Development of Protocols and Quantification of Mycotoxin Contaminants of Bakery Flours in the Maltese Market

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To my mother,

Carmen

Abstract

Mycotoxins, secondary toxic metabolites produced by fungi under favourable conditions, have long raised concerns as contaminants in various flours, including wheat, wholemeal, rye, and maize, due to their detrimental effects on both human and animal health. This study addressed a knowledge gap by conducting the first survey of mycotoxin contamination in flours available in the Maltese market. The primary objective was to develop and validate in-house methods, while also identify and quantify mycotoxins present in flour samples sourced from supermarkets, milling facilities, and bakeries, specifically targeting products utilized in bread making and bakery processes. A survey in 16 flour samples was undertaken, utilizing high-performance liquid chromatography coupled with fluorescent and photodiode detectors (HPLC-FLR-PDA) for the quantification of mycotoxin. OTA, DON, AF, ZEA, T-2, HT-2, FUM, and PAT were the key mycotoxins examined. Notably, the co-occurrences of mycotoxins were widely observed; however, AFs and FUM were absent from all samples. While common mycotoxins, including DON, T-2, and HT-2, were detected, the first was found below the maximum limits specified by the EU. The presence of patulin aligns with the prevalence of *Penicillium* contamination on the Maltese Islands. This finding highlights the need for more awareness and targeted surveillance strategies to accurately assess the true extent of mycotoxin levels in products circulating within the Maltese market. In conclusion, this first study gives an indication of the types of mycotoxins present in Maltese flours. The implications extend to both public health and food safety fields, prompting further investigations and the implementation of robust surveillance measures to ensure the accurate monitoring and control of mycotoxin levels within the local food supply chain.

Keywords: wheat flour; mycotoxins; co-occurrence; HPLC; contaminants; surveillance; food safety

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List of Abbreviations

15-AcDON	15-acetyl-DON
1-AN	1-Anthronitrile
3-AcDON	3-acetyl-DON
ADM	Aminoglycoside Detection Medium
ADRBC	Antibiotic Dextrose Rose Bengal Chloramphenicol Agar
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
AOH	Alternariol
ASE	Accelerated Solvent Extraction
ATA	Alimentary Toxic Aleukia
a_w	Water Activity
βBP	β- Cyclodextrin Beard Polymer
BGC	Biosynthetic Gene Cluster
C18	Octadecylsilyl
CMA	Czapek's Modified Agar
CSI	Combined Stress Indexes
CYA	Czapek Yeast Autolysate Agar
CZA	Czapek's Agar

DCBC	Dichloran Rose Bengal Chloramphenicol
DES	Deep Eutectic Solvent
DF	Dilution Factor
DG18	Dichloran 18% glycerol agar
D-MAP	4-Dimethylaminopyridine
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DON-3-G	Deoxynivalenol-3-Glucoside
d-SPE	Dispersive Solid-Phase Extraction
E_2	Estradiol
EC	European Commission
EFSA	European Food Safety Authority
ELEM	Leukoencephalomalacia
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
EUFS	Energy Units Full Scale
FAO	Food and Agriculture Organization
FB_1	Fumonisins B_1
FB_2	Fumonisins B_2
FB_3	Fumonisins B_3
FB_4	Fumonisins B_4
FDA	Food Drug Administration
FHB	Fusarium Head Blight
FLR	Fluorescence detection

FUM	Fumonisins
G25N	Glycerol 25% Nitrate Agar
GC	Gas Chromatography
GKCH	Chloramphenicol Yeast Glucose Agar
HPLC	High-Performance Liquid Chromatography
HT-2	HT-2 Toxin
IAC	Immunoaffinity Column
IARC	International Agency of Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KBr	Potassium Bromide
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LFIA	Lateral Flow Immunoassay
LLE	Liquid-Liquid Extraction
LOD	Limits of Detection
LOQ	Limits of Quantification
MEA	Malt Extract Agar
MIP	Molecularly Imprinted Polymer
MY50G	Malt Yeast 50% Glycerol Agar
NSO	National Statistics Office
OPA	Phthaldialdehyde
OTA	Ochratoxin A
PAT	Patulin
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar

PDA	Potato Agar Dextrose
PLE	Pressurized Liquid Extraction
PMT	Photomultiplier
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
R^2	Co-efficient of Determination
RSDr	Intra-day Precision
RT	Retention Time
SDA	Sabouraud Dextrose Agar
SFE	Supercritical Fluid Extractions
SLE	Solid-Liquid Extraction
SPE	Solid Phase Extraction
T-2	T-2 Toxin
TFA	Trifluoroacetic Acid
TLC	Thin-Layer Chromatography
UV	Ultraviolet
VALDS	Vortex Assisted Low Density Solvent-Microextraction
WHO	World Health Organization
YES	Yeast Extract Sucrose Agar
YGCA	Yeast Glucose Chloramphenicol
ZEA	Zearalenone
λ_{em}	Emission
λ_{ex}	Excitation

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Chapter 1 – Introduction

1.1 Fungi and their metabolites; Mycotoxins

Fungi are ubiquitous organisms that occur naturally as part of the microflora in agricultural fields or stored crops. As a result, fungi like *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* spp., among others, invariably find their way into the food chain at various stages—before and after harvesting or during storage—leading to inevitable contamination of food and crops (Ksenija 2018). These fungal contaminants pose a dual challenge: they not only contribute significantly to food losses, but also have the potential to produce naturally occurring toxic by-products referred to as mycotoxins under favourable conditions (Pandey, Samota et al. 2023). The main mycotoxins are aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FUM), deoxynivalenol (DON), zearalenone (ZEA), trichothecenes type A (T-2, HT-2) and patulin (PAT) (Khodaei, Javanmardi et al. 2021). The amount of toxic secondary metabolites produced depends on various physical factors such as water activity (a_w), moisture, relative humidity, temperature and mechanical damage of cereals (Chhaya, O'Brien et al. 2022). Numerous species are able to produce mycotoxins, such as *Aspergillus flavus*, *Aspergillus carbonarius, Fusarium verticillioides* and *Fusarium graminearum* (El-Sayed, Jebur et al. 2022), known to produce AFs, OTA, FUM and ZEA, respectively. Optimal production conditions for OTA production can range from 10 – 30 °C and a_w of 0.85 – 0.98 in the case of *A. carbonarius* (Ksenija 2018).

These harmful secondary compounds exhibit strong chemical stability and resistance to heat. As a result, there is growing concern about their potential persistence in food products like bread even after undergoing multiple processing stages (Marin, Ramos et al. 2013). The cumulative effect of mycotoxins can lead to significant acute and chronic health impacts on humans and animals, particularly when ingesting food that harbours these chemical agents over an extended period (Chhaya, O'Brien et al. 2022). It is estimated that over five billion individuals are exposed daily, often through unknown pathways, to contaminated food with mycotoxins such as AFs on a regular basis (Marc 2022).

To date, Malta lacks mycotoxin testing and research, leaving a gap in our understanding of the levels of contamination in the local flour supply. Flour is one of the basic ingredients used in baking, and for the Maltese baking is mostly associated with bread, as once a Maltese scientist Sir Temi Zammit stated that 'There is nothing else that is eaten more than bread and I don't know what we would have done without it' (Buttigieg, Magro et al. 2023). This study aims to be the first in Malta to provide an understanding on the level of mycotoxin contamination in bakery raw materials namely flour and if the latter adheres to the European Commission food safety legislations and regulations. This study aims to highlight the importance of continuous mycotoxin surveillance and improvement in knowledge and occurrence data.

1.2 Aims and Objectives

This study aims to develop and validate mycotoxin analytical methods using High Performance Liquid Chromatography coupled with fluorescence and photodiode detectors to quantify

mycotoxins in flour. Specifically, this study will be the first survey ever completed to assess and quantify mycotoxins in various local flour samples of which most (wheat) are used to produce the Maltese bread and *ftira*[1], amongst other bakery uses to produce other products like cakes, pies and cookies.

The specific objectives for this study are:

- To develop analytical methods to detect AF, OTA, ZEA, HT-2, T-2, DON, PAT, FUM.
- To validate and optimize the analytical methods to achieve accuracy, linearity, matrix affects, LOD and LOQ corresponding to the regulations.
- To perform a survey on various flour types used in baking and detect and quantify the mycotoxins.

1.3 Research Design and Methodology

This study is the first in Malta to perform research on mycotoxin detection and quantification. Therefore, laboratory experimental work will focus on method development and validation on various mycotoxin analytical methods using HPLC-FLR-PDA. Recovery experiments will be performed to assess the whole testing process including sample preparation, which involves extraction and clean-up using immunoaffinity columns (IACs). The analytical methods will also be tested in terms of selectivity, linearity, matrix effects, accuracy, limits of detection (LOD)

[1] The Maltese *ftira* is a type of sourdough bread which was the first ever local product inducted on the UNESCO's Intangible Cultural Heritage of Humanity list (Buttigieg, Magro et al. 2023).

and limits of quantification (LOQ) to fulfil the requirements of Commission Regulation (EC) No. 401/2006. Then, a surveillance study will be performed on all flour samples collected from supermarkets, milling facilities and bakeries. During this study multiple limitations may be encountered such as portability and practical issues based on the matrix effect, sample type and preparation and also calibrations in relation to the equipment being utilised.

Chapter 2 – Literature Review

2.1 History of cultivation and production of cereals

The first known records of wheat cultivation date back to 10000 - 8000 B.C.E and it is believed to have been first cultivated in the Tigris and Euphrates River Valley. Moreover, information from Egyptian tombs and surviving Chinese documents dating back to 5,000 and 2700 B.C.E, respectively, show an early understanding of harvesting and processing of wheat (Finnie, Atwell 2016a). Presently, human civilization still relies on cereals and cereal-derived products as a staple food of choice in daily diets (Laskowski, Górska-Warsewicz et al. 2019) mainly due to the relatively cheap cost and high nutritional value (Alconada, Moure et al. 2019, Rosell 2011). Cereals provide 18% of the calories and 20% of the protein content in human daily dietary requirements (Royo, Soriano et al. 2017).

Currently, China is the largest producer of cereal crops, producing 615 million metric tons in 2020 (Figure 1; FAOSTAT 2023). European countries, Germany, France and Poland, as well as those within the Mediterranean region, Turkey and Ukraine, also produce a significant amount of cereals. Middle eastern countries such as Lebanon, Jordan and Egypt cultivate and consume a large portion of durum wheat (*Triticum durum* L.) available worldwide since it is a key raw material to produce flat breads, bulgar and couscous (Pasqualone, Vurro et al. 2022, Royo, Soriano et al. 2017). Wheat (*Triticum aestivum* L.) and variations, such as durum wheat (*Triticum durum* Desf.), are the heart of Mediterranean food culture and heritage. Bread and pasta are a favourite food of choice within the region which are largely wheat-based recipes.

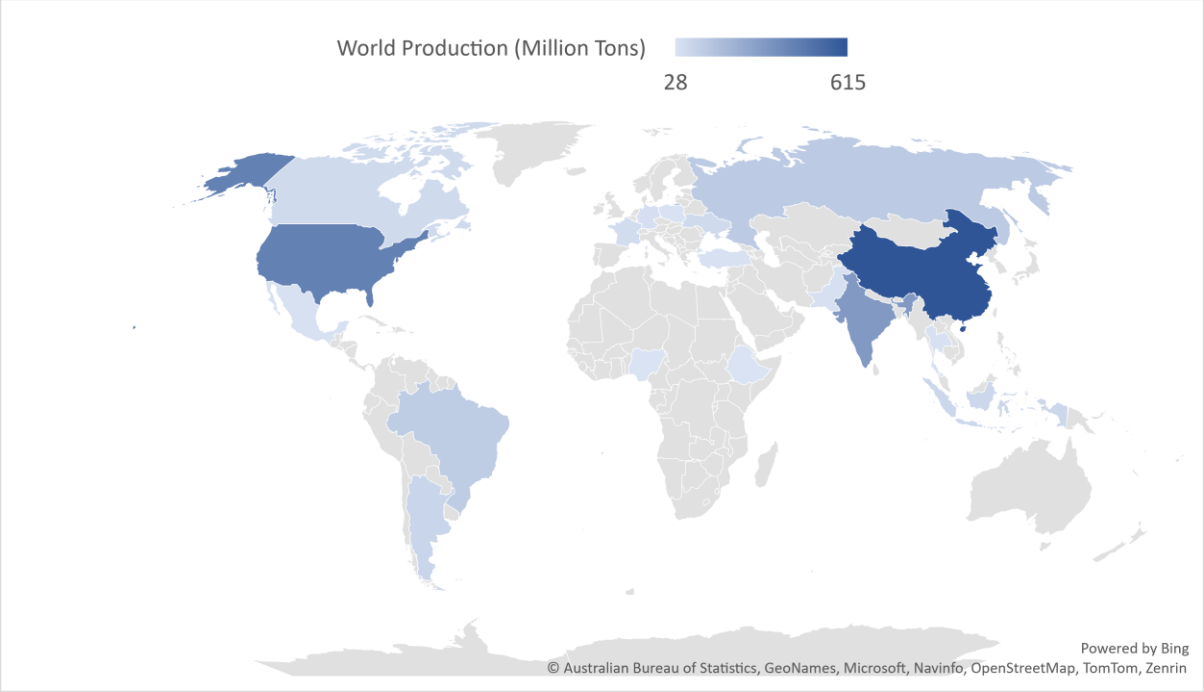

Figure 1. Total production for the top 20 countries for cereals of barley, oats, millet, sorghum, maize, rice, rye and wheat during the year of 2020 (FAOSTAT, 2023).

From the latest available data for worldwide production of key crops used in breadmaking, published by the Food and Agriculture Organization (FAO) of the United Nations, almost 2000 million tonnes of wheat and maize (corn) were produced in 2021. Whilst other cereals such as barley, rye, oats and millet were produced in lesser amounts (145, 13, 22 and 30 tonnes, respectively; FAO 2023). The production of bakery cereal crops from 2014 to 2021 are presented in Figure 2. Maize was the only crop that has been increasing in production amount, while other crops such as wheat, barley, oats, millet and rye were kept at same range. This is not in line with the global challenge the world faces, to increase global supply of wheat production and other cereals to meet the global demand, as the human population rises (Lama 2020).

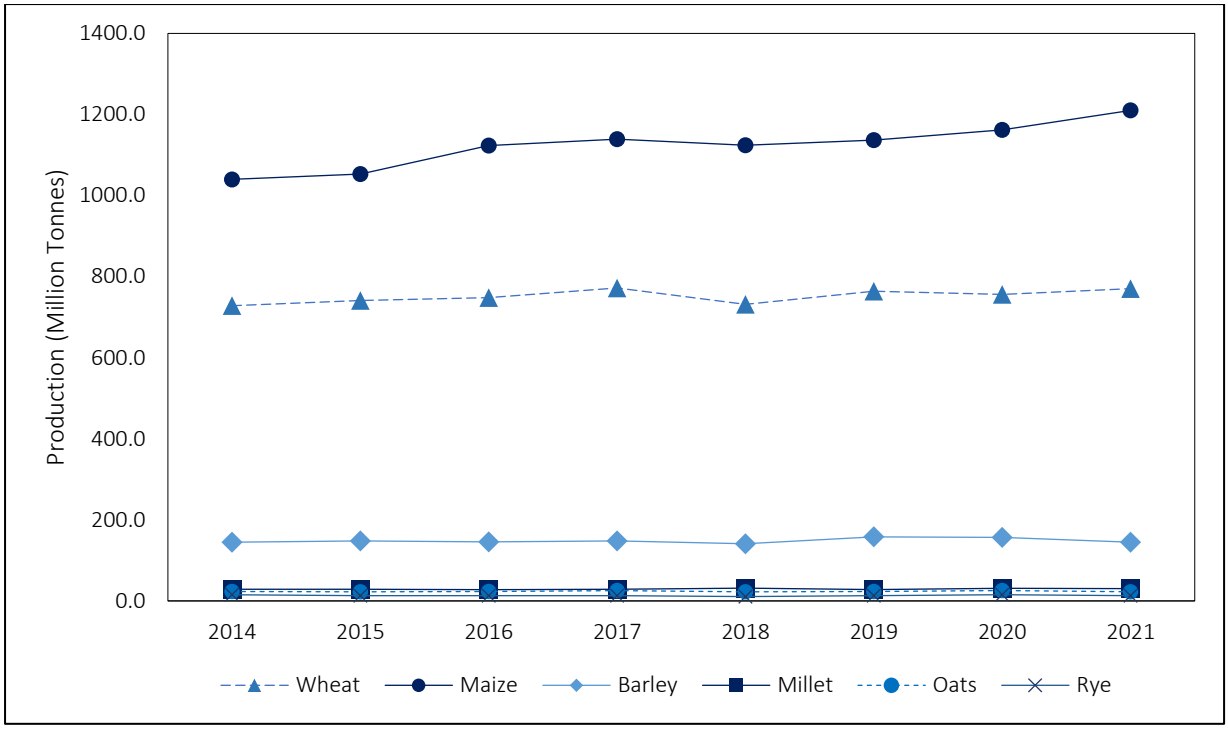

Figure 2. World Cereal Production of bakery crops from recent years (FAOSTAT, 2023).

However, in the past couple of years wheat production has suffered stressors such as climate change, COVID-19 pandemic, and territorial conflicts. For instance, the Russian invasion of Ukraine has greatly threatened the global wheat supply and prices of commodities, which caused them to soar. Russia and Ukraine are two of the largest producers, exporting over 25% of the world's wheat supply. Shortage of wheat considerably affected the demand and supply chains which were already impacted by the COVID-19 pandemic through farm production, processing, transport and logistics (OECD 2020, Swanson 2022). Furthermore, recent studies on climate change show that the mean global temperatures are set to rise by 5 °C and rainfall to decrease approximately 30% within the next decades resulting in a rise of natural disasters, such as heat waves and droughts, triggering land instability and havoc within food supply chain (Royo, Soriano et al. 2022). Heat stress and water scarcity, associated to changes in pest reproduction patterns as consequence of climate change, will cause a decline in wheat yield and quality. Zampieri, Toreti et al. (2020) performed high-resolution climate model simulation

using combined stress indexes (CSI) to quantify crop production climate resilience of the top ten wheat producers in the Mediterranean and the Middle Eastern region. The study highlighted that wheat production in the Mediterranean region is expected to suffer significant losses at increased climate temperature of +1.5 °C every two or three years, in other possible scenarios where the temperature increases by 2 °C or more the situation would be even more catastrophic.

2.2 From cereals to flour: post-harvest of bakery raw material

After cultivation and harvesting, bakery cereals such as wheat, barley, millet, maize, rye and oats are transported from the farms to milling facilities. In undeveloped countries this process is highly labour intensive, when compared to developing countries, where mechanical harvesters (combined harvesters) are used (FAO 1999). Through the years, some efficiency started to be implemented to accelerate the process and pestle and mortar started to be used, eventually using the same principle but on a larger and more efficient way, large rotating stone disks were used to crush the wheat kernel using animals to do most of the heavy labour (Finnie, Atwell 2016b).

Nowadays, technological advancements have automated the milling process. Dry milling involves three general operations: cleaning, tempering, and milling. Taking wheat as an example, the cleaning process involves the unloading of the grain, from trucks or ships which are conveyed into a mill elevator and left into grain storage tanks. A small sample is taken for quality checks of the lot to verify if the quality meets the set standards, the process can begin. Debris and unwanted materials such as stones and insects are removed by magnetic, milling

and disk separators, aspirators, scorer, gravity table and dry stoner (Finnie, Atwell 2016b, Papageorgiou, Skendi 2018).

Tempering is the second phase for the milling procedure, where a known amount of water is added to the wheat to toughen the bran and facilitating the separation of the endosperm and the germ. The moisture content is strictly regulated for the different types of wheat as this factor has a significant impact on overall quality. During this phase, the temperature is adjusted to 50 °C or lower to avoid affecting the functionality of gluten (Finnie, Atwell 2016b, Papageorgiou, Skendi 2018).

The actual milling is performed using machines called plansifters. Different forces (compression, abrasion, shear and impact) are employed during this process to obtain the desired particle size for the flour. The machine uses rollers, stone mills and air bubbles to successfully separate the germ and bran fractions from the endosperm (Cappelli, Olivia et al. 2020). Ball mills can also be used to produce modified wheat flour by using different methods of mechanical and thermal processes (Vogel, Scherf et al. 2018). Different sets of sieves are then used to separate different particles sizes. The smallest particles are what we call flour (Berghofer, Hocking et al. 2003). The final product is quality checked prior to packaging and transporting to retail. Quality control analysis includes odour tests, moisture, ash and protein content, pH tests and microbial testing and other tests relate to dough properties (Finnie, Atwell 2016c).

2.2.1 Wheat Flour

Wheat flour is an essential ingredient in bread making (Al-Defiery, Merjan 2015). It is mainly composed of starch and gluten, which give flour its functional properties (Guzman et al. 2022, Tian, Wang et al. 2022), but also consists of some non-starch polysaccharides and lipids (Goesaert, Brijs et al. 2005). The combination of four simple ingredients (flour, water, yeast and salt) plays a crucial role in forming the dough and affecting the final texture of the bread, flavour, and appearance (Dewettinck, Van Bockstaele et al. 2008). Bread remains universally one of the most important basic foods consumed (Sami, Abedi et al. 2020). Its low cost and high caloric value contribute to its persistent popularity hence, the need to constantly improve its quality and shelf-life (Garcia, Bernardi et al. 2019).

2.3 Bread Consumption and Local Practices

Bread making is an integral part of the heritage in various countries, including France, Italy, Greece, Egypt, and Malta, all of which share Mediterranean cultures or have significant ties to Mediterranean traditions. The rich tradition of bread making in Malta is deeply intertwined with the cultural and culinary heritage of the island, playing a central role in celebratory occasions, religious events, and everyday life, making it an essential symbol of Maltese identity and community. To this day, most bakers use recipes passed onto them from their mentors or families. This was also communicated verbally through our research on the subject by various bakers who were willing to divulge first-hand information about their methods and recipes. Generally, Maltese bakers use the same dough to produce the two main types of bread, which

are the Maltese Bread and the Maltese ftira (Figure 3). The traditional process for crafting Maltese bread and ftira commences with mixing water (~32.5 % w/w) and salt (~0.1 % w/w) to dissolve the salt. While mechanically kneading the mixture, wheat flour (~64.75 % w/w) is gradually incorporated. Simultaneously, dry yeast (1.5 % w/w) is introduced into the ongoing process. Then after numerous processes of fermentation, cutting, rounding, modelling and baking the final product is produced. Subsequently, following multiple stages of fermentation, cutting, shaping, and baking, the final product is crafted (Buttigieg, Magro et al. 2023).

Figure 3. The Maltese bread and *ftira* (Malta Independent 2020, Xuereb, 2012).

In 2018, the National Statistics Office (NSO) published data showing 'bread and cereals' as the most popular commodity consumed in Malta (27.5 %) when compared to other foods such as fish, fruits, and vegetables. Figure 4 illustrates the food purchases for an average person daily in terms of energy values (NSO 2018). Different lifestyle factors might affect the results since the Maltese individuals, like most inhabitants of the Western countries, prefer a quick, relatively cheap snack for lunch at their respective workplace or home (Buttigieg, Magro et al. 2023).

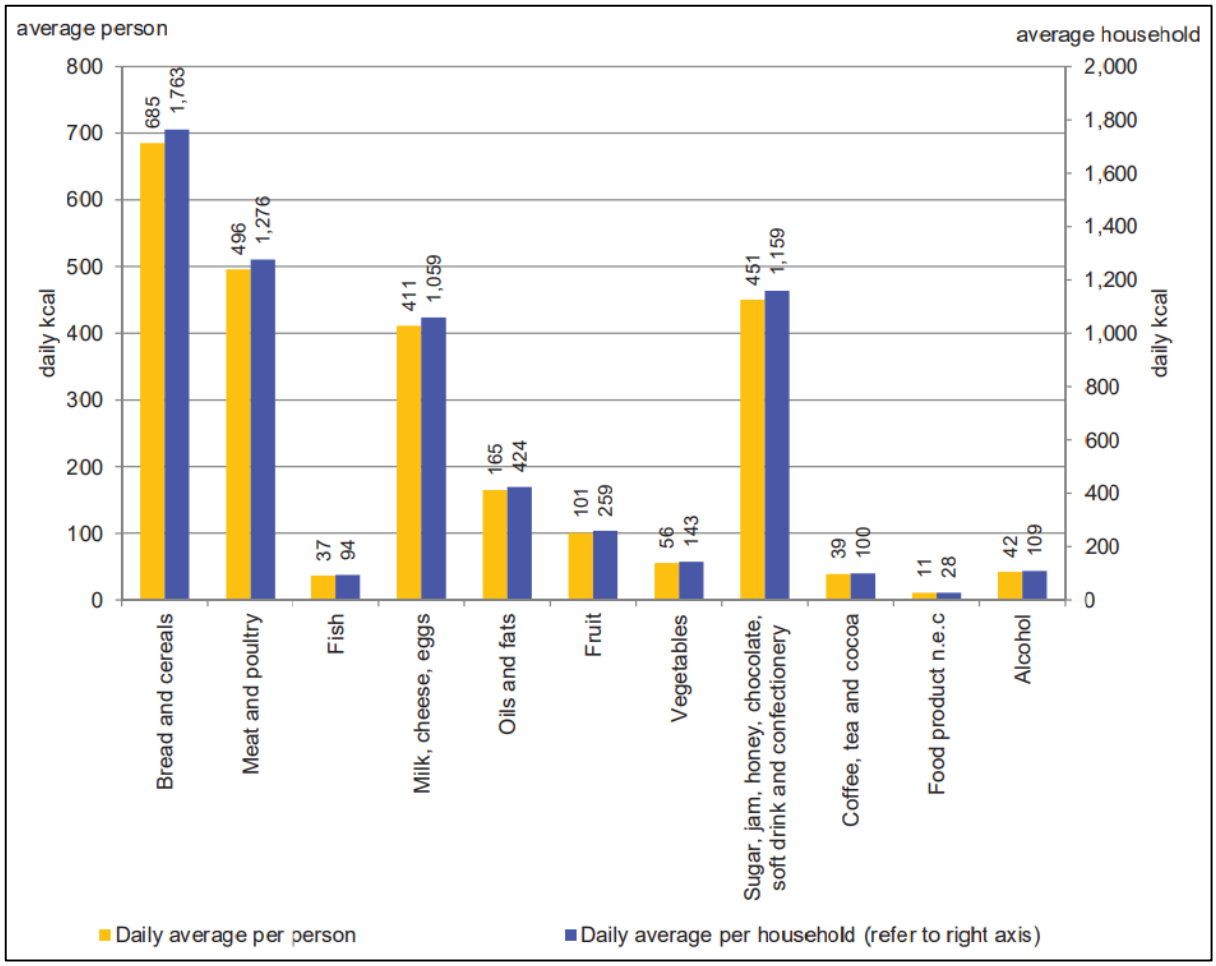

Figure 4. Food purchases in terms of calories by the average person and by the average Maltese households (NSO 2018).

The flour used to bake Maltese bread is composed of a mixture of soft and hard wheat in the ratio of 40:60, respectively. Most of the wheat consumed in the country is imported mainly by two local companies, Federated Mills Plc. and Vernons Limited. However, the milling process is carried out locally (Magri 2022). As a result, the variation seen in Maltese breads, in either the appearance, taste or quality, is mostly recipe-dependent used during the kneading process rather than the different flour types and quality (Buttigieg, Magro et al. 2023).

2.4 Fungal Contamination in cereals, flour and bread – An Inevitable Problem

Microbial contamination in food commodities leading to spoilage remains a global concern and has the greatest economic impact, but also threatens yield, quality, and general food safety (Adeyeye 2016).

Filamentous fungi are ubiquitous microorganisms which are capable of growing in a wide range of environmental conditions, with some species capable of withstanding extreme conditions, even those implemented commercially during food processing including thermal and chemical regimes. Fungi are also able to multiply uncontrollably increasing the biomass and therefore causing further cross-contamination (Snyder, Worobo 2018). Pitt and Hocking (2022) reported that food spoilage due to fungal contaminants might be responsible for 10-20 % of global food losses and waste. Food commodities such as cereals and cereal-based products can be contaminated at any point in the production chain: cultivation, harvesting, transport, processing, packaging, and storage (Al-Defiery, Merjan 2015). The widespread level of fungal contamination is remarkable due to their effortless dispersal promoted by favourable climate conditions such as temperature, humidity and windy environments (Gabarty, El Nour 2016, Kasprzyk 2008, Snyder, Worobo 2018).

It estimated that within the next two or three decades the yield of crops would most probably decrease by more than 25%, and given the exponential rise of the human population, the predicted crop production will not satisfy the world demands (Asseng, Ewert et al. 2015, Royo, Soriano et al. 2017). In addition, yield losses are predicted because of fungal contamination in

cereals in a range of 15-20 % causing losses up to 50 % (Różewicz, Wyzińska et al. 2021). In 2019, 22 % of wheat yield losses were exclusively due to fungal diseases (Kayim, Nawaz et al. 2022).

The temperatures within the Mediterranean average around 18 – 20 °C during the winter months and around 28 – 33 °C during the summer months, however, are set to constantly increase due to global warming. Hence, conditions are becoming more ideal for fungal infections which favour higher temperatures, humidity, moisture content and rainfalls (Liu, Van der Fels-Klerx 2021, Różewicz, Wyzińska et al. 2021). For instance, *Fusarium* spp. infections are expected to increase in Europe (Liu, Van der Fels-Klerx 2021, Nnadi, Carter 2021).

Cereals can have several sources of contamination from which fungi can accumulate and increase in microbial load, including soil, water, harvesting bins, machinery, tools and composted manure (Oliveira, Zannini et al. 2014). Wind patterns can further boost contamination since fungal spores are ubiquitous with easy dispersal resulting in contamination of cereals and their derived products (Schmidt, Horstmann et al. 2016). The main fungal pathogens to grains are *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp. and *Penicillium* spp. which also can produce mycotoxins (Alkuwari, Hassan et al. 2022).

Some fungal contaminants have the ability to produce toxic compounds which contaminate various agricultural food products which pose a significant health risk to humans and animals (Sarmast, Fallah et al. 2021). The main mycotoxigenic species which are able to invade and

contaminate cereals are categorized into field and storage based on the environment they occur. *Fusarium* spp., *Alternaria* spp., *Cladosporium* spp. and *Helmintosporium* spp. are normally found in the field, while *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp. and *Rhizopus* spp. show higher incidence during the storage (Minutillo, Ruano-Rosa et al. 2022). Nevertheless, fungal contamination and mycotoxin production are both unavoidable and challenging for the food industry (Oliveira, Zannini et al. 2014, Sarmast, Fallah et al. 2021).

The most important disease caused in cereals such as wheat and barley is Fusarium head blight (FHB) (Alconada, Moure et al. 2019). FHB causes spoilage in wheat, lowering yield and quality while also causing significant mycotoxin contamination compromising the safety and security of the food for both humans and animals (Nnadi, Carter 2021). This disease is primarily caused by *Fusarium graminearum* and *F. culmorum,* however several other species can cause FHB, i.e *F. avenaceum, F. poae, F. equiseti, F. langsethiae, F. sporotrichioides* and *F. tricinctum*. *Fusarium* spp. are known to produce highly toxic metabolites, such as trichothecenes (deoxynivalenol (DON), T-2, and HT-2) and zearalenone (ZEA) (Drakopoulos, Kägi et al. 2020). DON, for example, is known to cause vomiting, abdominal pain, fever and headaches, while ZEA is known to affect the reproductive system in particular oestrogen hormones in addition to risk of hepatocarcinoma and might also affect the immune system (Escrivá, Font et al. 2015, Teixido-Orries, Molino et al. 2023).

Table 1 compiles the main fungal contaminants in various cereal grains, which have been reported through various research work, where the majority cause spoilage and disease to the cereal but also produce mycotoxins.

Table 1. Occurrence of fungal contaminants in cereal grains reported in several countries by different methods of analysis.

Cereals	Location	Sampling	Main Fungal Contaminants (Frequency, %)	Quantitative Data	Method of Analysis	References
Wheat	Tunisia	*n = 24*	*Aspergillus* spp. (54.17%), *Penicillium* spp. (41.67%), *Alternaria* spp. (70.83%), *Fusarium* spp. (9.52%) *Eurotium* spp. (62.5%), *Cladosporium* spp. (29.17%), *Rhizopus* spp. (4.17%)	Not specified	Direct Plating Techniques (PDA) & DNA Based Techniques to detect Fusarium and Aspergillus toxigenic strains	Jedidi, Soldevilla et al. (2018)
Barley	Tunisia	*n = 20*	*Aspergillus* spp. (70%), *Penicillium* spp. (75%), *Eurotium* spp. (65%), *Alternaria* spp. (65%), *Fusarium* spp. (25%), *Rhizopus* spp. (25%), *Cladosporium* spp. (25%)	Not specified	Direct Plating Techniques (PDA) & DNA Based Techniques to detect Fusarium and Aspergillus toxigenic strains	Jedidi, Soldevilla et al. (2018)
Maize	Tunisia	*n = 21*	*Aspergillus* spp. (76.19%), *Penicillium* spp. (38.10%), *Fusarium* spp. (19.05%), *Alternaria* spp. (14.29%), *Cladosporium* spp. (20%), *Rhizopus* spp. (14.29%)	Not specified	Direct Plating Techniques (PDA) & DNA Based Techniques to detect Fusarium and Aspergillus toxigenic strains	Jedidi, Soldevilla et al. (2018)
Wheat	Poland	*n = 129*	*Aspergillus* spp. (20%), *Penicillium* spp. (20%), *Alternaria* spp. (10%), *Fusarium* spp. (13%), *Mucor* spp. (11%), *Cladosporium* spp. (6%)	Mean for 129 samples = 1.43	Direct Plating Techniques (DRBC)	Stuper-Szablewska, Perkowski (2014)
Wheat	Turkey	*n = 90*	*A. candidus, A. clavatus, A. flavus, A. fumigatus, A. niger, A. orhraceus, A. wentii, P. expansum, P. viridictum, P. chrysogenum, Cladosporium* spp*., Rhizopus* spp*., Ulocladium spp., Alternaria alternata, Acremonium* spp*., Mucor* spp*.*	Not specified	Direct Plating Techniques (MEA)	Aran, Eke (1987)
Rice	Turkey	*n = 60*	*A. candidus*, *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. versicolor*, *P. chrysogenum*, *P. citrinum*, *P. expansum*, *P. cyclopium*, *Cladosporium* spp., *Rhizopus* spp., *Fusarium* spp,	Not specified	Direct Plating Techniques (MEA)	Aran, Eke (1987)
Maize	Turkey	*n = 21*	*A. flavus, A. niger, A. wentii, P. chrysogenum, P. citrinum, P. expansum, P. cyclopium,*	Not specified	Direct Plating Techniques (MEA)	Aran, Eke (1987)

			Acremonium spp.*, Cladosporium* spp.*, Eurotium* spp.*, Rhizopus* spp.			
Barley	Turkey	*n = 9*	*A. flavus, A. versicolor, P. chrysogenum, P. expansum,*	Not specified	Direct Plating Techniques (MEA)	Aran, Eke (1987)
Wheat	Brazil	*n = 150*	*Fusarium* spp., *Alternaria* spp.*, Epicoccum spp.* and *Cladosporium* spp.	Not specified	Direct Plating Techniques (PDA) & ITS-amplicon metabarcoding analysis	Tralamazza, Bemvenuti et al. (2016)
Wheat Barley	Lithuania	*n = 71*	*Fusarium* spp. (*F. avenaceum, F. graminearum*), *Alternaria* spp., *Ulocladium* spp., *Penicillium* spp., *Aspergillus* spp., *Bipolaris* spp.	Not specified	Dilution Plate Method (MEA)	Mackinaite, Kačergius et al. (2006)
Wheat	Kenya	*n = 104*	*Epicoccum* spp. (52.8%), *Alternaria* spp. (34%), *Fusarium* spp. (6.4%), *Aspergillus* spp. (<6.4%), *Penicillium* spp. (<6.4)	Not specified	Direct Plate Method (CZA and PDA)	Muthomi, Mutitu (2003)
Wheat	Lithuania	*n = 13*	*Fusarium* spp. (85%), *Alternaria* spp. (69%), *Penicillium* spp. (54%), *Verticillium* spp. (54%), *Aspergillus* spp., *Mucor* spp. and *Rhizopus* spp.	Ranged from 3.24 to 5.82 log CFU/g	Dilution Plate Method (PDA)	Zadeike, Vaitkeviciene et al. (2021)
Wheat	Germany	Not specified	*Fusarium* spp. (*F. culmorum, F. graminearum, and F. poae*)	Not specified	Not specified	Alisaac, Behmann et al. (2019)
Barley and Wheat	Denmark	*n = 500*	*Penicillium* spp., *Alternaria* spp., *Eurotium* spp., *Aspergillus* spp.	Not specified	Direct Plating Techniques	Andersen, Thrane (2006)
Wheat	Egypt	*n = 20*	*Aspergillus* spp., *Alternaria alternata*, *Cladosporium cladospoioides*, *Epicoccum nigrum*, *Penicillium chrysiogenum* and *Rhizopus nigricans*.	12-73 (CFU/10g)	Dilution Plate Method (CZA)	Hamdy, El-Rify et al. (2020)
Wheat	Algeria	*n = 200*	*Alternaria alternata, Alternaria infectoria, Fusarium acuminatium*	Not specified	Surface Disinfection and Direct Plate Method (PDA)	Mokhtar, Dehimat (2013)
Wheat	India	Not specified	*Aspergillus terreus, Aspergillus oryzae, Aspergillus glaucus* and *Syncephalastrum racemosum*	Not specified	Dilution Plate Method (SDA)	Lohar, Sonawane (2013)
Wheat	Poland	*n = 22*	*Fusarium* spp. (95.5%), *Aspergillus* spp. (81.8%), *Penicillium* spp. (72.3%), *Alternaria* spp. (22.7%), *Mucor* spp. (4.5%)	2.3 – 5.04 (log CFU/g)	Dilution Plate Method (GKCH)	Čonková, Laciaková et al. (2006)
Wheat	Slovakia	*n = 48*	*Fusarium* spp. (70.5%), *Penicillium* spp. (68,2%), *Aspergillus* spp. (61.4%),	2.8 – 3.80 (log CFU/g)	Dilution Plate Method (GKCH)	Čonková, Laciaková et al. (2006)

Rye	Poland	*n = 23*	*Cladosporium* spp. (45.5%), *Alternaria* spp. (34.1%), *Mucor* spp. (27.3%)	2.5 – 4.60 (log CFU/g)	Dilution Plate Method (GKCH)	Čonková, Laciaková et al. (2006)
Rye	Slovakia	*n = 4*	*Fusarium* spp. (86.9%), *Aspergillus* spp. (73.9%), *Penicillium* spp. (78.3%),	3.3 – 3.73 (log CFU/g)	Dilution Plate Method (GKCH)	Čonková, Laciaková et al. (2006)
Barley	Slovakia	*n = 8*	*Cladosporium* spp. (30.4%), *Mucor* spp. (17.4%), *Alternaria* spp. (13.0%), *Fusarium* spp. (75.0%), *Aspergillus* spp. (25.0%), *Penicillium* spp. (50.0%), *Alternaria* spp. (75.0%)	3.11 – 3.69 (log CFU/g)	Dilution Plate Method (GKCH)	Čonková, Laciaková et al. (2006)
Wheat	Iran	*n = 34*	*Penicillium* spp. (75.0%), *Alternaria* spp. (66.7%) and *Cladosporium* spp. (58.3%)	Not specified	Dilution Plate Method (SDA)	Joshaghani, Namjoo et al. (2013)
Grains (barley, oats, rye, triticale, flax, sunflower, wheat, millet, soybeans, and quinoa)	Sao Paulo, Brazil	*n = 45*	*Alternaria* spp. (26.7%), *A. niger* (21.4%), *Fusarium* spp. (17.8%), *A. flavus* (10.7%), *Cladosporium* spp. (6%), *Penicillium* spp. (8.9%), *Rhizopus* spp. (3.5%)	2.7 (log CFU/g)	Direct Plating Techniques (DG 18 and DRBC)	Santos, Bernardi et al. (2016)

Aspergillus flavus (11.9%), *A. amstelodami* (10.4%), *P. polonicum* (10.4%), and *Penicillium citrinum* (9%)

PDA – Potato agar dextrose; SDA – Sabouraud dextrose agar; DRBC - Dichloran Rose Bengal chloramphenicol; MEA – Malt extract agar; CZA - Czapek's Agar; GKCH - Chloramphenicol Yeast Glucose Agar; DG18 - dichloran 18% glycerol agar.

Both flour and bread are susceptible to fungal contamination inducing economic decline, reducing their shelf-life and increasing food waste (Sami, Abedi et al. 2020). Bread losses due to fungal spoilage within European countries have been estimated to €250 million per year (Garcia, Bernardi et al. 2019). While in the United Kingdom, households' losses due to the same problem have been reported to reach 66,000 tonnes equivalent to £72 million per year (Axel, Zannini et al. 2017). Freire (2011) estimated that approximately 10 % of bread produce in Brazil were lost due to fungal spoilage. In the United States alone, around 300 million dollars are lost each year due to wheat fungal spoilage and resulting mycotoxins (Schmidt, Horstmann et al. 2016). Awareness of the microbial load and degree of contamination in flour and bread has increased making the bread food chain put in more effort to protect the health of the consumer and mitigate food losses (Rahman, Islamet al. 2022).

The total microbial load and mycobiota of the raw, intermediate ingredients and final product depend on different factors such as geographical location, seasonal climate conditions, water availability, relative humidity level, product formulation and processing method (Garcia, Bernardi et al. 2019, Sabillón, Stratton et al. 2016). It is especially important to characterize and monitor the mycological contamination in raw ingredients to reduce carry-over contamination in bakery products and hence resulting in spoilage issues (Snyder, Worobo 2018). It is already well established that the mycological inoculum from agricultural crops such as cereals and grains is relatively high (Garcia, Bernardi et al. 2019). Figure 5 highlights the level of fungal contamination across the life cycle of bread and hence where along its shelf-life the latter can become spoiled. Safety concerns seek to develop further mycological assessments and quality assurance of food products especially in the baking industry (Tournas, Niazi 2017). Process changes are important to satisfy the needs of the consumers. Thus, bakery industry

tends to diversify their products and focus towards healthier and more nutritious bread (such as bread with high fibre content) and also try to innovate in order to extend the shelf-life of bread (Santos, Bernardi et al. 2016). However, when various types of flour are used to produce different forms of bread such as white, wholegrain, sourdough, flatbread, and wheat amongst others, the mixing of flour leads to increase the fungal load (Cornea, Ciuca et al. 2011, Garcia, Bernardi et al. 2019, Pitt, Hocking 2022).

