chapter I (1.4 and 1.5 respectively). Each samples was analysed in triplicate.

2.3 Statistical analysis and data collection

The results are expressed as mean ± standard deviation (µg/kg) unless otherwise specified. Statistical analysis were conducted with R (Development Core Team) version 4.3.2 and Microsoft Excel (Microsoft Corporation, 2019).

3. Results

3.1 Method performance

The analytical method performed well with almost all the analytes. The linearity was over R2 >0.95 for all the analytes. Recoveries were tested by fortifying blank raw samples at the spiking concentration studied. The LOD-LOQ for hamburgers were: AFB1 (0.092 - 0.305), AFB2 (0.012 - 0.04), AFG1 (0.168 - 0.555), AFG2 (0.022 - 0.07), FB1 (17.017 - 56.15), FB2 (21.77 - 71.86), FB3 (24.61 - 81.21), T2 (2.88 - 9.51), HT2 (2.07 - 6.83), OTA (0.305 - 1.008), ZEA (4.33 - 14.28). Mean recoveries were between 75% and 125% for all the mycotoxins tested in all the matrices. DON have too low recoveries and therefore was not included in the analysis. It is worth noting that the method validated initially as screening method for cereals and unique matrices (spices) was suitable also for the analysis of PBMAs.

3.2 Mycotoxins presence in PBMAs analysed

The results of the mycotoxin analysis in processed by-products of meat analogues (PBMAs) are summarized in Table 14. The findings indicate varied mycotoxin profiles across these matrices, highlighting the importance of monitoring multiple mycotoxins in food safety assessments.

3.2.1 Overall Mycotoxin Occurrence

FB2 emerged as the most frequently detected mycotoxin, present in 45% of all samples (9 out of 20). This was followed by FB1 detected in 25% of the samples (5 out of 20). A significant co-occurrence of FBs was noted, with 55% of the matrices showing the presence of at least one mycotoxin. No AFs, T2, HT2, ZEA, DON, OTA were found. No issues related to European Regulation were detected.

3.2.2 Hamburgers (n=10)

In the soy-burger samples analyzed, the fumonisins were detected with the following occurrences and concentration levels: FB1 was found in 40% of the samples (4 out of 10), with a mean concentration of 109.03 ± 57.06 µg/kg, making it the second most abundant mycotoxin in these products. FB2, the most common mycotoxin detected overall, was present in 50% of the hamburgers (5 out of 10), but it had the lowest mean concentration among the fumonisins at 63.807 ± 33.011 µg/kg. FB3, although least frequently detected (20%, 2 out of 10), exhibited the highest concentration, with a mean value of 216.365 ± 88.039 µg/kg.

3.2.3 Meatballs (n=5)

In the meatball samples, of which there are 5 unique samples, fumonisins were detected as follows: FB1 was found in 16.67% of the samples (1 out of 5), with a mean concentration of 135.858 ± 52.2 µg/kg. FB2 was present in 33.33% of the meatball samples (2 out of 5), showing a mean concentration of 69.74 ±15.16 µg/kg. FB3 was not detected. FB3 was not detected in any of the meatball samples. This analysis indicates a presence of fumonisins in meatballs, with FB2 as the most frequently detected, although its concentration is lower compared to FB1. The absence of FB3 in meatballs highlights the specific mycotoxin profile in this type of plant-based meat alternative.

3.2.4 Sausages (n=5)

FB2 was again a significant contaminant, detected in 40% of the sausage samples (2 out of 5), with a mean concentration of 68.36 ± 18.41 µg/kg. This reinforces the ubiquitous presence of FB2 across different matrices. No concentrations of FB1 and FB3 were detected.

Table 14 Mycotoxins detected in PBMAs analysed with the validated method. The results are expressed as mean ± standard deviation (n=3, µg/Kg). “H” is the abbreviation of hamburgers, “M” for meatballs, and “S” for sausages.

Matrices	AFB1	AFB2	AFG1	AFG2	FB1	FB2	FB3	HT2	OTA	T2	ZEA
H	-	-	-	-	90.306±11.156	58.844±3.958	215.947±3.93	-	-	-	-
H	-	-	-	-	-	68.301±6.951	216.784±4.383	-	-	-	-
H	-	-	-	-	106.141±27.668	64.558±12.618	-	-	-	-	-
H	-	-	-	-	110.466±21.567	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	129.225±43.62	-	-	-	-	-	-
H	-	-	-	-	-	61.246±4.016	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	66.086±14.466	-	-	-	-	-
M	-	-	-	-	135.858±52.2	73.092±19.619	-	-	-	-	-
M	-	-	-	-	-	66.386±12.488	-	-	-	-	-
M	-	-	-	-	-	-	-	-	-	-	-
M	-	-	-	-	-	-	-	-	-	-	-
M	-	-	-	-	-	-	-	-	-	-	-
S	-	-	-	-	-	-	-	-	-	-	-
S	-	-	-	-	-	-	-	-	-	-	-
S	-	-	-	-	-	67.222±18.414	-	-	-	-	-
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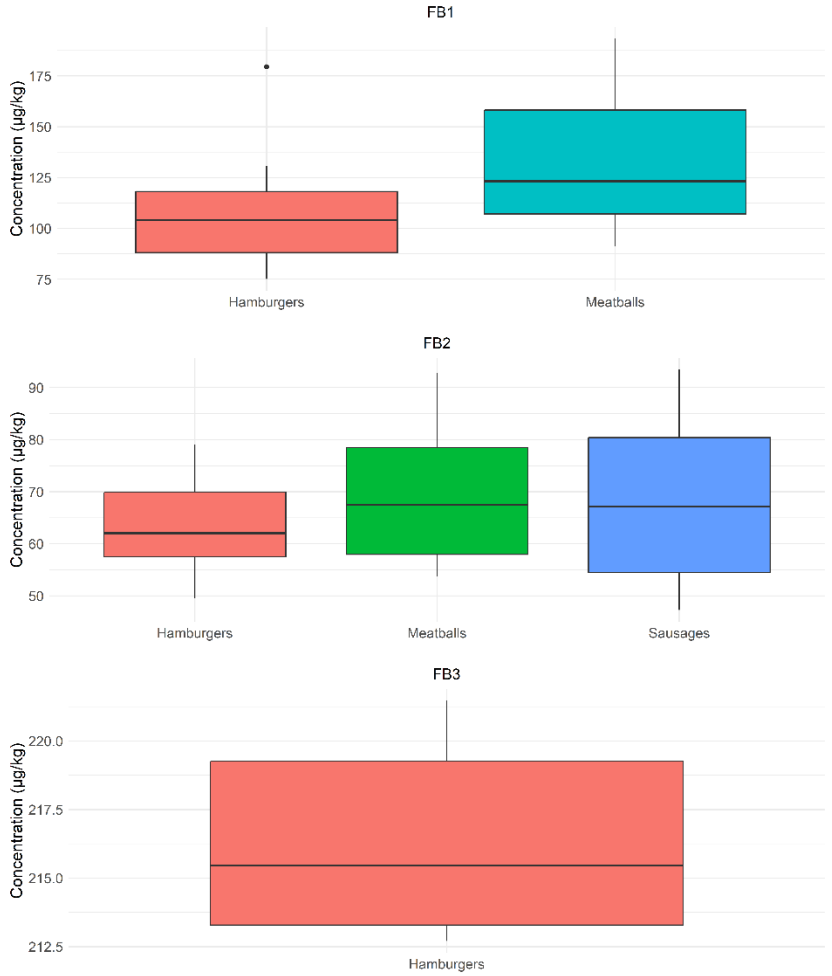

Fig. 12 Comparative Analysis of Mycotoxin Concentrations in Processed Food Products. This series of boxplots illustrates the distribution of FBs concentrations (FB1, FB2, FB3) across three different food matrices: Hamburgers (H), Meatballs (M), and Sausages (S). Each boxplot highlights the range, median, and potential outliers of FBs levels, emphasizing the variability of these contaminants in processed food products.

4. Discussion

This investigation into the prevalence of mycotoxins within plant-based meat alternatives (PBMAs) such as hamburgers, meatballs, and sausages has revealed a nuanced and differential distribution of these secondary metabolites across the tested foodstuffs. The complete absence of aflatoxins (AFB1, AFB2, AFG1, AFG2), Ochratoxin A (OTA), HT-2 toxin (HT2), T-2 toxin (T2), and Zearalenone (ZEA) contrasts sharply with findings from the literature, where the presence of aflatoxins has been reported in a significant proportion (38.5%) of similar samples (Augustin Mihalache et al., 2023).

The exclusive detection of fumonisins, with FB2 present in 45% of the samples, suggests a selective contamination pathway or a variance in susceptibility across PBMA types, potentially influenced by the nature of the raw materials utilized. Prior research has identified the presence of fumonisins in legumes, soybeans, and PBMAs (Garcia et al., 2016; Gutleb et al., 2015; Leblanc et al., 2005; Rodríguez-Carrasco et al., 2019; S. Zhang et al., 2021).

Particularly noteworthy is the work of Mihalache et al. (2023) (Augustin Mihalache et al., 2023), who reported fumonisins as the most prevalent mycotoxins in an assortment of PBMAs retailed in Italy, detecting FB1 in the vast majority (84.6%) and FB2 in 69.2% of the analyzed products. This finding diverges from our own, wherein FB2 was more frequently detected than FB1. Notably, our study did not yield data on FB3, a mycotoxin for which there is a paucity of regulatory and surveillance information in Europe.

Our research uncovered levels of fumonisins that exceeded those reported by Mihalache et al. (2023), yet despite this higher incidence, the concentrations remained beneath the maximum thresholds established by European Commission regulations, which range from 200 to 4000 micrograms per kilogram for FB1 and FB2, thus indicating no immediate health risks (Commission Regulation (EU) 2023/915, 2023). As FB3 lacks specific regulatory standards within the European context, our study underscores a gap in the current regulatory framework.

The disparities in fumonisin levels within the different matrices, and particularly the elevated concentrations in meatballs compared to hamburgers, could be attributable to the disparate compositions of the foundational materials. These

compositional differences have the potential to affect fungal proliferation and subsequent mycotoxin synthesis (Palumbo et al., 2020).

Another critical factor influencing mycotoxin emergence is the production process itself. For instance, reduced water activity within foodstuffs is known to inhibit fungal growth, thus potentially mitigating mycotoxin presence (Oviedo et al., 2010).

This study, therefore, highlights the imperative need for tailored mycotoxin monitoring and risk management protocols that are specific to the PBMA sector. Such bespoke approaches are essential for the maintenance of food safety and public health, particularly in the face of an ever-growing consumer shift towards plant-based diets. Future research endeavors should aim to dissect the complex interactions between food matrix composition, processing conditions, and mycotoxin contamination. Additionally, investigations into effective mycotoxin mitigation strategies for the PBMA industry will be of paramount importance to ensure the ongoing safety and integrity of these food products.

This study is not without limitations. The lack of data regarding the origin of the ingredients, specific processing steps, and the duration of storage prior to testing means that these potentially confounding factors were not accounted for in our analysis. Furthermore, the sample size (n=20) is too low to create a real assessment. Future research should aim to determine the factors that contribute to the observed differences in mycotoxin levels among PBMA types.

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Chapter III: Mycotoxins degradation in Microwave in PBMAs

1. Introduction

Mycotoxins are secondary metabolites primarily produced by *Aspergillus*, *Fusarium*, and *Penicillium* species and can contaminate a wide range of agricultural products under specific environmental conditions (Abdulkadar et al., 2004; Agriopoulou et al., 2020; El-Sayed et al., 2022). These compounds are noted for their toxic effects, including carcinogenesis, immune deficiency, organ damage, teratogenicity and hormonal imbalances (Omotayo et al., 2019; Ratnaseelan et al., 2018; Speijers & Speijers, 2004). Due to these hazardous effects, mycotoxins are regulated worldwide (López-García, 2022). In the European Union, the maximum levels (MLs) permitted in food matrices are established by Commission Regulation (EU) 2023/915 (Commission Regulation (EU) 2023/915, 2023), and the analytical methods must have the performance criteria set by Commission Regulation (EC.) No 401/2006 (Commission Regulation (EC) No 401/2006, 2014).