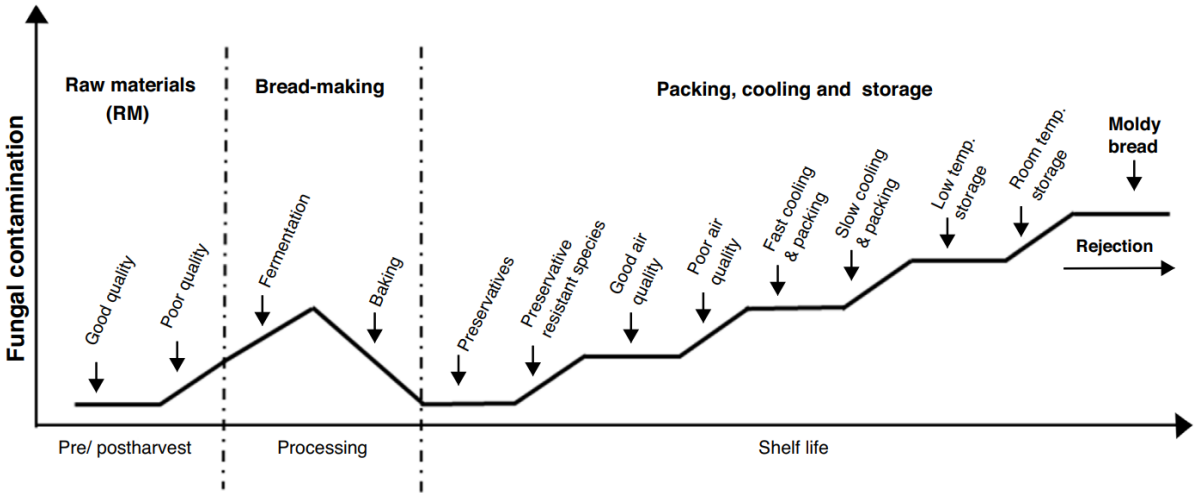

Figure 5. Levels of fungal contamination along the bread production chain for the product to end-up being spoiled (Garcia, Bernardi et al. 2019).

Whole wheat flour and its based products show higher risk of fungal contamination compared to other types. Although low water activity (a_w < 0.60) in flour does not support fungal growth (Magallanes, Simsek 2021), nonetheless when storage conditions change and the moisture content increases > 12%, xerophilic moulds such as *Fusarium* spp. and *Alternaria* spp. are able to grow (Tournas, Niazi 2017). Associated genera of known fungal spoilers in bakery products are *Penicillium* spp., *Aspergillus* spp., *Wallemia* spp., *Cladosporium* spp., *Mucor* spp., *Rhizopus* spp. and *Neurospora* spp. (Pitt, Hocking 2022, Garcia, Bernardi et al. 2019).

Garcia, Bernardi et al. (2019) identified that bread-making raw materials such as corn flour had significant fungal contamination including *Aspergillus* spp. and *Penicillium* spp. The same study also identified *Penicillium roqueforti*, which was prevalent in all bread types and raw materials used, and the species was also isolated from the air of the same bakery facility in cold processing and storage areas. The study highlights the possibility of aerosol dispersion within baking facilities, including the dispersal of flour particles, resulting in accumulation of fungal spores on the surface of equipment and fresh baked goods. Similarly, Santos, Bernardi et al. (2016) detected fungi in 100 % of whole flour (wheat and corn) samples and isolated fungal species such as *Penicillium polonicum* (16.8 %), *Aspergillus candidus* (15.2 %), *Penicillium commune* (8.8 %), *Fusarium* spp. (28.6 %) and *Aspergillus flavus* (11.9 %) amongst others which were detected in lower amounts. In the aforementioned study, the species isolated from flour were strongly associated with spoilage in bread. The frequent fungal contamination has an immediate impact on the bakery products. Besides, it is important to mention that bread is a great medium for fungal development due to its porous structure and adequate supply of oxygen (Svensson, Bucuricova et al. 2021). Table 2 compiles fungal contaminants from published studies which reported the occurrence or prevalence of fungal contaminants in flour and bread.

Table 2. Occurrence of fungal contaminants in flour samples.

Flour Type	Location	Sampling	Main Fungal Contaminants[2] (Frequency, %)	Quantitative Data[3]	Method of Analysis	References
Wheat Flour	Babylon	*n = 3*	*Aspergillus* spp. (21.3%) – *A. flavus*, *A. niger*, *A. orchraceus* *Penicillium* spp. (15.84%), *Fusarium* spp. (12.23%) Lower Frequency – *Rhizopus* spp., *Ulocladium* spp.	3.0 – 4.5 (log CFU/g)	Direct Plating Techniques (PDA)	Al-Defiery, Merjan (2015)
Wheat Flour	Isfahan, Iran	*n = 80*	*Penicillium* spp. (24.29%), *Cladosporium* spp. (20%), *Mucor* spp. (20%), *Aspergillus* spp. (19.29%), *Alternaria* spp. (3.57%), *Rhizopus* spp. (2.14%)	3.8 (log CFU/g)	Dilution Plate Method (PCA, YGCA, MEA) & DNA Based Techniques	Sami, Abedi et al. (2020)
Wheat Flour	Australia	*n = 81*	*Aureobasidium* spp., *Cladosporium* spp., *Alternaria* spp., *Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp., *Rhizopus* spp., *Mucor* spp.	/	Direct Plating Techniques (DRBC + DG-18)	Berghofer, Hocking et al. (2003)
Wheat Flour	Spain	*n = 26*	*Aspergillus* spp. (*A. candidus, A. flavus, A. fumigatus, A. ochraceus, A. versicolor, A. rubrum, A. niger*) and *Penicillium verrucosum*	Not specified	Direct Plating Techniques (DRBC + MEA + CYA + G25N)	Cabanas, Bragulat et al. (2008)
Wheat Flour Self-raising,	Washington, DC, USA	*n = 5*	*Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Eurotium* spp., *Fusarium* spp.	2.00 - 3.00 (log CFU/g)	Direct Plating Techniques (PDA + DG-18) & DNA Based Techniques for Identification	Tournas, Niazi (2017)
Wheat Flour unbleached	Washington, DC, USA	*n = 12*	*Alternaria* spp., *Aspergillus* spp. (*A. flavus*), *Fusarium* spp. (*F. graminearum*), *Penicillium* spp.	2.00 - 3.00 (log CFU/g)	Direct Plating Techniques (PDA + DG-18) & DNA Based	Tournas, Niazi (2017)

[2] Main fungal contaminants reported, and frequency data reported in %.
[3] Quantitative data reported in variable units as reported by various research studies.

					Techniques for Identification	
Wheat Flour (whole wheat)	Washington, DC, USA	*n = 5*	*Alternaria* spp., *Aspergillus* spp. (*A. flavus*), *Fusarium* spp., *Penicillium* spp., *Cladosporium* spp.	2.00 - 3.00 (log CFU/g)	Direct Plating Techniques (PDA + DG-18) & DNA Based Techniques for Identification	Tournas, Niazi (2017)
Wheat Flour, Barley Flour, Cake Flour	Hamedan Province, Iran	*n = 179*	*Aspergillus fumigatus* & *Aspergillus niger*	/	Direct Plating Techniques (ADRBC) & DNA Based Techniques for Identification	Ghafori, Habibipour et al. (2021)
Whole Wheat Flour	Sao Paulo, Brazil	*n = 50*	*Penicillium* spp. (38.2%), *Aspergillus* spp. (23.6%), *Aspergillus* spp. with Eurotium-type ascomata (19.1%)	3.1 (log CFU/g)	Direct Plating Techniques (DG 18 and DRBC)	Santos, Bernardi et al. (2016)
Whole Corn Flour	Sao Paulo, Brazil	*n = 5*	*Penicillium polonicum* (42.9%) and *Fusarium* spp. (28.6%)	4.8 (log CFU/g)	Direct Plating Techniques (DG 18 and DRBC)	Santos, Bernardi et al. (2016)
White Wheat Flour	Serbia	Not specified	*A. versicolor*, *C. cladosporioides*, *F. sporotrichoioides*, *P. aurantiogriseum, P. expansum*	DG18 - $6.0x10^1$ (CFU/g) MY50G - $3.0x10^1$ (CFU/g)	Direct Plating Techniques (DG 18 and MY50G, CYA)	Plavsic, Skrinjar et al. (2017)
Whole Wheat Flour	Serbia	Not specified	*C. cladosporioides, F. proliferatum, P. expansum*	DG18 - $1.3x10^2$ (CFU/g) MY50G - $4.0x10^1$ (CFU/g)	Direct Plating Techniques (DG 18 and MY50G, CYA)	Plavsic, Skrinjar et al. (2017)
Corn Flour	Serbia	Not specified	*A. flavus*, *A. niger, F. sporotrichoioides*, *F. proliferatum*, *P. commune*, *P. oxalicum, Rhizopus stolonifer*	DG18 - $3.4x10^2$ (CFU/g) MY50G - $2.7x10^2$ (CFU/g)	Direct Plating Techniques (DG 18 and MY50G, CYA)	Plavsic, Skrinjar et al. (2017)
Whole Buckwheat Flour	Serbia	Not specified	*Alternaria alternata*, *A. fumigatus*, *C. cladosporioides*, *Chrysonilia sitophila*, *P. aurantiogriseum*	DG18 - 5.0x102 MY50G - 3.0x101	Direct Plating Techniques (DG 18 and MY50G, CYA)	Plavsic, Skrinjar et al. (2017)
Wheat Flour	Qena City, Egypt	*n = 29*	*Aspergillus* spp. (58.2%) (*A. flavus* (27.8%), *A. niger* (14.6%), *A. parasiticus* (7.2%)), *Penicillium* spp. (15.2%), *Mucor circinelloids* (7.2%)	6.7 – 1356.9 (ATC/g)	Dilution Plate Method (CZA)	El-Shahir (2021)

Wheat Flour	Turkey	*n = 12*	*A. candidus, A. clavatus, A. flavus, A. fumigatus, A. niger, P. chrysogenum, Fusarium spp., Eurotium spp.*	Not specified	Direct Plating Techniques (MEA)	Aran, Eke (1987)
Wheat Flour Type "00'	Calabria, Italy	*n = 3*	*Alternaria spp. - Alternia infectoria, Aspergillus spp., Aspergillus fasiculatus, A. oryzae, A. clavatus, Chaetomium globosum., Cladosporium sp., Epicoccum nigrum, Fusarium oxysporum, Mucor sp., Penicillium aurantiogriseum, Penicillium sp., Penicillium albocoremium, Penicillium chrysogenum, Rhizopus oryzae, Penicillium citrinum*	Not specified	Direct Plating Techniques (PDA) & ITS-amplicon metabarcoding analysis	Minutillo, Ruano-Rosa et al. (2022)
Wheat Flour "0"	Calabria, Italy	*n = 3*	*Alternaria sp., P. griseofulvum, P. verrucosum, P. aurantiogriseum, P. viridicatum, P. polonicum, Penicillium sp., Cladosporium sp., Arthrinium arundinis*	Not specified	Direct Plating Techniques (PDA) & ITS-amplicon metabarcoding analysis	Minutillo, Ruano-Rosa et al. (2022)
Wholemeal Flour	Calabria, Italy	*n = 3*	*Alternaria sp., Penicillium sp., Penicillium aurantiogriseum, Penicillium allii-sativi, P. chrysogenum, Penicillium griseofulvum*	Not specified	Direct Plating Techniques (PDA) & ITS-amplicon metabarcoding analysis	Minutillo, Ruano-Rosa et al. (2022)
Wheat Flour	Jeddah, Saudi Arabia	*n = 50*	*Aspergillus* spp. (70%), *Penicillium* spp. (30%), *Eurotium* spp. (14%), *Fusarium oxysporum* (20%) and *Alternaria alternata* (18%)	224, 116, 109, 75, 64	Direct Plating Techniques (MEA and DRBC)	Gashgari, Shebany et al. (2010)
Various Flours (Mainly wheat and corn)	Italy	*n = 40*	*Aspergillus spp., Fusarium spp., Penicillium* spp.	Not specified	Plating Dilutiuon Technique (SDA and DG18) & ITS DNA Phylogenetic studies	Sacco, Donato et al. (2020)
Wheat Flour	Egypt	*n = 30*	*Aspergillus flavus*, *A. nigri* and *Penicillium* spp.	$8.3x10^2$ (CFU/g)	Dilution Plate Method (CMA, YES, ADM)	Abo-Dahab, Abdel-Hadi et al. (2016)

Wheat flour	Egypt	n = 20	*Aspergillus flavus*, *A. niger*, *A. versicolor*, *Penicillium ducluxi* and *Rhizophus nigricans*	22 - 281 CFU/10g	Dilution Plate Method (CZA)	Hamdy, El-Rify et al. (2020)
Yam Flour	Nigeria	*n = 3*	*A. flavus* (25.0%), *A. niger* (17.0%), *Rhizopus* spp. (17.0%), *Geotrichum* spp. (8.0%), *Yeast* spp. (8.0%), *Penicillium* spp. (17.0%) and *Paecilomyces* spp. (8.0%)	1.21×10^{12} - 1.6×10^{12} (CFU/g)	Dilution Plate Method (PDA)	Okafor, Eni (2018)
Wheat Flour	Nigeria	*n = 3*	*A. flavus* (33.0%), *A. niger* (11.0%), *Rhizopus* spp. (11.0%), *Paecilomyces* spp. (11.0%), *yeasts* (11.0%) and *Geotrichum* spp. (11.0%)	1.16×10^{12} - 3.51×10^{13} (CFU/g)	Dilution Plate Method (PDA)	Okafor, Eni (2018)
Plantain Flour	Nigeria	*n = 3*	*A. flavus* (38.0%), *A. niger* (17.0%), *Rhizopus* spp. (17.0%), *Paecilomyces* spp. (17.0%), and *yeasts* (17.0%)	2.02×10^{12} - 9.30×10^{13} (CFU/g)	Dilution Plate Method (PDA)	Okafor, Eni (2018)
Wheat Flour	Tabriz City, Iran	*n = 89*	*Aspergillus* spp. (50%) (*A. niger* and *A. fumigatus*), *Fusarium* spp. (18.0%), *Acremonium* spp. (14.5%), *Mucor* spp. (7.0%), *Penicillium* spp. (3.5%)	$>1 \times 10^4$ (CFU/g)	Dilution Plate Method (YCGA)	Rezazadeh, Pirzeh et al. (2013)

PDA – Potato agar dextrose; PCA – Plate count agar; YGCA - Yeast Glucose Chloramphenicol Agar; YCGA: Yeast Carbon Base Agar; SDA – Sabouraud dextrose agar; DRBC - Dichloran Rose Bengal chloramphenicol; MEA – Malt extract agar; CZA - Czapek's Agar; GKCH - Chloramphenicol Yeast Glucose Agar; DG18 - dichloran 18% glycerol agar; CYA - Czapek Yeast Autolysate Agar; G25N - Glycerol 25% Nitrate Agar; ADRBC - Antibiotic Dextrose Rose Bengal Chloramphenicol Agar; MY50G - Malt Yeast 50% Glycerol Agar; CMA - Czapek's Modified Agar; YES - Yeast Extract Sucrose Agar; ADM - Aminoglycoside Detection Medium.

2.5 Introduction to mycotoxins

Mycotoxins are secondary toxic metabolites produced naturally by some species of filamentous fungi. The main species producers of such toxins are *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium* (Chiotta, Fumero et al. 2020, Sarmast, Fallah et al. 2021). The word mycotoxin originated from the Greek words “myke” which means fungus and “toxicum” that means toxin (Atungulu, Mohammadi-Shad 2019, Stanciu, Banc et al. 2015).

Currently, there is description of around 400 to 500 known mycotoxins (Gurikar, Shivaprasad et al. 2023, Palumbo, Crisci et al. 2020, Tang, Liu et al. 2022), however the most studied are AFs, OTA, ZEA, FUM, PAT, T-2 and HT-2 (Kabak 2009, Sarmast, Fallah et al. 2021). Mycotoxins vary greatly in molecular structures and, as a result, exhibit different chemical and physical properties (Krska, Schubert-Ullrich et al. 2008). Mycotoxins are produced in a wide range of food and feed (Tola, Kebede 2016), posing as a serious threat to the agricultural industry, economy, and trade (Luo, Du et al. 2021).

The level of mycotoxin is one of the main factors which determines if the food is safe for the consumer. The Food and Agriculture Organization (FAO) has published a concerning report, indicating that approximately 25 % of global agricultural food commodities are contaminated with mycotoxins (Zhou, Li et al. 2016). However, Eskola, Kos, et al. (2020) have raised questions about this figure. They conducted an in-depth analysis by compiling data mainly from JECFA (Joint FAO/WHO Expert Committee on Food Additives) and other literature sources. Their findings suggest that the global prevalence of mycotoxin contamination in food crops varies

significantly, depending on several factors. These factors include the specific mycotoxin of concern, the analytical methods utilized to quantify it, and the way results are reported. According to their research, the prevalence of detected mycotoxins in food crops ranges from approximately 60 % to 80 %. In Europe and the United Kingdom, between 2010 and 2019, it was estimated that around 75 million tonnes of wheat (equivalent to 3 billion Euro) were lost due to FHB invasion and, consequently, exceeding threshold limit DON (750 µg/kg; European Commission 2006a, Johns, Bebber et al. 2022). The presence of these chemical hazards in the food chain are mostly dependent on favourable climatic conditions such as temperature, humidity and moisture content, a_w, pH (Janik, Niemcewicz et al. 2021), inadequate storage conditions (Seo, Jang et al. 2021), poor harvest and post-harvest practices such as transport and handling (Giannioti, Albero et al. 2023).

Since most of the fungal contaminants reported (Table 1 and Table 2) are known to be mycotoxin producers, it is possible to presume that naturally occurring mycotoxins also contaminate breadmaking raw materials (Karlovsky, Suman et al. 2016). Especially, if farmers fail to implement fungal growth or mycotoxin mitigation strategies to limit and control such production at pre- and post-harvest or during storage (Nada, Nikola et al. 2022). Due to this ongoing problem, farmers and businesses in the food industry suffer from extensive economical losses (Imade, Ankwasa et al. 2021). In table 3 the main types of mycotoxins within each group and producing species are summarized.

Table 3. Major mycotoxins and the respective microorganism source and common matrices contaminated.

Mycotoxin		Genus / Species	Commodity	Reference
Aflatoxins (B1, B2, G1, G2)	AFB_1 AFB_2 AFG_1 AFG_2	*Aspergillus flavus* *Aspergillus parasiticus*	Maize, wheat, rice, peanut, ground nuts, spices, figs	Kumar, Pathak et al. (2021)
Ochratoxin A	OTA	*Aspergillus ochraceus* *Aspergillus carbonarius* *Penicillium verrucosum* *Aspergillus niger*	Cereals, wine, grapes, coffee, milk, cocoa, herbs	Kumar, Mahato et al. (2020)
Fumonisin (B1, B2)	FB_1 FB_2	*Fusarium verticillioides,* *Fusarium proliferatum*	Maize, maize products, sorghum	Ferrigo, Raiola et al. (2016)
Zearalenone	ZEA	*Fusarium graminearum* *Fusarium verticillioides* *Fusarium incarnatum*	Cereals, cereal products (wheat, barley, maize)	Ropejko, Twaruzek (2021)
Patulin	PAT	*Penicillium expansum* *Bysochlamis nívea* *Aspergillus clavatus*	Apples, apple juice, and concentrate, pears, peaches, grapes, apricots, mango, cherry, corn, strawberries	Mahato, Kamle et al. (2021)
Trichothecenes Type A – T-2, HT-2	T-2 HT-2	*Fusarium langsethiae,* *Fusarium sporotrichioides* *Fusarium poae*	Cereals, cereal products	Pleadin, Vulic et al. (2017), Stanciu, Juan et al. (2019)
Trichothecenes Type B – Deoxynivalenol	DON	*Fusarium graminearum* *Fusarium culmorum*	Cereals, cereal products such as flour, pasta and bread	Nagl, Schatzmayr (2015)
Alternariol	AOH	*Alternaria alternata*	Fruits and fruit products, wine and beer, grains and their products	Escrivá, Oueslati et al. (2017)

2.5.1 Metabolism for production of mycotoxins

Mycotoxins are classified as secondary metabolites in fungi; thus, it is important to distinguish primary and secondary metabolites since both are categorised as bioactive compounds (Al-Fakih 2014). The primary metabolism represents the compounds produced by fungi for their growth and development, while secondary metabolites are produced as part of their defence or survival mechanism and expelled from the cells at some point of the development (Daley, Brown et al. 2017).

Figure 6 shows the complex regulation mechanism involved in the biosynthesis of sterigmatocystin by *Aspergillus nidulans*. The biosynthetic gene cluster (BGC) for sterigmatocystin is well-known for the regulation of its production. The diagram (Figure 8) summarizes the structure and encoded genes involved. White light is known to inhibit the BGCs for aflatoxins and sterigomatocystin, while on the other hand blue light stimulates the production of alternariol and altertoxin by *Alternaria alternata* (Keller 2019; Kolawole, Meneely et al. 2021).

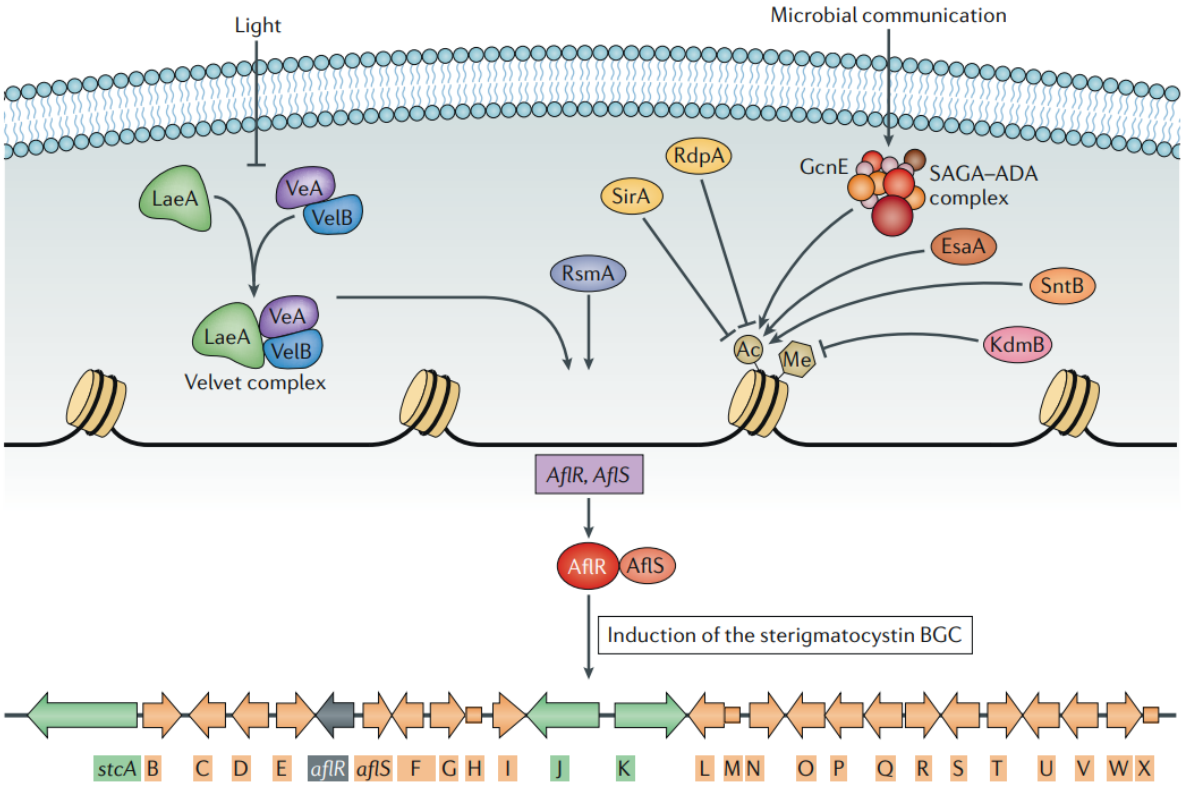

Figure 6. Biosynthetic gene cluster for the regulation of sterigmatocystin produced by *Aspergillus nidulans* (Adapted from Keller 2019).

2.5.2 The stability of Mycotoxins to heat and food processing.

Mycotoxins are both chemical and heat stable, with most remaining stable at temperatures of 80 – 121 °C (Kabak 2009, Tang, Liu et al. 2022). Therefore, they can remain present as food contaminants after thermal processing (Castilla-Fernández, Racio-Bautista et al. 2022, Suman 2021) such as boiling, frying, pasteurization and milling used in baking (Bullerman, Bianchini 2007). Nevertheless, several factors can affect the fate of mycotoxins after thermal processing such as the initial level of contamination, the heating temperature and duration of thermal processing alongside to pH, moisture content, ionic strength of food, type and concentration of mycotoxin present, and level of heat permeation during processing (Schaarschmidt, Fauhl-Hassek 2021). Vidal, Marin et al. (2014) evaluated changes in concentration of DON and OTA

in the mixing of raw breadmaking ingredients and sourdough after the breadmaking processes (flour, fermented dough and bread). In this study it was found that both mycotoxins were reduced by 21-44 %, and confirmed OTA was as stable after breadmaking processes. Moreover, other studies found that DON is also extremely thermostable, as it can resist temperatures between 170 to 350 °C (Kamle, Mahato et al. 2022). In fact, Vidal, Sanchis et al. (2017) studied the stability of DON and deoxynivalenol-3-glucoside (DON-3-G) during baking of small doughs composed of wheat flour and showed that DON content was reduced by 40 %, while DON-3-glucoside concentration increased by >100 % during baking. This validates that the levels of DON and DON-3-G can change while being heated, and there's even strong evidence that DON-3-G can even increase after the baking process.

2.6 Effect of mycotoxins on human and animal health

Studies have shown mycotoxins to be cyto-, nephron- and neurotoxic, carcinogenic, mutagenic, immunosuppressive, and to have estrogenic effects (Krska, Schubert-Ullrich et al. 2008). Directly consumption of contaminated sources may lead to a condition known as ‘mycotoxicosis’ or acute poisoning. However, the main danger of mycotoxins is the cumulative or chronic effect, which occurs when there is a continuous exposure over time. It is important not to underestimate the acute or chronic effects of these harmful metabolites when inhaled, in contact through the skin and especially when ingested. Mycotoxins have also been linked to cause immunodeficiencies as they can enter the lymphatic system and blood stream (Awuchi, Ondari et al. 2022).

2.6.1 Ochratoxins A

Ochratoxins (OTA) are produced primarily by the genera *Penicillium* and *Aspergillus* (Bui-Klimke, Wu 2015) as described in Table 3. Chemically, OTA is a derivative from the condensation of the amino group of L-phenylalanine with the carboxy group ochratoxin alpha ((3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-carboxylic acid; figure 7).

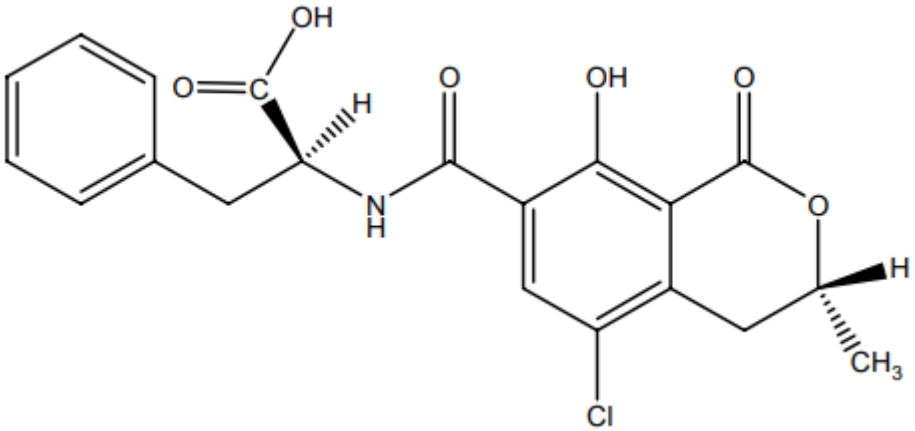

Figure 7. Chemical structure of ochratoxin A, chemical name L-Phenylalanine, N-[[(3R)-5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl]carbonyl]- (adapted from CAS, 2013).

The relevance of OTA is due to its ability to contaminate a wide range of food commodities including cereals, coffee, beer, wine, grapes, dairy, cured meat products, and even meat or dairy from animals consuming OTA-contaminated grains (El Khoury, Atoui 2010, Kumar, Mahato et al. 2020). OTA is a group 2B carcinogen (possible carcinogen), as classified by the International Agency of Research on Cancer (IARC) (IARC 1993, Wang, Hua et al. 2022).

OTA contamination represents a serious issue, especially since it is regarded as nephrotoxic, hepatotoxic, immunotoxic, teratogenic, mutagenic, genotoxic and to cause reproductive

problems, as well as problems in the blood-brain barrier (Jackson, Ryu 2017, Marin, Taranu 2015, Pfohl-Leszkowicz, Manderville 2007, Schwartz 2002). In view of the new studies and findings, in 2020, the European Commission (EC) has asked the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain to evaluate the risk of OTA to human health in food aiming to update the scientific opinion established in 2006. The panel recommendations were, that more scientific data especially those relating to occurrence and toxicity of modified OTA are required. It is also highlighted that the current assumptions or measures taken are more likely to overestimate the risks of OTA to humans and animals (CONTAM, Schrenk et al. 2020).

2.6.2 Aflatoxins

Aflatoxins (AFs) are produced mainly by *Aspergillus flavus* and *A. parasiticus* (Marchese, Polo et al. 2018), however, new species are being discovered as aflatoxigenic such as *A. bombycis* (Peterson, Ito et al. 2019). At least 13 AFs analogues were identified, however five major analogues of AFs are well known and studied, namely aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_1) and aflatoxin M_1 (AFM_1). AFB_1 is known to be the most toxic (CONTAM, Schrenk et al. 2020). *A. flavus* is able to produce only the analogues B (AFB_1, AFB_2), while *A. parasiticus* produces both G and B analogues (AFG_1, AFG_1, AFB_1 and AFB_2) (Martins, Sant'Ana et al. 2017). These toxic compounds can be found contaminating food at the consumer level especially in hot and humid climates which promote growth and dissemination of AFs producing species (Mahato, Lee et al. 2019). Chemically, AFs are bifuranocoumarin derivatives with a cyclopentenone in AFB_1 and AFB_2 or a δ-lactone ring in AFG_1 and AFG_2 (Figure 8).

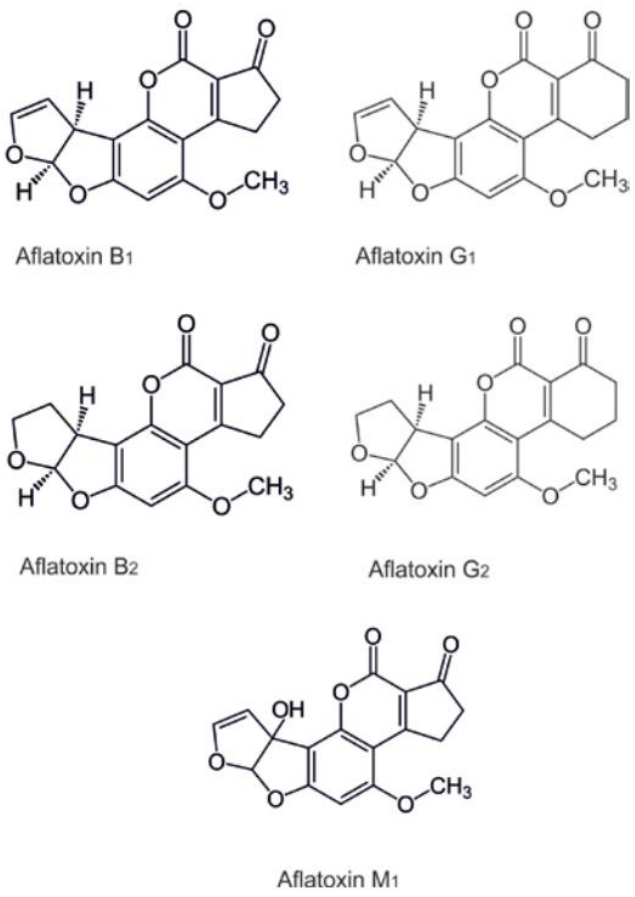

Figure 8. Chemical structure of Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2 (adapted from IARC 2016).

Contamination of food products with AFs have serious health related consequences for both humans and animals. Consumption of AFs has been associated with immunotoxicity, teratogenicity and hepatotoxicity (Alameri, Kong et al. 2023, Kumar, Mahato et al. 2017). AFB_1 is classified by the International Agency for Research on Cancer as group 1 (carcinogenic to humans) while AFM_1 is classified as group 2B (possibly carcinogenic to humans) (IARC, 2012). Between January and June 2004, the Ministry of Health in Kenya, along with collaborators, detected 317 instances of sudden liver failure in the eastern region of the country. Out of these cases, 125 individuals unfortunately passed away during the course of the illness. Such illnesses were mostly related to high concentrations of aflatoxins in maize (Azziz-Baumgartner,

Lindblade et al. 2004). From a survey study, performed in Sicily (Italy), 73 milk and 24 dairy products samples were tested for the presence of AFM_1. The latter metabolite was detected in 48 and 42 % of milk and dairy products samples, respectively (Santini, Raiola et al. 2013), which were above the EC directive limit of >0.05 ng/kg (European Commission 2006a)

2.6.3 Deoxynivalenol

Deoxynivalenol (DON) is a type B trichothecene usually produced by *Fusarium graminearum* and *Fusarium F. culmorum* (Mishra, Srivastava et al. 2020) and it is closely related to the presence of FHB. DON is one of the most prevalent mycotoxins found in cereals (wheat, barley, oats, rye, corn) (Mahato, Pandhi et al. 2022). Europe, the Middle East, America, Asia and Africa are all affected by DON contamination especially in wheat, maize (Topi, Babic et al. 2021), barley, rice and oats (Golge, Kabak 2020). DON (Trichothec-9-en-8-one, 12,13-epoxy-3,7,15-trihydroxy-, (3α,7α)-) is characterized as a tetracyclic sesquiterpene (Nagy, Fejer et al. 2005), soluble in polar solvents such as water, ethanol and acetonitrile and is also stable at low pH (Li, Gao et al. 2023). DON has numerous derivatives (Figure 9) which are also toxic. Some of the main symptoms of DON are nausea, vomiting, diarrhoea, abdominal pain and loss of appetite (Patriarca, Pinto 2017). DON can also cause DNA damage to humans by activating the tumour-suppressor gene p53 and is therefore regarded to be genotoxic since it induces cell death (Bensassi, Golli-Bennour et al. 2009). DON is also considered to be neurotoxic to animals as studied by Wang, Chen et al. (2020), however, IARC categorised DON as a group 3 (evidence of carcinogenicity is inadequate in humans) based on toxicological data (IARC, 1993, Stanciu, Juan et al. 2019).

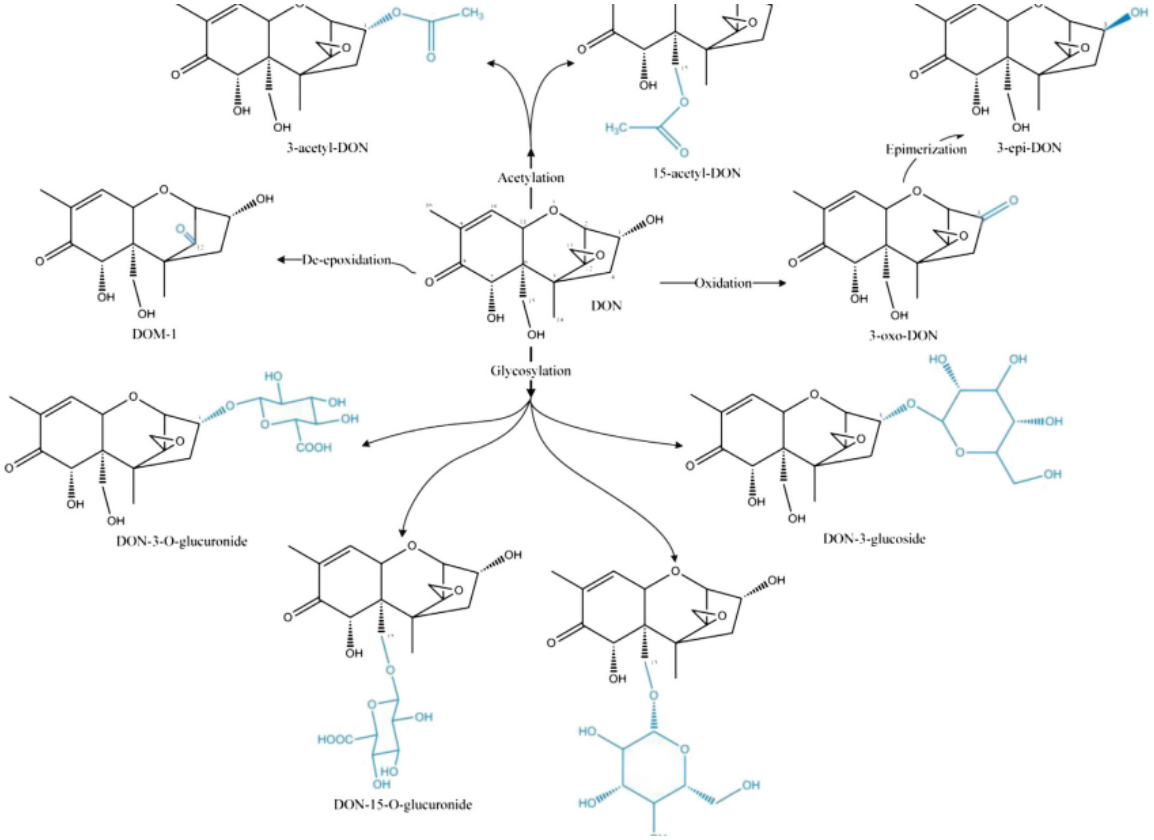

Figure 9. Structural formula of DON and its derivatives based on reactions of reduction, oxidation, acetylation and glycosylation (adapted from Ran, Wang et al. 2013).

DON is strongly associated with the infection of FHB and the toxin is the key virulence factor and gives the pathogen a competitive advantage, especially under stress conditions. DON associated with FHB, was detected in nearly 50 % of cereals in Europe (DSM World Mycotoxin Survey 2021). Good farming practice is one of the most fundamental factors to mitigate the occurrence of FHB and thus DON production. It is particularly important the timing of fungicide application, tillage, and crop rotation to reduce the risk of FHB. However, other independent factors, which are out of the farmers control, such as shift in climatic conditions and FHB resistance to the fungicide applied can affect the levels of infection and DON in the product (Kamle, Mahato et al. 2022). The timing for application of fungicide to control FHB is challenging and when applied incorrectly, the mycotoxin content can increase due to the stress

triggered by the pathogen. Since FHB is caused by a complex of several different *Fusarium* species with differences in pathogenicity, toxigenicity, and fungicide sensitivity, the application for fungicide may work excellently against some pathogens but might not be effective against other species (Leplat, Friberg 2013).

2.6.4 T-2 toxin and HT-2 toxin

T-2 toxin (T-2) and HT-2 toxin (HT-2) are type A trichothecenes, which are primarily produced by *Fusarium sporotrichioides and F. langsethiae* (Isidro- Sánchez, Cusack et al. 2020, Kiš, Vulic et al. 2021). Unlike DON, type A trichothecenes may be produced in cold climate in temperatures as low as -2 °C and a_w of 0.88 (Li, Wang et al. 2011, Pleadin, Vulic et al. 2017). Type A trichothecenes are commonly found in different cereals such as maize, oats, barley, rice and wheat (Foroud, Baines et al. 2019). T-2 and HT-2 are linked to numerous harmful effects (EFSA, Arcella et al. 2017) such as immunosuppression as result of a decrease in white blood cell count, weight loss, reduction in plasma glucose and cytotoxic changes in the liver and stomach (Boško, Pernica et al. 2022). Moreover, T-2 toxin is the leading cause of Kashin-Beck disease, also known as alimentary toxic aleukia (ATA) (Patriarca, Pinto 2017). However, due to the lack of data on T-2 toxin, the IARC classified it as group 3 (evidence of carcinogenicity is inadequate in humans) (IARC, 1993).

The basic structure of all trichothecenes is a tetracyclic sesquiterpene (Figure 10). The type A are characterised by an esterified or free hydroxyl group at C-8, or an unsubstituted C-8 (EFSA, Arcella et al. 2017). T-2 toxin (Trichothec-9-ene-3, 4, 8, 15-tetrol, 12, 13-epoxy-, 4, 15-diacetate 8- (3-methylbutanoate), (3α, 4β, 8α)-) (CAS, 2023) is insoluble in water but highly soluble in

organic solvents such as methanol, ethanol and acetone. T-2 toxin is proved to be resistant to milling processes (Pleadin, Vulic et al. 2017) and other thermal processes of around 151 °C, albeit it can be inactivated at around 200 °C (Zhang, Liu et al. 2022). *In vivo*, T-2 is transformed into HT-2 (Trichothec-9-ene-3,4,8,15-tetrol, 12,13-epoxy-, 15-acetate 8-(3-methylbutanoate), (3α,4β,8α)-) (CAS, 2023) rapidly after the ingestion (Marin, Ramos et al. 2013).

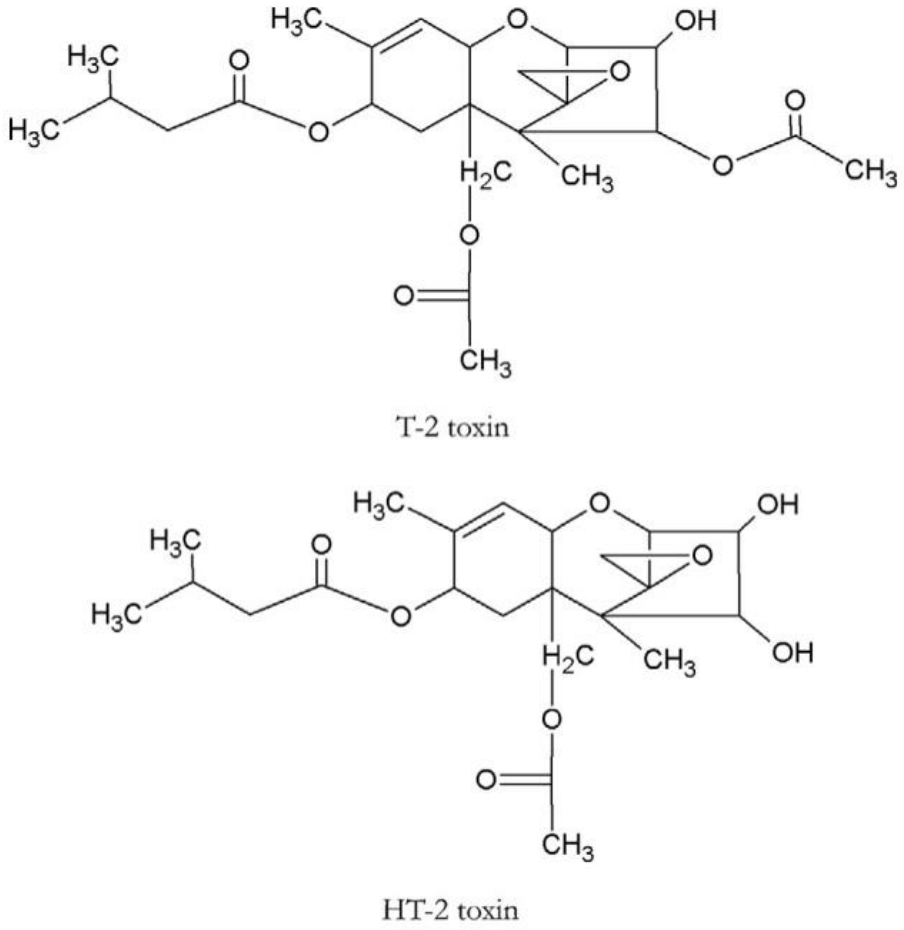

Figure 10. Chemical structure of T-2 and HT-2 (adapted from Marin, Ramos et al. 2013).

2.6.5 Fumonisins

Fumonisins (FUM) are produced by *Fusarium* species, particularly *F. verticillioides* and *F. proliferatum* frequently found to contaminate maize and maize-based goods and can co-contaminate with other forms of FUM (Kamle, Mahato et al. 2019) The most known within this group are Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), Fumonisin B_3 (FB_3) and Fumonisin B_4 (FB_4). FB_1 (Figure 11) is the most important analogue due to its toxicity and occurrence in a range of food commodities when compared to FB_2, FB_3 and FB_4 (Cendoya, Chiotta et al. 2018). FB_1 also differs structurally from the other toxins within the same group. The IARC has categorized FB_1 in the group 2B (possibly carcinogenic to humans). However, the main harmful effects of FUM are inflicted on equines that can develop leukoencephalomalacia (ELEM) and porcine pulmonary oedema when ingesting or exposed to FB_1 (Queiroz, Alves et al. 2012). FUMs are also linked with causing oesophageal carcinoma in humans (Patriarca, Pinto 2017).

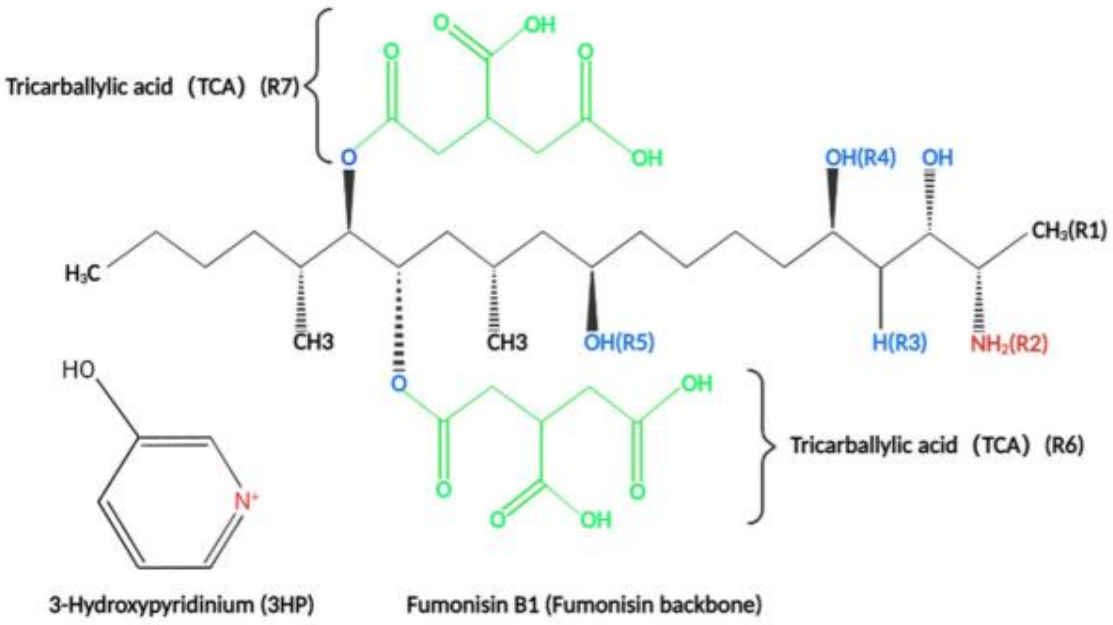

Figure 11. The chemical structure of FB_1, showing the fundamental framework of 20 carbon atoms accompanied by multiple carboxyl groups, hydroxyl groups and ester bonds which are distributed on both sides of this core backbone structure (adapted from Qu, Wang et al. 2022).

2.6.6 Zearalenone

Zearalenone (ZEA) (6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone), shown in Figure 12 (Mostrom 2011) is a non-steroidal mycotoxin produced by *Fusarium graminearum* amongst other species (Ropejko, Twaruzek 2021). ZEA and its derivatives are typically found in maize, especially when there is growth of pathogenic rots caused by *Fusarium* spp. such as the ear rot (*Giberella zeae*) and head blight in wheat and barley (Zhang, Feng et al. 2018). ZEA has a thermal stability of around 160 °C. This mycotoxin and its derivates have a chemical structure similar to the human 17-β-oestradiol (E_2) and, as a result, when ingested, it can mimic its function resulting in inhibition of natural steroid hormone synthesis (pseudo hypoestrogenism), hence interrupting endogenous estrogenic response and production (Hueza, Raspantini et al. 2014). Nonetheless, ZEA is classified as a group 3 (IARC 1993) (not classifiable as to its carcinogenicity to humans) by the IARC, however it has shown to be genotoxic, hepatoxic, carcinogenic and immunosuppressive (Lee, Ryu 2017). ZEA disturbs haematological cells and blood haemostasis (Ropejko, Twaruzek 2021) and can also cause significant damage to liver cells which may develop into liver carcinoma (Zheng, Wang et al. 2018).

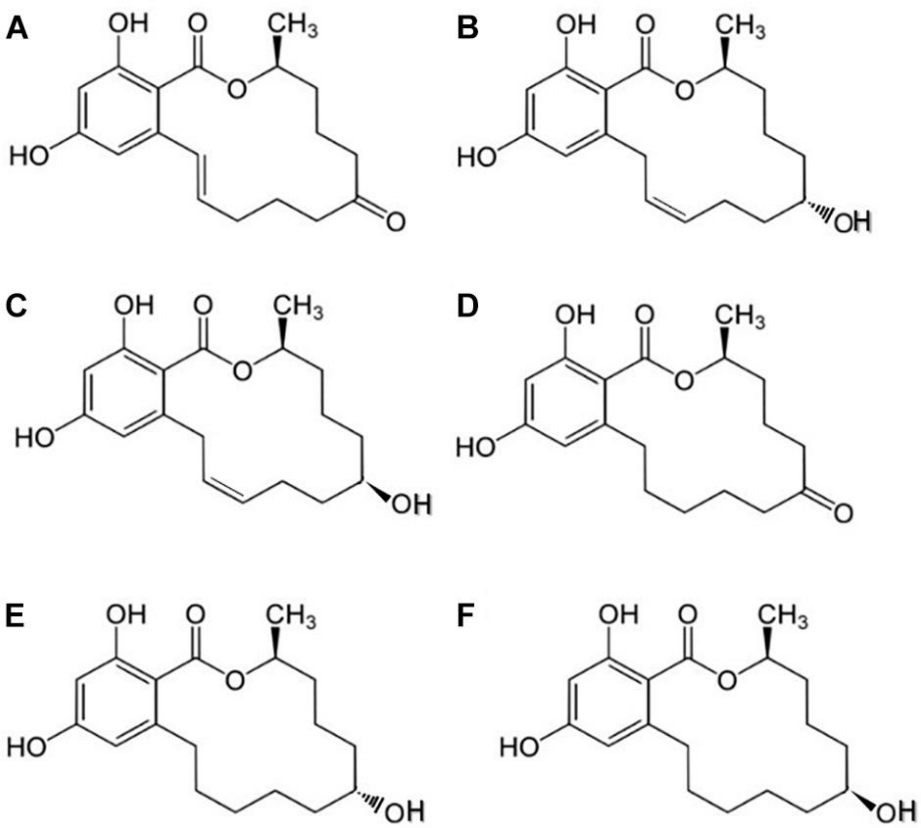

Figure 12. Chemical structures of ZEA and its derivatives: (A) zearalenone (ZEA), (B) α-zearalenol (α-ZEA), (C) β-zearalenol (β-ZEA), (D) zearalanone (ZAN), (E) α-zearalanol (α-ZAL), and (F) β-zearalanol (β-ZAL) (adapted from Zhang, Feng et al. 2018).

2.6.7 Patulin

Penicillium expansum is the most common microorganism associated with the production of PAT. PAT is a polyketide lactone (4-hydroxy-4H-furo [3,2-c]pyran-2(6H)-one), shown in Figure 13, often found in fruit and fruit derived products, due to the blue rots caused by *Penicillium* spp. Some species within the *Aspergillus* and *Byssochlamys* genus also can produce PAT (Assunção, Martins et al. 2016, Mahato, Kamle et al. 2021). The IARC listed PAT as a group 3 carcinogen, but more data is required to determine the full extent of the hazardous nature of this compound (Bacha, Li et al. 2023). PAT has also been linked with acute toxicity such as nausea, vomiting, diarrhoea, genotoxicity and immunotoxicity (Pal, Singh et al. 2017). PAT is

often detected in fruits like apples, pears, and grapes (Mandappa, Basavaraj et al. 2018), but a recent study carried out by Tanti (2023; unpublished data) identified *P. expansum* in wheat flour samples, locally.

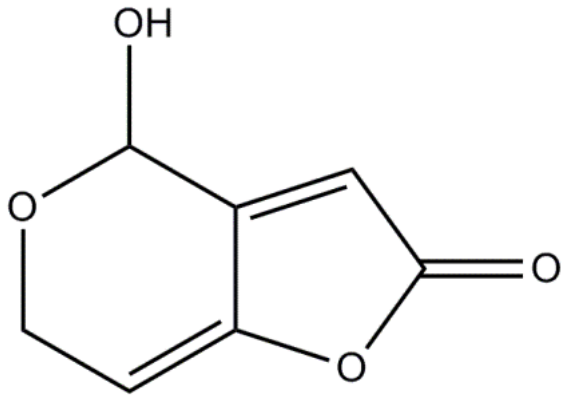

Figure 13. Chemical structure of patulin (adapted from Galaverna, Dall'Asta 2012).

2.7 European legislations and regulations on mycotoxins

Since the Middle Ages, documents dating back to 900 - 1800_{AD} describe transmission of ergotism, proving the existence of fungal toxins even back then existed. The preceding disease caused a significant number of deaths across Europe (Singh, Kumari 2022). Through all these years, mycotoxins and their harmful effects have been somewhat neglected (Pitt, Miller 2017). The discovery of fungal toxins was rather unconventional as in 1960s, 100,000 turkeys who ate feed contaminated by *A. flavus* died in an epidemic known as the Turkey X-disease (El-Sayed, Jebur et al. 2022), the compound that caused this disease was later identified as aflatoxin. Since the discovery of mycotoxins, health authorities have recognised the harmful health effects caused to humans and animals. As a result, in 2003 national guidelines on mycotoxins started to be published by 100 different countries and served to implement regulations restricting the

exposure of mycotoxins to humans and animals. However, only when the European Union (EU) and countries like Australia and New Zealand put up a joint effort to regulate the levels of mycotoxins, other countries started to fully adhere and comply to the set requirements mainly in other to avoid rejections of food imported to the EU (Van Egmond, Schothorst et al. 2007). As noted in Figure 14, the Commission Regulation (EC) No. 466/2001 was the first legislation to set the maximum levels for certain contaminants in foodstuffs, which included mycotoxins. Moreover, in order to standardise the methods of sampling and analysis for mycotoxins found in foodstuff, the European Commission passed a Commission Regulation (EC) No. 401/2006 stabilising official guidelines to Member States on how to perform mycotoxin surveys on food and animal feed. Sampling methods for cereals and related products were specified for AFB_1, total AFs (sum of AFG_1, AFG_1, AFB_1 and AFB_2), *Fusarium* toxins (DON, ZEA and FUM) and OTA. Later, the EC also identified the need for setting maximum levels for mycotoxins in certain foodstuffs and passed a Commission Regulation (EC) No. 1881/2006 to protect the public's health, assuring food safety and mitigating harmful effects to the consumer. The regulation from 2006, was based on the available toxicological and exposure data of mycotoxins. Afterwards, hazard and exposure assessments were done as a form of risk assessment which influences the process of establishing the regulations, later published by the EU. Over 30 amendments of the Commission Regulation (EC) No. 1881/2006 were carried out to either update or add new maximum levels for emerging mycotoxins, or due to additional data to substantiate claims for regulation. Figure 14 shows a timeline of key events for the regulation of mycotoxins, showing a history of key events to limit the risks and protect the consumers health and the general safety of food. Table 4 provides a summary of the minimum and maximum tolerable amounts in cereal and cereal derived products by the Food Drug

Administration (FDA) (FDA 2022) and the EU (European Commission 2006a, Ksenija 2018, Yu, Pedroso 2023).

Table 4. Maximum allowed limits of major mycotoxins in cereals and other food matrixes for food consumed by humans (European Commission 2006a, Ksenija 2018, Yu, Pedroso 2023).

Major Mycotoxins	Food Matrix	EU (EC 1881/2006) (µg/kg)	US FDA (µg/kg)
Aflatoxins (Total)	Cereals, cereal products, rice, nuts / dried fruits, spices	4 - 15	20
AFB_1	Cereals, cereal products, rice, nuts / dried fruits, spices	2 - 12	20
ZEA	Unprocessed cereals, cereal flour, unprocessed maize, bread	50 - 200	Not Regulated
DON	Wheat products, cereal, cereal products	750 - 1750	1 - 10
Fumonisins (FB_1, FB_2)	Unprocessed maize, maize flour	1000 – 2000[1]	2000 - 4000
OTA	Cereal, cereal products	3 - 5	Not Regulated
Patulin	Fruit and apple juices, apple products and infant food	10 – 50	50
T-2 / HT-2	Cereals, cereal products	Not Regulated	Not Regulated

[1] Sum of FB_1 + FB_2

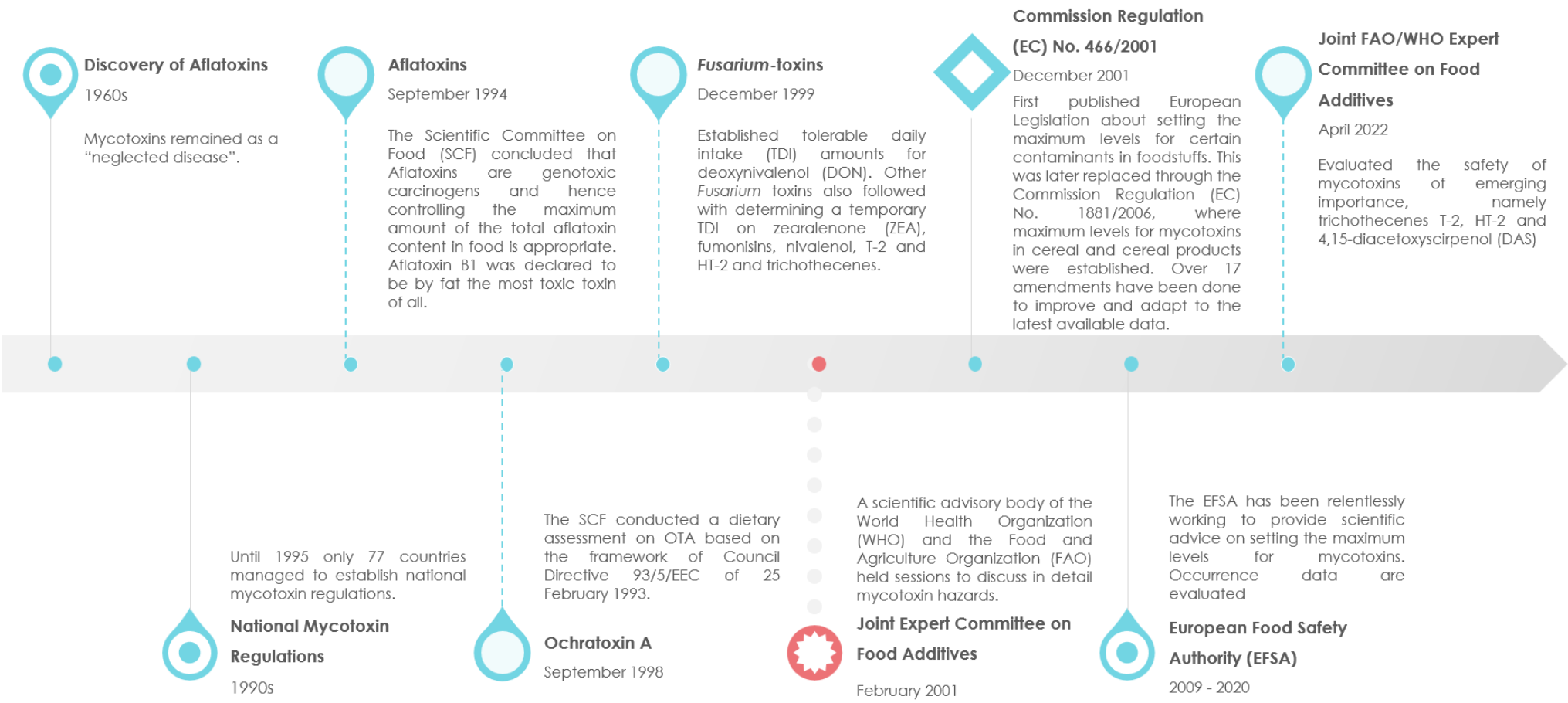

Figure 14. Timeline of events leading to improved legislation and regulation of mycotoxins across Europe and worldwide.

2.8 Major mycotoxins in flour

2.8.1 Occurrence of mycotoxins in flour

Table 5 is a collection of data from various publications which have detected and quantified various mycotoxins and their co-occurrence in different flour. Mycotoxin co-occurrence in food is increasing in significance amongst researchers and therefore it is important to first gather credible evidence from which authorities can issue new regulations to which safeguard the consumer (Santos, Pizzutti et al. 2021).

Table 5. Occurrence of mycotoxins in different flour samples.

Flour Type	Sampled Origin	Country	Contaminant Organism	Total Samples (n) and % Positive Samples	Mycotoxins detected = Range of Quantified toxins	Clean-up Technique	Analytical Method	Reference
Wheat Flour	Bakery	Brazil	-	*n = 39* DON - 100% ZEA - 2.6%	DON - 76.7 - 3630.2 µg/kg ZEA - 26.7 µg/kg	QuEChERS	UPLC-MS/MS	Lanza, Silva et al. (2019)
Corn Flour	Mills / Market	Iran	-	*n = 40* ZEA - 35% AFB1 - 62.5% OTA - 15%	DON - 65.6 – 519 ng/g ZEA - 33.7 – 3170 ng/g AFB1 - <LOQ – 1060 ng/g T-2 - <LOQ – 13.1 ng/g OTA - <LOQ – 299 ng/g	QuEChERS	UHPLC-MS/MS	Amirahmadi, Shoeibi et al. (2017)
Wheat Flour	Wheat silos	Iran	-	*n = 100* AFB1 - Not Specified AFB2 - Not Specified AFG1 -Not Specified AFG2 - Not Specified	AFB1 - 0.26 - 0.80 ng/g AFB2 - 0.27 - 0.34 ng/g AFG1 - 0.49 - 0.62 ng/g AFG2 - 0.45 - 0.75 ng/g	IAC	HPLC	Ghasemi-Kebria, Joshaghani et al. (2013)

Whole Wheat Flour	Supermarkets	China	-	*n = 35* DON - 100% ZEA - 17.14% 3-AcDON - 28.57% 15-AcDON - 17.14%	DON - 44.64 - 924.61 µg/kg ZEA - <LOD - 16.88 µg/kg 3-AcDON - <LOD - 54.88 µg/kg 15-AcDON - <LOD - 23.72 µg/kg	multi-IAC	HPLC-DAD-FLD	Zhang, Pei et al. (2019)
Refined Whole Flour	Supermarkets	China	-	*n = 50* DON - 62% 3-AcDON - 6% 15-AcDON - 2%	DON - <LOD - 401.83 µg/kg 3-AcDON - <LOD - 21.02 µg/kg 15-AcDON - < LOD - 14.73 µg/kg	multi-IAC	HPLC-DAD-FLD	Zhang, Pei et al. (2019)
Spelt Wheat Flour	Retail	United Kingdom Germany	-	*n = 22* DON - 51% ZEA - 86% OTA - 98% T-2 / HT-2 - 64%	DON - 33 µg/kg ZEA - 3.5 µg/kg OTA - 2.6 µg/kg T-2 / HT-2 - 1.2 µg/kg	ELISA	ROSA-M Reader	Wang, Hasanalieva et al. (2020)
Common Wheat Flour	Retail	United Kingdom Germany	-	*n = 20* DON - 48% ZEA -89% OTA - 99% T-2 / HT-2 - 58%	DON - 63 µg/kg ZEA - 3.8 µg/kg OTA - 3.2 µg/kg T-2 / HT-2 - 1.4 µg/kg	ELISA	ROSA-M Reader	Wang, Hasanalieva et al. (2020)
Wheat Flour	Mill	Portugal	-	*n = 2*	AFB1 - <LOD µg/kg AFB2 - <LOD µg/kg AFG1 - <LOD µg/kg AFG2 - <LOD µg/kg OTA - <LOD µg/kg	IAC	HPLC-FLD	Cardoso, Fernandes et al. (2019)
Whole Wheat Flour	Mill	Portugal	-	*n = 1* AFB1 - 0% AFB2 - 0% AFG1 - 0% AFG2 - 0% OTA - 0%	AFB1 - <LOD µg/kg AFB2 - <LOD µg/kg AFG1 - <LOD µg/kg AFG2 - <LOD µg/kg OTA - <LOD µg/kg	IAC	HPLC-FLD	Cardoso, Fernandes et al. (2019)

Rye Flour	Mill	Portugal	-	*n = 2* AFB1 - 0% AFB2 - 0% AFG1 - 0% AFG2 - 0% OTA - 0%	AFB1 - <LOD µg/kg AFB2 - <LOD µg/kg AFG1 - <LOD µg/kg AFG2 - <LOD µg/kg OTA - <LOD µg/kg	IAC	HPLC-FLD	Cardoso, Fernandes et al. (2019)
Whole Rye Flour	Mill	Portugal	-	*n = 2* AFB1 - 0% AFB2 - 0% AFG1 - 0% AFG2 - 0% OTA - 0%	AFB1 - <LOD µg/kg AFB2 - <LOD µg/kg AFG1 - <LOD µg/kg AFG2 - <LOD µg/kg OTA - <LOD µg/kg	IAC	HPLC-FLD	Cardoso, Fernandes et al. (2019)
Wheat Flour	Bakery & Markets	Egypt	*A. flavus* *A. parasiticus*	*n = 29* AFB1 - 78% AFB2 - 71% AFG2 - 36%	AFB1 - 13.416 - 9229.343 µg/L AFB2 - 2.639 - 152.668 µg/L AFG2 - 6.391 - 79.507 µg/L	Culture-based	TLC HPLC-DAD-FLD	El-Shahir (2021)
Wheat Flour Type 450 **Maize Extract** **Starch** **Corn germs**	Post-harvest	Romania	*F. graminearum* *F. culmorum*	*n = 69* Total AF - 45.4% ZEA - 7.1% OTA - 6.8% DON - 42.9%	Total AF - <1.75 - 82.94 µg/kg ZEA - <1.75 - 7.05 µg/kg OTA - <2.50 - 6.72 µg/kg DON - <18.5 - 1269.94 µg/kg	Not Specified	ELISA Ridascreen®	Gagiu, Mateescu et al. (2018)
Wheat Flour	Supermarkets	Portugal The Netherlands	-	*n = 17* ZEA - 31.6%	ZEA - 7.4-15.3 µg/kg	IAC	LC-FLD	Aldana, Silva et al. (2014)
Maize / Corn Flour	Supermarkets	Portugal The Netherlands	-	*n = 12* ZEA - 50%	ZEA - 5.9-111.7 µg/kg	IAC	LC-FLD	Aldana, Silva et al. (2014)
Mixed-Flour	Supermarkets	Portugal The Netherlands	-	*n = 13* ZEA - 35.2%	ZEA - 5.4-39.4 µg/kg	IAC	LC-FLD	Aldana, Silva et al. (2014)

				n = 35 DON - 3% NEO - 3% ZEA - 6% ENA - 9% ENA1 - 11% ENB - 80% ENB1 - 17%	DON - 190 µg/kg NEO - 38 µg/kg ZEA - 51 - 73 µg/kg ENA - <LOQ µg/kg ENA1 - 7 µg/kg ENB - 3 - 60 µg/kg ENB1 - 7 - 15 µg/kg			
Wheat Flour	Markets	Romania	-			Not Specified	LC-MS/MS	Stanciu, Juan et al. (2016)
Wheat Flour	Supermarket	Serbia	-	*n = 14* DON - 88.7% ZEA - 33.3% T-2 - 26.7%	DON - 17.5 - 976 µg/kg ZEA - 1.9 - 21.1 µg/kg T-2 - 9.8 - 26.9 µg/kg	IAC	UHPLC/HESI-MS/MS	Skrbic, Zivancev et al. (2012)
Wheat Flour	Flour Processors	Nigeria	*Aspergillus* spp.	*n = 50* Total AF - Not Specified	Total AF - 0.47 - 28 µg/kg	Not Specified	ELISA	Wartu, Whong et al. (2017)
Wheat Flour	Market	Spain	-	*n = 25* NIV - 36% DON - 20% AFB2 - 4% AFG1 - 8% AFG2 - 4% OTA - 12% ZEN - 4% BEA - 24%	NIV - <LOQ - 105 µg/kg DON - 45 - 367 µg/kg AFB2 - 2 µg/kg AFG1 - 0.53 - 0.72 µg/kg AFG2 - 1 µg/kg OTA - <LOQ - 3.5 µg/kg ZEN - 39.3 µg/kg BEA - 150 - 720 µg/kg	Matrix solid phase dispersion	LC-MS/MS	Rubert, Soler et al. (2011)
Corn Flour	Market	Spain	-	*n = 9* NIV - 11% FB2 - 22% ZEN - 11%	NIV - 92 µg/kg FB2 - 230 - 468 µg/kg ZEN - 70.5 µg/kg	Matrix solid phase dispersion	LC-MS/MS	Rubert, Soler et al. (2011)
Rice Flour	Market	Spain	-	*n = 3* BEA - 100%	BEA - 327 - 575 µg/kg	Matrix solid phase dispersion	LC-MS/MS	Rubert, Soler et al. (2011)

				n = 3				
Oats Flour	Market	Spain	-	NIV - 0% DON - 33.3% AFB2 - 33.3% BEA - 66.6%	NIV - Not detected DON - 153 µg/kg AFB2 - 1.60 µg/kg BEA - 226 - 325 µg/kg	Matrix solid phase dispersion	LC-MS/MS	Rubert, Soler et al. (2011)
				n = 88				
Wheat Flour	Mills / Market	Denmark	-	NIV - 27.3% DON - 85.22% HT-2 - 23% T-2 - 7.14% ZON - 33.33%	NIV - 20 - 172 µg/kg DON - 20 - 527 µg/kg HT-2 - 10 - 33 µg/kg T-2 - 10 - 10 µg/kg ZON - Not detected - 2	IAC	GC-ECD	Rasmussen, Ghorbani et al. (2003)
				n = 69				
Rye Flour	Mills / Market	Denmark	-	NIV - 45% DON - 7.25% HT-2 - 42.3% T-2 - 48% ZON - 6.66%	NIV - 20 - 48 µg/kg DON - 20 - 257 µg/kg HT-2 - 10 - 70 µg/kg T-2 - 10 - 193 µg/kg ZON - Not detected - 2 µg/kg	IAC	GC-ECD	Rasmussen, Ghorbani et al. (2003)
				n = 33				
Durum Wheat Flour	Mills / Market	Denmark	-	NIV - 100% DON - 100% T-2 - 90%	NIV - 20 - 440 µg/kg DON - 20 - 2591 µg/kg T-2 - 10 - 153 µg/kg	IAC	GC-ECD	Rasmussen, Ghorbani et al. (2003)
Wheat Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 12* AF - 50% OTA - 91.7%	AFB1 - 0.03 - 0.72 µg/kg AFB2 - 0.03 µg/kg AFG1 - 0.03 - 6.60 µg/kg OTA - 0.80 - 3.02 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Whole Wheat Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 3* OTA - 33%	OTA - 1.06 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)

Corn Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 3* AF - 100% OTA - 100%	AFB1 - 0.16 - 22.40 µg/kg AFB2 - 0.27 - 0.57 µg/kg OTA - 0.81 - 4.76 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Rusk Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 3* OTA - 100%	OTA - 0.89 - 2.19 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Rice Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 1* OTA - 100%	OTA - 0.94 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Soy Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 1* AF - 100% OTA - 100%	AFB1 - 0.15 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Gluten-free Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 1* OTA - 100%	OTA - 0.88 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Rye Flour	Supermarket	Turkey	*A. flavus* *Penicillium viridicatum* *A. niger* *A. ochraceus*	*n = 1* OTA - 100%	OTA - 1.37 µg/kg	IAC	HPLC-FLD	Demirel, Sariozlu (2013)
Wheat Flour	Supermarket	China	-	*n = 46* DON - 91.3% NIV - 21.7%	DON - 59.9 µg/kg (Mean) NIV - 1.9 - 20.5 µg/kg	Modified QuEChERS	UHPLC-MS/MS	Zhou, Li et al. (2016)

2.9 Analysis of mycotoxins

Surveys on mycotoxin content are important to protect human health, ensure food safety, and maintain the quality and integrity of food products. Identification and quantification of mycotoxins includes various pre-analytical and analytical steps, equally critical, as they can significantly affect the accuracy and reliability of the result (Razzazi-Fazeli, Reiter et al. 2011). Therefore, it is essential that all steps are carried out in the proper manner so that mycotoxin results can be determined consistently and with assurance (Janik, Niemcewicz et al. 2021). Analysis of mycotoxins involves a robust flow of work which includes sampling of food matrix of interest and sample preparation which involves extraction and clean-up and quantification by analytical approaches.

2.9.1 Sampling

Sampling methods have been introduced through EU legislations and should be followed in order to achieve satisfactory representative sampling and compliance. Aiming to standardise the sampling process, the EU published a Commission Regulation (EC) No. 401/2006. A latter legislation provides guidelines for the methods of sampling to control the levels of *Fusarium*-toxins (DON, ZEA, FBs), OTA, total AFs and AFB_1 in cereal and their derivate products (European Commission 2006b). The most recent amendment of these criteria was updated in 2014 through the Commission Regulation (EC) No. 519/2014. The guidelines emphasise the categorisation of lots depending on their size, and that sampling regulations should vary depending on this factor. For instance, sampling for cereals and sub-products for lots ≥50 tonnes require 100 incremental samples with an aggregate weight of 10 kg, while lots of <50

tonnes require between 3 – 100 increment samples of a minimum 1 kg. The legislation specifies that sampling at retail stage should contain a minimum aggregate weight of 1 kg (European Commission, 2014). A dynamic sampling plan is crucial since mycotoxins are produced heterogeneously within the matrices and consequently, taking a random sample from a particular lot should represent the overall contamination and indicate what is present within the whole lot (Miraglia, De Santis et al. 2006, Zhao, Chen et al. 2023). Sampling bias is an element which can also affect the process and as a result is mitigated through incremental sampling (Withaker 2003). Additionally, the level of skill of the researcher/analyst or surveillance team, the equipment, and resources available also affects sampling (Janik, Niemcewicz et al. 2021). The importance of sampling should not be underestimated since it is of fundamental importance, and poor compliance would lead to numerous problems across the agro-food chain and compilation of mycotoxin data. In fact, Whitaker (2007) stated sampling as the largest source of variability, more than the sample preparation and the analytical phase itself. Complications of incorrectly sampling may result in under-reporting mycotoxin levels resulting in the matrix to be deemed as safe to the consumer. Furthermore, poor sampling also risks false data in monitoring and surveillance reporting, misleading and incorrect data reporting, and eventually wrongful amendments to regulations and legislations (Maestroni, Cannavan 2011, Janik, Niemcewicz et al. 2021).

2.9.2 Sample Preparation

Generally, the first steps involved in the sample preparation, after the sampling stage, is milling or grinding a portion of the sample that will be the tested (Kumphanda, Matumba et al. 2021). The milling/griding step is routinely performed with whole cereals such as wheat or maize

kernels. Albeit, when dealing with flour, only homogenisation is required. Homogenisation is performed by constant agitation of the sampling bag/recipient until the sample is homogenous; meaning that if there are mycotoxins present in the main lot, irrespective from which part the sub-sample and the analytical sample is taken, the same estimation of contaminants should be present. The analytical sample is a smaller portion which should be representative of the primary sample, as summarized in Figure 15.

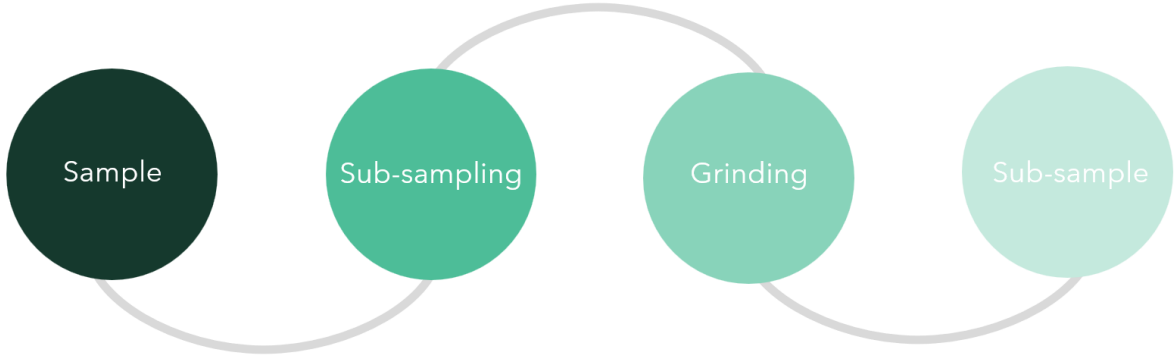

Figure 15. Flow of sample preparation prior to extracting. Sampling is performed from which a sub-sample is taken to the laboratory. Sample is milled or grinded and a sub-sample from which the analytical sample will be taken is made sure to be homogenous and therefore reducing the variation ratio (Janik, Niemcewicz et al. 2021, Nakhjavan, Ahmed et al. 2020).

2.9.3 Analyte Extraction

Once the flour, or other fine-ground sample, are homogenised extraction pre-treatment can be performed. Some studies described a pre-homogenisation step by mixing the solid phase (sample) with water to form a slurry prior to the solvent extraction (Janik, Niemcewicz et al. 2021). Dos Santos, Pizzutti et al. (2021) prepared a slurry by mixing wheat flour samples with water in a 1:1.5 (w/w) ratio, which was then homogenised, and a known quantity of the slurry was mixed with the extraction solvent to extract the desired mycotoxins. The main extraction,

however, is done by the addition of suitable organic solvents which are designed to extract and recover the maximum amount of the analyte with the highest efficiency (Singh, Kumari 2022). The solvents selected depend on the food matrix and the physical properties of the analyte (Razzazi-Fazeli, Reiter 2011). The most common chemical solvents used to extract mycotoxins from various matrices are methanol, acetonitrile, and formic acid amongst others (Zhao, Chen et al. 2023). For mycotoxins which are polar like DON, water is the most effective way for the extraction, differently of other hydrophobic toxins that require non-polar organic solvents. A different approach is to add extraction solvents in mixtures of acetonitrile-water or methanol-water at different proportions directly to the ground sample (Agriopoulou, Stamatelopoulou et al. 2020). Nevertheless, variability in recovery is expected depending on the ratio of organic solvent: water used. Such variations are linked to either the absorption of water into the dry matrix or interference with the matrix compounds. The use of internal standards can be one way to solve matrix problems in the method (Seo, Jang et al. 2021).

Nowadays researchers are trying to utilise green chemicals such as deep eutectic solvent (DES), as reported by He, Zhou et al. (2019), to extract aflatoxins in rice samples. DES is considered safer, cost-efficient, and environmentally friendly than other organic solvents such as chloroform (Jeong, Jin et al. 2018, Tan, Li et al. 2016, Vaz, Silva et al. 2020) and toluene (Amirahmadi, Shoeibi et al. 2017).

2.9.3.1 QuEChERS

QuEChERS or 'Quick, Easy, Cheap, Effective, Rugged and Safe' is a simple, fast and economical extraction technique, which provides accurate, reproducible and satisfactory performance for the extraction of mycotoxins from various complex matrices such as nuts, wine and cheese amongst others (Agriopoulou, Stamatelopoulou et al. 2020; Alcántara-Durán, Moreno-Gonzalez et al. 2019; Hamed, Moreno-Gonzalez et al. 2016; Zhang, Chen et al. 2018). It was first developed for the analysis of pesticides, but the method has been adopted by the mycotoxin scientific community for the analysis of multi-mycotoxin analysis as a screening-analytical option (Pantano, La Scala et al. 2021; Pereira, Fernandes et al. 2015; Varga, Ladanyi et al. 2020).

QuEChERS involves two main steps: the first involves the partitioning of an aqueous and organic layer after they reach equilibrium and extraction via a salting-out effect; the second step employs several combinations of porous sorbents and salts to remove matrix interfering substances, a procedure called dispersive solid-phase extraction (d-SPE) (Perestrelo, Silva et al. 2019). Pascari, Weigel et al. (2023) highlights the use of such extraction method for the detection and quantification of ZEA, α-ZEL, β-ZEL, ZEN-14-S, α-ZEL-14-S and β-zearalenol-14-sulfate (β-ZEL-14-S) from oat and wheat flour samples. The method showed good recovery levels for ZEA (72.4 – 85.7 %) and for other mycotoxins (90.7 – 95.6 %). Sospedra, Blesa et al. (2010) was also successful in analysing multiple mycotoxins in type-A and B-trichothecenes in wheat flour by QuEChERS which proved to be effective.

2.9.3.2 LLE

Liquid – Liquid Extraction (LLE) is a type of extraction method which is essentially based on the solubility of the toxins in an immiscible organic phase or in an aqueous phase (Janik, Niemcewicz et al. 2021). Thus, allowing the extraction of the analyte of interest through one solvent while leaving contaminants and unwanted compounds in the other phase (Turner, Subrahmanyam et al. 2009). This extraction option has its own pitfalls, especially when dealing with multi-mycotoxin extractions since this technique is vulnerable to low sensitivity, non-reproducibility and poor recovery while being very challenging when it comes to co-eluting of matrix components (Nakhjavan, Ahmed et al. 2020). This technique is also highly laborious and time consuming and in some instances one can suffer from loss of sample by adsorption onto the glassware (Turner, Subrahmanyam et al. 2008). LLe is usually adopted for liquid samples such as wine, milk and juices (Iqbal 2021). Fliszár-Nyúl, Szabó et al. (2020) used LLE method to extract alternariol (AOH) from red wine and tomato juice using β-cyclodextrin bead polymer (BBP) to bind the analytes. The results were rather promising, but authors pointed out that further testing is required to optimize this extraction method.

2.9.3.3 SLE

Solid-Liquid Extraction (SLE) is one of the most common extraction methods used for solid matrices such as cereals and cereal products. It involves weighing a homogenised sample, followed by the addition of an extraction solvent and agitation at high speed (Janik, Niemcewicz et al. 2021). The main advantages of SLE are that it is straightforward and can be performed using small volumes of solvent. However, its main limitation is evidently related to the matrix effects (Agriopoulou, Stamatelopoulou et al. 2020). Several studies based on this method of extraction, Karami-Osboo, Maham et al. (2013) described SLE as efficient for the

determination of DON. The authors used 20 g of wheat flour mixed at high speed (494 G-force; *g*) for 3 minutes with 100 mL of distilled water, followed by filtration, and centrifugation (3 minutes at 1372 *g*). The filtrate was passed through immunoaffinity column (IAC) as part of the clean-up procedure. To extract OTA from corn flour, Agül, Kara (2014) used 10 g of maize flour plus 200 mL of sodium bicarbonate into a blender and mixed for 2 minutes at high speed to homogenise. As for ZEA, Aldana, Silva et al. (2014) used 20 g of flour, first mixed with NaCl and then with 50 mL of acetonitrile:water (90: 10, *v/v*). The supernatant obtained after centrifugation (15 minutes at 2500 *g*) ensured extraction of the mycotoxin. Similarly, Mottaghianpour, Nazari et al. (2021) used salt and acetonitrile: water (80: 20, *v/v*) to extract aflatoxins from wheat and rice flour.

Other methods described for the extraction of mycotoxins are accelerated solvent extraction (ASE), vortex assisted low density solvent-microextraction (VALDS-ME), supercritical fluid extractions (SFE) and pressurized liquid extractions (PLE) (Agriopoulou, Stamatelopoulou et al. 2020; Janik, Niemcewicz et al. 2021). The advantages and disadvantages the main extraction techniques used up to date for quantification of mycotoxins are summarized in Table 6.

Table 6. Extraction methods, solvents used, advantages and disadvantages of such processes.

Method	Solvent	Advantages	Disadvantages	References
QuEChERS	Acetonitrile, acetonitrile/acetic acid, acetonitrile/citric acid, acetonitrile/formic acid	Quick, simplistic, economical, reproducible, and efficient	Procedures require modifications for optimization	(Al-Jaal, Salama et al. 2019; Gonzalez-Curbelo, Socas-Rodriguez et al. 2015; Perestrelo, Silva et al. 2019; Yang, Li et al. 2020)
LLE	Hexane, cyclohexane	Suitable for preparing on a small-scale	Time consuming, prone to loss of sample by adsorption onto the glassware, analyte might not always be pure	(Miklós, Angeli et al. 2020; Pereira, Fernandes et al. 2015; Song, Ediage et al. 2013; Turner, Subrahmanyam et al. 2009)
SLE	Acetonitrile/water, methanol/water	Reduced amounts of liquid used as a solvent	Further clean-up steps are necessary	(Leite, Freitas et al. 2020; Miklós , Angeli et al. 2020; Turner, Subrahmanyam et al. 2009; Xie, Chen et al. 2016)
ASE or PLE	Acetonitrile/water, acetonitrile/methanol	Short turn-around-time, low amounts of solvent used for extraction, automated	Expensive	(Alvarez-Rivera, Bueno et al. 2020; D'Arco, Fernandez-Franzon et al. 2008; Miklós , Angeli et al. 2020; Zhang, Dou et al. 2018)
VALDS-ME	Toluene, 1-octanol, Na_2SO_4, water	Simple, fast, effective	Requires a lot of specialized optimizations	(Somsubsin, Seebunrueng et al. 2018)

SFE	Supercritical CO_2 fluid, acetonitrile, acetone, ethanol	Fast, low solvent volumes used, extraction of temperature sensible analytes	Costly equipment, low recoveries	Zhang, Dou et al. 2018; Turner, Subrahmanyam et al. 2009; Woo, Ryu et al. 2019)

QuEChERS, Quick, Easy, Cheap, Effective, Rugged and Safe; LLE, Liquid-Liquid Extraction; SLE, Solid-Liquid Extraction; ASE, Accelerated Solvent Extraction; PLE, Pressurized Liquid Extraction; VALDS-ME, Vortex Assisted Low Density Solvent–Microextraction; SFE, Supercritical Fluid Extraction

2.9.4 Clean-up

The clean-up process is an inseparable-obligatory step required after extraction (Razzazi-Fazeli, Reiter 2011) to provide selective properties by removing unwanted substances which cause interference and facilitates the purification of mycotoxins of interest. Clean-up also limits the matrix effect phenomenon (Janik, Niemcewicz et al. 2021). Through clean-up, the sensitivity and specificity are increased, contributing to the improvement in the determination, accuracy, and precision of the method (Desmarchelier, Tessiot et al. 2014). The two main clean-up techniques currently used are solid phase extraction (SPE) and immunoaffinity columns (IAC).