The consumption of plant-based foods and meat alternatives (PBMAs) has increased significantly in recent years due to a convergence of factors related to health, environmental concerns, and ethical considerations (Banach et al., 2022; El-Sayed et al., 2022; X. Lin et al., 2023; Y. Wang et al., 2022). The environmental impact of meat production, particularly in terms of greenhouse gas emissions and animal welfare, is a major factor in the shift towards plant-based eating. It is projected that PBMA products will see further growth in the future, and some studies have projected that the market value of these products will be $95 billion by 2030 (Sha & Xiong, 2020).

Mycotoxin contamination in these products, both pre-harvest and post-harvest, is a prevalent issue due to various factors (X. Lin et al., 2023). Raw ingredients such as cereals (soybeans), which are commonly used in these product types, are particularly susceptible to mycotoxin contamination (Augustin Mihalache et al., 2022; Irakli et al., 2017b; Kunz et al., 2020; Niyibituronsa et al., 2018). The contamination of these substances in PBMAs has been documented by various researchers.. Aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), zeranol (ZEA), enniatin, and beauvericin were found in products that are already

commercialized in the European market (Arroyo-Manzanares et al., 2019; Augustin Mihalache et al., 2023; Cicero et al., 2020; Kunz et al., 2020). Mihalache et al. *(2023)* reported the co-occurrence (84.6%, 11/13) of different mycotoxins in soy-burger, soy-meat and soy-steak, particularly FBs that were the most frequently detected, followed by tentoxin (TEA), OTA, and AFB1 (Augustin Mihalache et al., 2023).

Similar results were reported by Carrasco et al. (2019) where the co-occurrence of mycotoxins was 94.4% (17/18) with the presence of up to six mycotoxins in the same soy-based burger (Rodríguez-Carrasco et al., 2019). However, to date, these products and some of their most common ingredients (soybeans, peas) are not regulated by European Regulations (Augustin Mihalache et al., 2023; Commission Regulation (EU) 2023/915, 2023; Kyriakopoulou et al., 2019). Mycotoxins are heat resistant; therefore, their presence in food matrices cannot be eliminated with the most common cooking method. This characteristic varies significantly depending on the specific type of mycotoxins. AFs are highly stable at high temperatures and can withstand normal food cooking processes such as boiling and baking (Kumar et al., 2017b; Méndez-Albores et al., 2004). FBs are more susceptible to heat treatment, while ZEA, OTA, DON, and T2 are more stable (Sobral et al., 2019). However, the degradation in the cooking processes depends on the analyzed matrices (Aiko & Mehta, 2015; Kabak, 2009; Milani & Maleki, 2014; Sobral et al., 2019). Th of this study is to evaluate the impact of the microwave cooking method on veggie burgers and some of the ingredients that can compose them. Carrots, zucchini, soybeans, and potatoes were used to make a homemade veggie burger that was compared with purchased burgers. Each matrix was fortified and cooked with the condition reported in the package of the purchased burger (60 s at 800 W). Furthermore, each matrix was cooked to a "Max" condition that is 50% more than of the time labelled in the package (90 s at 800 W). The analysis was conducted with liquid chromatography-tandem mass spectrometry (LC-MS/MS) that allows the analysis of different mycotoxins in different matrices with a single run (Azaiez et al., 2014b; Kunz et al., 2020; Malachová et al., 2018; Pantano et al., 2021).

2. Materials and Methods

2.1 Chemicals and materials

All the solvents used for the analyses were of LC-MS grades (>99.9%). Methanol and acetonitrile were purchased from Sigma-Aldrich (Amsterdam, Holland). The Milli-Q sys-tem (Millipore Burlington, MA, USA) was used to obtain the ultrapure water.

Aflatoxins mix (AFB1, AFB2, AFG1, AFG2) and fumonisins mix (FB1, FB2, FB3) were purchased from Romer Labs (Getzerdorf, Austria). HT2, T2, OTA, and OTA-d5 were pur-chased from HPC Standards GmbH (Cunnersdorf, Germany). The extraction and purifica-tion procedures were conducted with Supel QuE Citrate (EN) Tube (55227-U, 4 g $MgSO_4$, 1 g NaCl, 0.5 g sodium citrate dibasic sesquihydrate, and 1 g sodium citrate tribasic dihy-drate) and Supel QuE P.S.A. (primary, secondary amine, EN) Tube (55228-U, 0.9 g $MgSO_4$ and 150 mg of Supelclean P.S.A.).

2.2 Sample collection

The zucchini, carrots, potatoes, soybeans, and purchased soy burgers used in this study were acquired from a retail vendor in Palermo, Southern Italy. For uniformity, the carrots, potatoes, and zucchini were sliced using a vegetable cutting machine. The soybeans were homogenized using a Büchi B-400 vertical mixer (Flawil, Switzerland). Homemade burgers were prepared by combining carrots, potatoes, zucchini, and soybeans, which were first individually prepared and then combined in a homogenized mixture. This preparation ensured a consistent and even blend of ingredients in each burger. All the matrices were stored at -10 °C until analyses. Raw matrices were analyzed, and no mycotoxins were found.

2.3 Cooking procedure

The microwaving parameters were based on the instructions provided on the packaging of the purchased soy burgers, which recommended cooking for 1 minute at 800 watts ("Min" condition). An alternative condition tested was cooking for 90 seconds at 800 watts ("Max" condition). This was the maximum power allowed in the microwave used. These were the only conditions examined, as extending the cooking time resulted in the burning of the

hamburgers. Five grams of each food sample was microwaved separately under these conditions. The materials were placed in a 50 mL falcon tube and cooked with 5 mL of water. The cap was partially open to prevent an explosion or material leakage. The samples were prepared and fortified following the methodology outlined in Pantano et al. (2021)(Pantano et al., 2021). The initial mycotoxin concentrations in the matrices were as follows: ochratoxin A (OTA) at 3.0 µg/kg, aflatoxins (AFB1 at 1.6 µg/kg, AFG1 at 1.6 µg/kg, AFB2 at 0.4 µg/kg, AFG2 at 0.4 µg/kg), zearalenone (ZEA) at 75 µg/kg, fumonisins (FB1, FB2, and FB3 each at 400 µg/kg), T-2 toxin at 25 µg/kg, HT-2 toxin at 25 µg/kg, and OTA-d5 at 3.0 µg/kg. The spiking procedures were done on the surface of the products. Solutions containing the same amount of mycotoxins were cooked in the same conditions to test the degradation in water. Temperatures were measured immediately after the cooking with a calibrated electronic contact thermometer (IKA™ ETS-D5, Staufen Germany).

2.4 Extraction procedure and instrumental analyses

The instrumental equipment was a Thermo Fischer Ultra High-Performance Liquid Chromatography (UHPLC) system (Thermo Fisher Scientific, California, CA, USA) with of an ACCELA 1250 quaternary pump and an ACCELA autosampler. The column used was a Thermo Scientific Hypersil Gold reversed-phase UHPLC column (50 mm, 2.1 mm ID, 1.9 µm). The chromatography run, the instrumental condition and the extraction procedure were performed as described previously by Pantano et al. (2021) (Pantano et al., 2021). Briefly, 5 g of the samples were weighted in a falcon tube of 50 mL and 10 mL of bidistilled water and 10 mL of an acetonitrile/formic acid (2%) solution were added to the sample. It was vortexed for 15 minutes and then left to rest at -20 °C for another 15 minutes. Next, Tube (55227-U) was added, followed by shaking for 1 min and centrifugation at 5000 rpm for 10 min. The supernatant was then transferred to Tube (55228-U), shaken for 1 min, and centrifuged for 5 min at 5000 rpm. Finally, 3 mL of the supernatant was evaporated at 40°C and redissolved in 600 µL of a 50/50 v/v methanol/water solution, rendering the sample ready for injection. All the analyses were performed in triplicate. Results obtained from the instrument (µg/L) were

converted to (µg/Kg) with a conversion factor of 0.4.

2.5 Method performance and data accuracy

Linearity was assessed with matrix-matched standard overs the concentrations of 0.8-2-4-8 (µg/L) for AFG1 and AFB1, 0.2-0.5-1-2 (µg/L) for AFG2 and AFB2; 200-500-1000-2000 (µg/L) for FB1, FB2, FB3; 12.5-31.25-62.50-125 (µg/L) for T2 and HT2, 1.5-3.75-7.50-15 (µg/L) for OTA and 37.50-93.75-187.50-375 (µg/L) for ZEA. Specificity was assessed by zanalyzing 5 blanks for each matrices and no interferences were found. Forty blank samples were zanalyzed for each groups, n=20 for zucchini and carrots and n=20 for potatoes, soybeans, and hamburger purchased. LODs (limits of detection) and LOQs (limits of quantification) were determined by identifying the lowest analyte concentrations at which the signal-to-noise ratios equalled 3 and 10, respectively. The linearity was over R^2 >0.95 for all the analytes. Recoveries were tested by fortifying blank raw samples at the spiking concentration studied. The LOQ-LOQ for zucchini-carrots (µg/kg) were: AFB1 (0.335 - 1.105), AFB2 (0.03 - 0.098), AFG1 (0.367 - 1.212), AFG2 (0.074 - 0.243), FB1 (62.922 - 207.644), FB2 (55.943 - 184.613), FB3 (67.265 - 221.974), T2 (6.74 - 22.26), HT2 (5.66 - 18.68), OTA (0.43 - 1.42), ZEA (8.28 - 27.34). For soybeans hamburgers-potatoes-soybeans were: AFB1 (0.092 - 0.305), AFB2 (0.012 - 0.04), AFG1 (0.168 - 0.555), AFG2 (0.022 - 0.07), FB1 (17.017 - 56.15), FB2 (21.77 - 71.86), FB3 (24.61 - 81.21), T2 (2.88 - 9.51), HT2 (2.07 - 6.83), OTA (0.305 - 1.008), ZEA (4.33 - 14.28). Mean recoveries were between 75% and 125% for all the mycotoxins tested in all the matrices.

2.6 Statistical analyses

Samples were named depending on the matrix (Carrots, Zucchini, Soybeans, Potatoes, H. homemade, H. purchased) and grouped by the cooking condition in "Min" and "Max". All variables were tested for normal distribution using the Shapiro-Wilk test.

The statistical analysis was conducted in two primary phases. There are differences between matrices analysis (Carrots, Zucchini, Soybeans, Potatoes, H. homemade, H. purchased) and within matrices (Max-Min). The first was

evaluated with the Kruskal-Wallis H test, while the second depended on the normal distribution. When normally distributed, the Welch Two Sample t-test was used to evaluate differences in means between groups (Max-Min) among the same matrices. For variables that don't meet normality distribution, the Mann–Whitney U test was used instead. A Principal Component Analysis (PCA) was conducted after the data were scaled with Pareto-Scaling (Cumbo et al., 2022; Galluzzo et al., 2019). Kaiser-Meyer-Olkin (KMO) test showed an MSA of 0.5, and the p-value of Bartlett's Test of Sphericity was <0.05. The number of principal components (PCs) to keep was assessed with Kaiser-Harris criterion, Cattell Scree test and parallel analysis as described in Kabacoff (2021) (Kabacoff, 2011). Two PCs were kept because they were enough to explain the 78.8% of the variance. Statistical analyses were conducted with R®4.1.2 software (freeware available on https://cran.r-project.org/). All tests were performed with a 5% significance level.