2.9.4.1 Immunoaffinity columns (IAC)

IAC use an antigen-antibody principle to capture the mycotoxins-target in the test sample passing through (Agriopoulou, Stamatelopoulou et al. 2020). IACs use polyclonal or monoclonal antibodies able to act as the specific antigen towards the mycotoxins (Razzazi-Fazeli, Reiter 2011). During the cleaning up process the interfering substances are washed off, while the mycotoxin remains bound to the antibodies and later on are eluted using a miscible solvent such as pure methanol to break the antigen-antibody-mycotoxin bond. IACs are widely used for mycotoxin quantification since they are both used to detect one or multiple mycotoxins in a food test sample (Fitzgerald, Leonard et al. 2017). Such columns are highly sensitive, selective and are a great validation purification tool since they are fairly easy to master after adequate training (Janik, Niemcewicz et al. 2021). The technique can be quite time-consuming especially when the flow (mostly by gravity) is not steady. Through the years, IACs evolved, as researchers and commercial companies have identified the need for multi-

mycotoxin IACs (Wilcox, Donnelly et al. 2015). However, one important constraint is their limited ability to absorb mycotoxins, especially if the level of mycotoxins is greater than the binding capacity of the column (Castegnaro, Tozlovanu et al. 2006). Thus, since all of the antibodies are used up, mycotoxins are washed off along with unwanted substances, leading to false results (Agriopoulou, Stamatelopoulou et al. 2020). Another constraint arises from the presence of matrix compounds that can interfere with the antibodies (Janik, Niemcewicz et al., 2021). For instance, fatty acids found in the matrix can cause a masking effect, impeding the binding of FUM to the IAC antibodies (Castegnaro, Tozlovanu et al. 2006). In their evaluation, Gonçalves and Stroka (2016) investigated the cross-reactivity of DON and its conjugates, including DON-3-G, 3-acetyl-DON (3-AcDON), and 15-acetyl-DON (15-AcDON). They conducted tests using the DONPREP™ IAC from R-Biopharm, along with three other commercial IAC. The results indicated that the DONPREPTM IAC exhibited cross-reactivity with DON-3-G and 3-AcDON conjugates but failed to retain 15-AcDON. This outcome poses a challenge, as it may lead to an underestimation of DON, the target mycotoxin of interest, due to limited antibody availability for retaining the main target metabolite. Additionally, IAC are still expensive especially when dealing with a large sample number (Liu, Liu et al. 2018). Pisciottano, Imperato et al. (2020) adopted IAC for clean-up in their determination of T-2 and HT-2 toxins, using EASI-EXTRACT™ T-2 and HT-2 IAC from R-Biopharm (Darmstadt, Germany), from cereal and cereal derived products. The authors noted that the clean-up method was effective and achieved high recovery rates.

2.9.4.2 Solid phase extraction (SPE)

Unlike IAC, SPE make use of solid absorbents packed in cartilages (Singh, Mehta 2020) and these adsorbents are used to capture the mycotoxins while any contaminants are washed away similarly to IACs (Janik, Niemcewicz et al. 2021). Conventional strong anionic cartilages such as octadecylsilyl (C18), as well as novel carbon magnetic carbon nanomaterial are among the most common absorbents used with some level of specificity towards certain mycotoxins, for example, carbon nanotubes were employed to extract trichothecenes from maize, wheat and rice (Agriopoulou, Stamatelopoulou et al. 2020, Dong, Si et al. 2015). While this technique is known for its efficiency, reproducibility, and short turnaround time, it still has some limitations. The main limitation is the inability to use a single cartilage for multi-mycotoxin detection, thus, this method can lead to wastage, and it is generally more laborious. Additionally, the efficiency of SPE can be affected by several variables such as the type of solvent or condition of the sample including its ionic strength and pH (Zhang, Dou et al. 2018). Cao, Li et al. (2021) utilized an on-line Solid Phase Extraction (SPE) technique to quantify DON and its metabolites in corn and wheat flour. The method demonstrated a broad linear range, excellent precision, low limit of detection (LOD) ranging from 0.1 to 0.2 μg/kg, and high recoveries for the three target analytes ranging from 86.5 % to 99.7 %.

2.9.5 Analytical Determination of Mycotoxins

There are various methods which can be utilised for the detection of mycotoxins such as high-performance liquid chromatography (HPLC), Liquid Chromatography-tandem mass spectrometry (LC-MS/MS), thin-layer chromatography (TLC), gas chromatography (GC)

amongst others which offer rapid technologies such as enzyme-linked immunosorbent assay (ELISA), lateral flow immunoassay (LFIA) and biosensors (Janik, Niemcewicz et al. 2021).

2.9.5.1 High Performance Liquid Chromatography (HPLC)

HPLC is one of the most powerful analytical approaches used to quantify mycotoxins since it offers high sensitivity and selectivity (Keskin, Eyupoglu 2023). An HPLC system has several key components such as a high-pressure pump, a column, injector, a detector, or a combination of detectors connected to appropriate software for accurate data collection and analysis. Several detectors can be adapted for the analysis such as ultraviolet (UV), fluorescent (FLR) and photodiode array (PDA) detectors (Alshannaq, Yu 2017). Of course, the analysis and instrumentation settings for the HPLC equipment are highly dependent on the mycotoxin being analysed because of its unique physical and chemical properties. For the separation of analytes, reversed-phase C18 column is routinely used for mycotoxins. A C18 column, also is a type of chromatography column commonly used for the separation of mycotoxins. It is named after its stationary phase, which is made up of a hydrophobic material consisting of long alkyl chains bonded to a silica or polymer support. The "C18" indicates that there are 18 carbon atoms in these alkyl chains. Reversed-phase chromatography is a technique where the stationary phase is nonpolar (hydrophobic), and the mobile phase (solvent) is polar. When a sample is introduced into the column, compounds within the sample will interact differently with the stationary phase based on their polarity. Polar compounds tend to spend more time in the mobile phase and move through the column more quickly, while nonpolar compounds interact more strongly with the hydrophobic stationary phase and take longer to elute.

The mobile phase, with optimised composition and pH is also set-up to ensure satisfactory separation of compounds to assure good peak resolution and quantification. UV and FLR detectors rely on the presence of a chromophore in the metabolite of interest for their detection, OTA, AFG_1 and AFB_1 are great examples of chromophores since they can be detected directly via HPLC-FLR. On the other hand, mycotoxins such as FB_1 require derivatisation prior to their separation and detection due to the lack of chromophore in the molecule. Derivatisation is based on the principle of a chemical reaction to generate a chromophore, or fluorescent particle, to create a signal in the detection system. Derivatisation can be performed before analysis (precolumn derivatisation) or between the column separation and detection processes (post column derivatisation) (Janik, Niemcewicz et al. 2021) and can be performed by a physical or chemical reaction. One can connect an in-line photochemical reactor to the HPLC-FLR setup to identify AFG_1 and AFB_1. When exposed to UV light at a specific wavelength of λ 254 nm, these substances emit the same level of fluorescence as when they undergo an electrochemical reaction for derivatisation (Miklós, Angeli et al. 2020). To enhance the inherent fluorescence characteristics of AFG_1 and AFB_1, a specific electrochemical reaction can be performed, involving trifluoroacetic acid (TFA) (Akiyama, Goda et al. 2001), potassium bromide (KBr) (Gilbert, Vargas 2003) or iodine. Figure 16 shows a chromatogram of AFs standards peaks detected using HPLC-FLR.

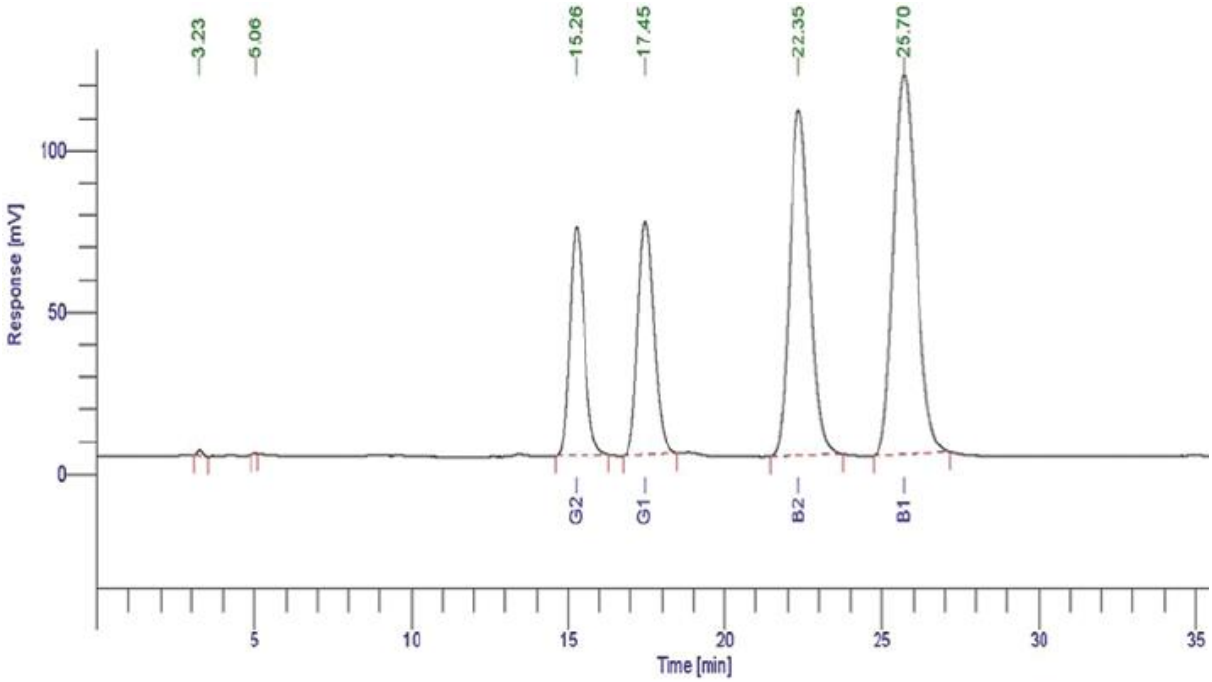

Figure 16. AFG_2, AFG_1, AFB_2 and AFG_1 standards detected after spiking cornmeal (Sirhan, Tan et al. 2014)

2.9.5.2 Liquid Chromatography associated to tandem Mass Spectrometry detection (LC-MS/MS)

LC-MS/MS is another quantitative method for the detection of mycotoxins and often regarded as the benchmark technique especially when coupled with LC-MS or LC-FLR (Iqbal 2021). Mass spectrometry uses mass to charge ratios to determine the chemical composition of the analyte (Zhao, Chen et al. 2023). LC-MS/MS is the preferred method for mycotoxin analysis among researchers due to several advantages. First, it offers a LOD, making it highly sensitive in detecting even trace amounts of mycotoxins. Additionally, LC-MS/MS is cost-effective and reliable, as evidenced by studies conducted by Janik, Niemcewicz et al. (2021). Another significant benefit is its ability to identify both non-fluorescent and fluorescent toxins simultaneously (Singh, Mehta 2020). Furthermore, the method requires minimal to almost no clean-up steps, which simplifies the analytical process (Sulyok, Stadler et al. 2020). Despite

these advantages, using LC-MS/MS for mycotoxin analysis comes with certain challenges. One of the main issues is the lack of practical guidance and documentation, particularly concerning multi-analyte approaches and matrix-effect validation. This can make it challenging for researchers to adapt protocols on this analytical equipment effectively. Moreover, when covering a large number of analytes, especially those near the limit of quantification (LOQ), manual inspection becomes necessary, leading to laborious and time-consuming data evaluation (Sulyok, Stadler et al. 2020). LC-MS/MS is a promising method for mycotoxin analysis due to its high sensitivity, cost-effectiveness, and ability to identify different types of toxins. However, researchers should be aware of the challenges related to method adaptation, multi-analyte approaches, and data evaluation, which can impact the efficiency and practicality of using LC-MS/MS in mycotoxin analysis. Nonetheless, adhering to European regulations, LC-MS/MS remains a recommended approach for the detection of multi-mycotoxins and co-occurrence monitoring in food, as emphasised by Varga, Fodor et al. (2021).

2.9.5.3 Validation of Analytical Method

Prior to the validation of the method a series of steps need to be caried out. Firstly, it is important to optimise the chromatographic conditions by adjusting the instrumental method parameters (column temperature, injection volume, gradient program (if required) and flow rate). Then, set the optimal emission (λ_{em}) and excitation (λ_{ex}) wavelengths to detect the mycotoxin of interest. To quantify the analyte, different concentration standards are prepared using a known pure solution with 4–6 point calibration curve. In order to validate the method, parameters such as linearity, recovery, repeatability, LOD, limits of quantification (LOQ) need to be considered. Linearity is checked from the calibration curve from the co-efficient of

determination (R^2). Demirel and Sariozlu (2013) employed a validation approach by introducing known levels of AF at three different concentrations into blank wheat flour samples during recovery experiments. Accuracy and repeatability also represent import parameters to guarantee good method development. The LOQ is defined as the lowest level of spiked sample that could be quantified with appropriate accuracy and precision. Once validation is complete, analysis of food samples can proceed. As for positive samples containing mycotoxins, their concentrations can be quantified by plotting the peak areas of the calibration curve of chemical reference substance against the corresponding concentrations. The analyte's concentration is determined by extrapolating the calibration curve to the x-intercept and considering corresponding dilution factor and sample size.

Chapter 3 – Materials and Methods

3.1 Chemicals and reagents

The standards of AF (AFB_1, AFB_2, AFG_1, AFG_2; 1000 ng/mL), DON (100 µg/mL), FUM (FB_1, FB_2; 100, 30 µg/mL), OTA (1000 ng/mL), ZEA (25 µg/mL), T-2 (100 µg/mL), HT-2 (100 µg/mL) and PAT (25 µg/mL) were purchased from R-Biopharm AG (Darmstadt, Germany). Ethyl acetate (99.8%), glacial acetic acid (HPLC Grade, 99.7%), diethyl ether (HPLC Grade, 99%), phosphate buffered saline (PBS) tablets, sodium chloride; >=99.5%, sodium bicarbonate (99%), sodium tetraborate decahydrate (99%), trifluoracetic acid (99%), 4-(dimethylamino) pyridine (99%), toluene (HPLC certified, 99%) and Phthaldialdehyde (OPA) 98%, were obtained from ThermoScientific™ (Dartford, United Kingdom). 1-Anthroylnitrile (10 mg) was purchased from LGC Standards GmBH (Wesel, Germany). Sodium phosphate monobasic monohydrate; >=99.0% and hexane for HPLC; >=97.0% were purchased from Sigma-aldrich (St. Gallen, Switzerland). Ortho-phosphoric acid; 85% was purchased from Lach-Ner Ltd (Tovarni, Czech Republic). The HPLC Grade acetonitrile, ultrapure water and methanol were purchased from Carlo Erba Reagents (Milano, Italy).

3.2 Materials and equipment

For sample clean-up, an immunoaffinity column rack and its corresponding accessory pack, containing 10 glass barrels and 10 syringes were purchased from R-Biopharm AG (Darmstadt, Germany). The IACs were purchased from R-Biopharm AG (Darmstadt, Germany), corresponding to, EASI-Extract™ Aflatoxin, FUMONIPREP™, DONPREP™, OCHRAPREP™, EASI-

EXTRACT™ Zearalenone, EASI-EXTRACT™ T-2 & HT-2 and EASIMIP™ PATULIN. The HPLC consumables were purchased from Agilent Technologies™ (Frankfurt, Germany): column Eclipse Plus C18 (4.6 x 100mm, 5µm), Guard column Eclipse Plus C18 (4.6 x 12.5 mm, 5µm), Guard cartridge holder (High perf. ZORBAX Guard fittings kit), 1.5 mL amber vials with screw pre-slit PTFE caps and amber 4 mL vials with caps. Gilson™ nylon syringe filters 0.22µm 13mm were purchased from Technoline Ltd (Gzira, Malta). The following equipment were utilized throughout the study: dry heating block (Fried Electric, Holon, Israel), Clifton™ Cyclone Vortex Mixer (Fisher Scientific™, Leicestershire, United Kingdom), centrifuge (Centurion Scientific Ltd, Chichester, United Kingdom).

3.3 Sampling

Samples of flour (*n* = 16) were purchased or obtained from local supermarkets, bakeries, and flour mills in Malta. The description of each sample is shown in Table 7. The samples collected were all regarded to be at commercial stage, thus 1 kg of each sample were acquired, as specified in the European Regulation 401/2006 (European Commission, 2006b) for sampling. Sampling was performed randomly, choosing the packages available on the supermarket's shelves, or as provided by the baker or flouring mill facility. The main sample was homogenised and divided into 7 bags with 25 g of flour to be specifically used for each mycotoxin (*n = 112*). The sub-samples were stored at 4-6 °C until analysis. An extra flour sample was purchased from the supermarket. The flour selected was not milled or packaged in Malta and therefore based on this selection-criteria, this sample was selected as the blank. In order to confirm, that the

latter can be used as a blank, sample preparation and analysis was performed for each mycotoxin method, as the below account, to confirm that the flour was mycotoxin-free.

Table 7. List of collected flour samples used in the survey.

Sample code	Flour Type	Flour Origin	Country Origin
1	Soya	Supermarket	unknown
2	Wheat	Mill	unknown
3	Potato	Supermarket	Finland
4	Wheat	Supermarket	unknown
5	Rye	Supermarket	unknown
6	Carob	Supermarket	unknown
7	Wheat	Bakery	unknown
8	Brown Rice	Supermarket	Italy
9	Wholemeal	Supermarket	Lithuania
10	Spelt	Supermarket	Austria
11	Pumpkin	Supermarket	Ukraine
12	Wheat	Bakery	unknown
13	Wheat	Bakery	unknown
14	Wholemeal	Supermarket	unknown
15	Flour ‘’00’’	Supermarket	unknown
16	Plain flour	Supermarket	unknown

3.4 Preparation of working standard solution and spiked samples

AFs working solutions were prepared in methanol at a concentration of 250 ng/mL, hence consisting of 62.5 ng/mL of each analogue AFB_1, AFB_2, AFG_1, AFG_2. Similarly, OTA working solutions were prepared in methanol at a concentration of 250 ng/mL. The working solutions for ZEA were diluted in acetonitrile to 1000 ng/mL from the stock solution. A working standard solution was also prepared for FUM at 20 µg/mL of total concentration (FB_1 + FB_2), which was prepared in acetonitrile: methanol: water (25: 25: 50, *v/v/v*). DON working solution was prepared in methanol: water (15: 85, *v/v*) at a concentration of 5000 ng/mL, while that of Patulin was prepared in acetic acid: water (0.1: 99.9, *v/v*) consisting of a concentration of 250 ng/mL. The working solution of T-2 and HT-2 was prepared in acetonitrile containing a total concentration of 1000 ng/mL. The spiked concentrations formulated for recovery experiments were: ZEA at 60, 160, 160 ng/g; AFs total concentrations at 10, 20, 30, 40 ng/g; OTA at 25 and 50 ng/g; DON at 100, 500, and 1000 ng/g; PAT at 25 and 50 ng/g; FUM at 1250, 2500 and 5000 ng/g; and T-2/HT-2 at 50 and 100 ng/g.

3.5 Instrumental apparatus for mycotoxin analysis

The detection and quantification of the analytes was caried out using a Waters® Alliance 2695 HPLC system with autosampler (200 µl loop), and quaternary pump (Milford, USA), coupled with a Waters® 2475 Multi λ Fluorescence (FLR) and Waters® 2996 Photodiode array (PDA) (Milford, USA). The system allowed temperature control in both autosampler and column oven. The separation was achieved using reverse phase Zorbax Eclipse Plus C18, 4.6 x 100 mm,

5 µm column (Agilent, USA) preceded by a guard column Zorbax Eclipse Plus C18 4.6 x 12.5 mm, 5 µm (Agilent, USA).

3.6 Method for DON quantification

3.6.1 Sample Preparation

The following protocol was adapted from R-Biopharm (2022a) (available at https://food.r-biopharm.com/wp-content/uploads/P50_DONPREP-IFU-V18_2022-05.pdf). 5 g of flour and 1 g of sodium chloride were weighed and transferred into a 50 mL polypropylene tube. To assess the recovery, 5 g of a blank (mycotoxin free) sample of flour was artificially contaminated in three levels of DON standard (*n = 3*; 100, 500 and 1000 ng/g), homogenised and extracted as per protocol. The spiking of the blank flour was done by pipetting the known standard concentration equivalent to the amount of flour (in grams) to contain the desired concentration in ng/g. This was followed for each mycotoxin analysis. In each sample and spiked flour, 40 mL of water was added and mixed at high speed by using a vortex for 2 min and centrifuged at 1792 *g* for 10 minutes at 4 °C. Both spiked and flour samples were filtered through filter paper. Then, 2 mL (equivalent to 0.25 g of sample) of filtrate was passed through the DONPREP® immunoaffinity column by gravity. The column was washed with 10 mL of water at a flow rate of 5 mL per minute. Air was passed to remove any residual liquid and the analyte was eluted from the column at a flow rate of 1 drop per second using 1.5 mL of methanol and collected in a 4 mL amber glass vials. The eluate was evaporated to dryness in a heating block at 60 – 70 °C. Afterwards, samples and spiked solutions were reconstituted with 1 mL of

methanol: water (15: 85, *v/v*), vortexed for 20 s and filtered into a 1.5 mL amber vial. The samples were analysed according to the conditions in the sub-heading 3.6.2.

3.6.2 Instrumental Conditions

The separation of DON was achieved with isocratic delivery of mobile phase with water: methanol (85: 15, *v/v*) at a flow rate of 0.5 mL/min. The column temperature was set at 40 °C, and the samples were kept at 5 °C in the injection module. The run time was set as 15 min, while the injection volume ranged between 20 – 100 µl for both samples and standards. The detection of DON was achieved in a PDA detector (item 3.5), set to operate at an absorbance of 220 nm at 1.2 nm resolution with sampling rate of 1 point/sec and filter constant set to "fast". A five-point calibration curve was performed for DON through serial dilution from the working solution. The different concentrations of standard ranged from 31.25 to 500 ng/mL were diluted in methanol: water (15: 85, *v/v*).

3.7 Method of aflatoxin quantification

3.7.1 Sample Preparation

The following protocol was adapted from R-Biopharm (2021a) (available at https://food.r-biopharm.com/wp-content/uploads/RP71_RP70N_EASI-EXTRACT-AFLATOXIN-V17_2021-06-1.pdf). 5 g of flour sample and 1 g of sodium chloride were weighed and transferred into a 50 mL polypropylene tube. The spiked samples (*n = 4*) were prepared in different concentrations of 10, 20, 30, 40 ng/g. Then, 10 mL of methanol: water (80: 20, *v/v*) was transferred to each

tube (samples and spiked) vortexed at high speed for 2 minutes, then centrifuged at 1792 *g* for 10 minutes at 4 °C. Moreover, 2 mL of filtrate was diluted in 14 mL of PBS and vortexed for 20 seconds. Next, 3.2 mL of the filtrate (equivalent of 1 g of sample) was passed through the EASI-EXTRACT® Aflatoxin immunoaffinity column by gravity. The column was washed by passing 20 mL of PBS. After the washing step, air was passed to remove any residual liquid. The analyte was eluted using 2 x 1.5 mL of methanol and collected into 4 mL amber glass vials. The samples were evaporated to dryness in a heat block at 60 °C and derivatisation was performed for each sample and standard (item 3.7.2).

3.7.2 Derivatisation of Aflatoxins

200 µl of hexane was added into each vial with dried extract and vortexed for 30 seconds. Then, 50 µl of trifluoroacetic acid was added and immediately vortexed for 30 seconds and left to react for 5 minutes. Next, 950 µl of acetonitrile: water (10: 90, *v/v*) was used to stop the derivatisation reaction. The vial was vortexed for 30 seconds and rested for 10 minutes to allow phase separation. The upper layer (~200 µl) was removed. The sample was collected using a 1 mL plastic syringe and filtered through nylon filters into a 1.5 mL amber vials. The samples were then injected into the HPLC System (item 3.5).

3.7.3 Instrumental Conditions

The mobile phase for AFs quantification was composed of water: methanol (60: 40, *v/v*) at a flowrate of 1 mL/min in an isocratic delivery. The temperature of the column was set at 25 °C,

while the samples were maintained at 5 °C. The run time of the method was set as 15 min and the injection volume were set at 10 - 50 µl for both samples and standards. The detection was performed in a multi wavelength detector (Waters® 2475; item 3.5). The following channels of λ_{ex} and λ_{em} to detect and quantify AFs: for AFB_1, AFB_2, AFG_1 and AFG_2 a were set to λ_{ex} 365 nm and λ_{em} 440nm. The detector was configured for data rate as 1 point/ sec, photomultiplier (PMT) gain of 10 and sensitivity of 10 000 EUFS. A seven-point calibration curve in the range 0.1953 – 12.5 ng/mL was injected for AFs analysis to quantify the spiked and any positive samples from the surveillance study.

3.8 Method for ochratoxin A quantification

3.8.1 Sample preparation

The following protocol was adapted from R-Biopharm (2021b) (available at https://food.r-biopharm.com/wp-content/uploads/P14_OCHRAPREP-V21_2021-06-1.pdf). 5 g of flour samples and blank spiked samples (*n = 2*; 25, 50 ng/g) were weighed into 50 mL polypropylene tube and 40 mL of acetonitrile: water (60: 40, *v/v*) was added to all sample tubes. The samples were vortexed at high speed for 2 minutes and then centrifuged at 1792 *g* for 10 minutes at 4 °C. Subsequently, 2 mL of extract was diluted with 34 mL of PBS solution. The diluted extract of each flour and spiked sample were vortexed at high speed for 30 seconds to ensure proper homogenisation prior passing through the column. Then, 2.4 mL of diluted extract (equivalent of 0.5 g of sample) was passed through the OCHRAPREP® immunoaffinity column by gravity. The column was washed with 20 mL of PBS solution, then, air was passed through to remove any residual liquid and impurities. The samples were then eluted using 2 x 1.5 mL of acetic acid:

methanol (2: 98, *v/v*) and were collected in 4 mL amber glass vials. Approximately 1 mL of the eluted sample was filtered into a 1.5 mL amber vial and injected in the HPLC.

3.8.2 Instrumental Conditions

The OTA separation was carried out with isocratic mobile phase mixed by the equipment consisting of acetonitrile: water: acetic acid (56: 42: 2, *v/v/v*) at a flow rate of 1 mL/min. The samples were kept at 5 °C in the injector module, while the temperature of the column was set at 40 °C. The method run time was set as 10 min and the injection volume was programmed for 20 – 50 µl for both samples and standards. The fluorescence detection (item 3.5) was set λ_{ex} 333 nm and λ_{em} 443 nm. An eight-point calibration curve was prepared ranging from 0.195 - 100 ng/mL.

3.9 Method for Zearalenone quantification

3.9.1 Sample Preparation

The following protocol was adapted from R-Biopharm (2021c) (available at https://food.r-biopharm.com/wp-content/uploads/RP91_RP90_EASI-EXTRACT-ZEARALENONE-V21_2021-10-2.pdf). 5 g of flour samples and blank wheat flour were weighed in a 50 mL polypropylene tube. The blank samples were spiked with ZEA standard (*n = 4*; 60, 60, 160, 160 ng/g) and homogenised. Then, 25 mL of acetonitrile: water (75: 25, *v/v*) was added to the tubes, vortexed at high speed for 2 minutes and then centrifuged at 1792 *g* for 10 minutes at 4 °C. 4 mL of the extract was diluted in 16 mL of PBS solution. The pH was measured and adjusted to 7.4 using

2M sodium hydroxide when necessary. Subsequently, 5 mL of diluted extract (equivalent to 1 g of sample) were passed through the EASI-EXTRACT® ZEARALENONE immunoaffinity column. The extract passed through the column by gravity to ensure maximum capture of the toxin by the antibody. The column was then washed with 20 mL of PBS to remove any unwanted material. Air was passed through the column to remove any residual liquid. The analyte was eluted with 2 x 1.5 mL of acetonitrile. The flour samples and spiked samples were evaporated to dryness in a heating block at 60 °C. Then, 1 mL of acetonitrile: water (50: 50, *v/v*) was added to resuspend the samples, vortexed for 30 s and additionally sonicated for 15 min. The samples were filtered through nylon filters into a 1.5 mL amber vial and injected into the HPLC System (item 3.5).

3.9.2 Instrumental Conditions

The mobile phase for ZEA analysis consisted of acetonitrile: water: methanol (46: 46: 8, *v/v/v*) delivered isocratically at a flow rate of 1 mL/min. The run time was set as 10 min, while the temperature of the autosampler was set at 5 °C and the temperature of the column at 40 °C. The injection volume varied between 20 – 100 µl for both samples and standards. The detection of the analyte was carried out using fluorescence detector (item 3.5) with λ_{ex} 274 nm and λ_{em} 445 nm. A six-point calibration curve ranging from 1.5 - 50 ng/mL was used to calculate the concentrations of ZEA.

3.10 Method for Patulin quantification

3.10.1 Sample Preparation

The following protocol was adapted from R-Biopharm (2021d) (available at https://food.r-biopharm.com/wp-content/uploads/2012/06/p250_easimip-patulin-v6_2021-06.pdf). 5 g of flour sample and blank samples were weighed in a 50 mL polypropylene tube. The blank samples were spiked with PAT standard (*n = 2*; 25, 50 ng/g) and homogenised. Then, 20 mL of HPLC water were added to each sample and spiked tube. The samples were vortexed for 20 s at high speed, then incubated for 2 hrs at 40 °C. The samples were centrifuged at 1792 *g* for 10 min and the extract filtered through a 0.45 µm nylon filter. Before passing the samples through the EASIMIP™ PATULIN, which are molecularly imprinted polymer (MIP), the columns were conditioned by passing 2 mL of acetonitrile at a flow rate of 1 mL/min not allowing the column to dry out. Next, 1 mL of ultrapure water was passed at 1 mL/min. Subsequently, 10 mL of extract (equivalent to 2.5 g of sample) was passed through the column at a flow rate of 0.5 mL/min. The flow rate was controlled to ensure maximum capture of the analytes. The columns were washed with 4 mL of 1% acetic acid, then dried out by passing air to remove any residual liquid. Then 500 µl of diethyl ether was passed through the column and air was passed through to remove any residual liquid. The analyte was eluted from the MIP column using 2 x 1mL of ethyl acetate which were collected in a 4 mL amber glass vials,10 µl of acetic acid was added to the eluate and vortexed for 20 s. The eluate was dried at 45 °C in a heating block. Right after complete drying, the samples were reconstituted with 1 mL of 0.1 % acetic acid, vortexed for 20 s and filtered through into a 1.5 mL amber vials and were injected in the HPLC system.

3.10.2 Instrumental Conditions

The mobile phase for PAT analysis consisted of acetonitrile: water: acetic acid (10: 90: 0.1, *v/v/v*) mixed by the system in an isocratic delivery at a flow rate of 1 mL/min. The run time of analysis was set as 10 min, while the injection volume was set at 20 – 100 µl. The detection used PDA detector (item 3.5) was set at an absorbance of 276 nm at 1.2 nm resolution. The column temperature was set at 30 °C and samples were kept at 5 °C. A five-point calibration curve for PAT from 6.25 - 100 ng/mL was used to quantify the concentration of toxin.

3.11 Method for T-2 and HT-2 quantification

3.11.1 Sample Preparation

The following protocol was adapted from R-Biopharm (2022b) (available at https://food.r-biopharm.com/wp-content/uploads/P43_EASI-EXTRACT-T-2-HT-2-IFU-V18_2022-05.pdf).

First, 5 g of sample along with 1 g of sodium chloride were transferred into a 50 mL polypropylene tube. The same was done for blank flour samples which were spiked with a mix of T-2 and HT-2 standards (*n = 4*; 50, 50, 100, 100 ng/g). Then, 50 mL of methanol: water (90: 10, *v/v*) was added and vortexed at high speed for 2 minutes, centrifuged at 1792 *g* for 10 minutes and 7 mL of the extract was diluted with 28 mL of ultrapure water. Next, 5 mL of diluted extract (equivalent to 1 g of sample) was passed through the EASI-EXTRACT® T-2 & HT-2 immunoaffinity column by gravity. The column was washed with 20 mL of ultrapure water and air was passed through the column to remove any residual liquid. The analyte was eluted from the column with 2 x 1.5 mL of methanol. The eluates were evaporated to dryness under

air at 60 °C in a heating block. Prior injection, the dried samples were derivatised with 4-dimethylaminopyridine (D-MAP) and 1-anthronitrile (1-AN) reagents, as described below.

3.11.2 Derivatisation of T-2 and HT-2

A stock solution of D-MAP reagent was prepared by weighing 20 mg of D-MAP into a glass jar. Then, 20 mL of toluene (equivalent to 1 mg/mL) was added. Similarly, 1-AN reagent was prepared using 5 mg in 5 mL of toluene (equivalent to 1 mg/mL). The working solution of D-MAP was diluted to a concentration of 325 µg/mL in toluene, and 1-AN to 300 µg/mL in toluene. The samples were reconstituted by adding 50 µl D-MAP (325µg/mL) and 50 µl 1-AN (300 µg/mL) and vortexed for 1 min. The mixtures were left to react at 50 °C in a heating block for 15 min. The vials were then placed inside an iced bath for 15 min then evaporated to dryness at 60 °C. The dried samples were reconstituted with 1 mL of acetonitrile: water (70: 30, *v/v*), vortexed for 20 s and filtered into a 1.5 mL amber vial. The samples were then injected into the HPLC System.

3.11.3 Instrumental Conditions

The mobile phase for the analysis of T-2 and HT-2 was composed of water and acetonitrile with gradient delivery at a flow rate of 1 mL/min. The gradient was programmed as the following: from 0 to 5 min at 70% acetonitrile, at 15 min acetonitrile was increased to 85% and kept until min 25, increased to 100% at min 27 and kept until min 32 then lowered to 70% from min 32 to 35 to allow re-equilibration. Consequently, the run time for analysis was set as 35 min, while the injection volume ranged at 10 – 20 µl. The temperature of the samples and column was

set at 5 °C and 40 °C, respectively. The fluorescence detector (item 3.5) was set at λ_{ex} 381 nm and λ_{em} 470 nm. A six-point calibration curve was prepared from 3.125 – 100 ng/mL, considering that the ratio if standards for T-2 and HT-2 were prepared at 1:1.

3.12 Method for Fumonisins quantification

3.12.1 Sample preparation

The following protocol was adapted from R-Biopharm (2022c) (available at https://food.r-biopharm.com/wp-content/uploads/P31_FUMONIPREP_IFU-V19_2022-05.pdf). In a 50 mL polypropylene tube, 5 g of sample and 1 g of sodium chloride were weighed, separately. Using a blank matrix (wheat flour) (*n = 3*), were spiked with three different concentrations of FB_1+ FB_2 by adjusting the working standard solution (20 µg/mL) to 1.25, 2.5 and 5 µg/g. Then, 25 mL of the extraction solvent consisting of acetonitrile: methanol: water (25: 25: 50, *v/v/v*) was added to each sample and spiked. The tube was then vortexed at high speed for 2 min. Subsequently, each sample was centrifuged at 1792 *g*for 10 min. Then, 10 mL of the filtrate was diluted with 10 mL of PBS solution. The diluted extract was filtered through glass microfibre filter paper. Then, 6 mL of filtrate was then passed through the FUMONIPREP® immunoaffinity column, by gravity. Afterwards, the column was washed with 10 mL of PBS. The analyte was eluted with 3 x 1 mL methanol passing through the column at a rate of 1 drop per second. The eluate was then dried on a heating block at 60 °C and reconstituted with 1 mL of methanol: water (50: 50, *v/v*) prior the injection in the HPLC.

3.12.2 Instrumental conditions and auto-sampling

The 0.1 M Borate Buffer was prepared by weighing 3.8 g of sodium tetraborate decahydrate in a glass jar and dissolving it in exactly 100 mL of HPLC water. The mobile phase for FUM quantification was composed of methanol: sodium monophosphate buffer 0.1M (70: 30, *v/v*). The mobile phase was pre-mixed, adjusted to pH = 3.35 with o-phosphoric acid, 85%, filtered with 0.22 µm nylon membrane and delivered isocratically at a flow rate of 1 mL/min. The derivatisation was carried out using the OPA-mix that was composed by 40 mg OPA (o-phthalaldehyde), 1 mL methanol, 5 mL disodium tetraborate 0.1mM and 50 µL b-Mercaptoethanol. The OPA-mix was freshly prepared, filtered into to a 1.5 mL vial and placed in the autosampler. The injector was programmed to draw 50 µl from the vial with sample/FUM standards and, in sequence, to draw 50 µl from the vial containing OPA-mix and was then injected in the system without delay. The temperature of the column was set at 40 °C, while the samples and the OPA-mix were maintained at 4 °C in the autosampler. The run time of the method was set as 30 min. The detection was performed in multi wavelength detector (Waters® 2475; item 3.5) set to λ_{ex} 335 nm and λ_{em} 440 nm. The detector was configured for data rate as 1 point/ sec, photomultiplier (PMT) gain of 1 and sensitivity of 10 000 EUFS. A six-point calibration curve in the range 0.24 – 3.85 µg/mL was injected for FB_1 analysis, while for FB_2 a calibration curve with a range of 0.20 – 3.3 µg/mL was set-up to quantify positive samples.

3.13 Data Analysis

Data acquisition and processing/integration of chromatograms were performed using Empower 2 Software (Waters; 2008). Microsoft Excel (Version 2307, Microsoft 365, US) was used to plot the calibration curves and consecutive calculations of analyte concentration. The correlation coefficient, y-intercept, slope of the regression line (Eq. 1), and residual sum of squares were assessed by plotting signals as a function of standard concentration. The estimation of LoD (Eq. 2) and LoQ (Eq. 3) was based on the standard deviation of the response and the slope of the calibration curve according to ICH Q2(R1) guidelines. The corresponding values for the analyte concentration were calculated by substituting the y values into the rearranged expression of the regression line (Eq. 4) and then corrected to the corresponding dilution factor (DF).

$$y = mx + c \tag{1}$$

$$\mathrm{LoD} = \frac{3.3\sigma}{m} \tag{2}$$

$$\mathrm{LoQ} = \frac{10\sigma}{m} \tag{3}$$

$$x = \frac{y - b}{m} \tag{4}$$

$$Accuracy = \frac{(Value\ accepted - Value\ Observed)}{Value\ accepted} x100 \tag{5}$$

$$Apparent\ Recovery\ (\%) = \frac{Calculated\ concentration}{Spiked\ concentration} x100 \tag{6}$$

Were:

x = analyte concentration

y = instrument response (peak area)

c = *y-intercept*

m = slope of linear regression

σ = standard deviation of residuals of calibration curve

Chapter 4. Results

4.1 Method development for the detection and quantification of mycotoxins

4.1.1 ZEA

For the quantification of ZEA, a seven-point calibration curve (0.78 – 50 ng/mL) was set-up by performing a serial dilution from the working solution (1000 ng/mL). The linear regression, (Figure 17), was obtained by plotting the standard concentration against the response (peak area) after HPLC analysis. The linearity was also depicted through a calibration curve, which had a good coefficient of determination, R^2 = 0.9971. The chromatograms of the calibration standards were shown in Figure 18. The retention time (RT) for ZEA was at 4.4 min. A summary of the peaks for the standards are described in Table 8. The intra-day precision (RSDr) was assessed by using a known concentration of ZEA standards at two different concentrations of 12.5 and 25 ng/mL, which were injected 4 times each. The RSDr ranged from 0.95 % to 1.04 % on the day of analysis, while the injection accuracy was 97.80 % ± 1.01 % and 96.40 % ± 0.91 %, respectively. The peaks for each injection are shown in Figure 19.

Furthermore, for method validation, matrix-matched spiked samples (*n = 4*) were analysed, at two different concentrations (60 and 160 ng/g) in duplicates to obtain the recovery levels. The lower recovery level was 59.80 % and the upper level was 91.20 %. The average recovery in percentage was calculated as 84.60 % ± 5 %, although the low recovery outlier was removed. Figure 20 shows the chromatograms for the ZEA recovery experiments. The limits of detection (LOD) and limits of quantification (LOQ) for ZEA were 3.51 and 10.63 µg/kg, respectively (Table 21).