The percentage lost of mycotoxins in hamburger was calculated with the following equations:

$$\%Lost = 100 * \frac{(\text{mean value found after cooking})}{\text{values fortified}} \quad (1)$$

3. Results

3.1 Impact of microwave cooking on mycotoxin degradation

In this study, a total of 14 categories of samples were analysed. Controls (Max-Min), Carrots (Max-Min), Zucchini (Max-Min), Potatoes (Max-Min), Soybeans (Max-Min), H. homemade (Max-Min), and H. purchased (Max-Min). The mean values of mycotoxins found are shown in Table 1 and multivariate analysis in Figure 4, while the impact of degradation of AFs (AFB1, AFB2, AFG1, AFG2), FBs (FB1, FB2, FB3), OTA, T2, HT2, and ZEA in soy burgers are shown in Figure 5. The differences between the matrices with the same condition are shown in Figure 1-3. In all the conditions waters added vapored completely, temperatures recorded ranged from a minimum of 90°C to a maximum of 98.3°C in food matrices and 82.5°C-80.3°C in control (Table 1).

3.1.1 Aflatoxins

In the analysis of Aflatoxins (AFs), zucchini under the 'Min' condition demonstrated a high persistence of AFs, with AFB1 reaching 1.42 ± 0.066, AFB2 at 0.344 ± 0.021, and AFG1 at 1.50 ± 0.023. Carrots, also under the 'Min' condition, exhibited notable levels of AFB1 at 1.54 ± 0.04 and AFG1 at 1.45 ± 0.105. Potatoes under the 'Max' condition exhibited the lowest AFB1 level at 0.324 ± 0.023 and a lower AFG1 level at 1.13 ± 0.048, suggesting a higher degree of AF degradation. In the case of hamburgers, homemade variants (H. homemade) under the 'Max' condition showed a reduced level of AFB1 at 0.56 ± 0.064 compared to the purchased ones (H. purchased), which registered at 1.15 ± 0.018 of AFB1. Regarding differences between cooking condition, the different treatment showed signifi-cant differences for AFB1 and AFG1 (4 on 6 matrices) while AFB2 and AFG2 were the least sensitive to prolonged cooking times (1 on 6 matrices).

3.1.2 Fumonisins

Zucchini under the 'Min' condition emerged with the highest FB1 concentration at 373.85 ± 3.392. Carrots also showed high FB1 levels under 'Min', peaking at 363.55 ± 45.391. Potatoes under 'Max' displayed considerably lower FB1 levels at 194.42 ± 1.141, suggesting greater FB1 degradation. Hamburgers, both purchased (H.purchased) and homemade (H.homemade), exhibited lower FB1 concentrations under 'Max', with purchased hamburgers at 56.47 ± 0.328 and homemade at 136.94 ± 2.385. This pattern was the same for FB2 and FB3, with zucchini and carrots showing higher concentrations than potatoes, yet zucchini's FB2 and FB3 levels did not reach the high levels of FB1. Both hamburger types also showed reduced FB2 and FB3 levels, consistent with FB1 trends. FBs were sensible to the prolonged heat treatment in almost all matrices, with FB2 and FB1 different between the conditions Min-Max in 100% and 66.6% of the matrices analysed.

3.1.3 HT2 and T2

Carrots, under the 'Min' condition, emerged with the highest HT2 toxin concentration, reaching 23.59 ± 1.42, suggesting a lower degradation rate of HT2 in carrots under milder cooking conditions. Zucchini and potatoes, under

the 'Max' condition, showed lower HT2 levels, with zucchini at 22.47 ± 0.105 and potatoes at 17.84 ± 0.276, indicating better degradation efficiency, especially in potatoes. For hamburgers, a difference in HT2 degradation was noted between purchased (H. purchased) and homemade (H. homemade) variants under 'Max'. P. hamburgers had a higher HT2 concentration of 22.27 ± 1.667, while homemade hamburgers showed a lower level at 11.71 ± 0.074, indicating more effective degradation in the homemade variety. The differences between the condition Min-Max was significant in 50% of the matrices.

Regarding T2 toxin, zucchini exhibited the highest level among the food items tested under the 'Max' condition, with a concentration of 23.52 ± 1.150. Carrots followed, showing 24.98 ± 0.483 under 'Min', while potatoes had a lower concentration at 24.28 ± 0.677 under 'Max'. In hamburgers, both purchased and homemade variants showed lower T2 levels compared to vegetables, with purchased hamburgers at 11.04 ± 1.510 and home-made at 10.26 ± 0.273 under 'Max'. The differences between the conditions of cooking were relevant for soybeans and purchased hamburgers.

3.1.4 OTA

Zucchini displayed the highest OTA concentration among the tested vegetables, peaking at 2.609 ± 0.061 under the 'Min' condition, suggesting lower degradation efficiency during microwave cooking. Carrots showed moderate OTA levels under 'Min', with a peak at 2.41 ± 0.146, indicating a moderate degradation rate. Soybeans under 'Max' had a peak OTA concentration of 1.485 ± 0.029, demonstrating slightly better degradation efficiency than zucchini but still retaining significant levels. Potatoes showed the lowest OTA level at 1.014 ± 0.007 under 'Max', suggesting a higher degradation efficiency. For hamburgers, both purchased and homemade varieties under 'Max' exhibited lower OTA levels than vegetables. P. hamburgers had a peak OTA level of 1.094 ± 0.066, significantly lower than most vegetable samples. H. hamburgers showed a slightly higher OTA concentration than purchased ones, reaching 1.346 ± 0.055, but still lower than the levels in vegetables. The differences between condition “Max” and “Min” were statistically significant in all the matrices except for both hamburgers.

3.1.5 ZEA

Zucchini, under the 'Min' condition, showed the highest concentration of ZEA among the tested food items, reaching a maximum of 51.862 ± 0.838. This suggests that zucchini is more prone to retaining high ZEA levels during microwave cooking. Following zucchini, carrots exhibited a notable ZEA level under 'Min', peaking at 33.734 ± 0.758, indicating a significant presence of ZEA post-cooking. Potatoes, however, demonstrated a lower ZEA level under the 'Max' condition, with a maximum of 16.988 ± 0.495, suggesting a higher degradation rate of ZEA. In hamburgers, both purchased (H. purchased) and homemade (H. homemade) variants under 'Max' showed lower ZEA concentrations compared to vegetables. P. hamburgers had a maximum ZEA level of 15.31 ± 0.918, significantly lower than that in vegetables. H. hamburgers also displayed lower ZEA levels, with a concentration of 19.682 ± 0.137. This pattern indicates that hamburgers, regardless of being purchased or homemade, tend to retain lower levels of ZEA when microwaved compared to vegetable samples. In carrots, zucchini, and potatoes there were statistically significant differences between Min-Max condition.

3.2 Statistical analysis

The results of the Kruskal-Wallis H test for analyzing the differences in mycotoxin levels across different food matrices showed that all mycotoxins were different between matrices ($p < 0.05$ for all the mycotoxins). Regarding hamburgers, the Wilcoxon rank-sum test results indicate that there are not significant differences in the levels of several mycotoxins between homemade and purchased hamburgers. Specifically, no significant differences were observed for mycotoxins cooked in the same condition between purchased and homemade hamburgers ($p \geq 0.100$). This finding indicates that the type of hamburger (homemade vs. purchased) does not significantly influence the mycotoxin content under the specific cooking conditions tested.

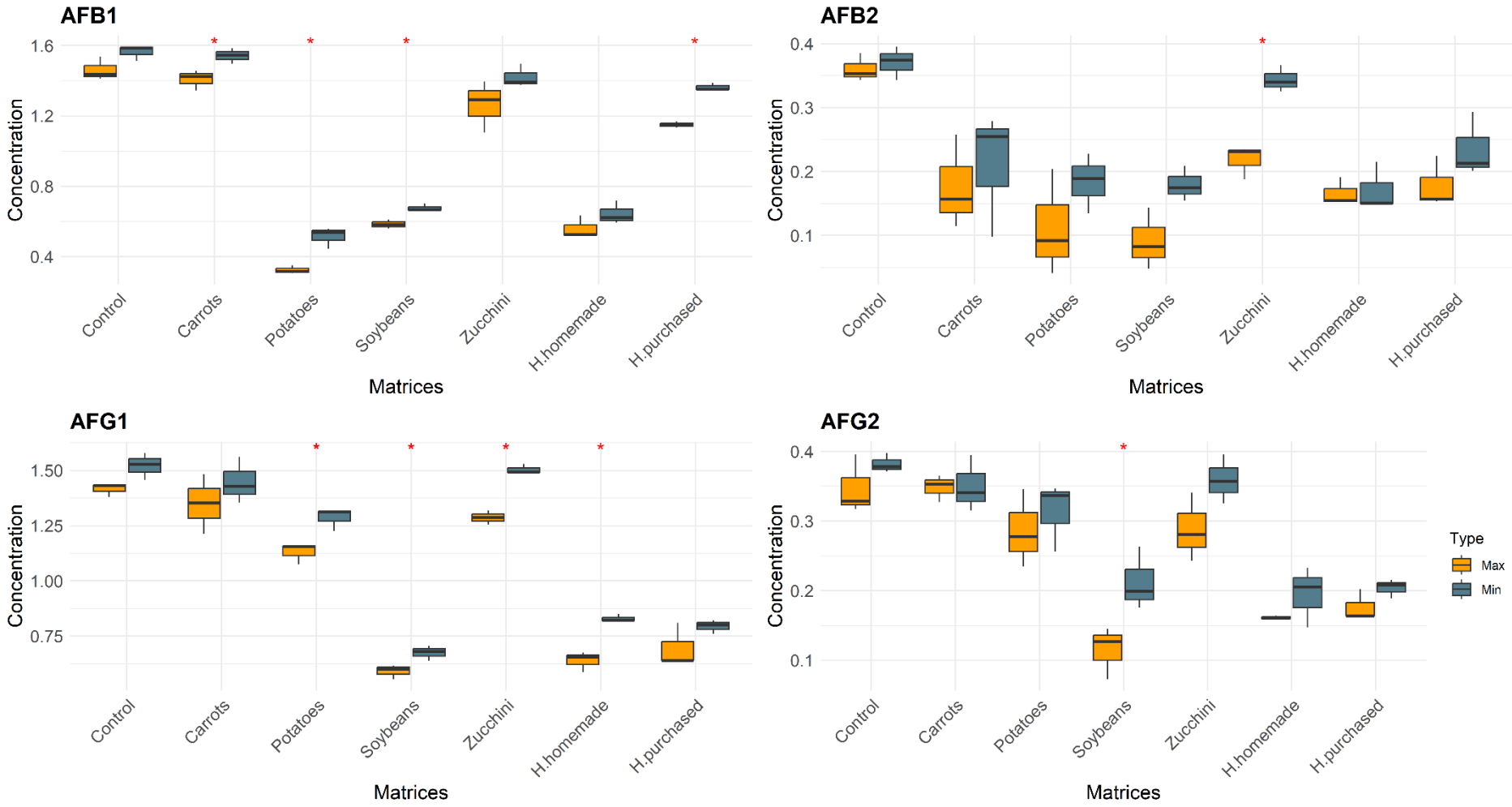

Fig. 13 Concentration of AFs after cooking procedure divided by matrices and colored fill by condition of cooking. "*" statistically significant differences ($p<0.05$).