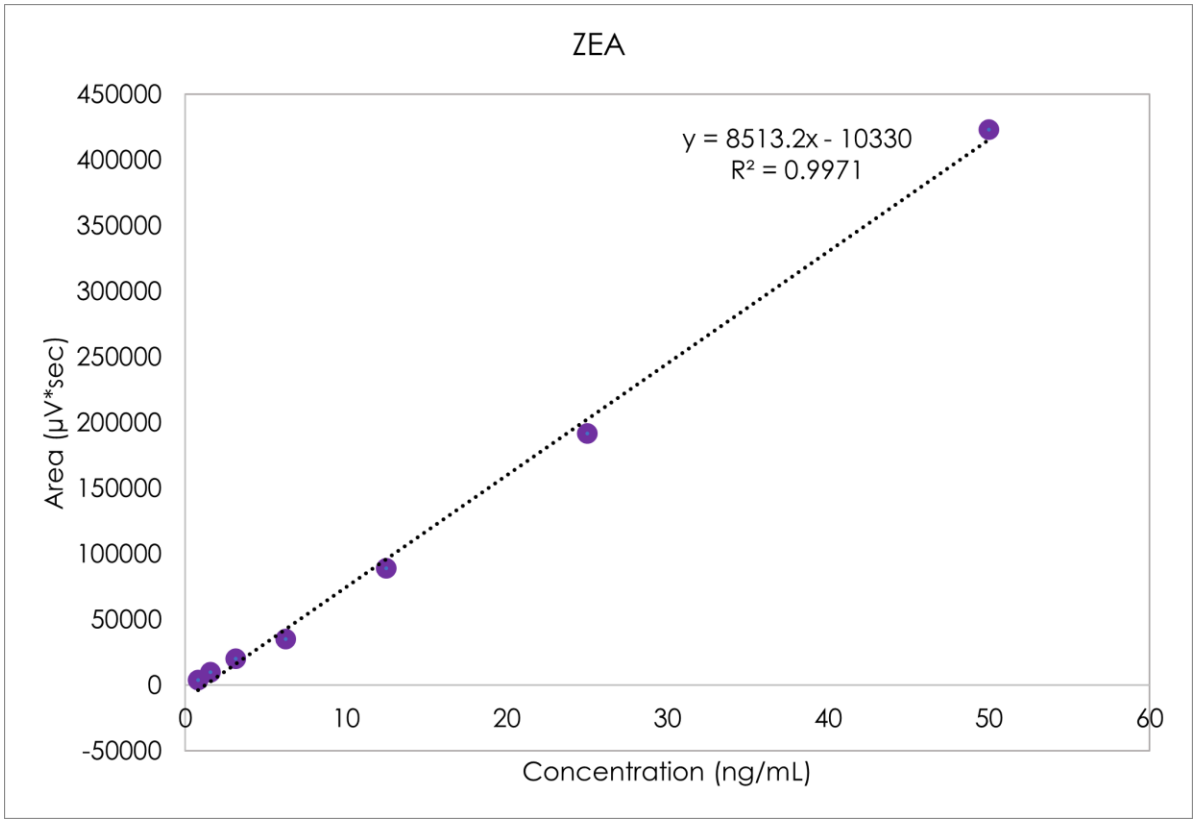

Figure 17. Seven-point calibration curve (0.78 - 50 ng/mL) serially diluted for the quantification of ZEA showing the corresponding linear regression (y= mx + c) and coefficient of determination (R^2).

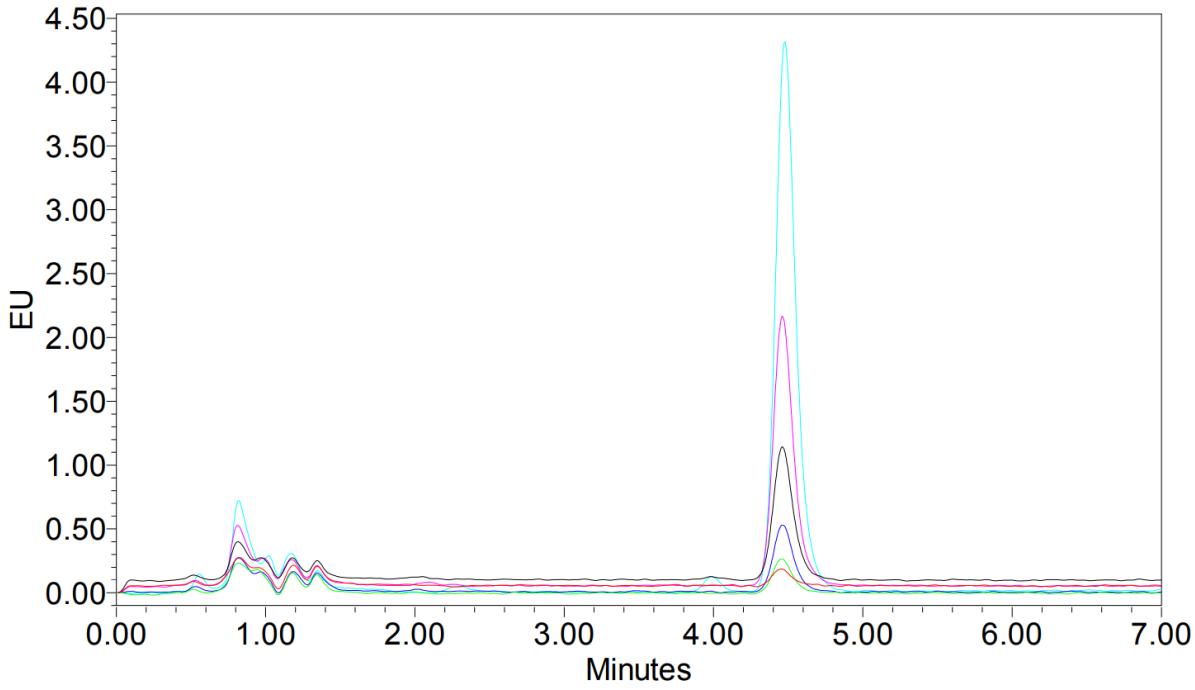

Figure 18. Overlapped chromatograms for the standards of ZEA ranging from 1.5 to 50 ng/mL. Detection was achieved at λ_{ex}= 274 nm and λ_{em}= 445nm.

Table 8. Peak summary for the calibration curve of ZEA, showing retention time, peak area, % area and height.

Concentration of ZEA (ng/mL)	Retention Time (min)	Area	% Area	Height
1.50	4.455	11916	100.00	1303
3.12	4.456	26233	100.00	2705
6.25	4.465	48809	100.00	5171
12.5	4.461	86195	100.00	9830
25.0	4.461	186809	100.00	20501
50.0	4.477	402028	100.00	42640

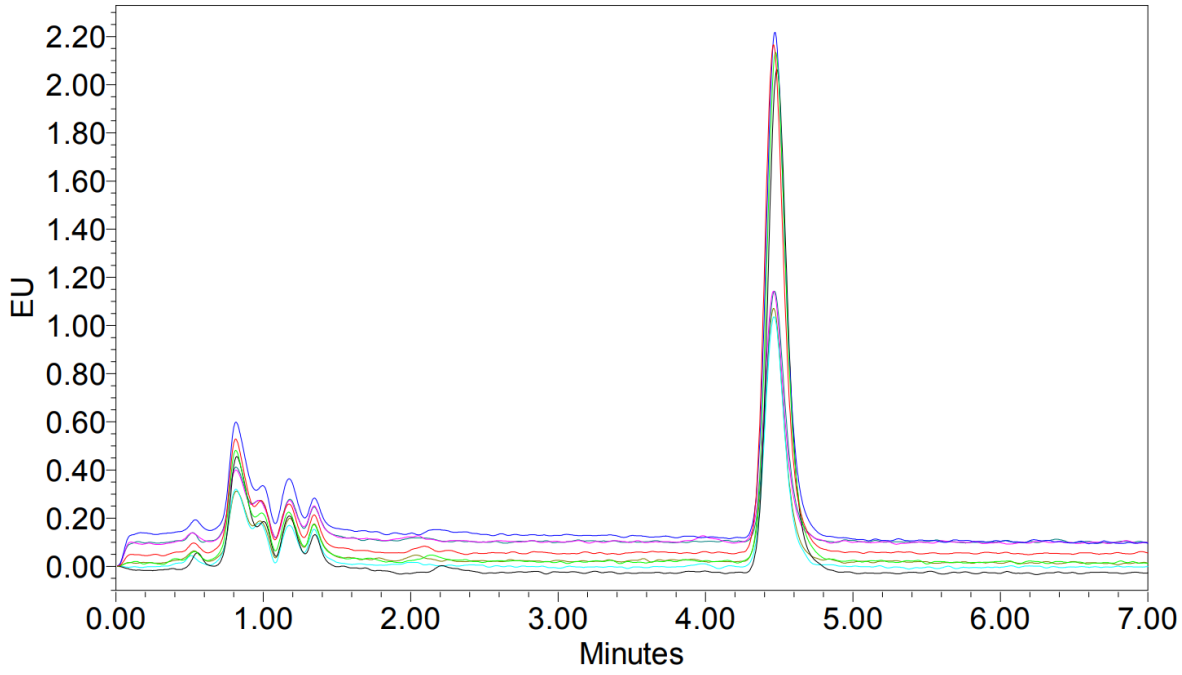

Figure 19. Overlapped chromatograms of the estimation of the intra-day precision with ZEA at 12.5 and 25 ng/mL injected 4 times each. Detection at λ_{ex}= 274 nm and λ_{em}= 445nm.

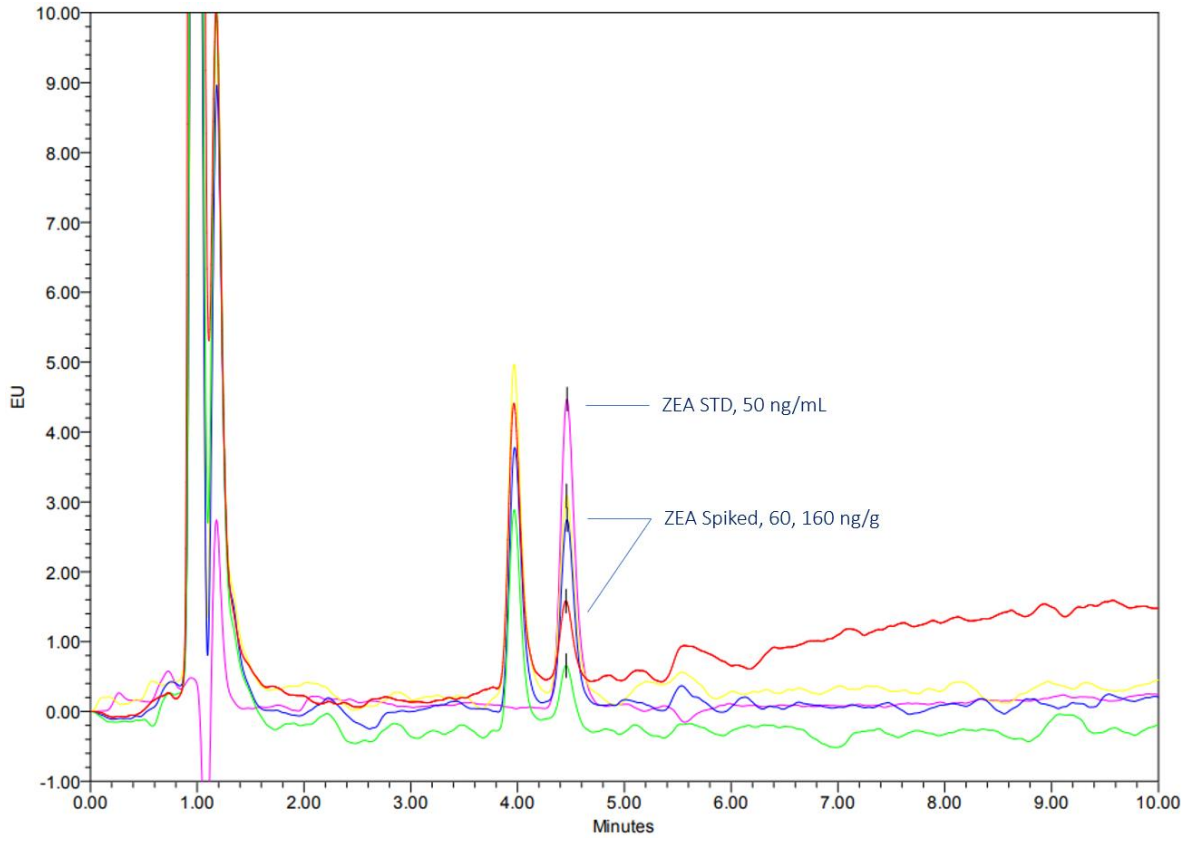

Figure 20. Overlapped chromatograms showing different spiking levels at different concentrations at 60, 160 ng/g in duplicates and the standard of ZEA (50 ng/mL).

4.1.2 OTA

The quantification of OTA was performed using an eight-point calibration curve (0.0488 – 6.25 ng/mL) set-up by serial dilution from the working solution (250 ng/mL). The linear regression, shown in Figure 21, demonstrates the standard concentration against the response (peak area). The linearity was also depicted through a calibration curve, which had a good coefficient of determination, R^2 = 0.9998. Figure 22 shows the peaks of OTA obtained when running known concentrations of the mentioned mycotoxin to calculate the LOD and LOQ of the analytical method. Moreover, OTA standard peaks ranging from 0.195 – 100 ng/mL can be seen at a retention time of ~2.8 minutes. Table 9 specifies the peak summaries of the calibration curve.

The intra-day accuracy was assessed by using a known concentration standard (25 ng/mL; Figure 23) injected 6 times, demonstrating accuracy of 95.30 % ± 2.92 %. The method validation was performed by analysing matrix-matched spiked samples (*n = 4*) at two different concentrations (25 and 50 ng/g) in duplicates to determine the percentage recovery levels. The lower recovery level for OTA was 72.10 %, while the highest was 83.30 %, and average 77.5 ± 5 %. The LOD and LOQ for OTA metabolite were 0.22 and 0.68 µg/kg, respectively. The chromatograms obtained from the recovery analysis for OTA are shown in Figure 24.

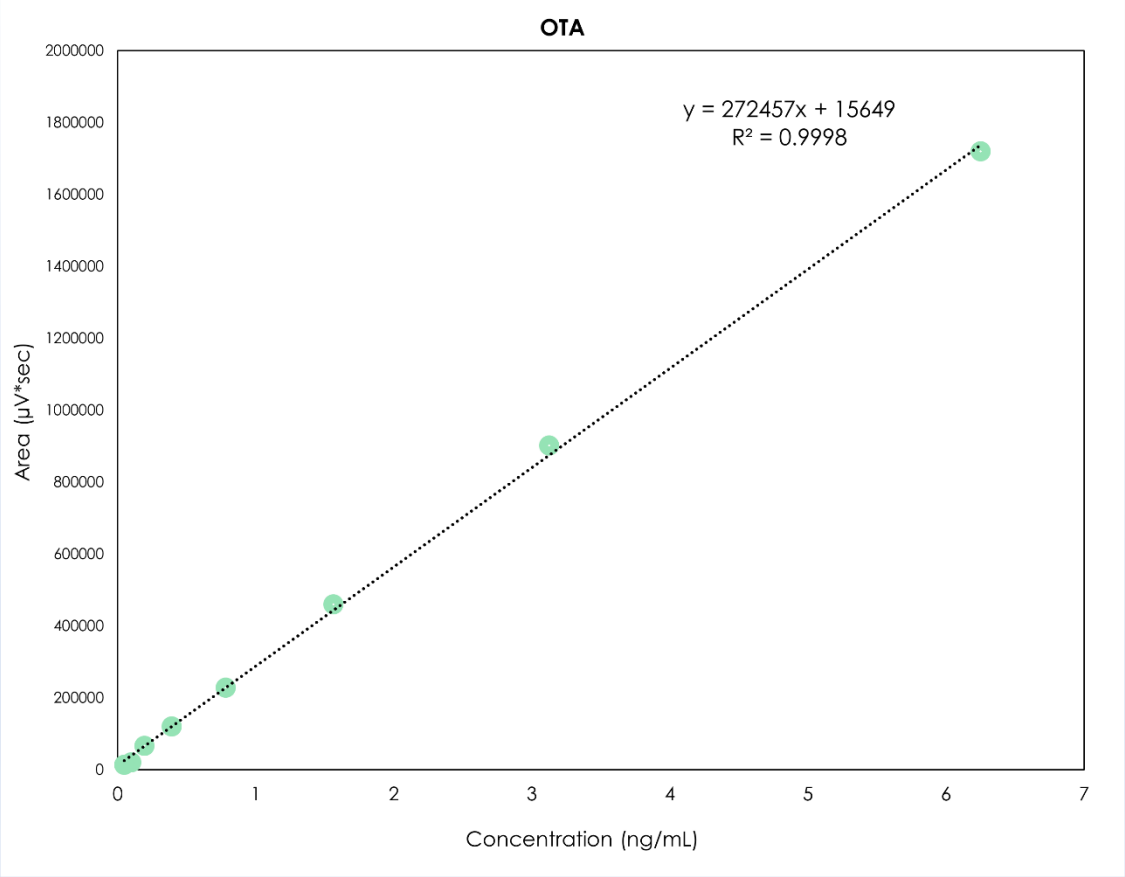

Figure 21. Eight-point calibration curve (0.0488 – 6.25 ng/mL) serially diluted for the quantification of OTA showing the corresponding linear regression (y= mx + c) and coefficient of determination (R^2).

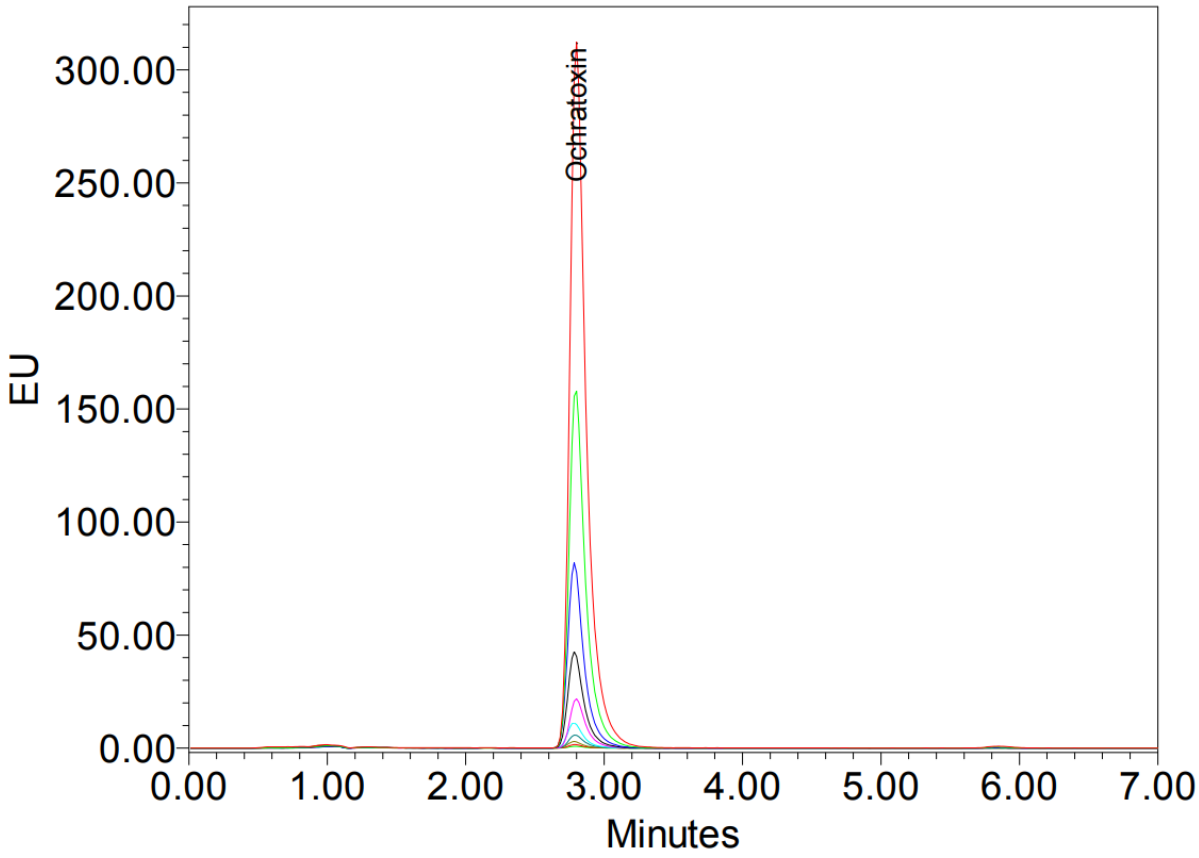

Figure 22. Overlapped chromatograms of the calibration curve of ochratoxin A (OTA) with concentrations ranging from 0.195 to 100 ng/mL.

Table 9. OTA calibration curve peak summary, showing retention time, area, % area and height.

Concentration of OTA (ng/mL)	Retention Time (min)	Area	% Area	Height
0.19	2.794	65684	100.00	8015
0.39	2.789	119697	100.00	15321
0.78	2.784	227705	100.00	28700
1.56	2.791	460015	100.00	57566
3.12	2.783	901758	100.00	111210
6.25	2.800	1719229	100.00	214561
12.5	2.786	3410371	100.00	423172
25.0	2.785	6503936	100.00	810562
50.0	2.796	12641944	100.00	1563454
100	2.801	24935831	100.00	3086575

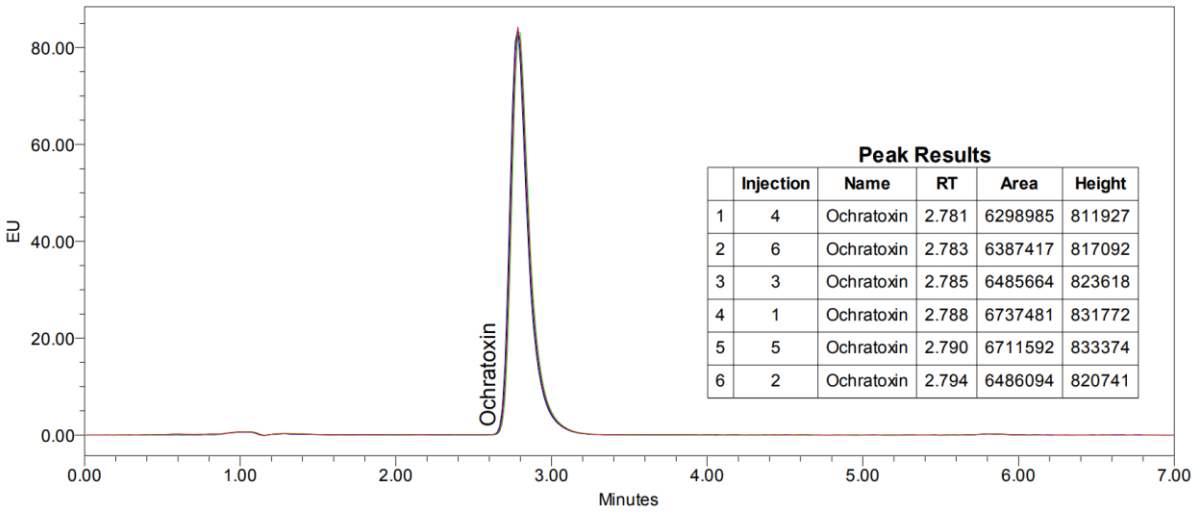

Figure 23. Intra-day precision as assessed using 25 ng/mL of known standard after injecting for six times.

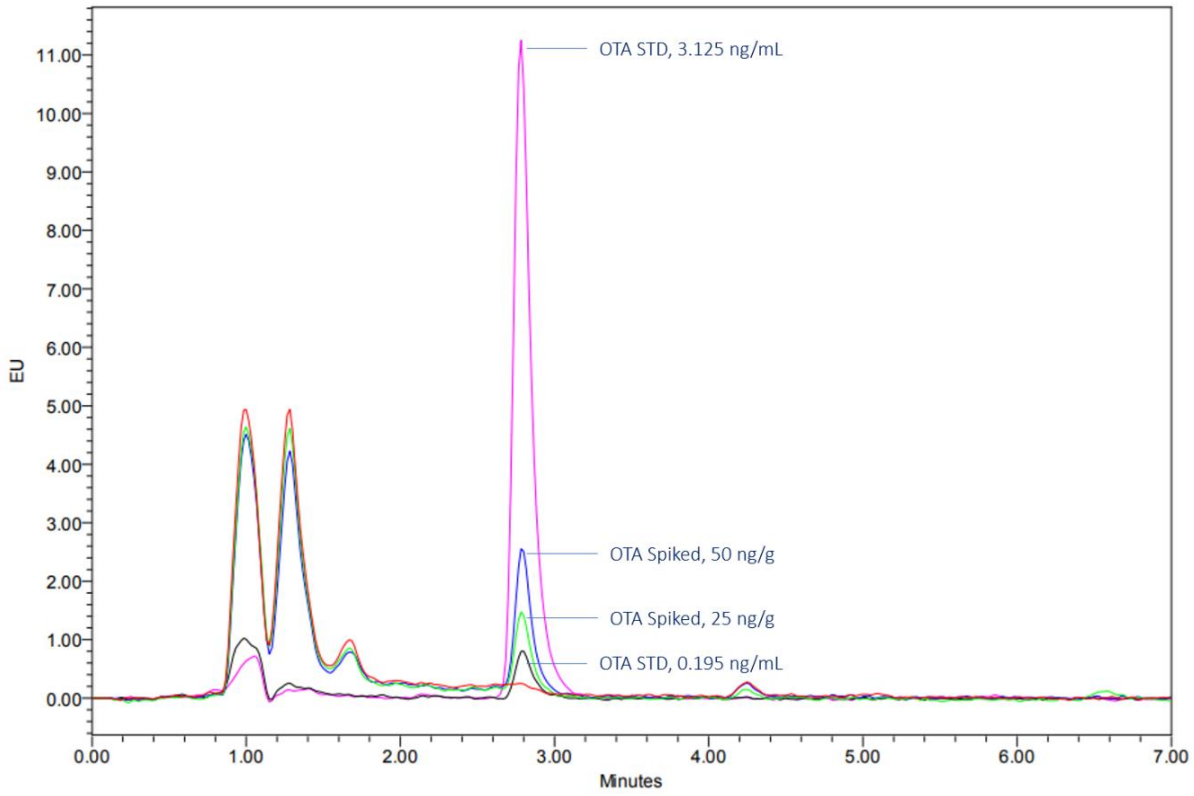

Figure 24. Overlapped chromatograms showing different spiking levels at different concentrations at 25, 50 ng/g in duplicates. OTA standard injections were included with this figure to contrast the RT of the standards to the spiked matrix samples.

4.1.3 DON

The quantification of DON was performed using a five-point calibration curve (31.25 – 500 ng/mL) set-up by serial dilution from the working solution (5000 ng/mL). The linear regression, (Figure 25), was obtained by plotting the standard concentration against the response (peak area) after HPLC analysis. The linearity showed a good coefficient of determination, R^2 = 0.997. Figure 26 shows the overlapped chromatograms of DON standards to obtain a calibration curve. DON standards were detected at retention time ~6 min. Table 10 shows the chromatographic peaks for the calibration curve. The intra-day accuracy (13.80 %) was assessed

by using 250 ng/mL of DON standard injected 4 times. The injection accuracy was 89.90 % ± 12.40 (Figure 27).

To assess recovery levels, three sets of matrix-matched spiked samples (*n = 3*) were subjected to analysis at varying concentrations (100, 500, and 1000 ng/g). The obtained recovery percentages were 96.20 %, 77.40 %, and 86.50 %, respectively. The average recovery percentage was computed as 86.70 ± 9 %. Chromatograms for the DON recovery experiments are presented in Figure 28. The LOD and LOQ for DON were found to be 116.20 and 352.12 µg/kg, respectively (Table 21).

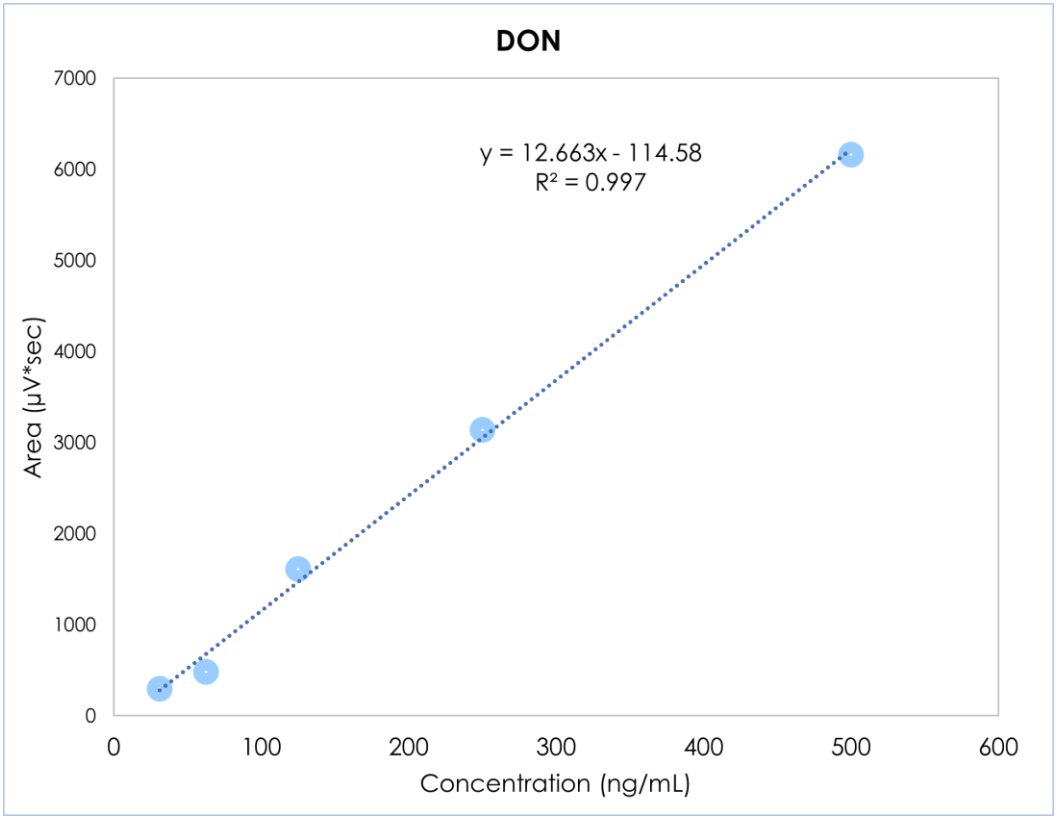

Figure 25. Five-point calibration curve (31.25 – 500 ng/mL) serially diluted for the quantification of DON, showing the corresponding linear regression (y = mx + c) and coefficient of determination (R^2).

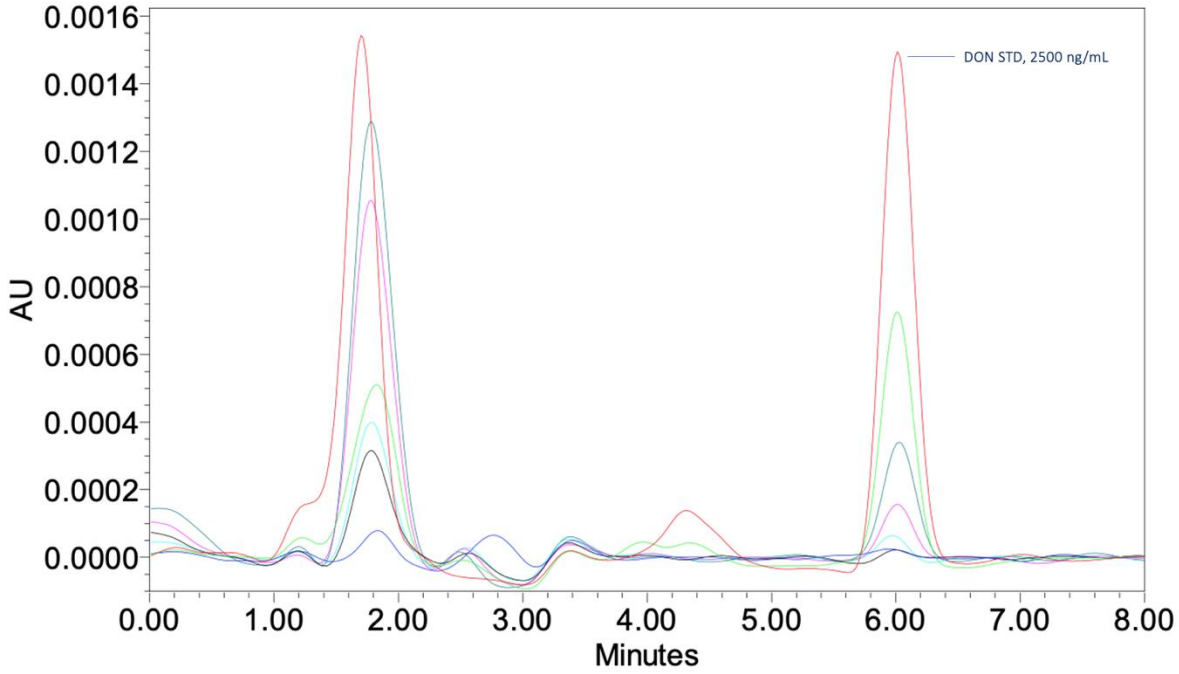

Figure 26. Overlapped chromatographic peaks for the standards of DON ranging from 31.25 to 2500 ng/mL, detection at absorbance of 220 nm at 1.2 nm resolution.

Table 10. Peak summary for the calibration curve of DON, showing retention time, peak area, % area and height.

Concentration of DON (ng/mL)	Retention Time (min)	Area	% Area	Height
31.25	5.950	291	100.00	19
62.5	6.003	450	100.00	30
125.0	5.976	1303	100.00	76
250.0	6.015	2746	100.00	158
500.0	6.029	6549	100.00	348
1250	6.014	13655	100.00	747
2500	6.017	27873	100.00	1519

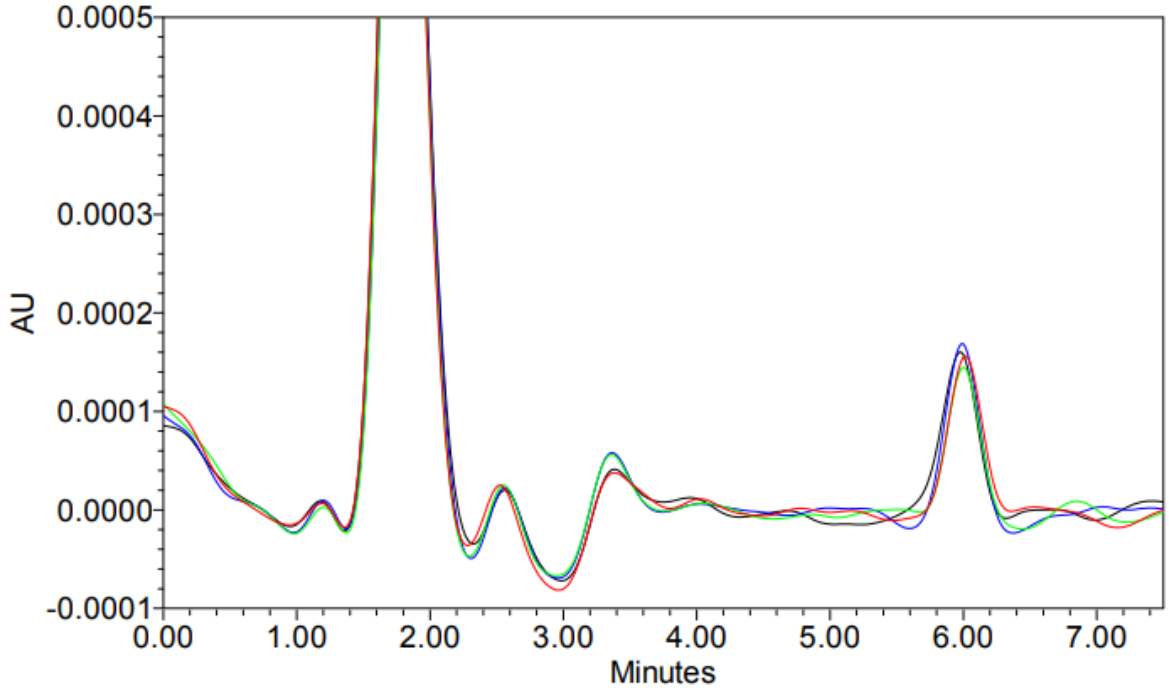

Figure 27. Overlapped chromatogram for DON intra-day precision using a known standard concentration of 250 ng/mL.

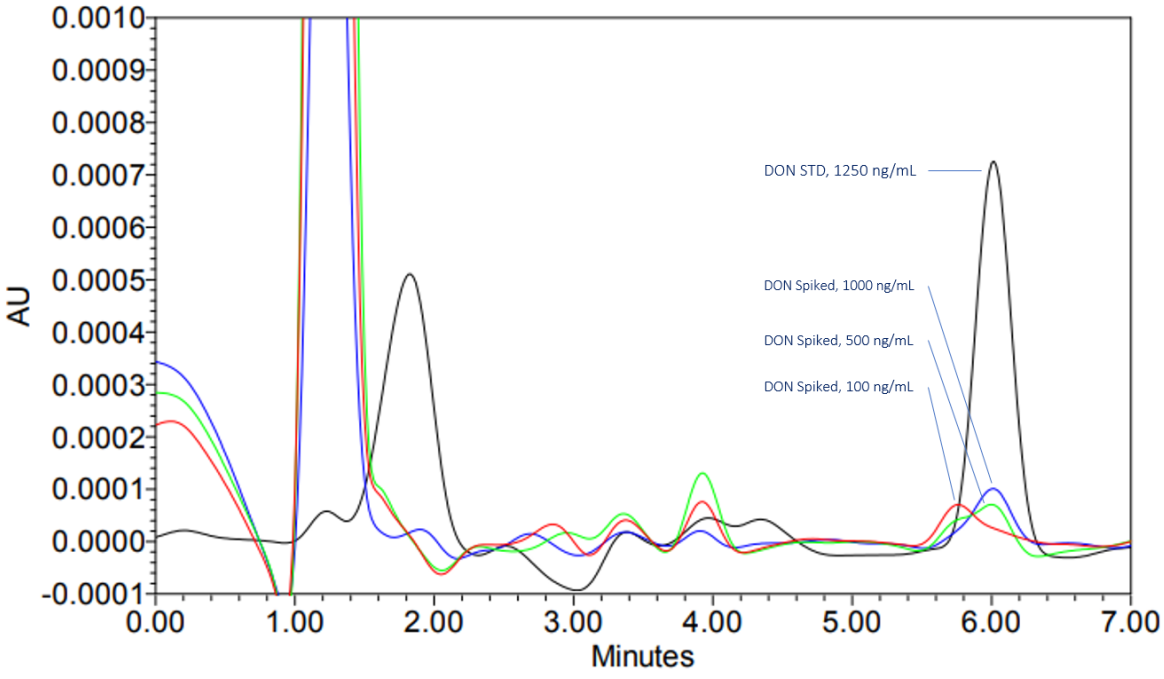

Figure 28. DON recovery analysis for method validation, showing three different concentrations, with variable overlapped peaks. A standard of concentration 1250 ng/mL was also included to compare the RT.

4.1.4 AFs

The quantification of AFs was performed using a seven-point calibration curve ranging from 0.195 ng/mL to 12.5 ng/mL for each AFs analogue (AFB_1, AFB_2, AFG_1, and AFG_2). Calibration curves are shown in Figures 29, 30, 31 and 32 for AFB_1, AFB_2, AFG_1, and AFG_2, respectively.

The calibration curves exhibited excellent linearity for all AFs analogues, as indicated by high co-efficient of determination values. Specifically, AFB_1 displayed an R^2 value of 0.996, AFB_2 had an R^2 value of 0.9997, AFG_1 showed an R^2 value of 0.9994, and AFG_2 demonstrated an R^2 value of 0.9992. The limits of detection and quantification were also calculated for each aflatoxin types. The LOD and LOQ for AFB_1 were 0.33 and 0.99 µg/kg, while for that of AFB_2 were 0.29 and 0.86 µg/kg, respectively. As for AFG_1 the LOD and LOQ 0.38 and 1.15 µg/kg and for AFG_2, 0.45 and 1.35 µg/kg.

In Figure 33, seven distinct peaks representing different concentrations of AFG_2, AFB_2, AFG_1, and AFB_1 standards ranging from 0.195 to 12.5 ng/mL are illustrated. The retention times, peak areas, % area, and heights for each peak are summarized in Table 11, 12, 13 and 14 for all four AFs, respectively. For AFG_2, the chromatographic peak is observed at a retention time of approximately 2.92 minutes. As the concentration of AFG_2 increases from 0.1953 ng/mL to 12.5 ng/mL, there is a corresponding increase in peak area from 13,110 to 1,618,945, which represents 100 % of the total area for all peaks in each chromatogram. Similarly, for AFB_2, the chromatographic peak appears at around 4.43 minutes retention time. The peak area increases

progressively from 47,126 to 3,186,729 as the concentration rises from 0.1953 ng/mL to 12.5 ng/mL, with all peaks representing 100 % of the total area.

The chromatographic peak for AFG_1 emerges at approximately 5.19 minutes retention time. The peak area rises from 33,828 to 2,820,317, corresponding to an increase in concentration from 0.1953 ng/mL to 12.5 ng/mL. Lastly, for AFB_1, the chromatographic peak is observed at around 8.61 minutes retention time. The peak area increases substantially from 85,381 to 5,749,693, as the concentration of AFB_1 increases from 0.1953 ng/mL to 12.5 ng/mL, with all peaks representing 100 % area. These calibration curves will be valuable for quantifying the concentrations of AFG_2, AFB_2, AFG_1, and AFB_1 in unknown samples tested in this study and during method validation while performing spiking and recovery analysis.

The intraday accuracy for AFG_2 was 103.09 %. The intraday accuracy for AFB_2 was 99.54 %, while the %RSD of 1.87 %. The intraday accuracy for AFG_1 was 106.41 %. The %RSD of 2.04 %. The intraday accuracy for AFB_1 is 102.51 %. The %RSD of 3.00 %. Figure 34 shows the intra-day precision using a known concentration of 6.25 ng/mL.

The recoveries of AFG_2, AFB_2, AFG_1, and AFB_1 were determined at various spiked concentration levels to assess the analytical method used. The spiked concentrations were 10, 20, 30, and 40 ng/g for each compound, and the recovery percentages obtained for each spiked concentration are summarized in the Table 15.

The validation of the analytical method was assessed through recovery experiments using matrix-matched wheat flour samples ($n = 4$) spiked with known concentrations of AFs standards

(10, 20, 30 ,40 ng/g). For AFG_2, the recoveries ranged from 88.61 % to 101.67 %, while for AFB_2, from 81.88 % to 154.36 %. As for AFG_1, the exhibited recoveries ranged from 82.03 % to 102.27 %, and for AFB_1, from 88.64 % to 103.48 %. The average recovery for AFG_2 was 94.01 % ± 4.91 %, while that for AFB_2 was 119.69 % ± 31.84 %. Moreover, the average recovery for AFG_1 was 90.15 % ± 8.56 %, while that of AFB_1 was 98.10 % ± 5.63 %. Figures 35 and 36 show the recovery chromatograms for AFs.