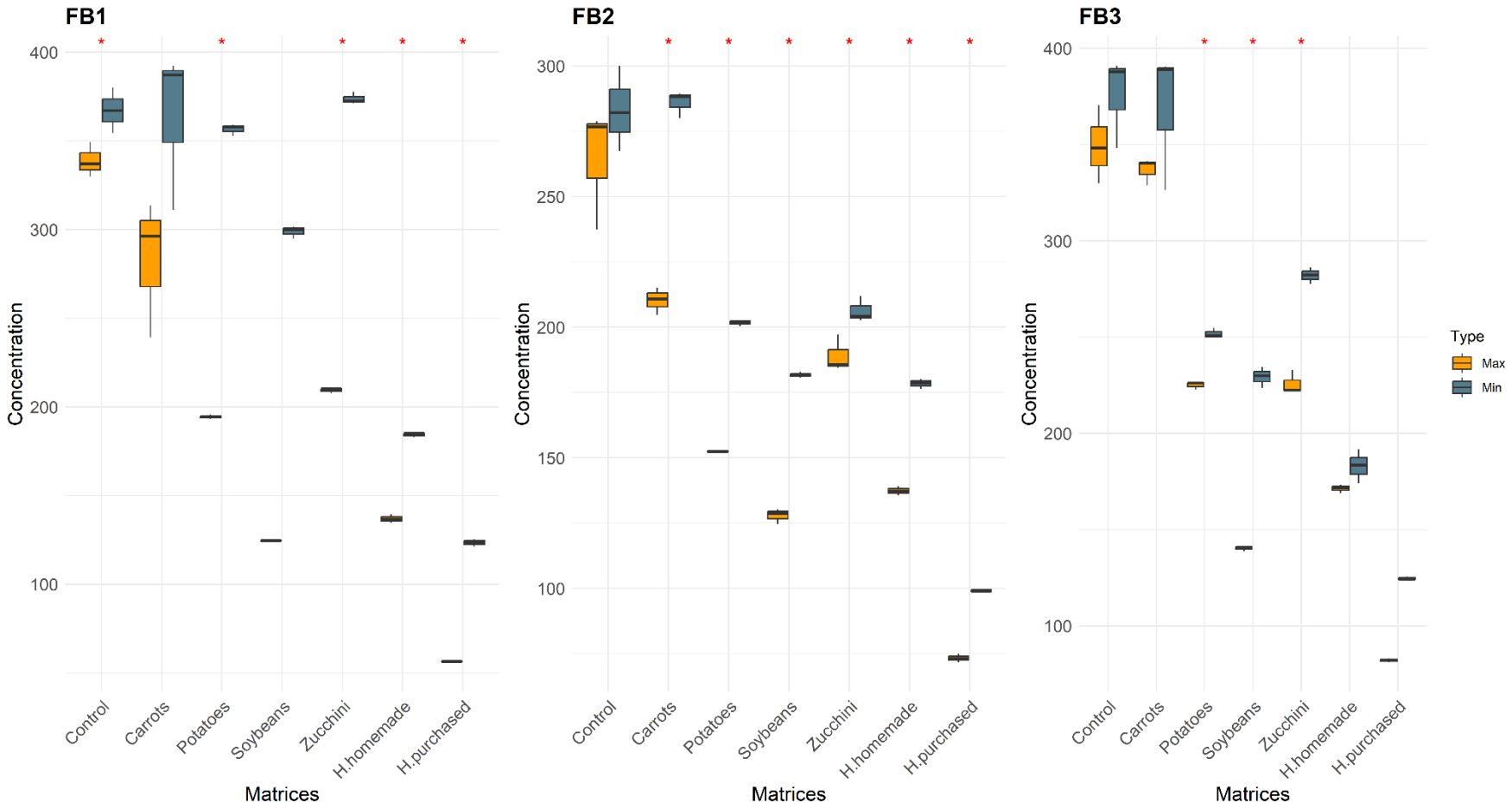

Fig. 14 Concentration of FBs after cooking procedure divided by matrices and colored fill by condition of cooking. "*" statistically significant differences ($p<0.05$).

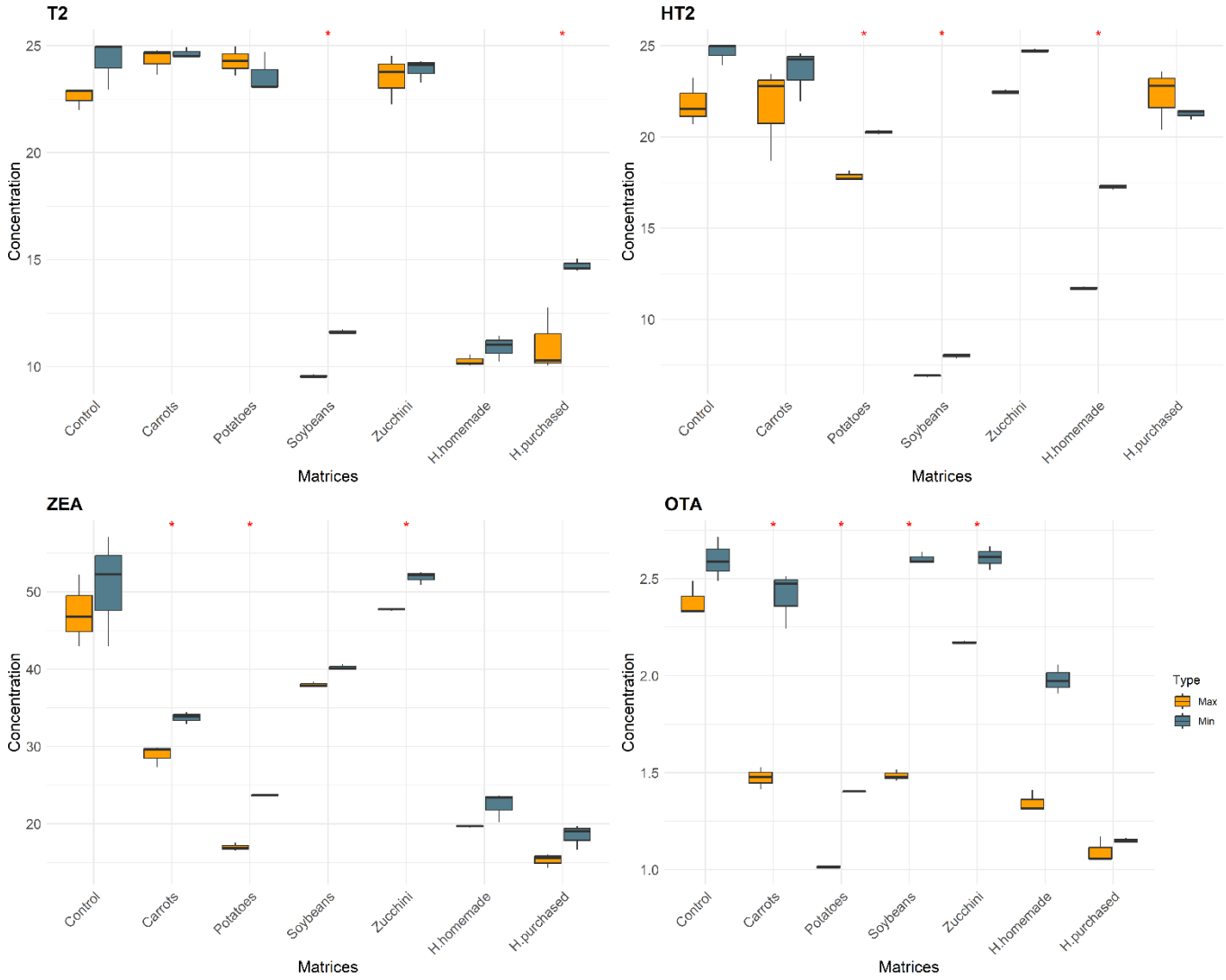

Fig. 15 Concentration of T2, HT2, OTA, and ZEA after cooking procedure divided by matrices and colored fill by condition of cooking. "*" statistically significant differences (p-value <0.05).

Table 15 Mycotoxin (expressed as mean ± standard deviation µg /Kg). In grey are colored the concentration that was statistically different between “Min”-“Max” con-ditions ($p<0.05$). “Control” refers to the aqueous standard solution of mycotoxins.

Analytes	Type	Carrots	Zucchini	Potatoes	Soybeans	H. purchased	H. homemade	Control	Spiked
AFB1	Max	1.41 ± 0.06	1.264 ± 0.147	0.324 ± 0.023	0.584 ± 0.026	1.151 ± 0.018	0.56 ± 0.064	1.462 ± 0.066	1.6
	Min	1.542 ± 0.044	1.422 ± 0.066	0.514 ± 0.06	0.676 ± 0.021	1.363 ± 0.022	0.637 ± 0.072	1.565 ± 0.045	
AFB2	Max	0.177 ± 0.07	0.218 ± 0.026	0.112 ± 0.083	0.091 ± 0.048	0.178 ± 0.04	0.166 ± 0.022	0.36 ± 0.022	0.4
	Min	0.211 ± 0.098	0.344 ± 0.021	0.184 ± 0.047	0.18 ± 0.027	0.236 ± 0.05	0.172 ± 0.037	0.371 ± 0.026	
AFG1	Max	1.35 ± 0.14	1.287 ± 0.031	1.13 ± 0.048	0.591 ± 0.032	0.697 ± 0.098	0.639 ± 0.045	1.417 ± 0.032	1.6
	Min	1.448 ± 0.105	1.503 ± 0.023	1.286 ± 0.051	0.675 ± 0.035	0.795 ± 0.031	0.83 ± 0.017	1.521 ± 0.062	
AFG2	Max	0.348 ± 0.02	0.288 ± 0.049	0.286 ± 0.056	0.115 ± 0.037	0.177 ± 0.022	0.161 ± 0.003	0.347 ± 0.043	0.4
	Min	0.35 ± 0.041	0.359 ± 0.036	0.313 ± 0.05	0.213 ± 0.045	0.204 ± 0.013	0.195 ± 0.044	0.382 ± 0.014	
FB1	Max	283.164 ± 38.94	209.443 ± 1.562	194.419 ± 1.141	124.545 ± 0.263	56.474 ± 0.328	136.944 ± 2.385	338.83 ± 9.794	400
	Min	363.552 ± 45.391	373.848 ± 3.392	356.692 ± 3.332	298.861 ± 3.558	123.584 ± 2.143	184.36 ± 1.335	367.165 ± 12.819	
FB2	Max	210.288 ± 5.34	189.176 ± 6.928	152.419 ± 0.305	127.898 ± 2.938	73.36 ± 1.599	137.405 ± 1.79	264.365 ± 23.388	400
	Min	285.901 ± 5.12	206.331 ± 4.959	201.751 ± 1.213	181.773 ± 1.063	99.296 ± 0.568	178.417 ± 1.959	283.163 ± 16.332	
FB3	Max	336.882 ± 6.99	225.779 ± 6.124	225.165 ± 2.141	140.381 ± 1.332	82.125 ± 0.876	171.33 ± 2.122	349.522 ± 20.247	400
	Min	368.691 ± 36.548	282.031 ± 4.314	251.728 ± 2.627	229.382 ± 5.42	124.756 ± 0.737	183.055 ± 8.718	375.667 ± 23.865	
HT2	Max	21.635 ± 2.57	22.467 ± 0.105	17.843 ± 0.276	6.9 ± 0.061	22.268 ± 1.667	11.715 ± 0.074	21.834 ± 1.298	25
	Min	23.595 ± 1.421	24.741 ± 0.092	20.258 ± 0.121	8.004 ± 0.135	21.27 ± 0.279	17.268 ± 0.151	24.646 ± 0.608	
T2	Max	24.356 ± 0.63	23.518 ± 1.15	24.277 ± 0.677	9.555 ± 0.078	11.037 ± 1.51	10.257 ± 0.273	22.607 ± 0.529	25
	Min	24.644 ± 0.247	23.885 ± 0.543	23.611 ± 0.941	11.631 ± 0.092	14.721 ± 0.295	10.893 ± 0.611	24.296 ± 1.171	
OTA	Max	1.473 ± 0.06	2.172 ± 0.008	1.014 ± 0.007	1.485 ± 0.029	1.094 ± 0.066	1.346 ± 0.055	2.384 ± 0.09	3
	Min	2.41 ± 0.146	2.609 ± 0.061	1.402 ± 0.003	2.602 ± 0.032	1.15 ± 0.013	1.979 ± 0.075	2.598 ± 0.115	
ZEA	Max	28.938 ± 1.39	47.744 ± 0.164	16.988 ± 0.495	37.978 ± 0.335	15.31 ± 0.918	19.682 ± 0.137	47.317 ± 4.679	75
	Min	33.734 ± 0.758	51.862 ± 0.838	23.745 ± 0.138	40.247 ± 0.349	18.482 ± 1.631	22.415 ± 1.943	50.767 ± 7.217	
Temp.	Max	93.5 ° C	95.2 °C	97.6°C	92°C	93.2°C	91°C	82.5°C	
	Min	94 °C	95.3 °C	98.3°C	94.3°C	91.8°C	90°C	80.3°C	

However, it is worth noting that the percentage of mycotoxins lost due to the cooking conditions (Figure 5) were different for all the mycotoxins tested.

Regarding PCA, The PC1 opposes individuals such as 'Carrots - Min'-'Zucchini - Min' (to the right of the graph, characterized by a strongly positive coordinate on the axis) to individuals such as 'H.purchased - Max'- 'Soybeans - Max', (to the left of the graph, characterized by a strongly negative coordinate on the axis). The group, including 'Zucchini - Min', shared high values for AFB2, ZEA, AFB1, OTA, T2, AFG1, and FB1. 'Carrots - Min' is characterized by high values for FB3 and FB2. 'H.purchased - Max' has values that do not differ significantly from the mean. 'Soybeans - Max' has low HT2, AFB1, AFB2, and T2 values. Regarding PC2, it opposes individuals such as 'H.purchased - Max', 'H.purchased - Min (to the top of the graph, characterized by a strongly positive coordinate on the axis) to individuals such as 'Carrots - Min'- 'Soybeans - Min' (to the bottom of the graph, characterized by a strongly negative coordinate on the axis). The group, including 'H.purchased - Max' and 'H.purchased - Min', has values that do not differ significantly from the mean. The group, including 'Soybeans - Min', has low HT2, AFB1, AFB2, and T2 values. The group, including 'Carrots - Min', has high values for FB3 and FB2. H. home-made, soybeans, and H. purchased are in the same parts of PC1. Therefore, they are characterized by a similar pathway in the degradation of mycotoxins.

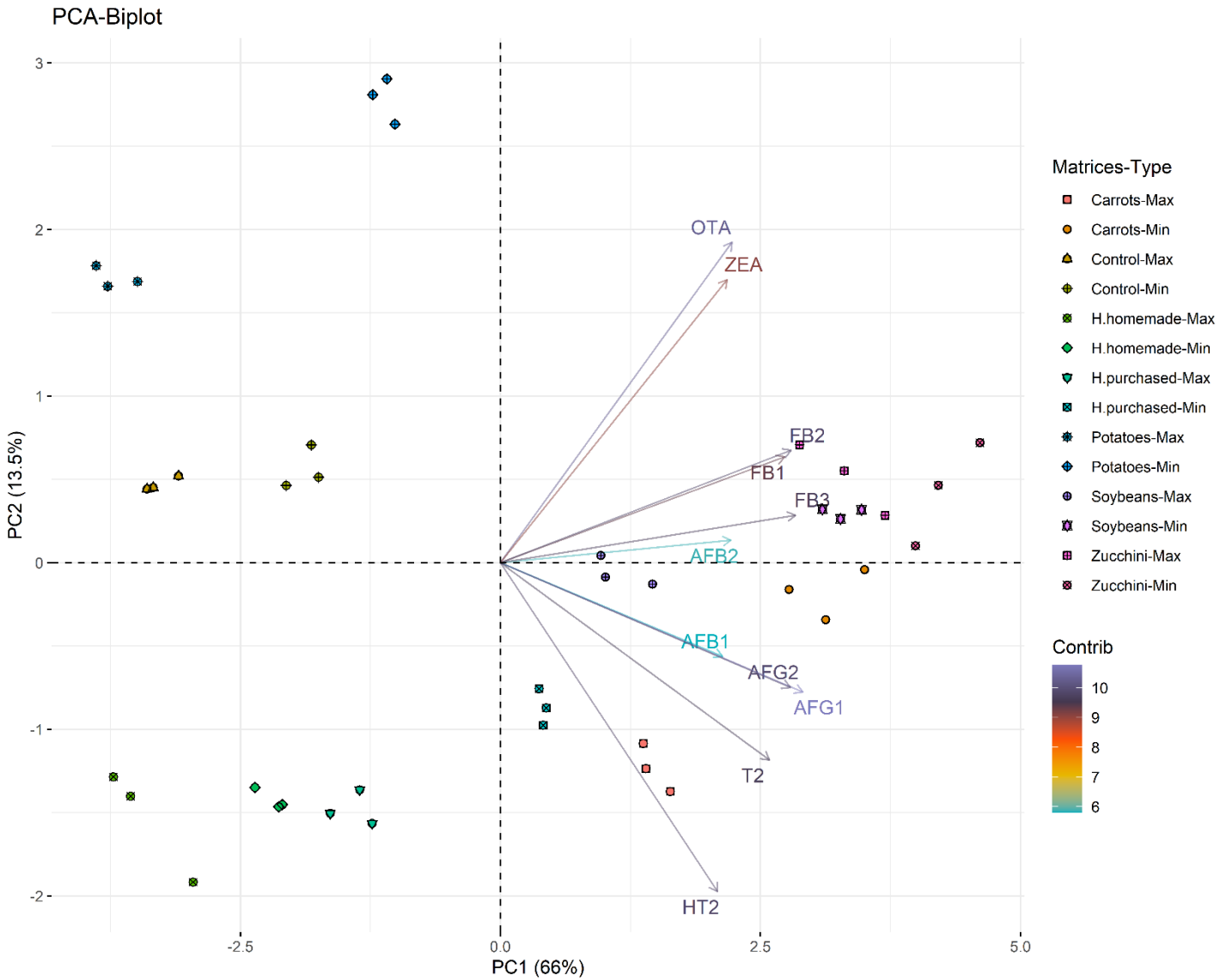

Fig. 16 PCA Variables are colored depending on the contribution to the PCs (purple>blue in decrescent order).

4. Discussion

4.1 Mycotoxin degradation difference for cooking conditions

There are different data about the degradation of mycotoxins due to heat treatment in various matrices (Kabak, 2009; Suman, 2021). Industries use cooking methods such as boiling to reduce AFs contents in cereals. For example, a significant reduction of AFB1 (around 94%) in maize was achieved by tortilla industries after nixtamalization, which involves boiling under alkaline conditions (Elias-Orozco et al., 2002). Other methods include roasting, bakery processing, ozone treatment, and UV irradiation (Suman, 2021). Each method can mitigate mycotoxin content, influenced by the food matrix, temperature, and treatment duration (Aiko & Mehta, 2015). Moisture content seems to be one of the key factor that can enhance the degradation of mycotoxins during food processing (Babaee et al., 2022; H. J. Lee et al., 2017; Sobral et al., 2019). Our analysis revealed a general trend where extended cooking times led to reductions in most mycotoxins, depending on the category. In terms of *Aspergillus* mycotoxins (AFB1, AFB2, AFG1, and AFG2), under the Max cooking condition, significant reductions in AFB1 and AFG1 were observed across most matrices, while AFB2 and AFG2 proved more heat stable. This finding contrasts with literature, which suggests that extremely prolonged cooking can enhance mycotoxins degradation (Herzallah et al., 2008; Sadeghi et al., 2020; Sobral et al., 2019). The addition of water in the falcon tube increases the degradation of AFs, affecting the stability of the lactone ring characteristic of AFs (Raters & Matissek, 2008). In fact, water in microwave heating can participate in the degradation of AFs and not act only as a solvents and the degradation does not depends only to heat treatment but also microwaves waves can participate to the degradation (Coomes et al., 1966; Gómez-Bombarelli et al., 2013; Samarajeewa et al., 1990; Y. Zhang et al., 2021).

For *Fusarium* mycotoxins (FB1, FB2, and FB3) significant reductions were observed across all matrices when subjected to the extended cooking time of 90 seconds. The additional 30 seconds of cooking at 800 W significantly reduced these mycotoxins in nearly all tested matrices, indicating their

thermolability, as reported in literature (Bullerman et al., 2002; D'Ovidio et al., 2007b). Interestingly, HT2 shows a slight decrease in all matrices except for hamburgers homemade and potatoes where degradation was more pronounced. The variability in response to microwave cooking among different mycotoxins and matrices aligns with literature reports of T2 and HT2 (Gbashi et al., 2019; Schwake-Anduschus et al., 2010). Specifically, the presence of water can enhance the degradation of T2 (Kuchenbuch et al., 2018; Sobral et al., 2019). Schmidt et al. (2017) demonstrated that 35% moisture in oat flour can significantly increase T2 degradation during processing over 50 % (Schmidt et al., 2017). OTA, and ZEA generally show a decrease under extended cooking, with statistical differences in most matrices, except for both hamburgers and soybeans, where the variations weren't statistically significant. This is contradictory to literature reports where a low percentage of OTA loss was observed after various cooking techniques like frying, boiling, and microwaving (H. J. Lee et al., 2017; Pleadin et al., 2014; Raters & Matissek, 2008; Sadeghi et al., 2020). The same applies to ZEA, a heat-resistant mycotoxin that withstands common cooking methods and is unaffected by moisture(Yu et al., 2022b).

4.2 Mycotoxin degradation in hamburgers

The microwave heating's role in reducing aflatoxins has been studied in cereals, with a maximum reduction of 32% after 10 minutes at 900 W (Herzallah et al., 2008). In chicken breast, characterized by higher moisture levels, the reduction of AFs post-microwave cooking was greater, exceeding 50% (50.7-78.6% AFB1, 46.2–84.6% AFG2) (Sobral et al., 2019). In our study, AFs degradation was higher in H hamburgers (AFB1 65%, AFB2 59.17%, AFG1-AFG2 60%) than in H. purchased (AFB1 28.13%, AFB2 55.83%, AFG1 56.43%, AFG2 56.67%) possibly due to the homemade hamburger's different composition and texture, which increased the exposure of AFs to heated water within the matrix. However, it is important to note that, despite the differences in loss percentages, the statistical analysis showed no significant difference in mycotoxins between homemade and purchased hamburgers under each condition.

FBs showed greater degradation in purchased hamburgers compared to

homemade ones. The degradation reaches a peak of 85.88% (FB1) in hamburgers purchased and cooked at maximum conditions, while the minimum reduction was observed in homemade hamburgers cooked at minimum conditions (53.91%). These percentages are in accordance with the literature where a reduction of up to 50% was observed in chips (frying and extrusion) (Milani & Maleki, 2014) and in chicken breast cooked in microwave (42%) (Sobral et al., 2019). However, FBs can bind food matrix components such as protein and these compounds are called "hidden fumonisins" and cannot be detected easily (Dall'Asta et al., 2009). The greater thermolability of FBs is also evident, as both cooking conditions consistently resulted in significant variations in their quantities within each matrix, with the exception of FB1 in soybeans and FB3 in carrots and hamburgers (grey in Table 1, Figure 2).

T2 and HT2 exemplified remarkable stability post-heat treatment, aligning with existing scholarly discourse that identifies these mycotoxins as resilient to thermal processes (Sobral et al., 2019). The attrition in HT2 levels varied, ranging from 10.93% (Max, H. purchased) to 53.14% (Max, H. homemade), indicating a differential thermal susceptibility. T2 exhibited less resistance to heat across all matrices relative to HT2. These findings are corroborated by the research of Kuchenbuch et al. (2018), who observed similar patterns in heat-treated biscuits and crunchy muesli in the oven. (Kuchenbuch et al., 2018).

In the case of ZEA, the observed degradation was pronounced, fluctuating between a minimum of 53.14% and a maximum of 79.59%. Notably, the degradation rates were consistently higher in H. purchased samples. This contrasts with existing literature that reports lower degradation rates for ZEA, typically characterizing it as a heat-resistant mycotoxin. In particular, ZEA degradation percentages reported were less than 20% in microwave chicken breast (Sobral et al., 2019) and fried potatoes (Sadeghi et al., 2020).