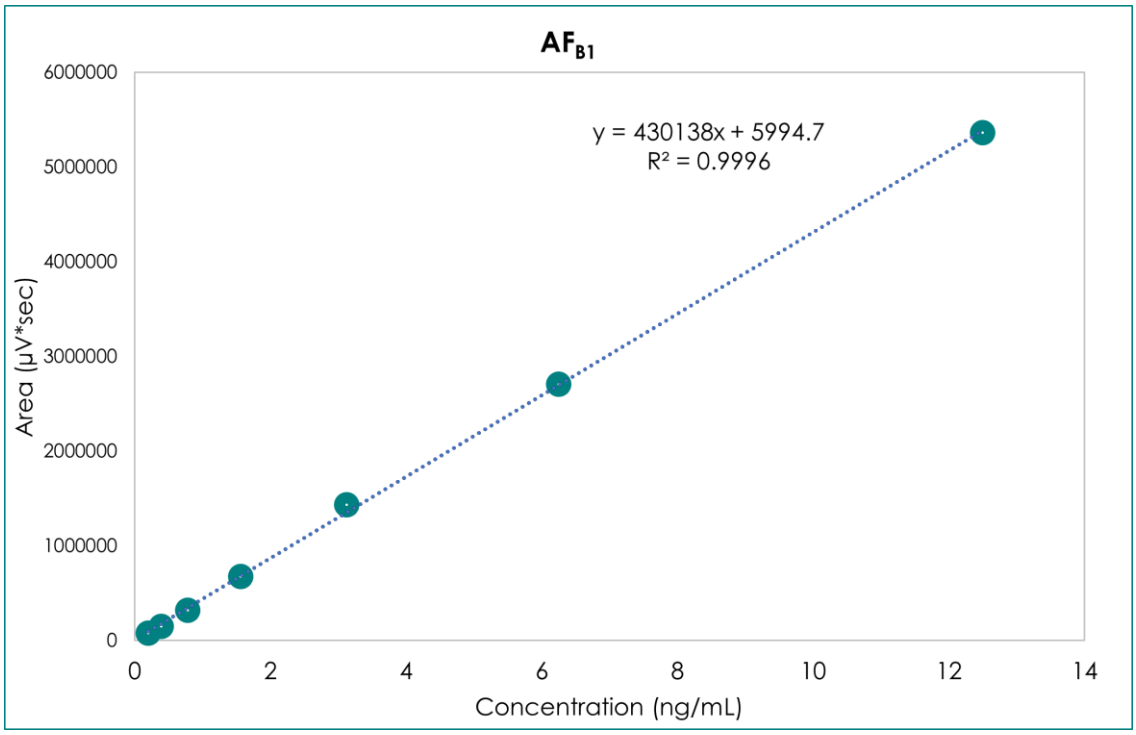

Figure 29. Seven-point calibration curve of range 0.1953 – 12.5 ng/mL, for AFB_1, also showing the linear equation and coefficient of determination R^2.

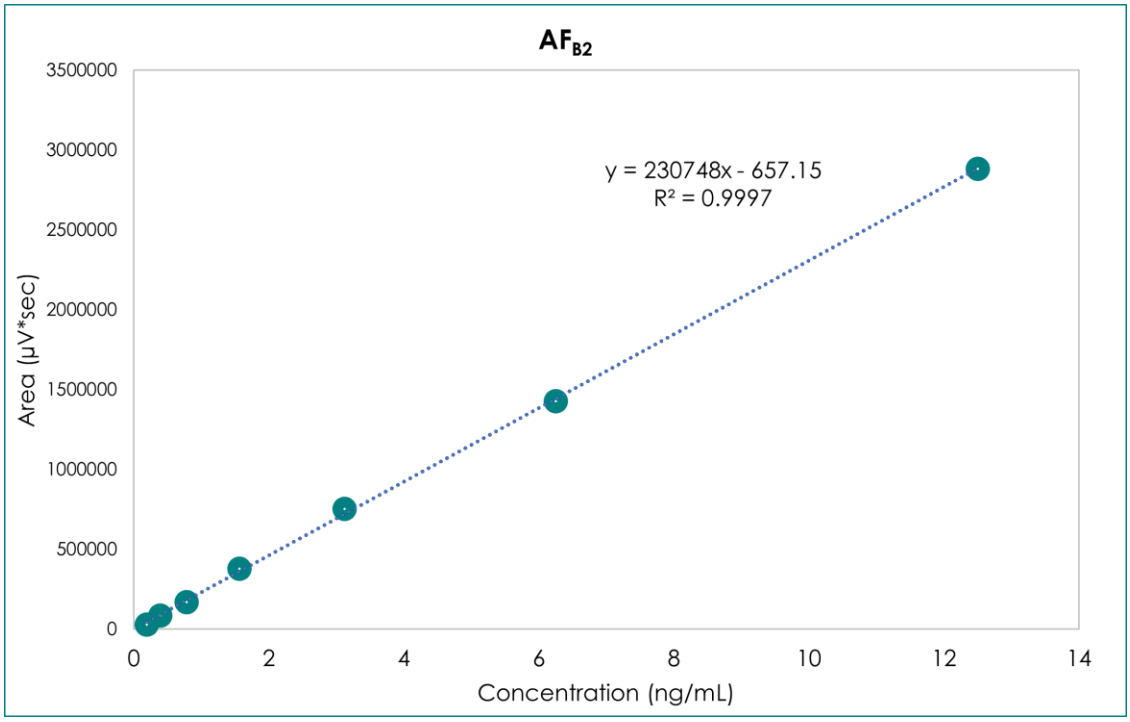

Figure 30. Seven-point calibration curve of range 0.1953 – 12.5 ng/mL for AFB_2 showing also the linear equation and coefficient of determination R^2.

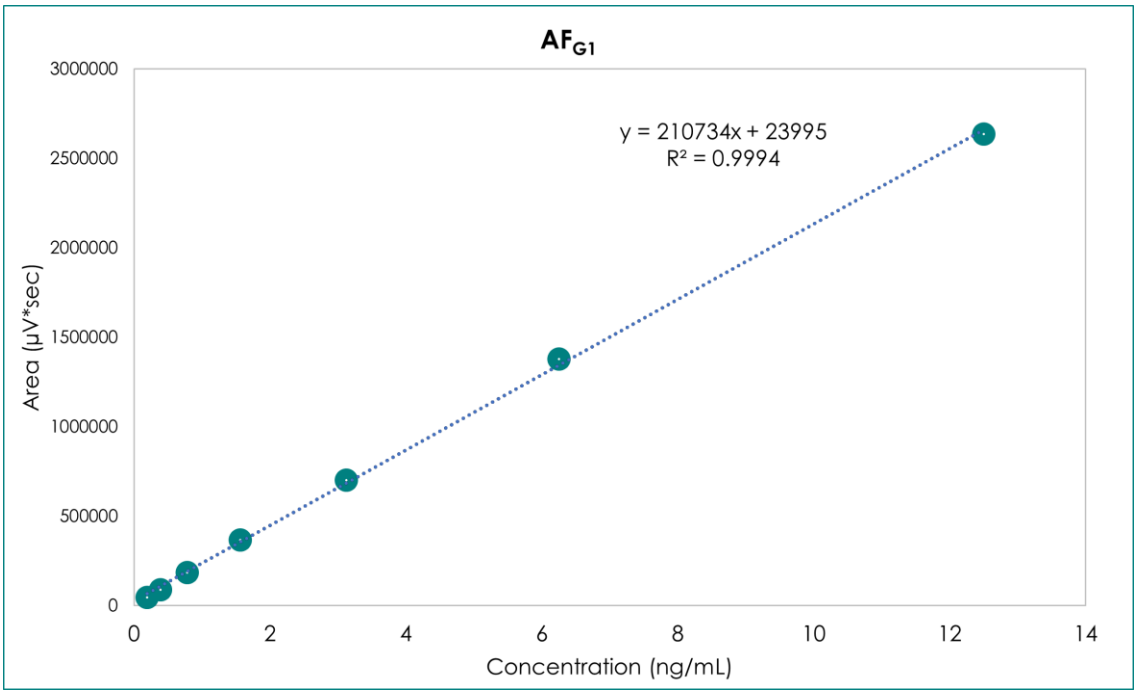

Figure 31. Seven-point calibration curve of range 0.1953 – 12.5 ng/mL for AFG_1 showing also the linear equation and coefficient of determination R^2.

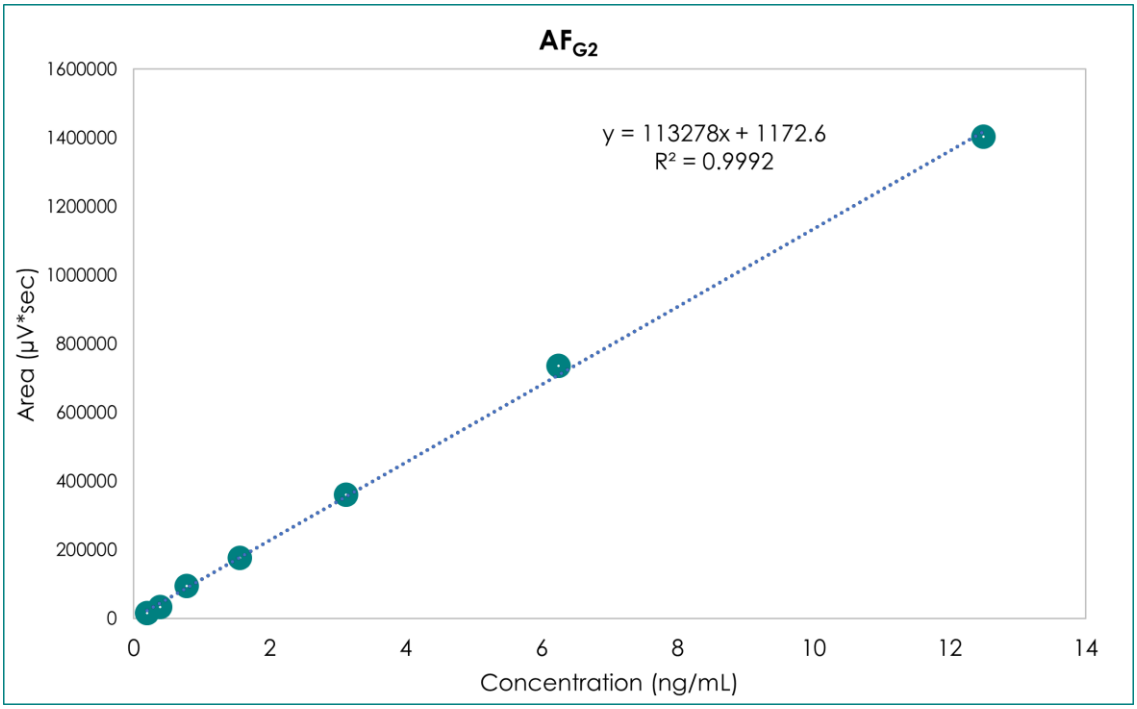

Figure 32. Seven-point calibration curve of range 0.1953 – 12.5 ng/mL for AFG_2 showing also the linear equation and coefficient of determination R^2.

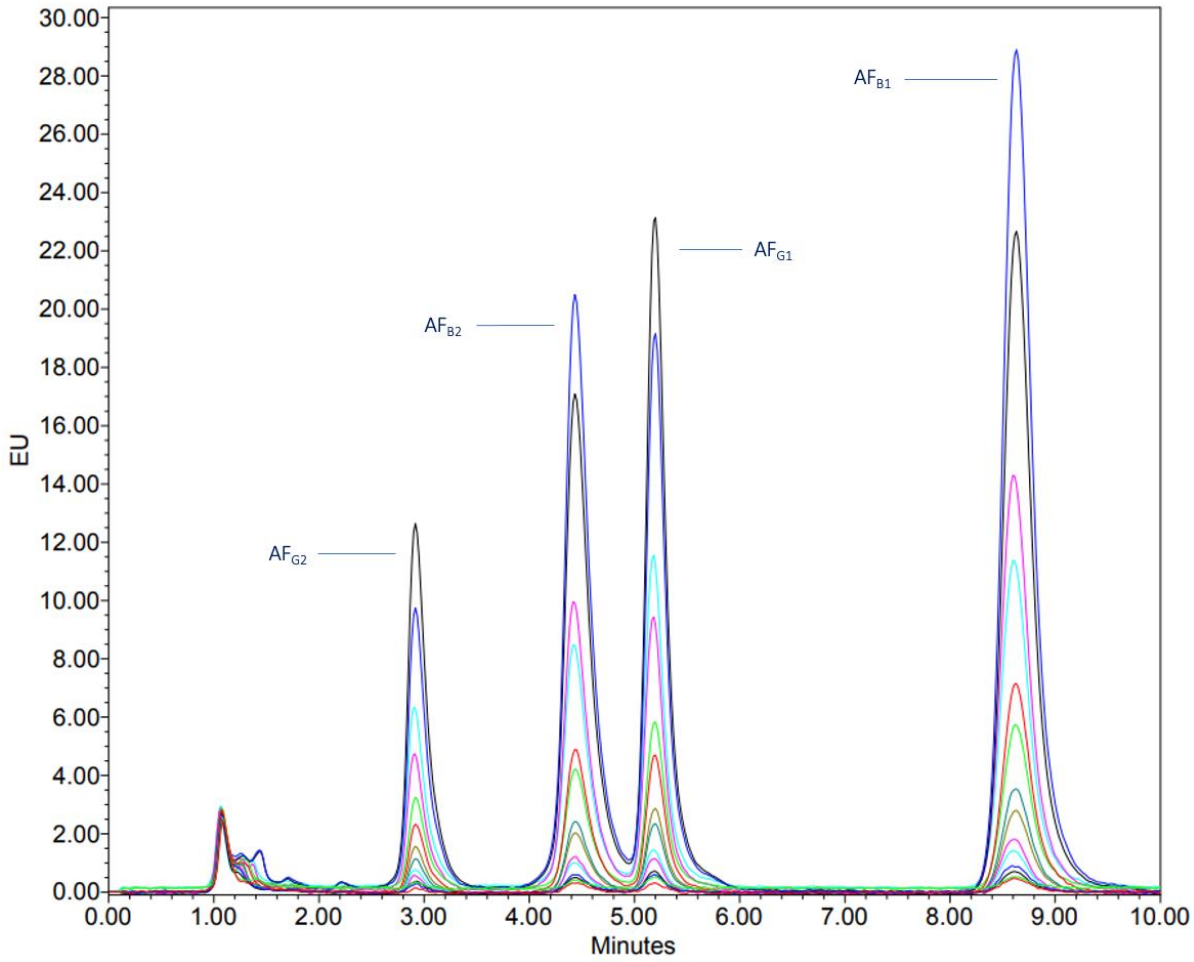

Figure 33. Overlapped chromatographic peaks for the standards of AFG_2, AFB_2, AFG_1 and AFB_1 ranging from 0.195 – 12.5 ng/mL with the FLR detector set at at λ_{ex} 365/362 nm and λ_{em} 440/455 nm.

Table 11. Peak summary for the calibration curve of AFG_2, showing retention time, peak area, % area and height.

Concentration of AF (ng/mL)	Retention Time (min)	Area	% Area	Height
0.1953	2.919	13110	100.00	1797
0.390	2.927	34917	100.00	3642
0.781	2.917	87295	100.00	7662
1.562	2.920	187236	100.00	15661
3.125	2.921	378945	100.00	30662
6.25	2.908	789522	100.00	61160
12.5	2.918	1618945	100.00	123847

Table 12. Peak summary for the calibration curve of AFB_2, showing retention time, peak area, % area and height.

Concentration of AF (ng/mL)	Retention Time (min)	Area	% Area	Height
0.1953	4.455	47126	100.00	3052
0.390	4.430	80273	100.00	5656
0.781	4.432	156743	100.00	11292
1.562	4.439	358550	100.00	23405
3.125	4.440	741617	100.00	47153
6.25	4.424	1495449	100.00	94960
12.5	4.436	3186729	100.00	196364

Table 13. Peak summary for the calibration curve of AFG_1, showing retention time, peak area, % area and height.

Concentration of AF (ng/mL)	Retention Time (min)	Area	% Area	Height
0.1953	5.196	33828	100.00	3324
0.390	5.189	79645	100.00	6701
0.781	5.181	175654	100.00	13945
1.562	5.195	357822	100.00	27662
3.125	5.194	720234	100.00	55172
6.25	5.181	1362832	100.00	108513
12.5	5.195	2820317	100.00	221868

Table 14. Peak summary for the calibration curve of AFB_1, showing retention time, peak area, % area and height.

Concentration of AF (ng/mL)	Retention Time (min)	Area	% Area	Height
0.1953	8.615	85381	100.00	4668
0.390	8.593	165415	100.00	8743
0.781	8.610	288184	100.00	16664
1.562	8.625	658457	100.00	34619
3.125	8.626	1402559	100.00	71118
6.25	8.607	2831885	100.00	142030
12.5	8.630	5749693	100.00	286704

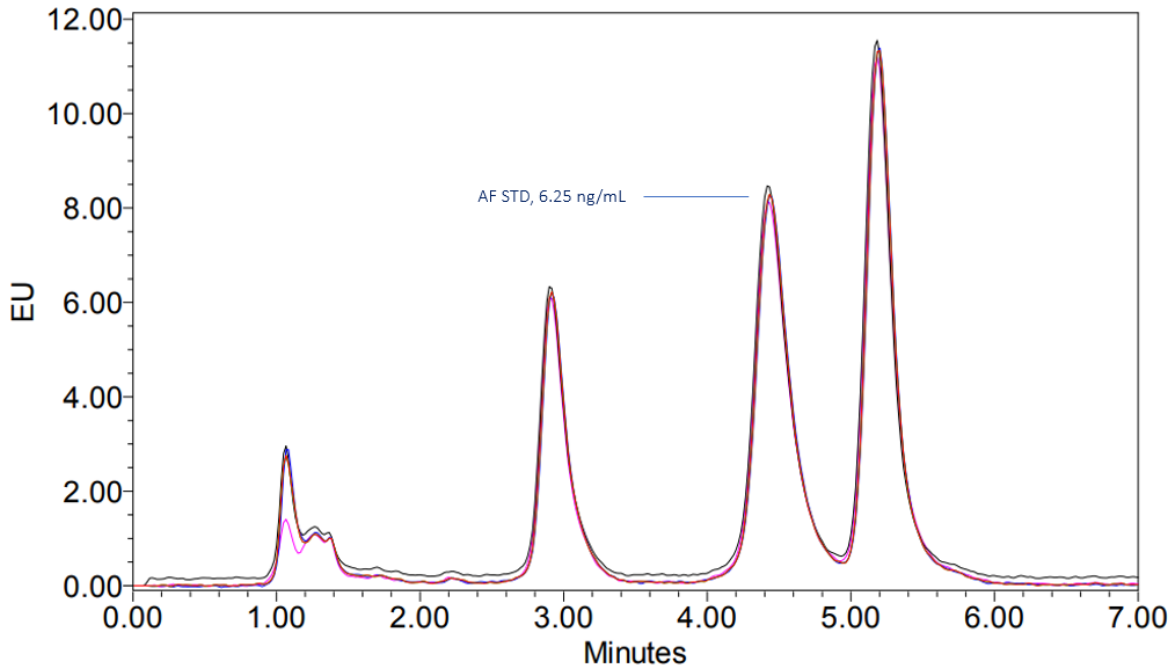

Figure 34. Overlapped chromatogram for AFs intra-day precision using a known standard concentration of 6.25 ng/mL.

Table 15. Summary for spiked experiments performed for the validation of aflatoxins.

Total Spiked Concentration (ng/g)	AFG_2 (%R)	AFB_2 (%R)	AFG_1 (%R)	AFB_1 (%R)
10	101.67	154.36	82.03	100.54
20	91.10	94.61	102.27	99.72
30	94.66	147.92	82.14	103.48
40	88.61	81.88	94.17	88.64

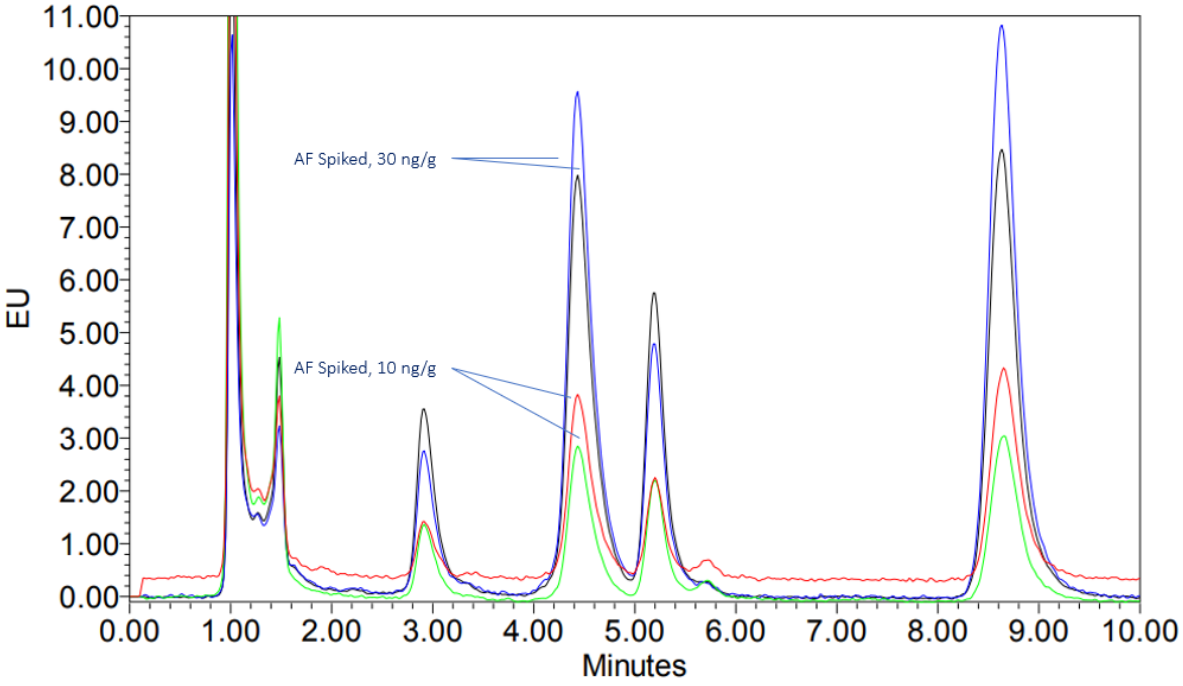

Figure 35. Spiked aflatoxin experiments for method validation at concentrations of 10, 30 ng/g.

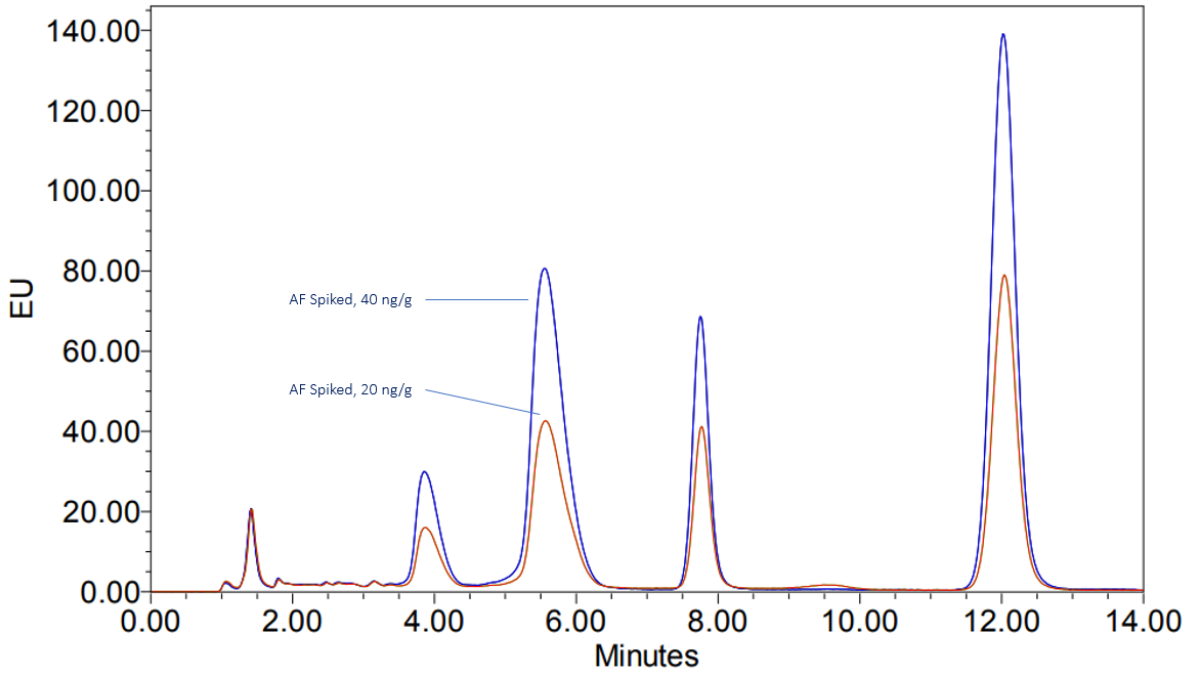

Figure 36. Spiked aflatoxin experiments for method validation, at concentrations of 20, 40 ng/g

4.1.5 PAT

For the quantification of PAT, a five-point calibration curve (6.25 – 100 ng/mL) was set-up by performing a serial dilution from the working solution (250 ng/mL). A linear regression, (Figure 37), was obtained by plotting the standard concentration against the response (peak area), demonstrated satisfactory linearity, indicated by a high coefficient of determination, R^2 = 0.9993. Figure 38 shows five distinct peaks which corresponding to the different concentrations of PAT standards serially diluted overlapped and normalized in the Empower software for better representation. The summary of the peaks are shown in table 16. The retention time for the peaks was at 4.0 min The intra-day accuracy of 6 consecutives injections of PAT standard solution of 25 ng/mL. The intra-day relative standard deviation (RSDr) was 5.17 %, and injection accuracy was 98.8 ± 5.10 %. The chromatograms obtained from each injection overlapped are shown in Figure 39.

Molecularly imprinted polymer (MIP) columns were employed for clean-up purposes, optimized specifically for apple juice puree. To evaluate the efficiency of the MIP columns in flour, two sets of matrix-matched spiked samples were analysed at concentrations of 25 and 50 ng/g. The recoveries were 40.5 % and 58.9 %, respectively. The average recovery percentage was 49.70 ± 19 %. The chromatograms for the PAT recovery are shown in Figure 40. Additionally, the LOD and LOQ for PAT were determined to be 3.94 and 11.94 µg/kg for 25 and 50 ng/g, respectively (Table 22).

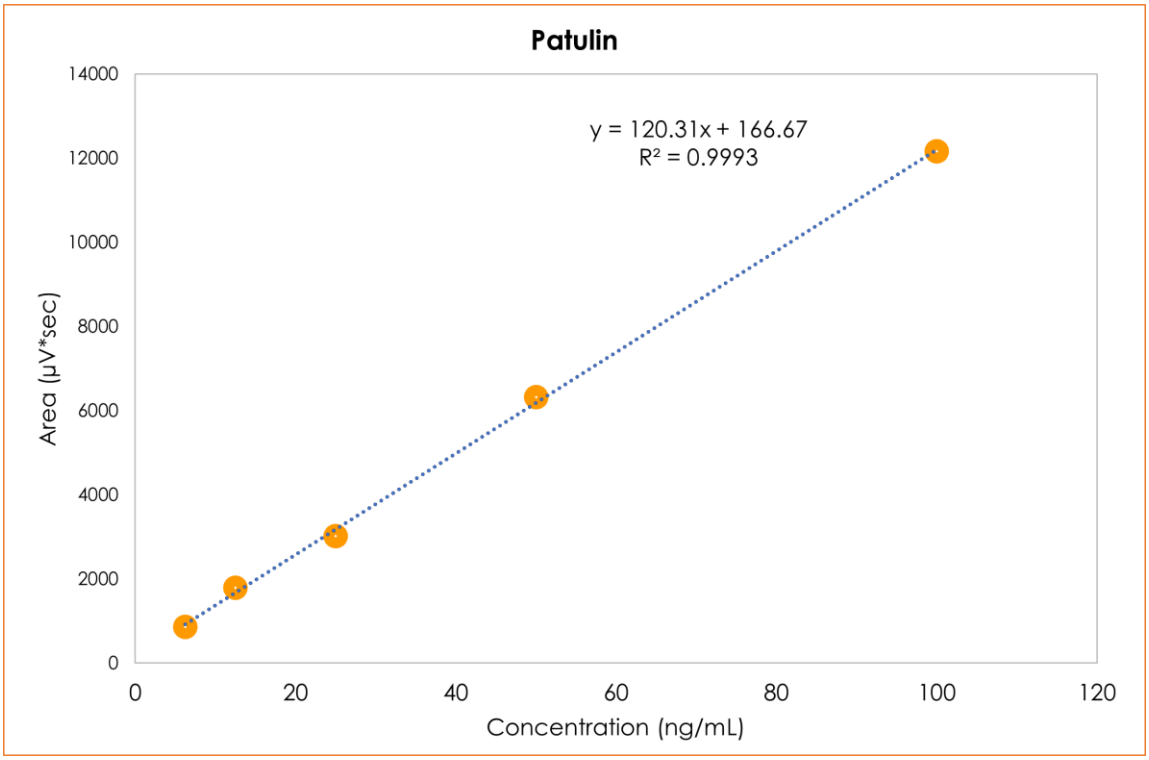

Figure 37. Five-point calibration curve ranging from 6.25 – 100 ng/mL using PAT standards, showing also the linear regression equation and coefficient of determination (R^2).

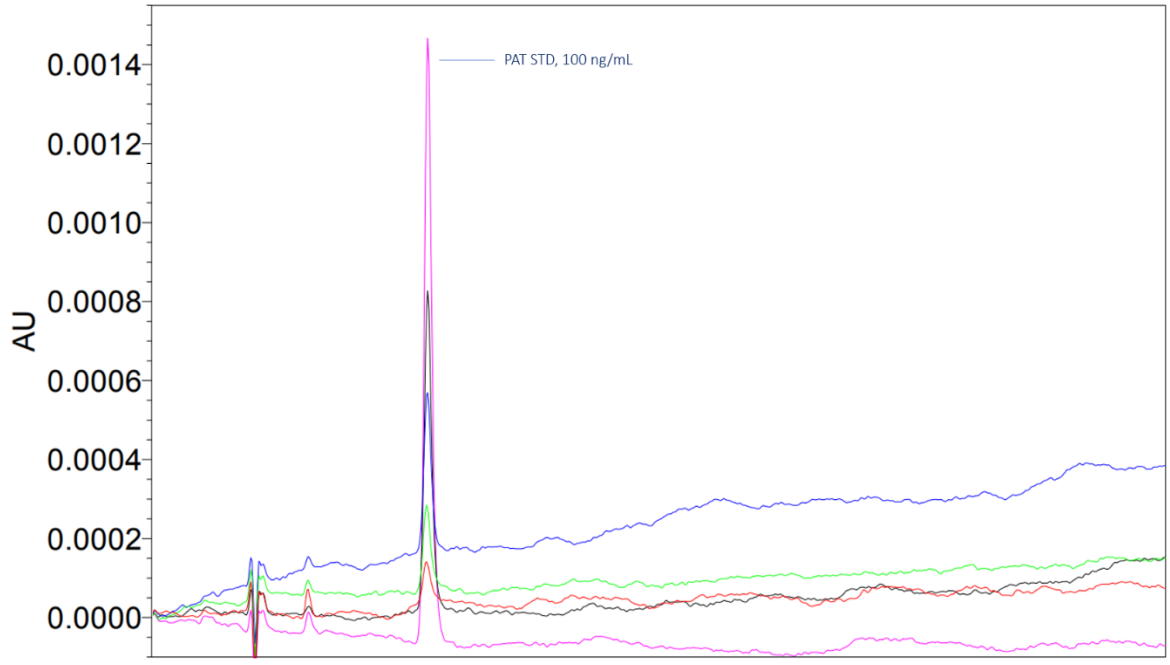

Figure 38. Chromatographic peaks for the standards of PAT ranging from 6.25 to 100 ng/mL, with the PDA detector set at 276 nm at 1.2 nm resolution.

Table 16. Peak summary for the calibration curve of PAT, showing retention time, peak area, % area and height.

Concentrations of PAT (ng/mL)	Retention Time (min)	Area	% Area	Height
6.25	4.065	817	100.00	100
12.5	4.068	1606	100.00	207
25.0	4.078	3114	100.00	400
50.0	4.083	6401	100.00	804
100.0	4.085	12156	100.00	1526

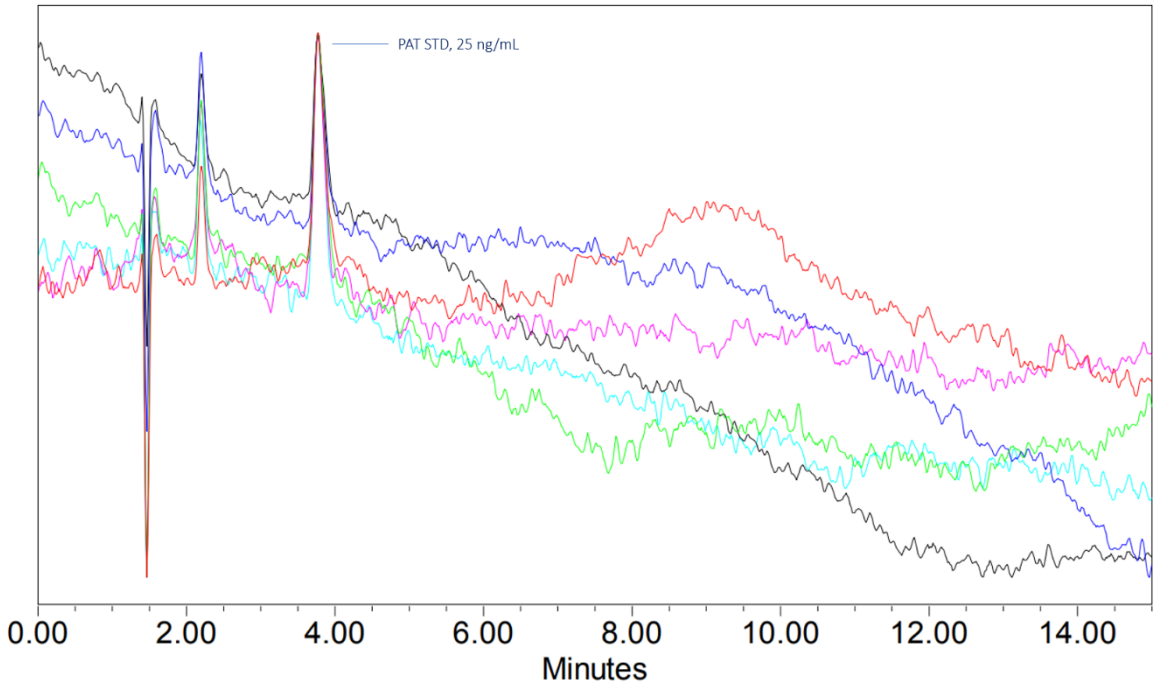

Figure 39. PAT method intra-day precision using a known standard concentration of 25 ng/mL.

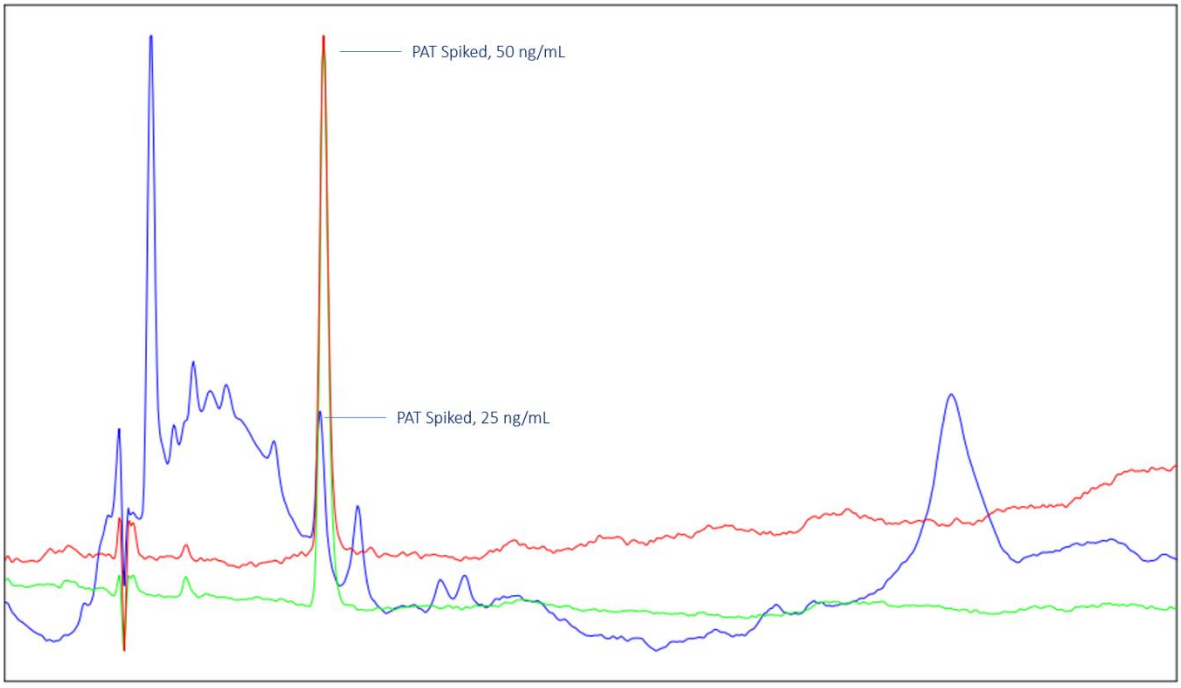

Figure 40. PAT recovery analysis for method validation using two different spiking concentrations of 25, 50 ng/g.

4.1.6 FUM

The quantification of FUM was performed with a 5-point calibration curve in the range of 0.24 – 3.85 µg/mL and 0.20 – 3.3 µg/mL, for FB_1 and FB_2 respectively. Subsequently, a linear regression, correlating the standard concentrations with the signal (peak areas) demonstrated good linearity, with high coefficient of determination, R^2 = 0.9987 and R^2 = 0.998 for FB_1 and FB_2, respectively (Figure 41 and 42).

The six overlapped chromatograms, representing different concentration of FB_1 standards ranging from 0.24 to 15.4 µg/mL are showed in Figure 43. The Tables 17 and 18 shows the summary for the peaks of FUM. The retention time of FB_1 was 8.8- 9.0 and ~10.8 min for FB_2. The accuracy of injection was assessed with a concentration of 5 µg/mL total FUM injected 4

times consecutively, as shown in Figure 44. The precision under the specified conditions was 6.90 % with injection accuracy was 84.0 % ± 5.80.

The recoveries of FB_1 and FB_2 were determined for each spiked concentration, as shown in Figure 45. The recovery for 5 µg/g of FB_1 was 85.60 %, while FB_2 was 94.60 %, for the concentration 2.5 µg/g, FB_1 had a recovery of 80.00 %, whereas FB_2 was 61.30 %, and for the concentration of 1.25 µg/g, the recovery was 80.70 % for FB_1 but FB_2 was not detected. The average recovery for FB_1 was 81.77% ±3 %, while for FB_2 the average recovery was 77.97 % ± 21 %. The LOD and LOQ for FB_1 were 0.72 and 2.19 µg/kg and for FB_2, 0.40 and 1.21 µg/kg, respectively.

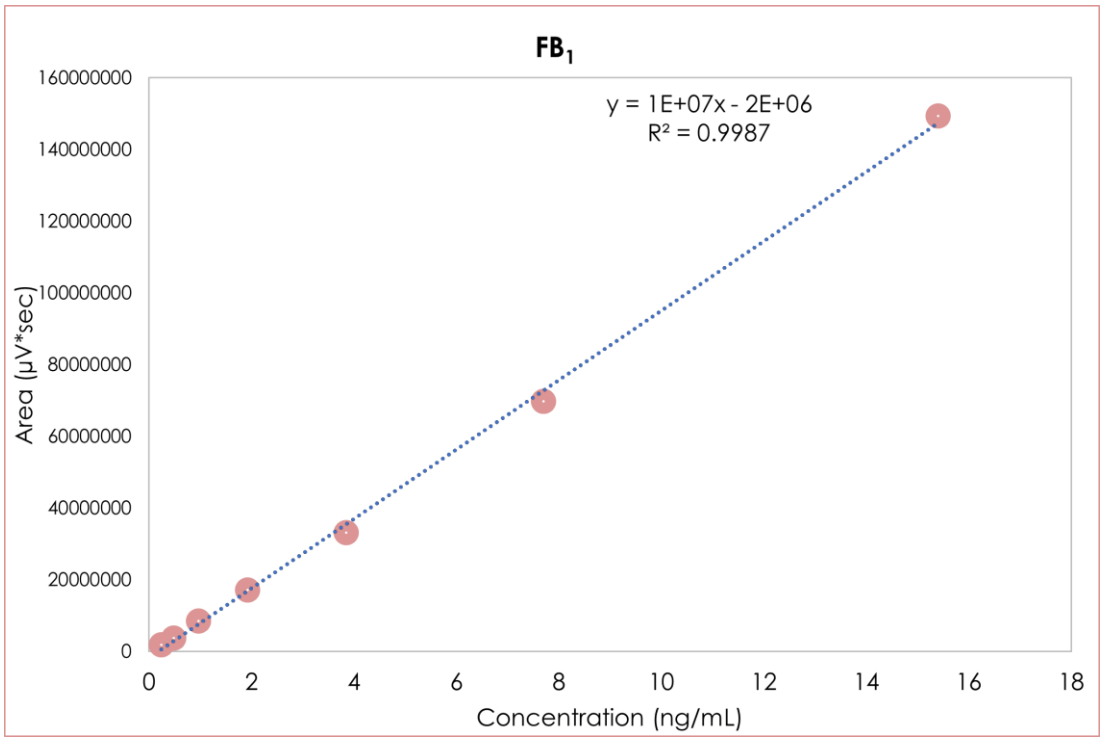

Figure 41. Five-point calibration curve ranging from 0.24 – 3.84 µg/mL for FB_1, showing the linear regression equation (y = mx + c) and coefficient of determination.

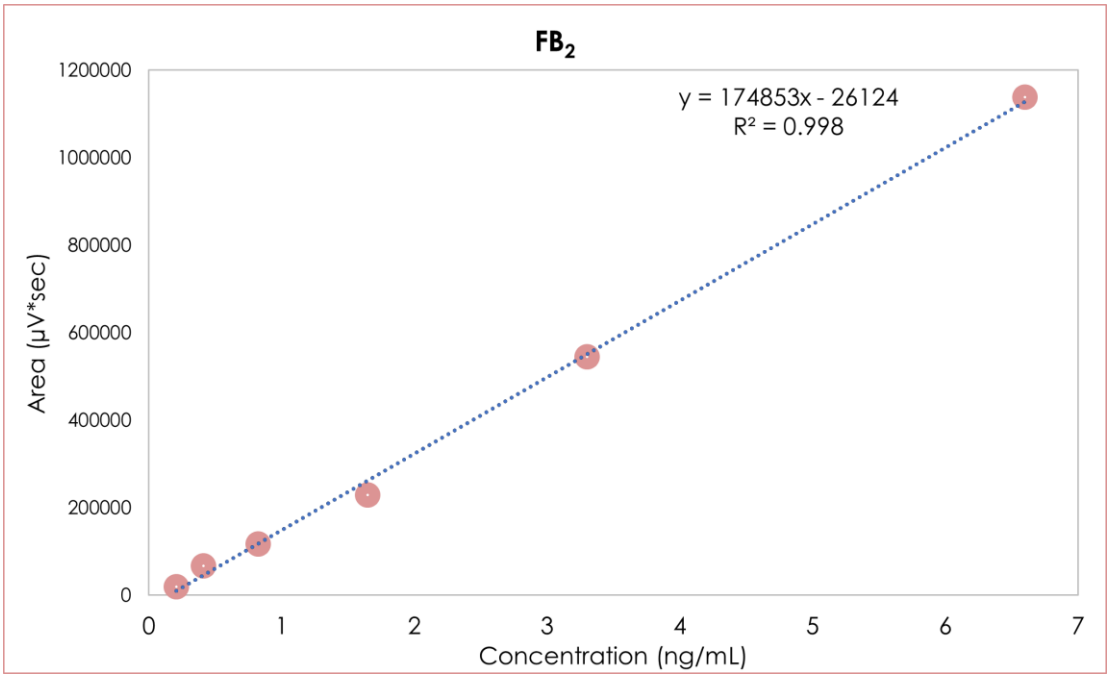

Figure 42. Five-point calibration curve ranging from 0.625 – 10 µg/mL for FB_2, showing the linear regression equation (y = mx + c) and coefficient of determination.