The degradation of OTA exhibited variability between homemade hamburgers (58.97% Max-56.43% Min) and purchased hamburgers (55.85% Max, 41.11% Min), with a marked increase in degradation across all conditions in the purchased variant. This observation stands in contrast to prior studies suggesting that increased moisture content may impede OTA degradation (Boudra et al., 1995). Investigations into microwave cooking of chicken breast

revealed OTA degradation percentages of approximately 39.1% and 25.1% at 700 W for 15 minutes (Sobral et al., 2019). Our findings seem more congruent with studies indicating a substantial 84% degradation of OTA in beans subjected to pressure cooking in water (Milanez & Leitão, 1996; Simionato et al., 2004), possibly attributable to a semi-open cap environment that simulates steam cooking due to the presence of 5 mL of water during the process.

These results underscore the significance of matrix-specific characteristics and food composition in influencing the thermal stability of mycotoxins. Each matrix demonstrated distinct mycotoxin profiles, necessitating further exploration of diverse matrices to elucidate the extent of mycotoxin contamination and its modulation during food processing. The Principal Component Analysis (PCA) furnished a nuanced perspective on the mycotoxin profiles across various food matrices and preparation methodologies. The observed distinct clustering of certain sample groups suggests unique patterns of myco-toxin contamination and degradation. For instance, soybeans and hamburgers, both purchased and homemade, aligned along the same trajectory on PC1, indicative of similar mycotoxin degradation patterns.

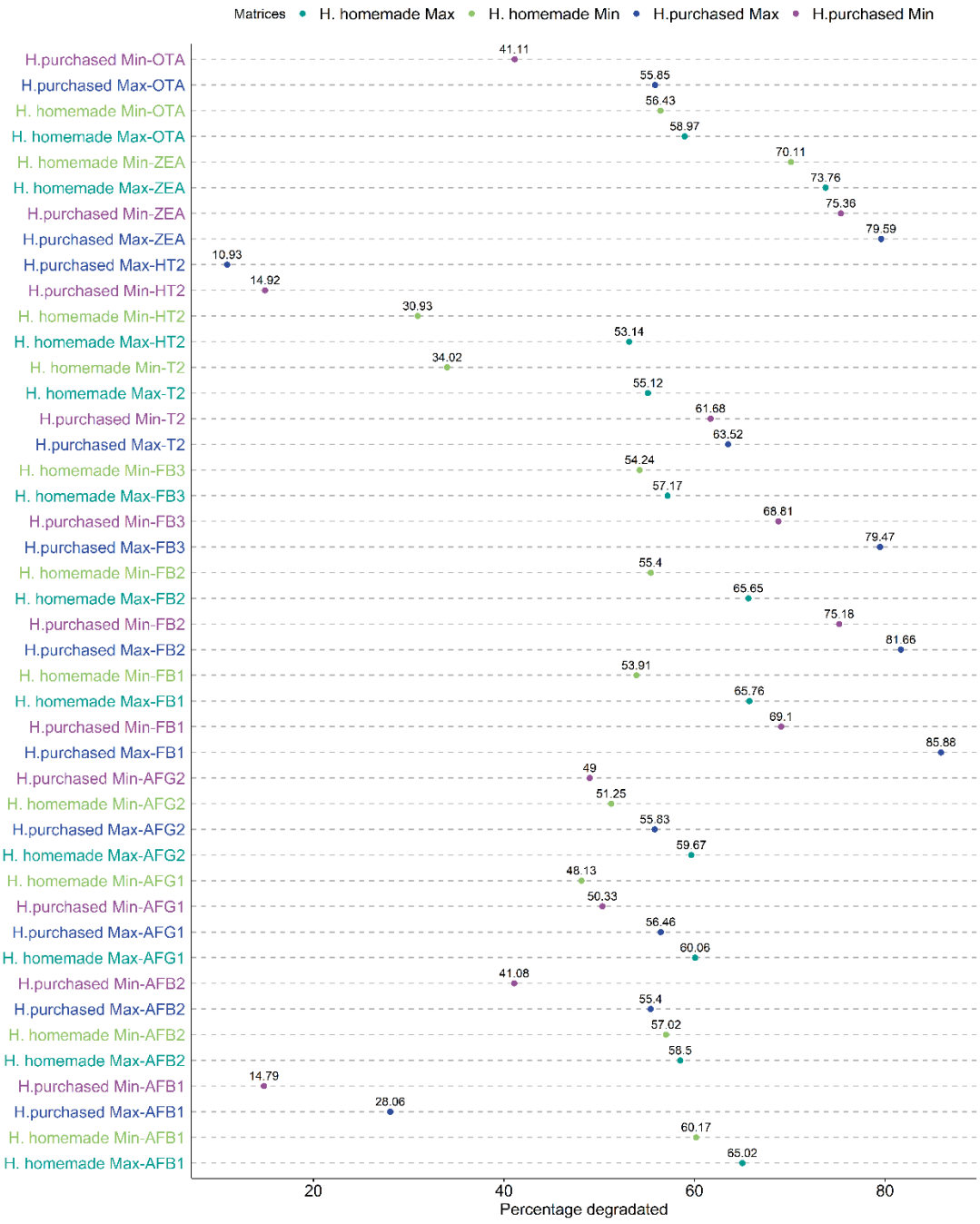

Fig. 17 Degradation expressed as percentage losses of mycotoxins analyzed. "Min" refers to 800 w for 60 s, while "Max" is 800 w for 90 s

4.3 Study limitations

The study conducted has several limitations. First, the method is not fully validated and parameters were not calculated for each matrix. Considering the matrix effect that can enhance or suppress the signal, it is important to fully validate a method that encompasses the analyses of PBMAs, ingredients

included. The study showed that each matrices is different, therefore other type of products must be studied (such as veggie-sausages and veggie-meatballs). Considering the plethora of the ingredients that can be used for these products (such as lupins), the matrices analyzed are few. Furthermore spiked samples cannot be indicative of naturally contaminated samples. Therefore, it would be beneficial to confirm these findings with real samples. The role of degradation due to temperature or microwave waves still unclear.

5. Conclusions

This study augments the current understanding of mycotoxin behavior in food matrices under cooking conditions, particularly focusing on matrices not yet regulated by European Regulation. The findings, highlighting the degradation of certain mycotoxins such as OTA, T2, HT2 during microwave cooking, contribute to the existing literature on the heat resistant nature of these compounds. Adding water during the microwave-cooking can help to reduce AFs in high percentages. There is a pressing need for additional re-search to facilitate a more nuanced comparison of food matrices and their degradation patterns, given the wide array of products available as PBMAs.

3. Final considerations

The experimental work conducted during my PhD (2020-2024) primarily focused on mycotoxin analysis in cereals and plant-based meat alternatives (PBMAs). The study commenced with the establishment and validation of a screening method compliant with European Regulation 401/2006. This method proved effective for analyzing aflatoxins (AFB1, AFB2, AFG1, AFG2), fumonisins (FB1, FB2, FB3), ochratoxin A (OTA), HT2, T2, zearalenone (ZEA), and deoxynivalenol (DON) in cereals and spices. Among the hundred samples analyzed, detectable amounts of OTA were found in two samples (maize and black pepper), both below the maximum levels (MLs) stipulated by regulation. Contrary to the high incidence reported in the literature, our study did not find a widespread occurrence of mycotoxins.

Further analysis of mycotoxins in PBMAs indicated that these products are generally safe, though it is noteworthy that the sample size was limited. No significant health concerns were identified. However, it is crucial to mention that, from the inception of the PhD project to the present (2020-2024), no specific European regulations have been formulated for PBMAs, despite their increasing consumption.

The investigation into mycotoxin degradation in common PBMA ingredients and in PBMAs themselves revealed that each matrix exhibits unique degradation patterns. Given the extensive variety of ingredients in these products, it is impractical to analyze each ingredient or product individually. Instead, focusing on the degradation patterns in key ingredients like soybeans and ensuring high levels of hygiene at the production onset are more effective strategies.

In terms of processing methods, our findings suggest that using water as a solvent in microwave cooking enhances the degradation of mycotoxins, particularly aflatoxins, where water may act as a chemical reactant in facilitating their breakdown.

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5. Publications

Data were exported using Publish or Perish, version 8.9.4554.8721, covering the period from 2020 to 2024. The retrieval date for this data was January 6, 2024. During this time, I authored 13 manuscripts.

1. A Cicero, FG Galluzzo*, G Cammilleri, ... (2020) **Development of a rapid and eco-friendly UHPLC analytical method for the detection of histamine in fish products**. *International Journal of …*, mdpi.com, cited by 10 (3.33 per year)

Abstract
We developed, validated, and confirmed with proficiency tests a fast ultra-high-performance liquid chromatography with diode array detector (UHPLC-DAD) method to determine histamine in fish and fishery products. The proposed method consists of two successive solid–liquid extractions: one with a dilute solution of perchloric acid (6%) and the second only with water. The instrumental analysis with UHPLC provides a very fast run time (only 6 min) with a retention time of approximately 4 min, a limit of quantification (LOQ) of 7.2 mg kg−1, a limit of detection (LOD) of 2.2 mg kg−1, a recovery around 100%, a relative standard deviation (RSD%) between 0.5 and 1.4, and an r2 of calibration curve equal to 0.9995. The method detected optimal values of the validation parameters and required a limited number of reagents in comparison to other methods reported in the literature. Furthermore, the method could detect histamine in a very short time compared with other methods. This method, in addition to being validated, precise, specific, and accurate, avoids wasting time, money, and resources, and limits the use of organic solvents.

Int. J. Environ. Res. Public Health **2020**, *17*(20), 7453; **https://doi.org/10.3390/ijerph17207453**

2. A Cicero, G Cammilleri, FG Galluzzo*, I Calabrese, ... (2020) **Histamine in fish products randomly collected in southern Italy: A 6-year study**. *Journal of Food …*, Elsevier, cited by 12 (4.00 per year)

Abstract
In total, 4,615 fresh and processed fish samples collected from 2010 to 2015 were analyzed for histamine by ultrahigh-performance liquid chromatography with diode array detection. Histamine levels were detected in 352 (7.6%) samples, with a maximum of 4,110 mg kg−1 and mean values of 908.9 ± 1,226.79 and 344.01 ± 451.18 mg kg−1 for fresh and processed fish samples, respectively. No histamine levels were found in canned tuna and smoked fish samples in contrast to most of the data reported in the literature. A low percentage (2.79%) of noncompliant samples was found. The highest mean values were found during 2011 and 2015 for fresh and processed fish samples, respectively, showing a significant ($P < 0.05$) difference between the sampling years. The histamine contents found in fresh fish samples were significantly higher ($P < 0.05$) than those of processed samples. Most of the positive samples came from street vendors, suggesting the need to improve inspection measures in these commercial categories to ensure fish product safety.

*Cicero, A., Cammilleri, G., Galluzzo, F. G., Calabrese, I., Pulvirenti, A., Giangrosso, G., ... & Ferrantelli, V. (**2020**). Histamine in fish products randomly collected in southern Italy: A 6-year study. Journal of Food Protection, 83(2), 241-248.*
https://doi.org/10.4315/0362-028X.JFP-19-305

3. L Pantano, L La Scala, F Olibrio, FG Galluzzo*, ... (2021) **QuEChERS LC–MS/MS screening method for mycotoxin detection in cereal products and spices**. *International Journal of …*, mdpi.com, cited by 29 (14.50 per year)

Abstract
We developed and validated a screening method for mycotoxin analysis in cereal products and spices. Ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) was used for the analysis. Dispersive solid-phase extractions (d-SPEs) were used for the extraction of samples. Ochratoxin A (OTA), zearalenone (ZEA), aflatoxins (AFLA; AFB1, AFB2, AFG1, AFG2), deoxynivalenol (DON), fumonisin (FUMO; FB1, FB2, FB3), T2, and HT2 were validated in maize. AFLA and DON were validated in black pepper. The method satisfies the requirements of Commission Regulation (EC) no. 401/2006 and (EC) no. 1881/2006. The screening target concentration (STC) was under maximum permitted levels (MLs) for all mycotoxins validated. The method's performance was assessed by two different proficiencies and tested with 100 real samples.

Int. J. Environ. Res. Public Health **2021**, *18*(7), 3774; **https://doi.org/10.3390/ijerph18073774**

4. FG Galluzzo, G Cammilleri, L Pantano, ... (2021) **Acrylamide assessment of wheat bread incorporating chia seeds (Salvia hispanica L.) by LC-MS/MS**. *Food Additives & …*, Taylor &Francis, doi:10.1080/19440049.2020.1853823, cited by 10 (5.00 per year)

Abstract
We examined the acrylamide content in samples of wheat bread with chia seeds added at different concentrations (2%, 5%, 7%, 10%) and cooked at predefined conditions (20 min at 200°C) by a validated LC-MS/M method after QuEChERS extraction. The acrylamide contents of the bread samples with added chia seeds were compared with control wheat bread samples. The highest acrylamide values were found in bread with 5% chia seeds, showing a mean value of 156.5 ± 115.4 µg/kg, followed by bread with 10% chia seeds (150.2 ± 103.8 µg/kg). About 6% of the bread samples with added chia seeds reached acrylamide levels above the benchmark level set by the EU Regulation. No significant differences in acrylamide values were found between control samples and bread with different percentages of chia seeds ($p > .05$). The results obtained provide a first report on the possible contribution of chia to the increase of acrylamide formation in bread.

*Francesco Giuseppe Galluzzo, Gaetano Cammilleri, Licia Pantano, Giovanni Lo Cascio, Andrea Pulvirenti, Andrea Macaluso, Antonio Vella & Vincenzo Ferrantelli (**2021**) Acrylamide assessment of wheat bread incorporating chia seeds (Salvia hispanica L.) by LC-MS/MS, Food Additives & Contaminants: Part A, 38:3, 388-395, DOI: 10.1080/19440049.2020.1853823*

5. FG Galluzzo, G Cammilleri, A Cicero, ... (2021) **The cold chain and the COVID-19 pandemic: an unusual increase in histamine content in fish samples collected in Southern Italy during lockdown**. *Food Quality and …*, academic.oup.com,

doi:10.1093/fqsafe/fyab031/6430763, cited by 2 (1.00 per year)

Abstract
Objectives: We analysed 900 samples of fresh (250) and processed (650) fish products collected in Sicily (Southern Italy) in 2020 during the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic (hereafter: COVID-19).
Materials and methods: The samples were divided temporally based on four phases relating to the various restrictions imposed by the Italian government in this period. The validated method of ultrahigh-performance liquid chromatography combined with a diode array detector was then employed for the analysis.
Results: The samples collected during the Phase I lockdown period and after it had ended (Phase II) revealed significant increases in the mean histamine levels: (41.89±87.58) mg/kg and (24.91±76.76) mg/kg, respectively. The 11 (1.3% of the total) fresh fish samples that were identified as being non-compliant with Regulation (EC) No.2073/2005 were only found during these two periods. All the processed samples were always compliant. The histamine values decreased as the restrictions eased, achieving a mean value of (11.16±9.3) mg/kg (Phase III).
Conclusions: There was an increase in the incidence of fish samples that were non-compliant with Regulation (EC) No.2073/2005 compared to previous surveillance data. These results provide a first report on the effect of lockdown measures on food safety and the cold chain. Our findings must cause food safety operators to intensify their controls over fresh fish products in such periods to safeguard consumer health. Further studies are required to evaluate whether the same trend would be observed with other food contaminants.

Francesco Giuseppe Galluzzo, Gaetano Cammilleri, Antonello Cicero, Licia Pantano, Andrea Pulvirenti, Andrea Macaluso, Nicola Cicero, Vittorio Calabrese, Vincenzo Ferrantelli, The cold chain and the COVID-19 pandemic: an unusual increase in histamine content in fish samples collected in Southern Italy during lockdown, Food Quality and Safety, Volume 5, **2021***, fyab031,* https://doi.org/10.1093/fqsafe/fyab031

6. V Cumbo, FG Galluzzo, G Cammilleri, A Mascetti, ... (2022) **Trace elements in stomach oil of Scopoli's shearwater (Calonectris diomedea) from Linosa's colony**. *Marine Pollution …*, Elsevier, cited by 1 (1.00 per year)

Abstract
Calonectris diomedea is a colonial Procellariiform breeding on Mediterranean islands. The stomach oil produced during chick rearing is a peculiar trait of this species. The composition of the stomach oil is likely to reflect the composition of the prey ingested and might reveal the contaminants uptake with prey becoming a possible tool for the marine pollution monitoring. We examined the concentration of 15 trace elements by ICP-MS and direct mercury analyser. The principal component analysis revealed a heterogeneous pattern of metal concentration, showing a significant separation between samples collected 20 and 70 days after hatching. The data obtained in this work give preliminary information on the feeding habits and breeding ecology of Linosa's colony of Scopoli's shearwater. The trace metals variability found suggest that the stomach oil may have a role as trophic markers to understand predator-prey

relationships and to have evidence on the accumulation of pollutants in the latter.

*Cumbo, V., Galluzzo, F. G., Cammilleri, G., Mascetti, A., Cascio, G. L., Giangrosso, I. E., ... & Ferrantelli, V. (***2022***). Trace elements in stomach oil of Scopoli's shearwater (Calonectris diomedea) from Linosa's colony. Marine Pollution Bulletin, 174, 113242.* https://doi.org/10.1016/j.marpolbul.2021.113242

7. G Cammilleri, V Calabrese, A Vella, ... (2022) **Essential and non-essential elements in white lupin (Lupinus albus L.) cultivated in Southern Italy**. *Natural Product …*, Taylor &Francis, doi:10.1080/14786419.2022.2107641, cited by 1 (1.00 per year)

Abstract
We assessed the presence of V, Cr, Ni, Cu, Zn, As, Se, Sb, Cd, and Pb in white lupin samples cultivated in Southern Italy by the validation of an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method. The ICP-MS method validation showed satisfactory values of linearity (r2 > 0.999), recovery (87.4–100.7%), repeatability, and reproducibility values. Zinc was the most abundant element; showing mean concentrations of 0.778 ± 0.09 mg/Kg wet weight (w.w.) and a maximum of 1.013 mg/Kg w.w., followed by copper (0.191 ± 0.05 mg/Kg w.w.). Among the non-essential elements, important levels of cadmium were found (0.017 ± 0.004 mg/Kg w.w.), with 28% exceeding the limits set by the EU Regulation. The results of this work confirm the role of white lupins and other legumes in reducing the pH of the soil, increasing the exchangeable forms of Cd. This work also provides the first data on the nutritional and antinutritional properties of white lupins cultivated in Italy.

*Gaetano Cammilleri, Vittorio Calabrese, Antonio Vella, Andrea Macaluso, Emanuela Bacchi, Licia Pantano, Francesco Giuseppe Galluzzo, Adriana Oddo, Giuseppe Giangrosso, Vincenzo Ferrantelli & Mariagrazia Brunone (***2022***) Essential and non-essential elements in white lupin (Lupinus albus L.) cultivated in Southern Italy, Natural Product Research, 38:1, 164-168, DOI: 10.1080/14786419.2022.2107641*

8. FG Galluzzo, V Cumbo, G Cammilleri, V Calabrese, ... (2022) **Fatty Acids Composition of Stomach Oil of Scopoli's Shearwater (Calonectris diomedea) from Linosa's Colony**. *Animals*, mdpi.com, cited by 1 (1.00 per year)

Abstract
Calonectris diomedea is a Procellariforms seabird having a very representative colony in Linosa Island (Southern Italy). The adult forms of C. diomedea produce a pasty oil from their proventriculus to feed their chicks during the rearing period. In this work, we examined the fatty acids composition of the stomach oil of C. diomedea from Linosa Island by gas chromatography with flame ionization detection (GC-FID). The samples were collected at 20 and 70 days after hatching. Twenty different fatty acids (FAs) were identified. Saturated fatty acids (SFA) were the most abundant in percentage (41.6%) at day 20 followed by polyunsaturated fatty acids (PUFA, 38.7%) and monounsaturated fatty acids (MUFA, 19.7%). MUFAs were the most abundant in samples collected at day 70 (53.8%), followed by SFAs (36.6%) and PUFAs (9.8%). Oleic acid (C18:1ω9) in the samples on day 70 was 4 times higher than that in the samples on day 20. The Principal Component Analysis (PCA)

verified a clear separation of the stomach oil samples in two groups, according to the day of sampling. The results obtained confirm the role of FAs analysis of stomach oil to understand the ecology and breeding behaviour of C. diomedea, highlighting a resemblance with signatures recorded in marine organisms of Linosa Island.

Animals **2022**, *12*(9), 1069; **https://doi.org/10.3390/ani12091069**

9. E Bacchi, G Cammilleri, M Tortorici, FG Galluzzo, ... (2022) **First Report on the Presence of Toxic Metals and Metalloids in East Asian Bullfrog (Hoplobatrachus rugulosus) Legs**. *Foods*, mdpi.com, cited by 2 (2.00 per year)

Abstract
We examined the presence of As, Cr, Cd, and Pb in 42 samples of farmed East Asian bullfrog (Hoplobatrachus rugulosus) from Vietnam and Thailand by inductively coupled plasma-mass spectrometry (ICP-MS). An estimation of the dietary intake and exposure to the toxic elements analysed was also carried out. The results showed very high As levels, with mean values of 0.094 ± 0.085 mg/Kg w.w. and a maximum of 0.22 mg/Kg. No significant differences were found for As contents between areas of production ($p > 0.05$). No detectable Cd contents were found in all the samples examined. The Pb concentrations of the East Asian bullfrog legs samples were below the European Commission's permitted levels. The Cr and Pb contents of the East Asian bullfrog produced in Vietnam were significantly higher than that produced in Thailand ($p < 0.05$). The target hazard quotient (THQ) ratio for Cr was not exceeded for all the samples analysed. In contrast, the benchmark dose lower confidence limit (BMDL) and THQ ratios for As were exceeded, indicating carcinogenic and non-carcinogenic risks for those who consume this type of food. The results of this work confirm the role of As-contaminated water absorption as an important source of arsenic for these adult organisms.

Foods **2022**, *11*(19), 3009; **https://doi.org/10.3390/foods11193009**

10. G Cammilleri, V Calabrese, L Accordino, ... (2023) **Toxic metals and total lipids comparison between wild and farmed fish of South mediterranean**. *Natural Product …*, Taylor &Francis, doi:10.1080/14786419.2022.2037588, cited by 4 (4.00 per year)

Abstract
A total of 151 wild and farmed fish samples of three species (*S. aurata*, *D. labrax* and *U. cirrosa*) from south Mediterranean were examined for cadmium, lead and mercury and total lipids detection to verify possible differences between wild and farmed fish. Mercury was detected only in S. aurata samples, with mean values of 0.056 ± 0.128 mg/kg and 0.031 ± 0.033 mg/kg for farmed and wild samples, respectively. The results verified no significant differences in Pb, Cd and Hg levels between wild and farmed fish samples ($p < 0.05$). The farmed fish samples showed higher total lipids contents than wild ($p > 0.05$). As far as we know, this work report first findings on the toxic metal presence in farmed Umbrina cirrosa, showing no detectable Hg, Cd and Pb values. The results of this work seems to confirm that the presence of toxic metals could be more related to the pollution the site than the type of production.