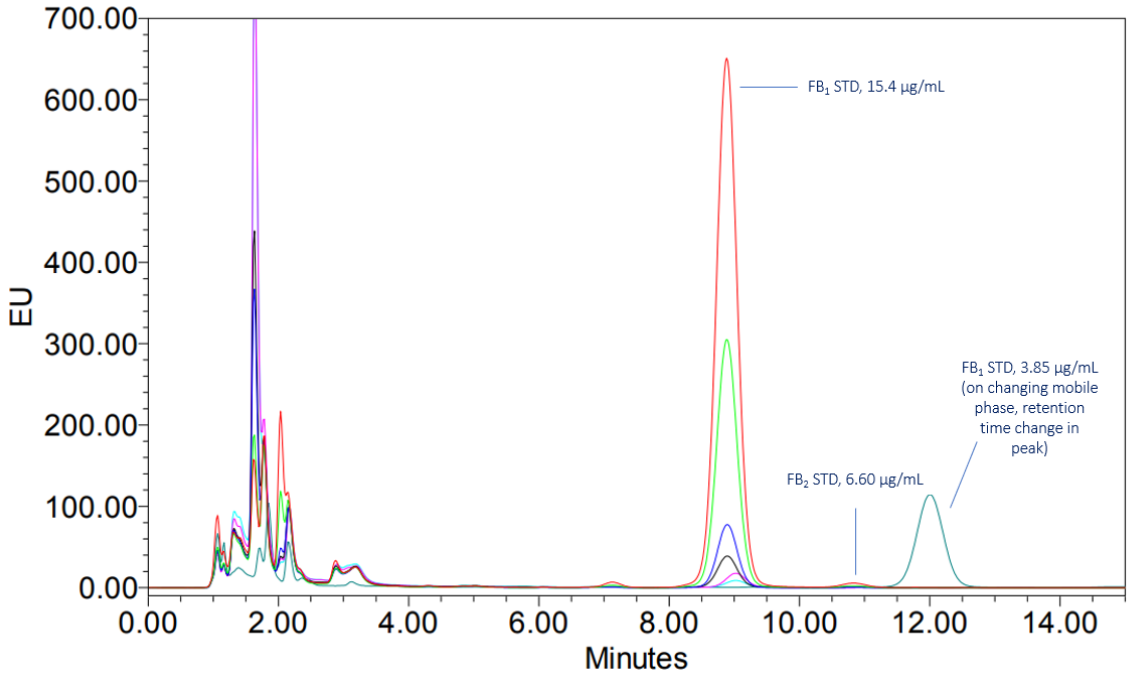

Figure 43. Chromatographic peaks for the standards of FB_1 and FB_2 ranging from 0.24 to 15.4 µg/mL and 0.20 to 6.6 µg/mL, respectively, with the FLR detector set at at λ_{ex} 335 nm and λ_{em} 440 nm.

Table 17. Peak summary for the calibration curve of FB_1, showing retention time, peak area, % area and height.

Concentration of FB_1 (µg/mL)	Retention Time (min)	Area	% Area	Height
0.24	9.022	1775207	100.00	81396
0.481	9.023	3760217	100.00	172802
0.962	8.893	8468594	100.00	382667
1.925	8.892	17131427	100.00	773325
3.85	8.886	33104559	100.00	1525019

Table 18. Peak summary for the calibration curve of FB_2, showing retention time, peak area, % area and height.

Concentration of FB_2 (µg/mL)	Retention Time (min)	Area	% Area	Height
0.206	10.822	19854	100.00	1235
0.412	10.838	66668	100.00	2685
0.825	10.847	117412	100.00	5066
1.65	10.892	228580	100.00	6827
3.3	10.831	544896	100.00	21749

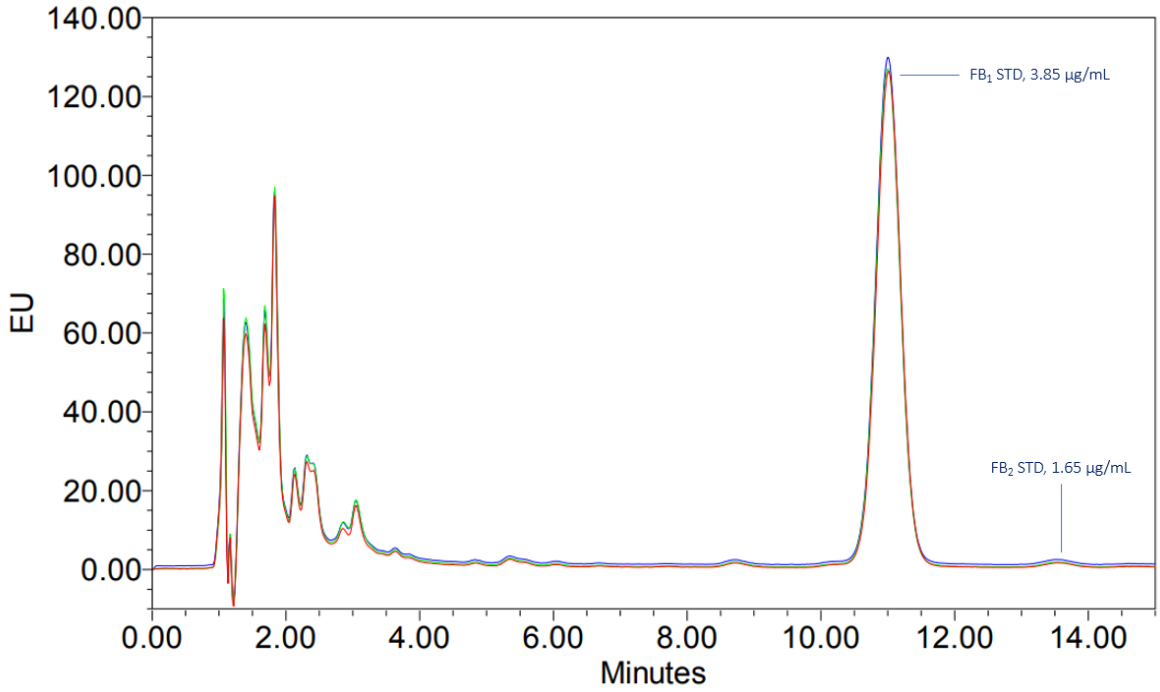

Figure 44. FB_1 and FB_2 method intra-day precision using a known standard concentration of 5 µg/mL.

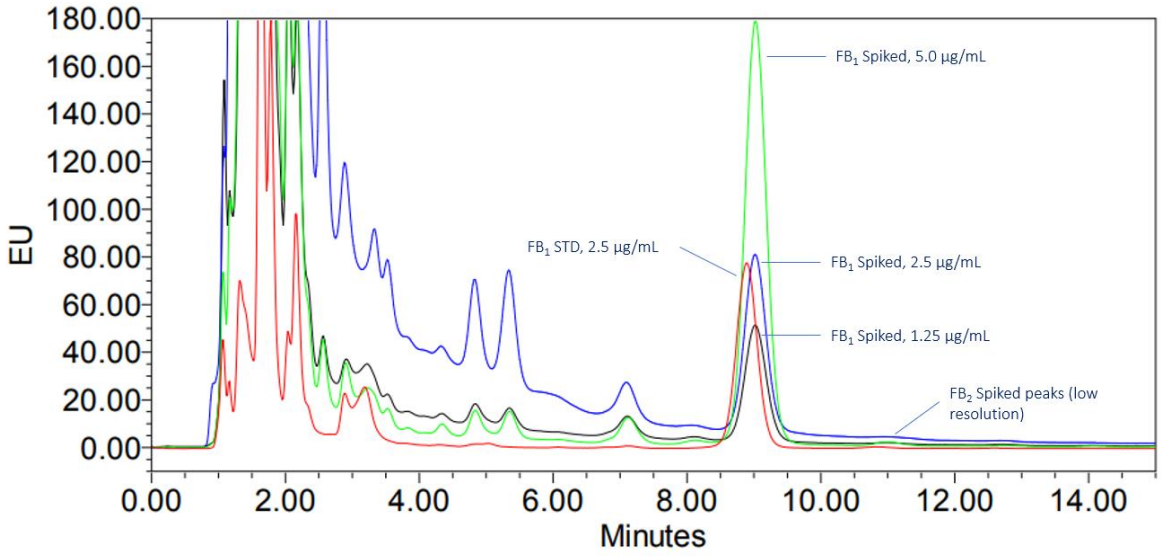

Figure 45. FB_1 and FB_2 recovery analysis for method validation, showing different concentrations of 1.25, 2.5 and 5.0 µg/g.

4.1.7 T-2 and HT-2

The calibration curve for quantification of T-2 and HT-2 mycotoxins ranged from 3.125 ng/mL to 100.0 ng/mL, for both analytes, the curve was serially diluted from the working solution (1000 ng/mL). The resulting calibration curves for both T-2 and HT-2 (Figure 46 and 47), showed good linearity by the high coefficient of determination, R^2 = 0.9998 and R^2 = 0.9981, respectively. The concentration of standards was lowered to 1.795 ng/mL to improve the sensitivity of detection of T-2 and HT-2 toxins.

Figure 48 shows the overlapped chromatograms of the serially diluted standards curve for both T-2 and HT-2. Table 19 and Table 20 describes the peak summary for the calibration curves of T-2 and HT-2 toxins, respectively. To assess the intra-day precision of the analytical method, a standard solution of T-2 and HT-2 at 62.5 ng/mL was injected five times on the same day (Figure 49). The accuracy for T-2 was 102.33 % ± 0.38 %, with an RSDr = 0.38 %. Similarly, for HT-2, the accuracy was 110.86 % ± 0.90 %, with RSDr = 0.81 %.

The validation of the analytical method was assessed through recovery experiments using matrix-matched wheat flour samples (*n = 4*) spiked with T-2 and HT-2 mycotoxins at 50 ng/g and 100 ng/g in duplicates for each level. For T-2, the individual recoveries ranged from 80.60 % to 99.30 %, while for HT-2, from 93.5 % to 105.6 %. The average recovery for T-2 was 92.4 % ± 8.00 %, and for HT-2, 97.50 % ± 5.00 %. The LOQ and LOD for T-2 were 1.41 and 4.29 µg/kg, respectively, while that of HT-2 were 2.41 and 7.31 µg/kg. Figure 50 shows the overlapped chromatograms for the recovery analysis for T-2 and HT-2.

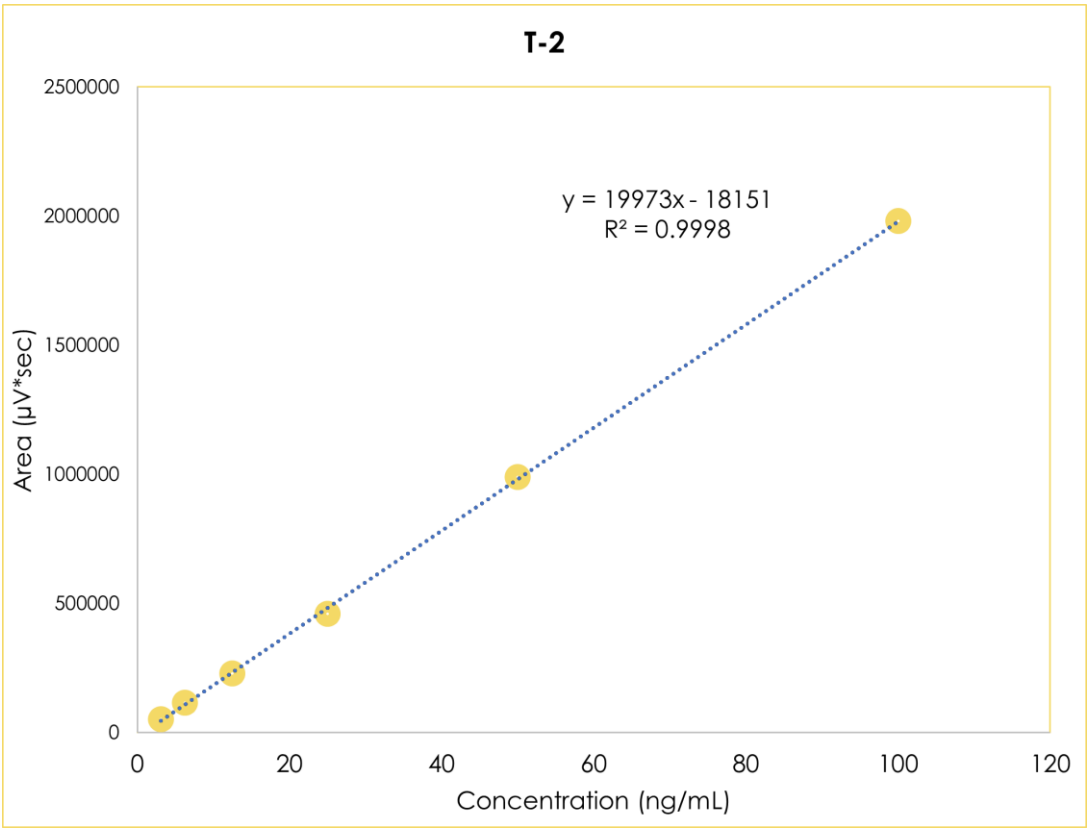

Figure 46. Five-point calibration curve ranging from 3.125 – 100 ng/mL for T-2, also showing the linearity, through the coefficient of determination (R^2) and the linear equation (y = mx – c).

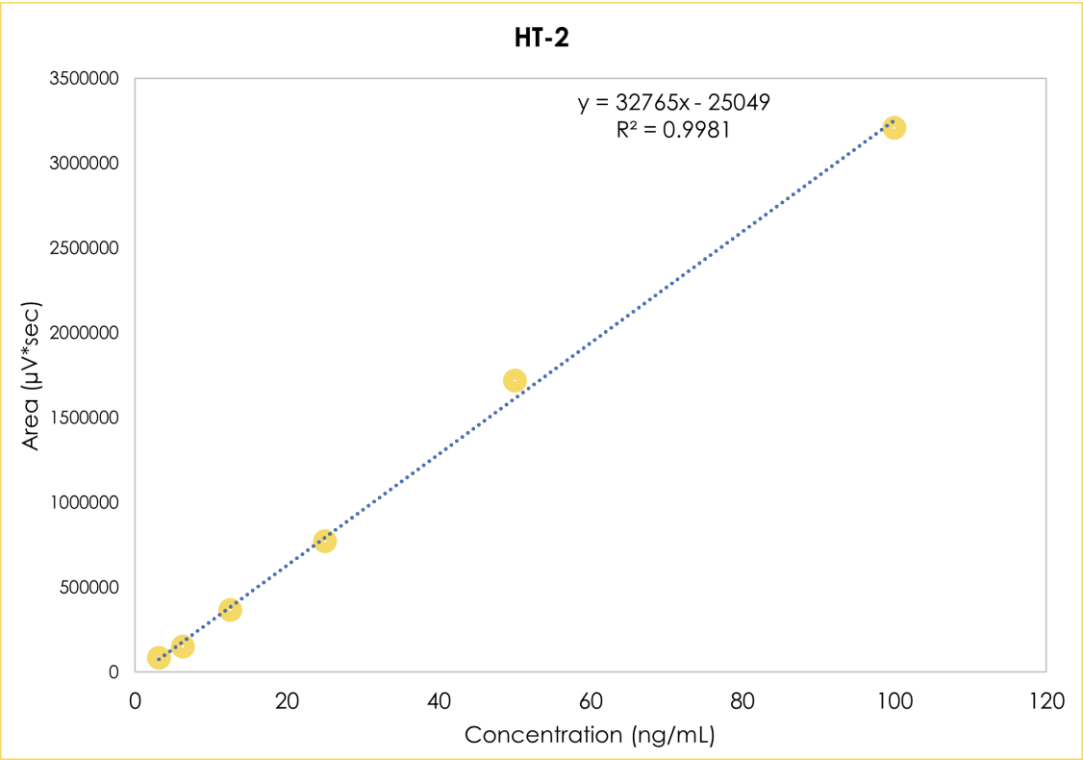

Figure 47. Five-point calibration curve ranging from 3.125 – 100 ng/mL for HT-2, also showing the linearity, through the coefficient of determination (R^2) and the linear equation (y = mx – c).

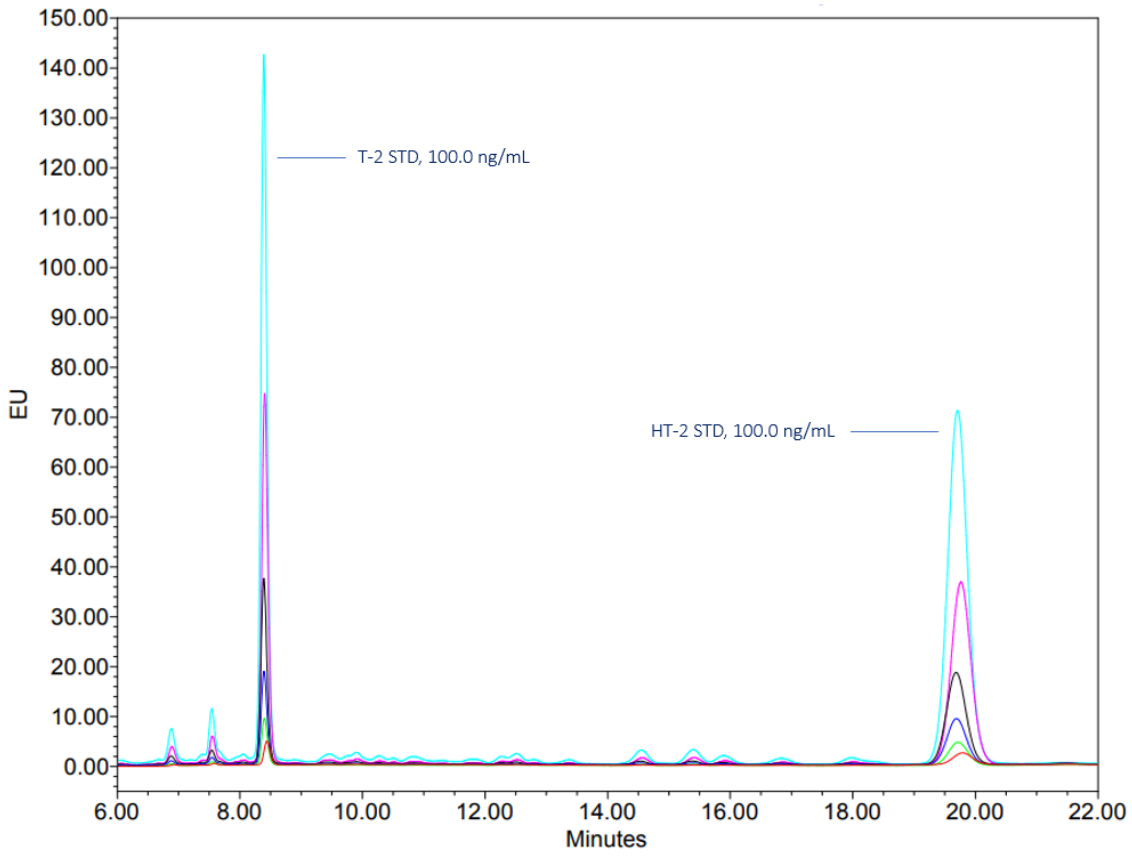

Figure 48. Chromatographic peaks for the standards of T-2 and HT-2 ranging from 3.125 – 100.0 ng/mL and 3.125 – 100.0 ng/mL, respectively, with the FLR detector set at λ_{ex} of 381 nm and an λ_{em} of 470 nm.

Table 19. Peak summary for the calibration curve of T-2, showing retention time, peak area, % area and height.

Concentration of T-2 (ng/mL)	Retention Time (min)	Area	% Area	Height
3.125	8.439	313188	100.00	46156
6.25	8.400	663241	100.00	93549
12.5	8.393	1292165	100.00	185238
25.0	8.390	2587593	100.00	369473
50.0	8.405	5261978	100.00	740866
100.0	8.390	9990151	100.00	1411205

Table 20. Peak summary for the calibration curve of HT-2, showing retention time, peak area, % area and height.

Concentration of HT-2 (ng/mL)	Retention Time (min)	Area	% Area	Height
3.125	19.792	542014	100.00	23240
6.25	19.719	1073132	100.00	46370
12.5	19.690	2087375	100.00	91625
25.0	19.685	4231728	100.00	184052
50.0	19.763	8508339	100.00	365888
100.0	19.707	16305025	100.00	707669

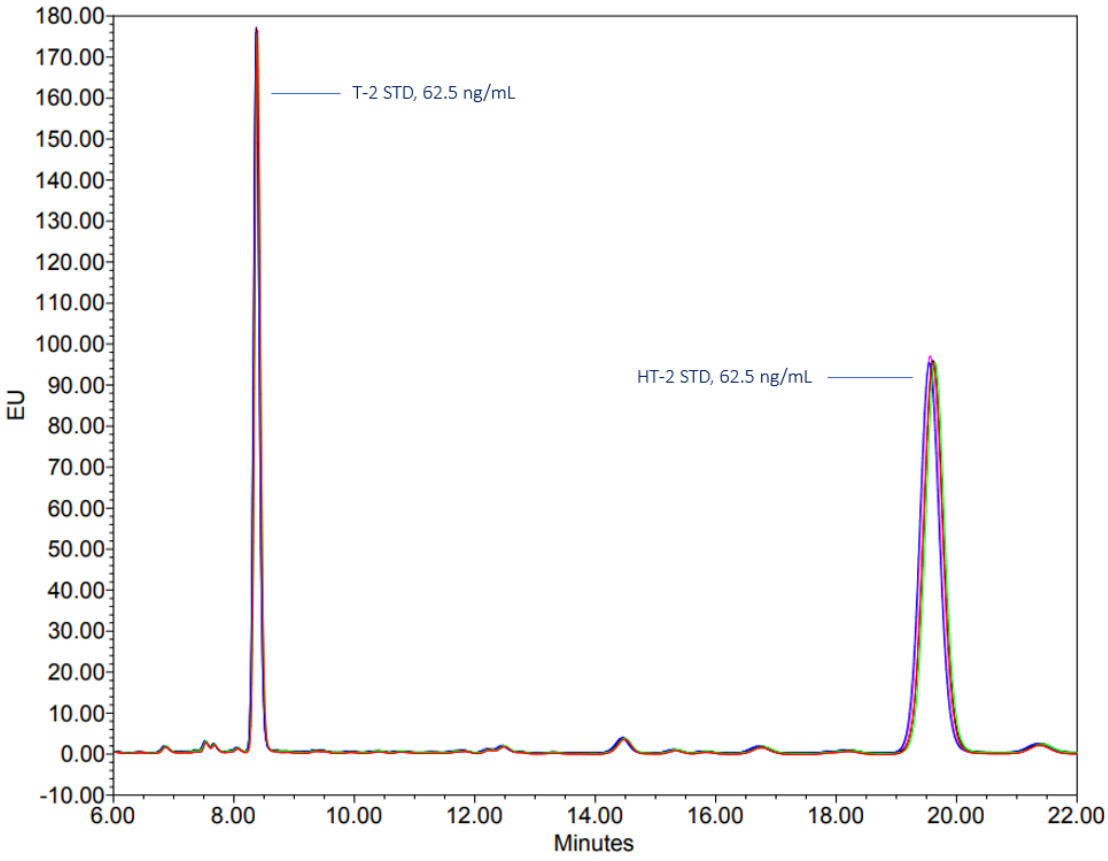

Figure 49. T-2 and HT-2 method intra-day precision using a known standard concentration of 62.5 ng/mL.

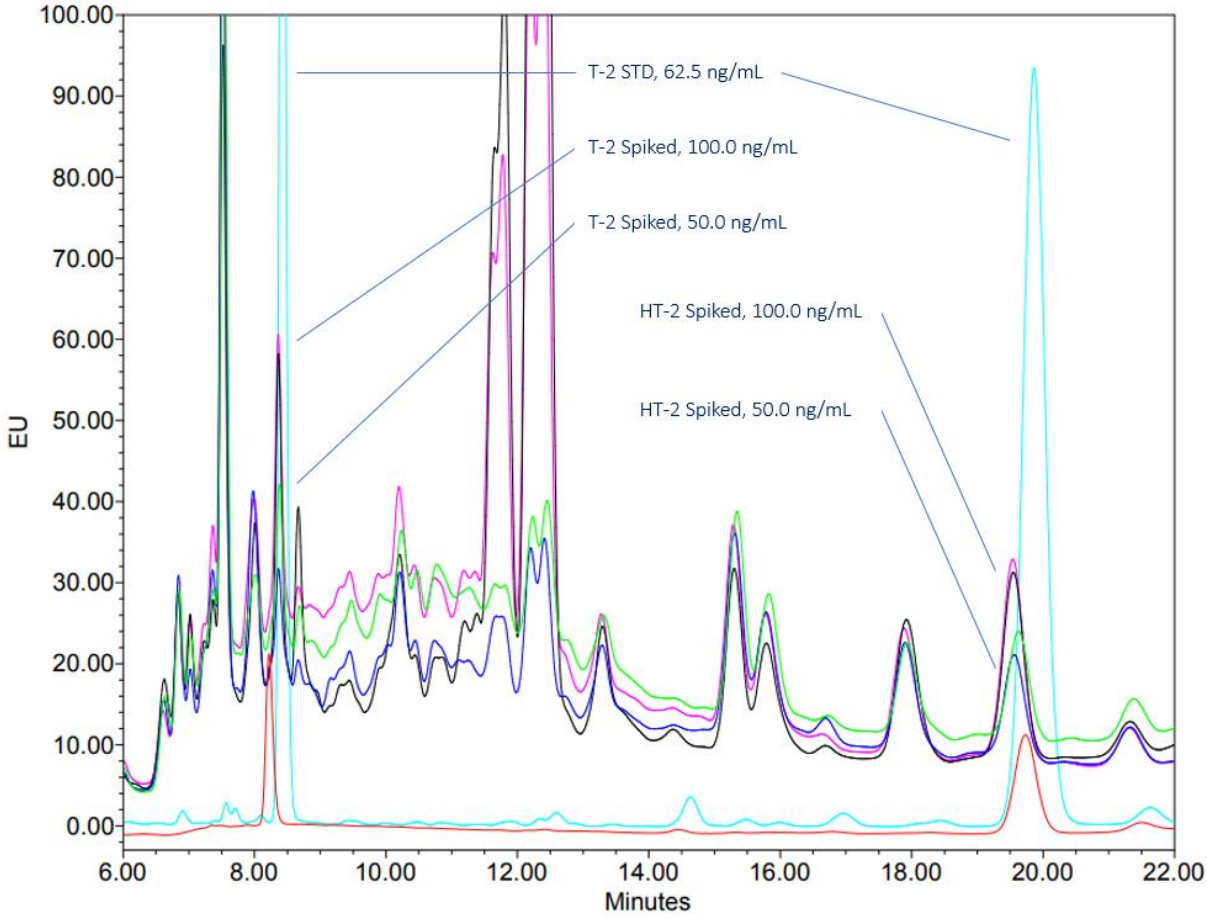

Figure 50. T-2 and HT-2 recovery analysis for method validation, showing peaks of T-2 and HT-2 with a slight variation of the retention time, but proportional to their spiked concentrations.

Table 21. Overview of the LOD, LOQ, recovery (%), injection accuracy and linearity (R^2) obtained in the method development for quantification of mycotoxins in flour.

Mycotoxin		Spiked Concentration (ng/g)	Average Recovery (%) ± RSD (%)	Average Accuracy (%) ± RSD (%)	R^2	LOD (µg/kg)	LOQ (µg/kg)
ZEA		60, 160, 160	85.52 ± 5.0	97.80 ± 1.04	0.9971	3.51	10.63
AFs	AFG_1	2.5, 5, 7.5, 10	90.15 ± 8.56	106.41 ± 2.04	0.9994	0.38	1.15
	AFB_1		98.10 ± 5.63	102.51 ± 3.00	0.9996	0.33	0.99
	AFG_2		94.01 ± 4.91	103.09 ± 1.90	0.9992	0.45	1.35
	AFB_2		119.69 ± 31.8	99.54 ± 1.87	0.9997	0.29	0.86
OTA		25, 50	77.50 ± 6.0	95.30 ± 2.92	0.9998	0.22	0.68
DON		100, 500, 1000	86.67 ± 9.0	89.92 ± 13.80	0.997	116.20	352.12
PAT		25, 50	49.70 ± 19.0	98.80 ± 5.17	0.9993	3.94	11.94
FUM	FB_1	1250, 2500, 5000	81.77 ± 3.0	84.02 ± 6.90	0.9987	721.38	400.70
	FB_2	1250, 2500, 5000	77.97 ± 21.0	93.86 ± 7.56	0.998	2186.00	1214.26
T-2		50, 100	92.44 ± 8.0	102.33 ± 0.38	0.9998	1.41	4.29
HT-2		50, 100	97.54 ± 5.0	110.86 ± 0.81	0.9981	2.41	7.31

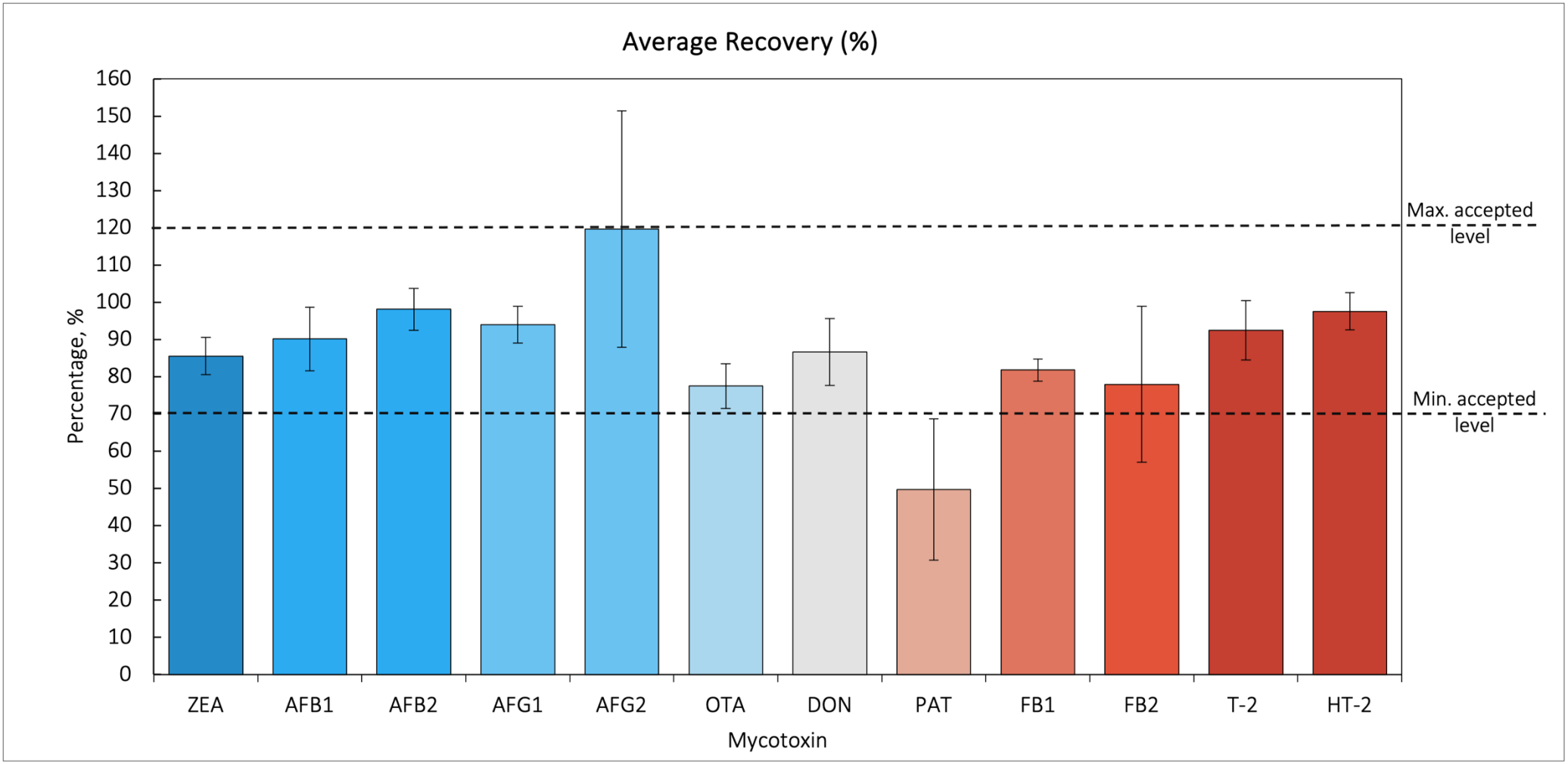

Figure 51. Average percentage recovery for all mycotoxins tested. Min. accepted level is 70 %, Max. accepted level is 120 % according to Commission Regulation (EC) No 401/2006. Bars represent the AV % ± RSD %.

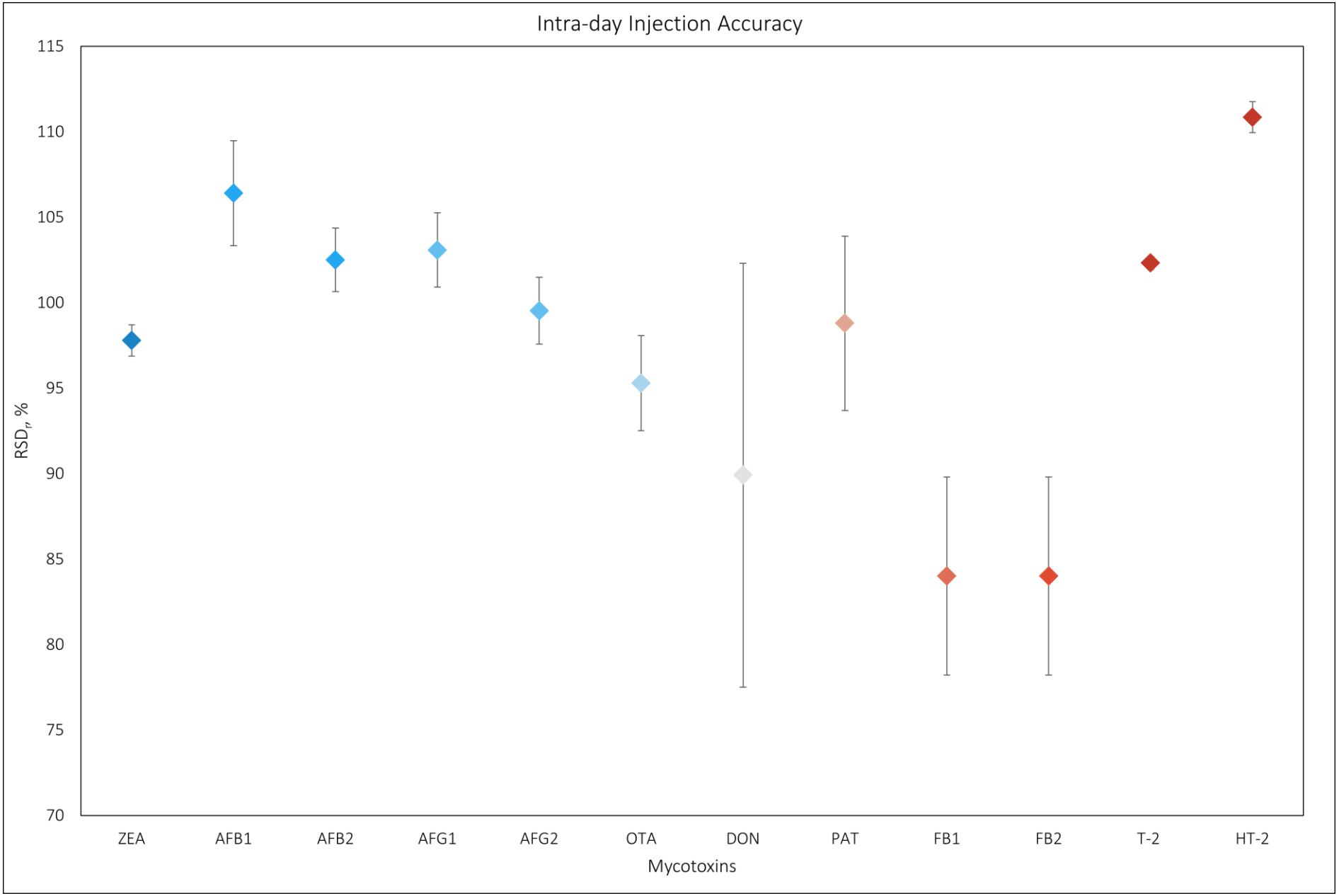

Figure 52. Intra-day percentage accuracy for all mycotoxins analysed. Values represent the AV % ± RSD %.

4.2 Detection and quantification of mycotoxins in flour samples

Figure 53 shows the co-occurrence combinations found to be contaminating the flour samples tested. Various combinations were detected, such as DON + PAT, DON + T-2, DON + T-2 + HT-2, PAT + HT-2, PAT + T-2, PAT + T-2 + HT-2 and ZEA + OTA + PAT + T-2 + HT-2. The results show the presence of various mycotoxins in flour samples, which are noted in Table 22. Based on the surveillance results for testing the presence of ZEA from the 16 samples tested, only one sample (sample 11) showed a detectable concentration (13.7 µg/kg). Figure 54 highlights the prevalence (composition) of mycotoxins within each sample as detected in this survey. Figure 55 illustrates all the overlapped chromatograms of the injections for ZEA contrasted with injection of different concentrations of standards. Similarly, sample 11 was the only sample with OTA present at a concentration of 60.0 µg/kg (Figure 56), while all the other tested samples were below the limits of detection for the mentioned analytical method. DON was detected in 6 out of 16 samples (37.5 %) while the remaining samples had concentrations below the limits of detection. Figure 57 shows the overlapped chromatograms of the samples injected to detect DON. The highest concentration was detected in sample 3 (324.55 µg/kg), while the lowest concentration was found in sample 5 (189.67 µg/kg). PAT showed the highest occurrence of all the samples surveyed (Figure 59), as 11 out of 16 (68.75 %) samples showed the metabolite as a contaminant. The range of detected PAT concentrations in the positive samples varied from 2.94 to 27.6 µg/kg. The highest PAT concentration was found in sample 10 (27.8 µg/kg), while the lowest positive concentration was found in sample 4 (2.94 µg/kg). Moreover, the surveillance results showed that none of the samples were contaminated with FUM or AFs, as shown in Figures 58 and 60, respectively. T-2 and HT-2 toxins were detected in various flour samples. T-2 was detected in 5 out 16 samples (2, 3, 6, 11 and 14) with

concentrations ranging from 2.53 to 111.42 µg/kg, while 6 out of 16 (1, 5, 9, 11, 12 and 15) showed detectable limits of HT-2 ranging from 2.45 to 9.75 µg/kg.

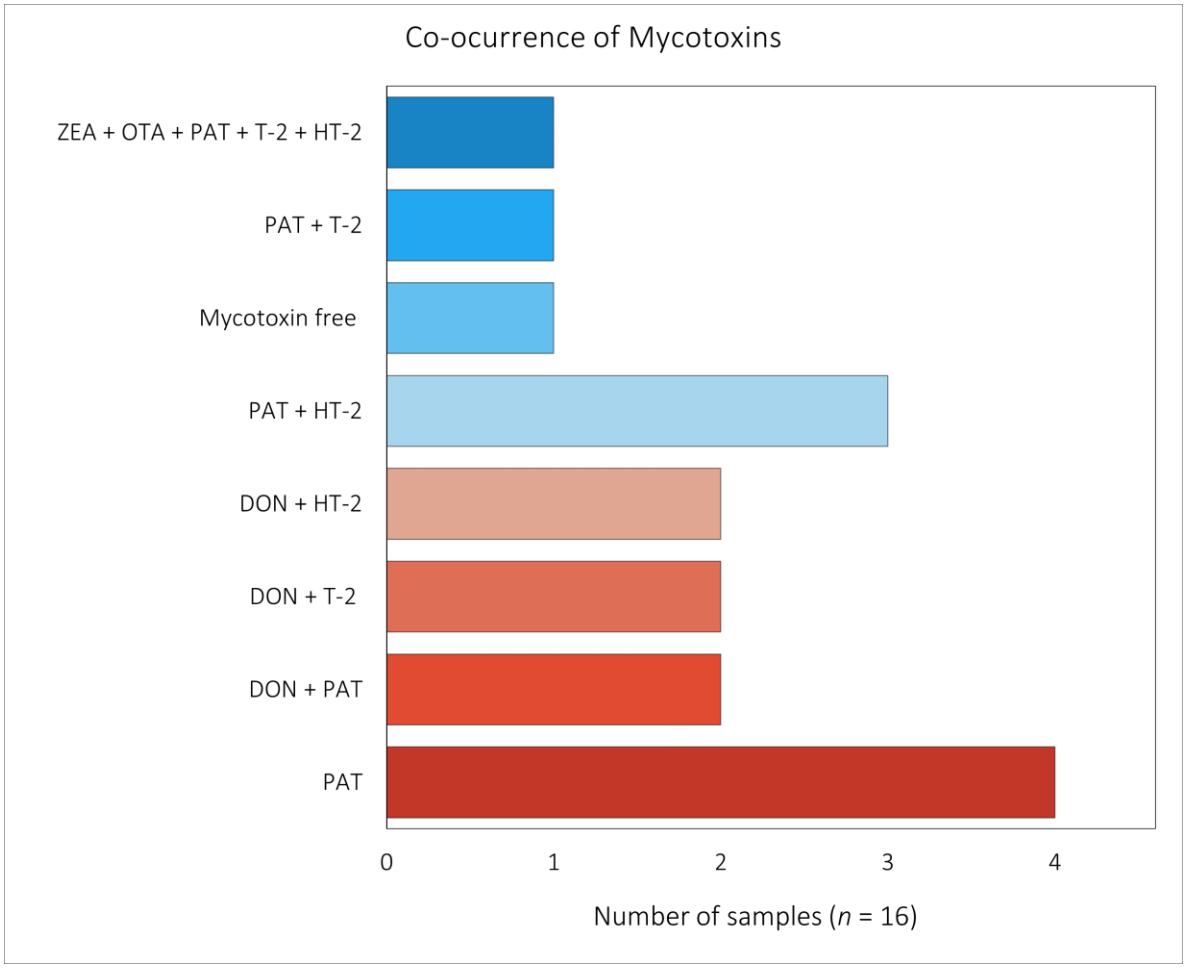

Figure 53. Co-occurrence of mycotoxins represented as number of positive samples for each mycotoxin.