*Gaetano Cammilleri, Vittorio Calabrese, Letizia Accordino, Licia Pantano, Aldo Migliazzo, Francesco Giuseppe Galluzzo, Vincenzo Parrino, Assunta Brunone, Gianluigi Maria Lo Dico, Emanuela Bacchi, Giuseppe Giangrosso, Pellegrino Francesco Calvacca & Vincenzo Ferrantelli (***2023***) Toxic metals and total lipids comparison between wild and farmed fish of South mediterranean, Natural Product Research, 37:13, 2232-2242,*
DOI: *10.1080/14786419.2022.2037588*

11. D Gambino, FG Galluzzo, L Cicero, R Cirincione, ... (2023) **Antibiotic Resistance Genes Carried by Commensal Escherichia coli from Shelter Cats in Italy**. *Veterinary sciences*

Abstract
Antimicrobial resistance is a widespread global health problem. The presence of resistant bacteria and antibiotic resistance genes has been demonstrated not only in humans but also in animals, including pets. Stray cats share the urban environment with people and pets. This may facilitate transmission of resistant bacteria and resistance genes between stray animals, people and domestic animals. Several studies have investigated the role of stray cats as a fecal carrier of ESBL-producing bacteria. However, there are many genes and resistance mechanisms that can be detected in commensal E. coli, which, because of its genetic plasticity, is considered an indicator for monitoring antibiotic resistance. In this study, rectal swabs were collected from stray cats from colonies and shelters in the city of Monza (Monza Brianza, Italy) to isolate commensal E. coli. Phenotypic tests, such as the minimum inhibitory concentration (MIC) and the double disc test (DDST), and molecular analyses to detect antimicrobial resistance genes (ARGs) were used to study the resistance of these isolates. The results obtained confirm that stray cats can carry ESBL-producing E. coli (6.7%) and genes conferring resistance to other important antibiotic classes such as tetracyclines and sulfonamides.

Vet. Sci. **2023**, *10*(12), 680; **https://doi.org/10.3390/vetsci10120680**

12. G Cammilleri, V Calabrese, L Pantano, ... (2023) **Polyphenols of white lupin (Lupinus albus L.) seeds cultivated in Southern Italy by a LC-HRMS method**. *Natural Product …*, Taylor &Francis, doi:10.1080/14786419.2023.2245535

Abstract
In this work we examined the contents of 14 polyphenols in white lupin (Lupinus albus L.) samples cultivated in Southern Italy by the optimisation and validation of a LC-HRMS method. The validation of the LC-HRMS method showed linearity results r2 > 0.989 and recovery values between 71 and 119% for a very wide range of concentrations. Ellagic acid was the most abundant polyphenol, with mean concentrations of 16271.86 ± 19798.53 µg/Kg, followed by apigenin (2749.51 ± 889.95 µg/Kg). A significant variability in ellagic acid contents was found between the areas of cultivation examined ($p < 0.05$). As far as we know, this work provides the first data on the polyphenols contents of white lupins cultivated in Italy. The comparison with other study confirms the role of the cultivation area for the determination of the polyphenol's contents. The study also confirms white lupins as a promising source of antioxidant and anti-inflammatory substances in a balanced diet.

*Gaetano Cammilleri, Vittorio Calabrese, Licia Pantano, Mariagrazia Brunone, FrancescoGiuseppe Galluzzo, Andrea Pulvirenti, Tilman Fritsch, Carmelo Bongiorno, Andrea Macaluso & Vincenzo Ferrantelli (***2023***) Polyphenols of white lupin (Lupinus albus L.) seeds cultivated in Southern Italy by a LC-HRMS method, Natural Product Research,*
DOI: 10.1080/14786419.2023.2245535

13. G Cammilleri, FG Galluzzo, A Pulvirenti, ... (2023) **Toxic metals in Loggerhead sea turtles (Caretta caretta) stranded freshly dead along Sicilian coasts**. *Veterinary …*, Taylor &Francis, doi:10.1080/01652176.2023.2169781

Abstract
Background: The Loggerhead sea turtle (Caretta caretta) is a marine reptile belonging to a monophyletic group of chelonians. As these animals are long-lived, they have the ability to accumulate pollutants. Aim: To collect epidemiological data on toxic metals in marine Loggerhead sea turtles. Materials and Methods: Forty Loggerhead sea turtles comprising 25 males and 15 females stranded freshly dead between 2013 and 2018 along the coasts of Sicily, Southern Italy, were examined for arsenic, cadmium, and lead accumulation in muscle and adipose tissues by means of a validated ICP-MS method. A modified K index as a growth condition factor, namely Fulton's K index, was used. Samples were tested in duplicate. A Wilcoxon rank sum test was carried out to evaluate metal contents differences between muscle and adipose tissues and between genders. Results: The Fulton's K index suggested a good body condition of the C. caretta recovered with mean values of 5.34 ± 3.40 (n = 40; ±SD). Detectable concentrations of lead were found in 70% of the samples analysed with mean values of 0.65 ± 1.67 mg/kg wet weight and 0.51 ± 1.29 mg/kg wet weight in muscle and adipose tissues, respectively. No significant differences in arsenic, cadmium, and lead were detected between genders. In addition, no significant correlation was found between modified K index and concentrations of arsenic, cadmium, and lead. Clinical relevance: Findings on muscle and adipose tissues suggest chronic exposure of Caretta caretta to high concentrations of especially lead which might negatively affect health and welfare of these marine turtles although body condition was good.

Gaetano Cammilleri, FrancescoGiuseppe Galluzzo, Andrea Pulvirenti, Licia Pantano, Vittorio Calabrese, Antonino Gentile, Valentina Cumbo, Andrea Macaluso, Vito Macaluso, Antonio Vella & Vincenzo Ferrantelli (***2023***) Toxic metals in Loggerhead sea turtles (Caretta caretta) stranded freshly dead along Sicilian coasts, Veterinary Quarterly, 43:1, 1-10, DOI:* 10.1080/01652176.2023.2169781

6. Posters

2023

1. “Determination of aflatoxins (AFB1, AFB2, AFG1, AFG2, AFB2) in pistachios collected in Southern Italy (Sicily)”. Francesco Giuseppe Galluzzo, Licia Pantano, Antonello Cicero, Vito Macaluso , Gaetano Cammilleri, Andrea Macaluso, Vincenzo Ferrantelli. World Mycotoxin Forum Anversa 8-11/10/2023

2. “Trace elements composition of marine collagen powder food supplements” Gaetano Cammilleri, Marina Tortorici, Francesco Giuseppe Galluzzo, Andrea Pulvirenti, Stefano Vullo, Salvatore Seminara, Andrea Macaluso. XIII Congresso Nazionale di Chimica degli Alimenti. Marsala 31/05/2023

3. “New methodological approaches in glyphosate’s research in cereals and its derivatives” Licia Pantano, Gaetano Cammilleri, Maria Drussilla Buscemi, Francesco Giuseppe Galluzzo, Andrea Macaluso, Tiziana Bertuglia, Nicola Cicero, Mariarita Pisano, Vincenzo Ferrantelli. XIII Congresso Nazionale di Chimica degli Alimenti. Marsala 31/05/2023

4. “A rapid LC-MS/MS after quechers method for the determination of pesticides in dead bees for honey production risk management”. Gaetano Cammilleri, Maria Drussilla Buscemi, Francesco Giuseppe Galluzzo, Licia Pantano, Emanuela Bacchi, Veronica Fiore, Andrea Macaluso, Nicola Cicero, Giovanni Lo Cascio, Barbara Randisi, Vincenzo Ferrantelli. XIII Congresso Nazionale di Chimica degli Alimenti. Marsala 31/05/2023

5. “Analyses of pesticides in honey, bee (Apis mellifera) and honeycomb in Southern Italy (Sicily) as strategies for monitoring pollution”. Gaetano Cammilleri , Francesco Giuseppe Galluzzo , Emanuela Bacchi , Maria Drussilla Buscemi , Licia Pantano , Valentina Cumbo , Stefano Vullo , Andrea Macaluso , Vincenzo Ferrantelli. Annual General Session of the World Assembly of Delegates of the World Organisation for Animal Health (WOAH, founded as OIE) took place on Sunday 21 to Thursday 25 May 2023, in Paris.

6. “Trace elements composition of marine collagen powder food supplements” Gaetano Cammilleri, Marina Tortorici, Francesco Giuseppe Galluzzo, Andrea Pulvirenti, Stefano Vullo, Salvatore Seminara, Andrea Macaluso. XIII Congresso Nazionale di Chimica degli Alimenti. Marsala 31/05/2023

7. “Use of terrestrial gastropods as bioindicators of contamination status environment of sicilian natural parks” Maria Drussilla Buscemi, Gaetano Cammilleri, Francesco Giuseppe Galluzzo, Lucia Pantano, Emanuela Bacchi, Andrea Macaluso, Nicola Cicero, Vincenzo Ferrantelli. XIII Congresso Nazionale di Chimica degli Alimenti.

Marsala 31/05/2023

8. “Erucic acid determination of commercial mustard collected in Southern Italy (Sicily) by GC/FID”. Vincenzo Ferrantelli, Giovanni Lo Cascio, Gaetano Cammilleri, Vittorio Calabrese, Licia Pantano, Francesco Giuseppe Galluzzo, Vito Macaluso, Andrea Macaluso. 16 - 20 October 2023 - Lima, Peru XXX CPQ SILAE CONGRESS INVITATION

9. “A study on the possible correlation between toxic metals levels and infastation of anisakis sp. in fish of mediterranean sea” Gaetano Cammilleri, Andrea Macaluso, Francesco Giuseppe Galluzzo, Stefano Vullo, Vincenzo Ferrantelli, Salvatore Seminara. 16 – 20 October 2023 - Lima, Peru XXX CPQ SILAE CONGRESS INVITATION

10. “Analysis of cadmium (Cd) levels in mussels (M. galloprovincialis) collected in Sicily”. Francesco Giuseppe Galluzzo, Gaetano Cammilleri, Calogero Alfano, Elisa Maria Messina, Andrea Macaluso, Vincenzo Ferrantelli. 16 - 20 October 2023 - Lima, Peru XXX CPQ SILAE CONGRESS INVITATION

2020-2022

11. "Screening on the presence of Ochratoxin A in dry-cured meat products collected in Southern Italy (Sicily) during 2019-2021". pubblicato agli atti del "The World Mycotoxin Forum" 15-18/05/2022 presso Parma P8

12. "Presence of ochratoxin A in red and white wines sourced from Southern Italy (Sicily)" pubblicato agli atti del "The World Mycotoxin Forum" 15-18/05/2022 Parma P7

13. "Analysis of the presence of ochratoxin A in coffee and coffee-based products collected from Southern Italy (Sicily) during 2019-2021" pubblicato agli atti del "The World Mycotoxin Forum" 15-18/05/2022 Parma P15

14. “In-house validation of an artificial digestion protocol for the detection of anisakidae larvae in fish products according to the ISO 23063-2:2021" 75°CONVEGNO SISVET Lodi, 15-18 giugno 50 P.

15. "Seasonal trend of Anisakidae infestation in silver scabbardfish collected in South Mediterranean" 75°CONVEGNO SISVET Lodi, 15-18 giugno 51 P.

16. "Histamine Concentration Assessment in fish samples commercialised in Sicily" 75°CONVEGNO SISVET Lodi, 15-18 giugno 52 P.

17. Poster "Toxic Metals in cetaceans stranded along South Mediterranea Coasts" del 75°CONVEGNO SISVET Lodi, 15-18 giugno 105 P.

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I am deeply indebted to my family for their endless love, patience, and belief in my capabilities. Their moral support and unwavering confidence in me have been my pillars of strength. To my friends, thank you for being there during the highs and lows, providing much-needed breaks, and always believing in me.

Lastly, I want to express my heartfelt appreciation to coffee and folk dances. Coffee helps me to survive in early mornings, providing the necessary fuel to keep me going. Folk dances offered a much-needed respite from the computer, insisting on human contact and physical activity, which was essential for maintaining my mental and emotional well-being.