Table 22. Mycotoxin surveillance studies in various flour samples.

Flour Sample	(ZEA), µg/kg	(OTA), µg/kg	(DON), µg/kg	(Afs), µg/kg	(PAT), µg/kg	(FUM), µg/kg	(T-2), µg/kg	(HT-2), µg/kg
1	<LOD[1]	<LOD	190.30	<LOD	<LOD	<LOD	<LOD	9.75
2	<LOD	<LOD	221.25	<LOD	<LOD	<LOD	89.02	<LOD
3	<LOD	<LOD	324.55	<LOD	<LOD	<LOD	111.42	<LOD
4	<LOD	<LOD	<LOD	<LOD	2.94	<LOD	<LOD	<LOD
5	<LOD	<LOD	189.67	<LOD	<LOD	<LOD	<LOD	2.50
6	<LOD	<LOD	<LOD	<LOD	1.09	<LOD	2.53	<LOD
7	<LOD	<LOD	222.20	<LOD	19.40	<LOD	<LOD	<LOD
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9	<LOD	<LOD	<LOD	<LOD	17.38	<LOD	<LOD	2.71
10	<LOD	<LOD	270.22	<LOD	27.75	<LOD	<LOD	<LOD
11	13.70	60.00	<LOD	<LOD	2.59	<LOD	18.62	2.45
12	<LOD	<LOD	<LOD	<LOD	6.10	<LOD	<LOD	5.58
13	<LOD	<LOD	<LOD	<LOD	11.64	<LOD	<LOD	<LOD
14	<LOD	<LOD	<LOD	<LOD	20.24	<LOD	<LOD	<LOD
15	<LOD	<LOD	<LOD	<LOD	7.11	<LOD	<LOD	3.32
16	<LOD	<LOD	<LOD	<LOD	2.36	<LOD	<LOD	<LOD

[1] <LOD, below limits of detection.

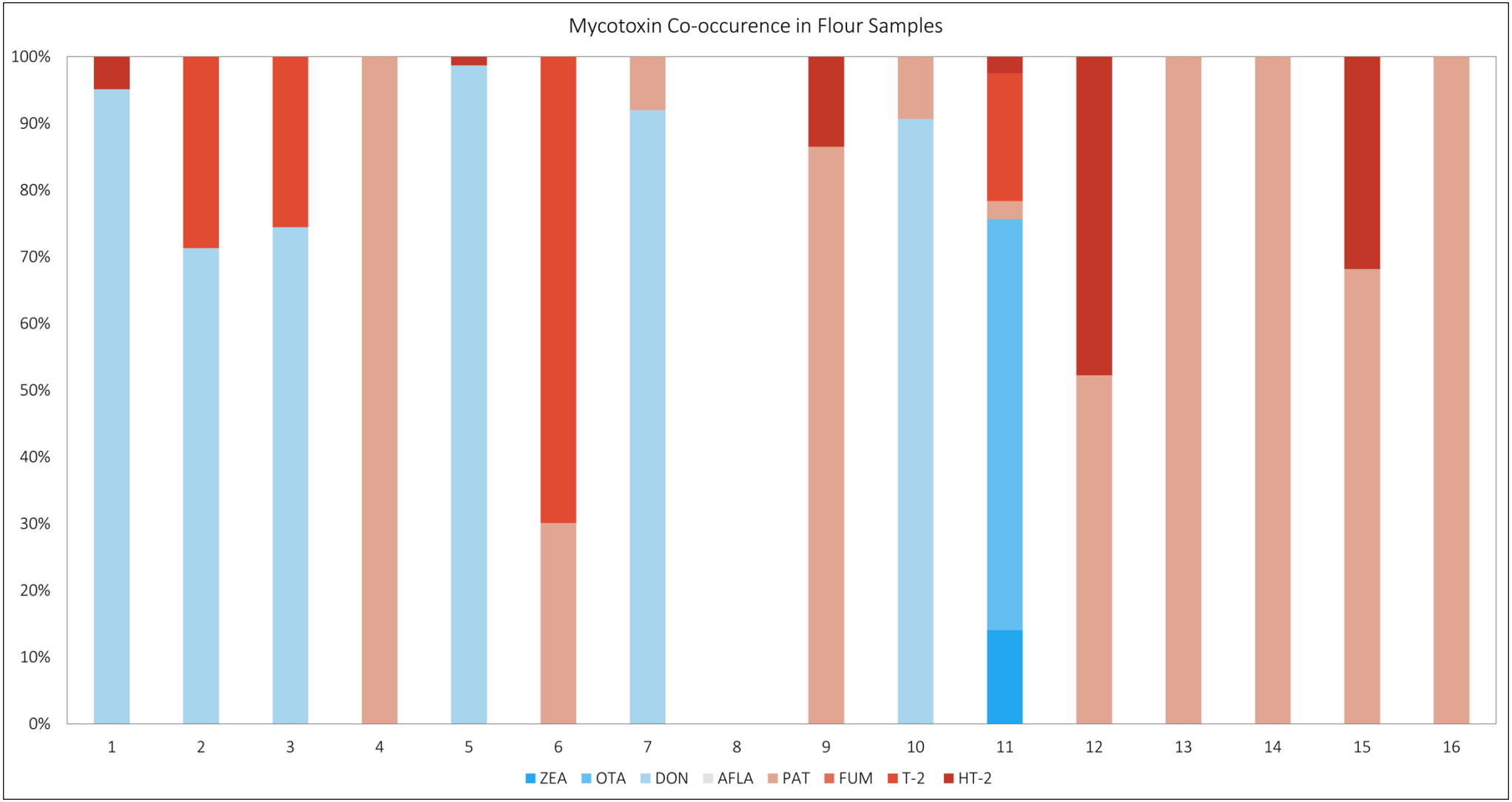

Figure 54. Percentage stacked column graph showing the occurrence of mycotoxins across the samples tested.

Chapter 5 – Discussion

To our knowledge, this study is the first to perform mycotoxin analysis in food and feed in Malta and therefore, method development and validation is required to guarantee the reliability of the analytical methods being performed. This study also attempted to perform a survey, to detect the mycotoxin contaminants in flour being sold in local supermarkets, milling companies, and used in bakeries to produce the Maltese bread and *ftira*.

5.1 Method development

During the in-house method development and validation, this study focused on four main quality criteria: linearity, accuracy, precision, sensitivity (LOD and LOQ) and apparent recovery. All the mycotoxins (ZEA, AF, OTA, DON, PAT, FUM, T-2, HT-2) analysed had good linearity, or coefficient of determination (R^2), ranging from 0.9970 to 0.9998. The accuracy and precision of the methods were generally close to or exceeding 100 %, indicating minimal bias in the quantification results. The intra-day precision (RSD_r) ranged from 0.38 to 5.8 %, with an outlier for DON at 12.4 %. Hence, 86 % of the in-house methods demonstrated good precision. The LODs for AFs (total and individual analogues) and OTA were below 0.5 µg/kg, while the LODs for ZEA, PAT, T-2 and HT-2 were <4 µg/kg. On the other hand, DON and FUM showed relatively high LOD and LOQ (116.20, 352.12 for DON and 721.38, 400.70 for FB_1, respectively). Various factors might contribute to variations in quantification, such as matrix effects, detector sensitivity, instrumental limitations, chemical properties of the molecules or even cross-reactivity of other compounds in the immunoaffinity columns. DON derivatives such as 3-AcDON, 15-AcDON are intermediate derivatives of DON during the biosynthesis of such

mycotoxin, which are known to co-exist. Such conjugates have a similar configuration which facilitates their binding to the antibody present in the IACs. Gonçalves and Stroka (2016), investigated the cross-reactivity between DON conjugates in four IACs from different suppliers since there are no specific IACs for DON derivatives. Authors concluded that only one IAC (DONtest) managed to retain all three derivatives, while two other IACs (DONneo, DONPREP) missed 15-AcDON, while another missed (DONaokin) both DON-3-G and 3-AcDON.

The methods developed in the current study showed reliable capacity for monitoring mycotoxin contamination in wheat flour following the maximum limits laid down by EU regulations EC No. 1881/2006 (European Commission, 2006). The achieved LOQ for DON (352 µg/kg) was within the regulation limits for wheat and sub-products (750 – 1750 µg/kg). The limits for OTA in unprocessed cereals and derivate products are 5.0 and 3.0 µg/kg, respectively, and the LOQ in the method developed was 0.68 µg/kg. For ZEA (LOQ 10.63 µg/kg) and FUM (LOQ 1600 µg/kg) the LOQs were also below the regulated limits for cereals and products: 50 -75 µg/kg 2000-400 µg/kg, respectively. In the case of total AFs, the limits established by the EU legislation for cereals and sub-products is 4 µg/kg, and the LOQ achieved by the in-house method was 1.15 µg/kg. As for AFB_1 the EU regulation sets a lower maximum limit (2 µg/kg) also reached by the method (LOQ 0.99 µg/kg). PAT is not regulated in cereals, but the regulation limit is 10-50 µg/kg in apples juices and derivates, and the LOQ was just below 12 µg/kg. However, considering the strictest maximum limits laid down by the EU regulation for mycotoxins in infants' food and special dietary products (DON 200 µg/kg; AFB_1 0.10 µg/kg, OTA 0.50 µg/kg, FUM 200 µg/kg, ZEA 20 µg/kg, PAT 10 µg/kg), the LOQs achieved show constraints and the methods need to be further improved.

In addition to the regulated mycotoxins, the present survey also developed a quantification method for T-2 (LOQ 4.29 µg/kg) and HT-2 (LOQ 7.31 µg/kg). Such toxins are not strictly regulated; however, they have indicative levels for surveillance of the sum of T-2/HT-2 set to 100 µg/kg for wheat and 50 µg/kg for other milling products as stated on the recommendation EC No. 165/2013 (European Commission, 2013). EFSA in a scientific report published in 2017, emphasized on the importance for more research on the occurrence of T-2 and HT-2 in food to established maximum limits of such toxins in different dietary foods (EFSA, Arcella et al. 2017).

The performance criteria according to regulation EC No. 401/2006 (European Commission 2006b) must satisfy the range of 50 to 120 % for AFB_1, AFB_2, AFG_1, AFG_2 when a concentration of < 1.0 µg/kg is used. The performance criteria for other mycotoxins are also specified; OTA and PAT from 50 to 120 %, PAT for a concentration of < 1 and < 20 µg/kg, respectively. The performance criteria for DON, FB_1 and FB_2, ZEA, T-2 and HT-2 is also specified as 60 to 110 %, 60 to 120 %, 60 to 110 %, and 60 to 130 %, respectively (European Commission 2006b). The apparent recoveries for the method validation varied from 77.97 to 119.70 %, demonstrating accurate quantification. The overall apparent recovery percentage met the official performance criteria specified in regulation EU No. 519/2014 (European Commission 2014). However, PAT, AFB_2 and FB_2 showed variation above the recommended ($RSD_{\%}$ = 20 %) for method validation therefore requiring further optimisation.

The recovery for PAT (49.70 %) was not within the acceptable criteria as stated above. The EASIMIP™ PATULIN columns utilise a molecularly imprinted polymer developed to capture PAT molecules and remove interferents from the sample. However, the protocol for its use was optimised for apple puree and apple juice, thus the application to other food matrices would need to be optimised as well. The sample matrix may have caused variation leading to low recovery values. Consequently, the use of such columns to perform sample preparation in flour is not advisable without further development in both extraction and column utilisation. The fluctuations in the recovery values may show matrix-effect and analytical variabilities or sample preparation inconsistencies.

Irakli, Skendi et al. (2017) performed method validation for the simultaneous determination of mycotoxins (DON, OTA, AFs and ZEA) in wheat. Recovery experiment results were <60 % when using concentrations of MeOH of less than 70 %. It was also noticeable that recoveries were further reduced when water was utilised instead of PBS for the washing step during the extraction protocol. However, good recoveries were achieved when using 100 % MeOH, as an extraction solvent, resulting in recoveries of more than 80 % in all mycotoxins extracted.

5.2 Mycotoxin survey in flour and their co-occurrence

The co-occurrence of mycotoxins in the samples assessed (Figures 53 and 54) showed that 2 or more contaminants were present in 11 out of 16 samples (68.75 %), while 4 out of 16 samples (25%) had only one mycotoxin. Sample 8 (brown rice flour) was the only sample free of any mycotoxins. Sample 1 (soya flour) and sample 5 (rye flour) were contaminated with DON + HT-

2, sample 2 (wheat flour) and sample 3 (potato flour) were both contaminated with DON + T-2, sample 4 (Indian gram flour), sample 13 (wheat flour), sample 14 (wholemeal flour) and sample 16 (plain flour) were all contaminated with PAT. Additionally, other combinations were observed in samples 7 and 10, which were wheat and spelt flour, both contaminated with DON + PAT. Sample 9 (wholemeal flour), sample 12 (wheat flour) and sample 15 (flour "00") were contaminated with PAT + HT-2. Sample 6 (carob flour) was contaminated with PAT + T-2, while sample 11 (pumpkin flour) had the most co-occurrence in ZEA + OTA + PAT + T-2 + HT-2.

Flour is the result of thorough processing of agricultural crops like wheat, maize, rye, and carob, thus, is susceptible to the simultaneous presence of mycotoxins due to its production process (Palumbo, Crisci et al. 2020). Mycotoxins typically persist through food processes (Smith, Madec et al. 2016). As a result, it is crucial to investigate and monitor the simultaneous occurrence of mycotoxins due to their added detrimental health impacts in human and animal (Grenier, Oswald 2011). The co-occurrence of mycotoxins in cereal grains is widely recognised and can be derived from three primary factors: contamination by multiple fungal species, mixture of various grain sources in animal diets, and capacity of most fungal species to produce multiple mycotoxins concomitantly. The combined effects of mycotoxins are somewhat unknown and understudied, as limited data is available (Smith, Madec et al. 2016). For example, wheat plants are prone to *Fusarium* infections, such as FHB, caused by a complex of strains increasing the likelihood of co-contaminants such as T-2, HT-2, ZEA and DON. Species of *Fusarium* spp., like *F. graminearum*, can co-produce the mycotoxins ZEA and DON (Schaarschmidt, Fauhl-Hassek 2018).

Sample 1 (soya flour), had co-occurrence of DON + HT-2, at 190.30 and 9.75 µg/kg, respectively, while sample 5 (rye flour), was contaminated by the same toxins but in slight lower levels, 189.67 and 2.50 µg/kg, for DON and HT-2, respectively. However, the contamination levels were below established maximum levels (MLs) for DON (Commission Regulation, 2006a). The MLs for DON in unprocessed rye should not exceed 1250 µg/kg, whereas rye intended for direct human consumption, such as rye flour, should not surpass 750 µg/kg (European Commission 2006a). DON stands out as a prevalent mycotoxin in grains and grain-based products in Central Europe. In fact, DON was discovered in 29 European countries which were studied, with an occurrence nearing 50% in the wheat-based food analysed (Johns, Bebber et al. 2022).

Globally, only 13 countries have officially documented acceptable, or suggested MLs, for T-2 and HT-2 toxins in food and/or animal feed products, with recommended levels varying from 25 to 1000 µg/kg (EFSA 2011). However, standardized MLs for T-2 and HT-2 toxins in food and feed items within the EU are yet to be established. In response, the EC has advised EU member states to collect reliable data on the annual fluctuations of T-2 and HT-2 toxins (as well as other *Fusarium* toxins) to facilitate the development of future MLs for food and feed products (Pernica, Kyralova et al. 2022).

The EFSA conducts numerous risk assessment studies and modelling frameworks using animal (pigs and chicken) and human case studies. Such assessments involve problem formulations, exposure assessments, hazard assessments and risk characterisation to investigate the component-based approach and provisional daily intake. Complex toxicokinetic models are

used from studies which reported the bioavailability parameters necessary for the intended modelling approach. In 2020, EFSA collaborated with numerous experts within the field to published an external scientific report which included a risk assessment study on multiple mycotoxin mixtures in animals and humans (Battilani, Palumbo et al. 2020).

The inherent co-occurrence of T-2 and HT-2 toxins prompted the initial establishment of a tolerable daily intake (TDI) threshold of 100 ng/kg body weight/day. This threshold was formulated by the European Food Safety Authority's Panel on Contaminants in the Food Chain (EFSA 2011) and was recently revised to 20 ng/kg body weight/day (EFSA 2017). Consequently, this raises concern in two samples tested, which are samples 2 (wheat flour) and 3 (potato flour), in which DON + T-2 were prevalent in both samples. The wheat flour sample is sourced from Malta's primary wheat importers, who supply a significant portion of local bakeries and restaurants. In a Times of Malta article written by Xuereb (2022), the origin of wheat imported to Malta mainly comes from countries like Hungary, Poland, Romania, and Bulgaria. Previously, Ukraine and Russia were major wheat suppliers, but the economic sanctions imposed by the European Union on Russia have made imports from these countries challenging.

Moreover, the levels of T-2 and HT-2 found in this survey raise further concern and the need for continuous monitoring regarding T-2 toxin levels in food is required. Varga, Fodor et al. (2021) performed a study by testing the presence of 12 mycotoxins in 54 wheat-based products such as wheat flour and whole wheat flour from Hungarian markets. DON was present in 47 out of 54 samples tested, while T-2 was present in 5 out of 54 samples. HT-2, 15-Ac-DON and DON-3-G were also detected in some samples. The average concentration determined for

DON in this study was 169 µg/kg, while the average reported by Varga, Fodor et al. (2021) was 236.4 µg/kg. Moreover, the average concentration for T-2 in this study was 55.39 µg/kg, with sample 2 and 3 containing the highest concentrations of 89.02 and 111.3 µg/kg, respectively. The same Hungarian survey also reported T-2 contamination, but only consisting of an average concentration of 4.5 µg/kg.

To date, no surveys reported mycotoxin contamination in potato flour. The sample used in the present survey originated from Finland and several studies reported elevated levels of T-2, HT-2, and DON mycotoxins in wheat and barley from Finland (Hietaniemi, Ramo et al. 2016, Van Der Fels-Klerx, Klemsdal et al. 2012). Given this context, the current study speculates that the occurrence of T-2 and DON mycotoxins in the sample of potato flour used in the survey might be attributed to potential carryover from the production facility.

Samples 4, 7, 9, 12, 13, 14, 15, and 16 were all wheat-based flour samples, whilst sample 6, 10 and 11 were carob, spelt and pumpkin-seed flour, respectively. These samples all showed PAT contamination between 2.59 to 27.75 µg/kg, some in co-occurrence of other mycotoxins (i.e, PAT + DON, PAT + HT-2, PAT + T-2, ZEA + OTA + PAT + T-2 + HT-2) and some other with only PAT contamination. Several studies have isolated *Penicillium* spp. in flour, which are known to be the main source of PAT producers (Loncaric, Sarkanj et al. 2021). A local study performed on flour, including wheat flour, showed presence *P. expansum* (Tanti 2023; unpublished data). Minutillo, Ruano-Rosa et al. (2022) also have noted that *Penicillium* spp. was the most abundant organism, and *Penicillium griseofulvum* which is a known PAT producer was identified. The same study states that they have isolated *Penicillium citrinum* from ''00'' flour,

which is known to produce citrinin (Kamle, Mahato et al. 2022) although this study did not test for the presence of citrinin in flour. Other species able to produce mycotoxin also were reported, as *Penicillium verrucosum*, which produces OTA, *P. commune* which produces cyclopiazonic acid and *P. chrysogenum* which produces roquefortine C (Minutillo, Ruano-Rosa et al. 2022). Although, no reports of PAT in flour are available numerous reports of *Penicillium* spp. contaminating flour are recorded (see table 2; Cabanas, Bragulat et al. 2008, Weidenbörner, Wieczorek et al. 2000). The EU regulates the presence of PAT in various food products including baby foods, apple juice, and alcoholic beverages like ciders, as well as fruit juices. The EU has established specific maximum allowable limits for PAT, which can vary between 10 to 50 µg/kg depending on the type of food product (European Commission 2006a).

In the survey performed, only 1 sample (11 - pumpkin flour) was contaminated with ZEA and OTA. The presence of ZEA or OTA in pumpkin flour has not been reported so far. Although, Escrivá, Agahi et al. (2022) counteracts our findings stating that pumpkin powder is associated with significant reduction the presence of AFB_1 and OTA bio-accessibility. There are numerous reports of ZEA contamination or co-occurrence in cereals and flours, although this study was not in accordance with such studies. Andre, Muller et al. (2022) also reported the occurrence of ZEA in wheat flour made in Switzerland, while Tima, Bruckner et al. (2016) reported various *Fusarium* toxins (DON, ZEA, T-2) to be contaminating wheat cereals harvested from Hungarian fields.

Several studies have reported the presence of OTA flour products (Bryla, Ksieniewicz-Woźniak et al. 2021, Elaridi, Yamani et al. 2019), however the present survey contradicted this trend.

OTA was detected in sample 11, at 60 µg/kg which is well above the EU MLs of 3.0 µg/kg in cereal processed products. It is important to continuously monitor such hazardous metabolite to truly ensure that OTA is not a common metabolite found in flour products due to its persistent stability through processes such as baking, where temperature and other factors do not reduce it (Milani, Heidari 2016). From this survey, AFs and FUM were not detected. Hence, the samples analysed in this study adhere to the EU regulations which state that the MLs for AFs in food should be between 2 µg/kg for AFB_1 and not more than 4 µg/kg for total AFs. Ghasemi-Kebria, Joshaghani et al. (2013) detected AFB_1, AFB_2, AFG_1 and AFG_2 in all wheat flour samples (*n = 100*). AFs are more prevalent in maize as detected by several studies such as Kibwana, Kimbokota et al. (2023), which detected AFs in 28% of maize flour samples (*n = 50*) from the region of Dodoma, Tanzania. FUM MLs are only specified for maize flour rather than wheat, and the sum of FB_1 and FB_2 should not be more than 1000 µg/kg to adhere to EU regulations. Tarazona, Gomez et al. (2020) detected FUM and AFs in co-occurrence in maize kernels in Spain.

The co-occurrence of mycotoxins is a subject that demands deeper exploration and additional data. For example, this study detected ZEA + OTA + PAT + T-2 + HT-2 in sample 11 and it is unknown if the food product is more harmful or still safe for consumption. This is because when multiple mycotoxins are present simultaneously, they can alter their toxicity to both humans and animals. Such deleterious effects can arise from various factors, such as antagonistic, additive, or synergistic interactions (Alassane-Kpembi, Schatzmayr et al. 2017).

5.3 Limitations of the study

This study achieved good results in linearity, accuracy, LOD and LOQ, but certain limitations affected its scope and accuracy. One of the main constraints was the restricted availability of flour samples as the wheat industry in Malta has very few suppliers making it extremely difficult to acquire a larger sample size. Time constraints also played a significant role further limiting the number of samples analysed in this study. The methods for DON and FUM require further method optimisation to lower the LOD and LOQ, while also increase the peak resolutions for FB_2. This could be done by changing the mobile phase or by also changing the ratio of OPA: STD to ensure that the chemical derivatisation step is occurring properly. Additionally, the IACs which were adapted for this study can detect mycotoxins and their conjugate forms such as α-ZEA, β-ZEA, α-Zearalenol and β-Zearalenol for ZEA. Conjugate cross reactivity, and competitive binding with the specific antibodies found inside the columns are two factors which can limit this study. The latter issue was highlighted by a recent study by Pascari, Weigel et al. (2023), who successfully detected various modified forms of ZEA mycotoxin using a multi-mycotoxin QuEChERS method for sample preparation. To improve the results, adapting a multi-mycotoxin IAC approach could have streamlined the analysis process saving both time and costs while enabling the comprehensive evaluation of a wider array of mycotoxins. The utilisation of IACs also posed challenges, primarily due to the complexity of the matrices being processed. The presence of numerous compounds in these matrices could potentially interfere with the antibodies and limit the adsorption capacity for the target toxins, as observed in prior research (Castegnaro, Tozlovanu et al. 2006). Moreover, the focus of the method development was exclusively on wheat flour composition, overlooking other potential matrices such as pumpkin, rye, and carob flour, which could have provided insights into matrix effects. The study also

involved derivatisation steps for mycotoxins like AFs and T-2 and HT-2 toxins, which were found to be labour-intensive and carried a risk of errors.

5.4 Future Research

A robust surveillance study can be performed to gather accurate data from samples collected incrementally over a period of 2 to 3 years. This way to exact amount of mycotoxins in imported raw materials can be quantified. Imported raw materials can also be tested for quality parameters such as moisture content and a_w to perform or model fungal contamination scenarios, between the storage period until they are milled. Moreover, it is also interesting to investigate the presence of modified or emerging mycotoxins. Future research should also aim to develop a multi-mycotoxin analysis and perform sample preparation in a single method, rather than adapting a method for every single mycotoxin, thus reducing time and solvent consumption. Other topics of interest related to mycotoxins could be applied further to improve knowledge such as predictive modelling, study the fate of mycotoxins after certain bakery processes, impact of climate change to mycotoxins levels and co-occurrence or synergistic effects of mycotoxins to human and animal health.

5.5 Conclusion

This study attempted to develop and validate seven methods for mycotoxin quantification. Moreover, the results showed that mycotoxins are also present in food products on the Maltese market and consequently it is important to closely adapt and monitor a surveillance

system to ensure that food products accessible to consumer obey EU regulations as stated in Commission Regulation (EC) No 1881/2006 (Commission Regulation, 2006).

In conclusion, this study contributed to increase the knowledge on mycotoxin contamination on flour which is generally used in Maltese bakeries and sold in supermarkets. The findings underscore the presence of mycotoxins in various flour types within the Maltese food market, emphasising the need for robust surveillance and regulatory measures in alignment with EU guidelines, as outlined in Commission Regulation (EC) No 1881/2006. Notably, the study revealed a significant exceedance of the permissible limit for OTA, with a concentration of 60 µg/kg detected, well surpassing the controlled threshold of 3.0 µg/kg. This research significantly contributes to our understanding of mycotoxin contamination in flour, a staple ingredient in Maltese bakeries and supermarkets. By shedding light on the extent of mycotoxin presence and emphasising the importance of vigilance in ensuring food safety. This study underscores the urgency of continuous monitoring and mitigation efforts within the food supply chain. Ultimately, the insights gained from this study have implications for both regulatory compliance and public health, guiding future strategies to minimise mycotoxin exposure and maintain the integrity of the food products available to consumers in Malta.

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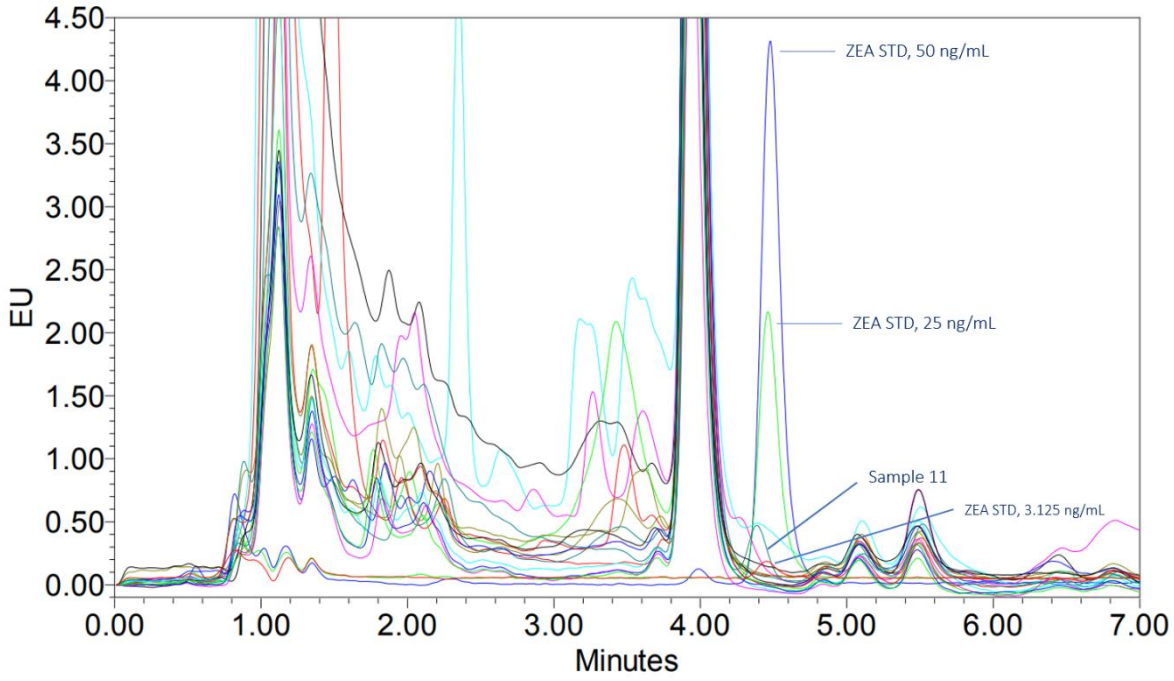

Figure 55. Overlapped chromatograms for ZEA surveillance in samples of flour used in this study.

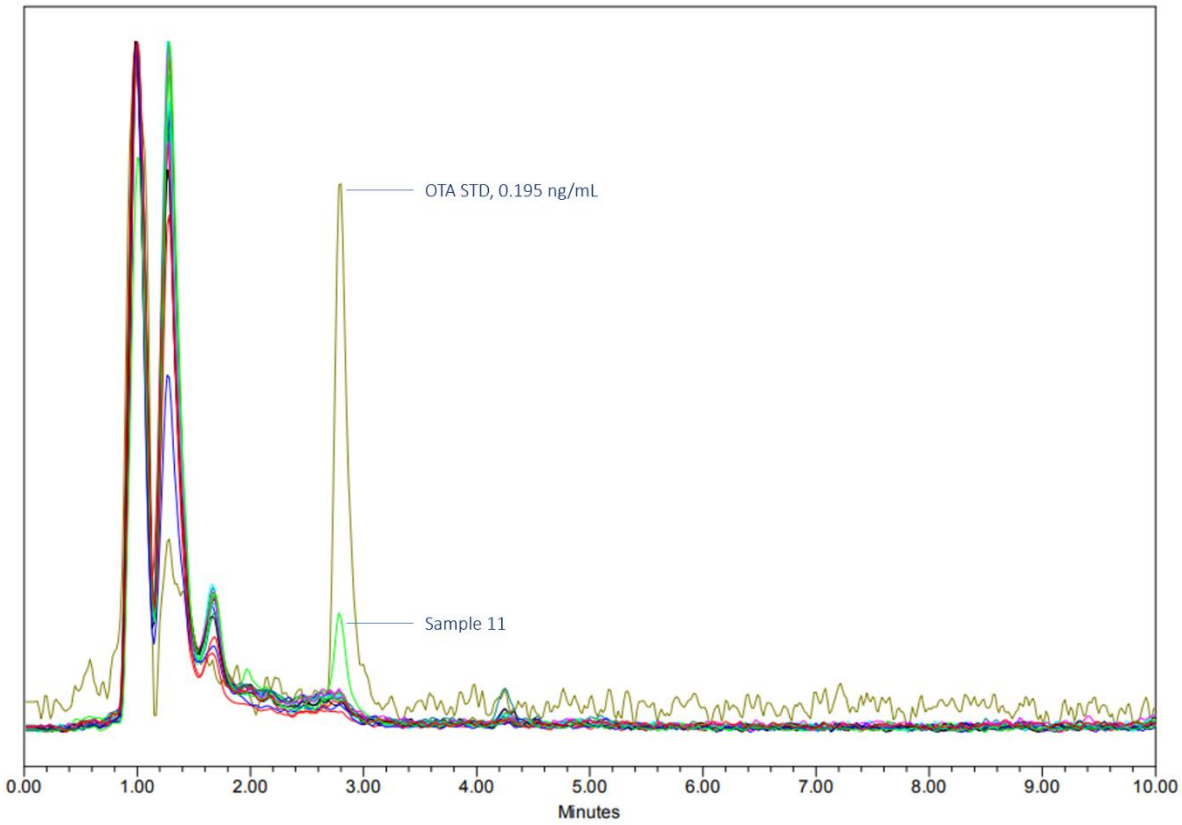

Figure 56. Flour samples screened for any OTA contamination.

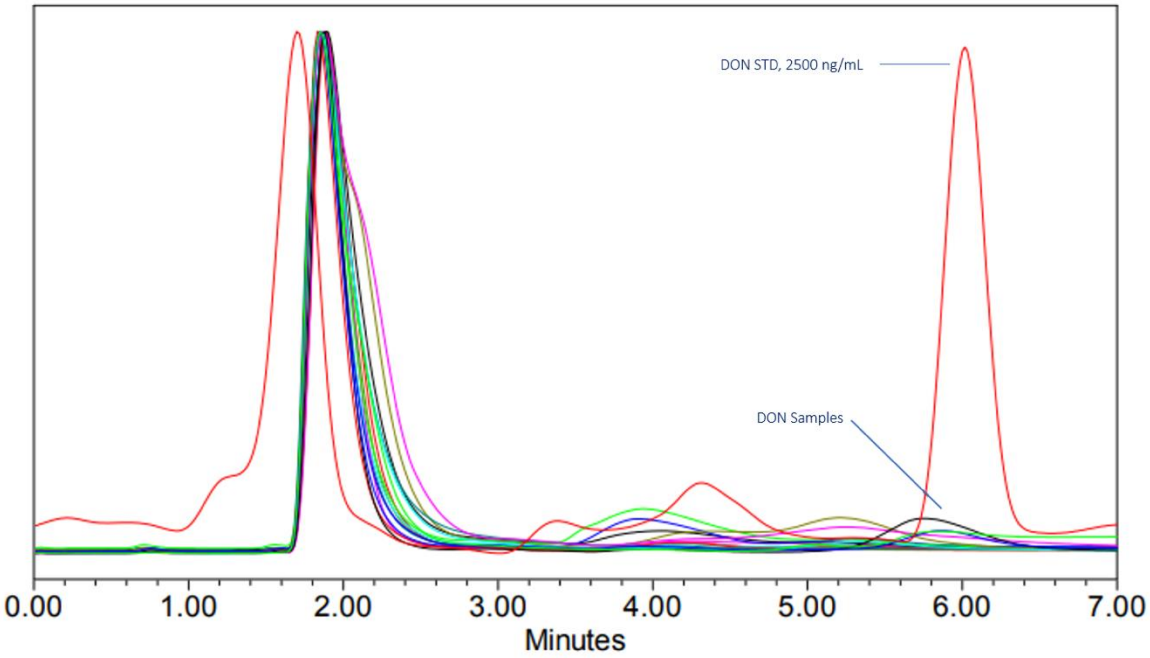

Figure 57. Surveillance study highlighting DON analysis in flour samples.

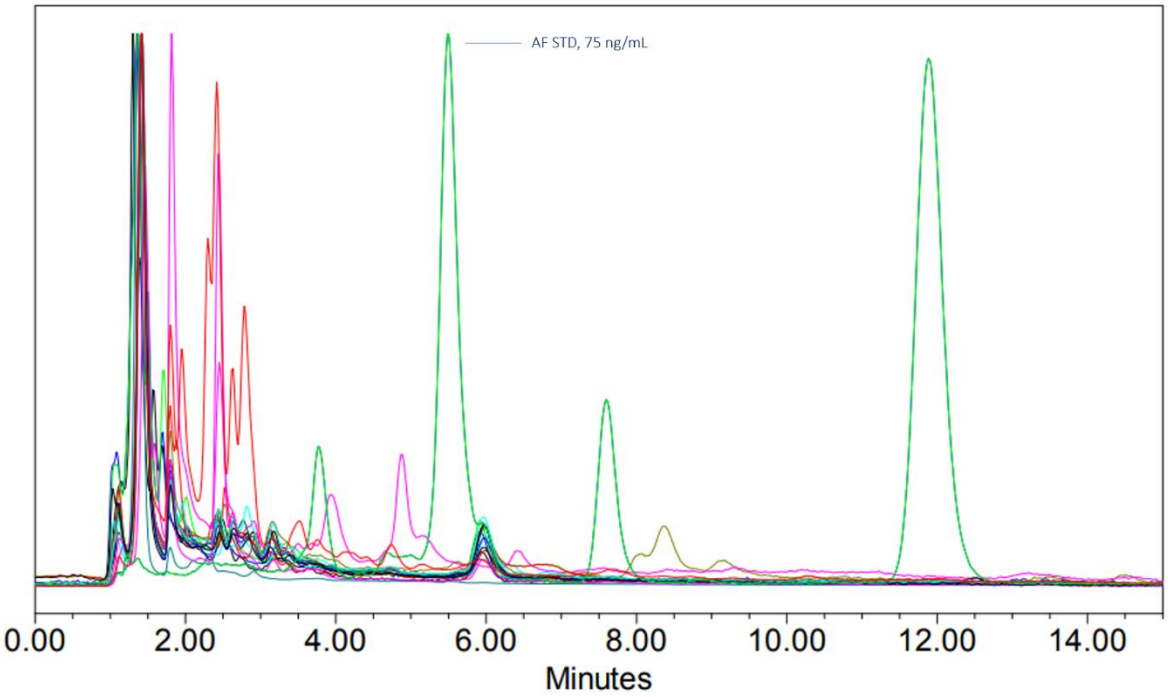

Figure 58. Flour samples analysis for the screening of Aflatoxins, where all samples were below the detectable limits of the analytical method.

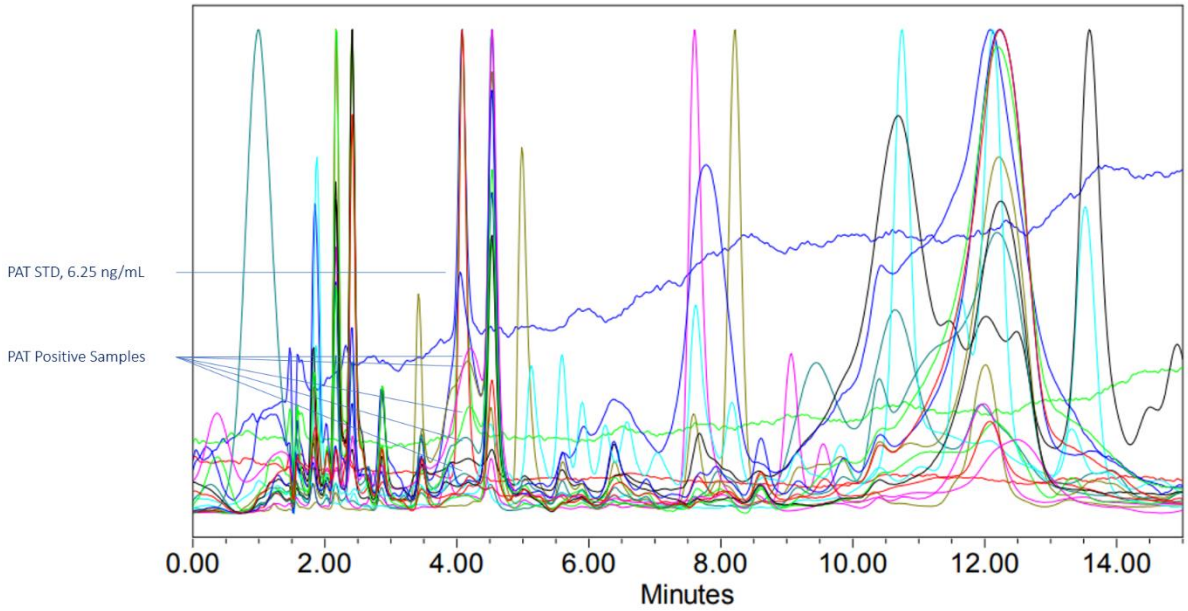

Figure 59. Multiple positive samples can be seen at the same retention time of the standard injected, which confirm the presence of patulin in various samples.

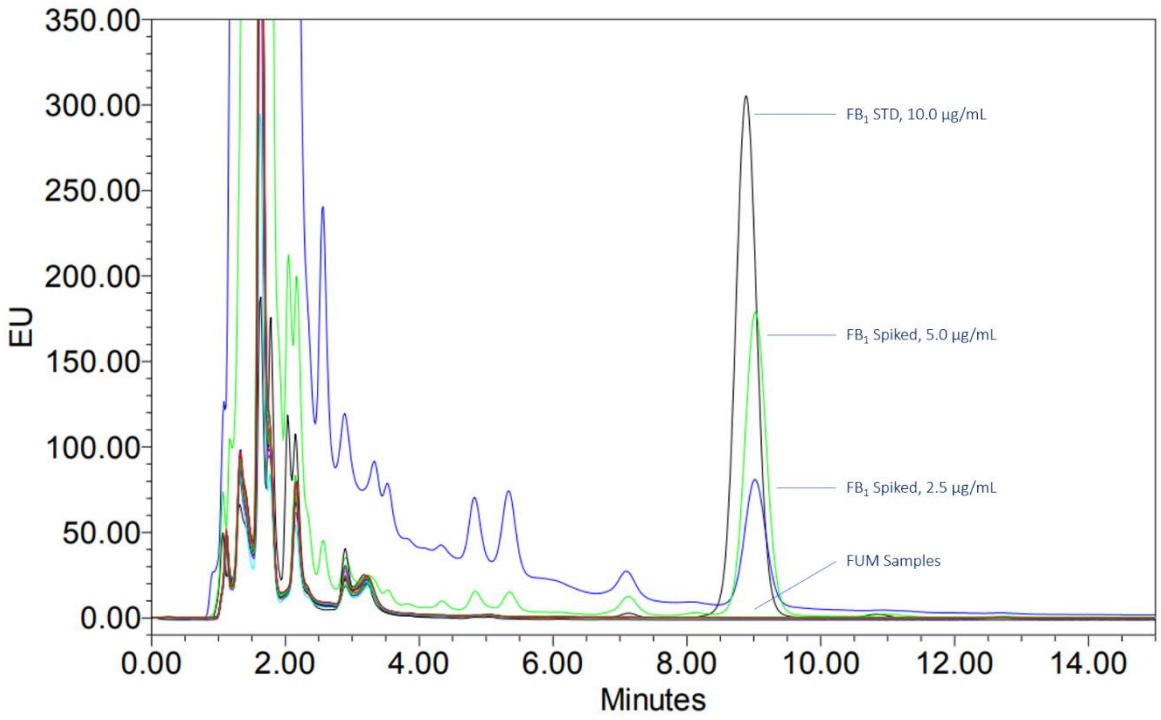

Figure 60. All samples tested for fumonisins were below the detectable limits.

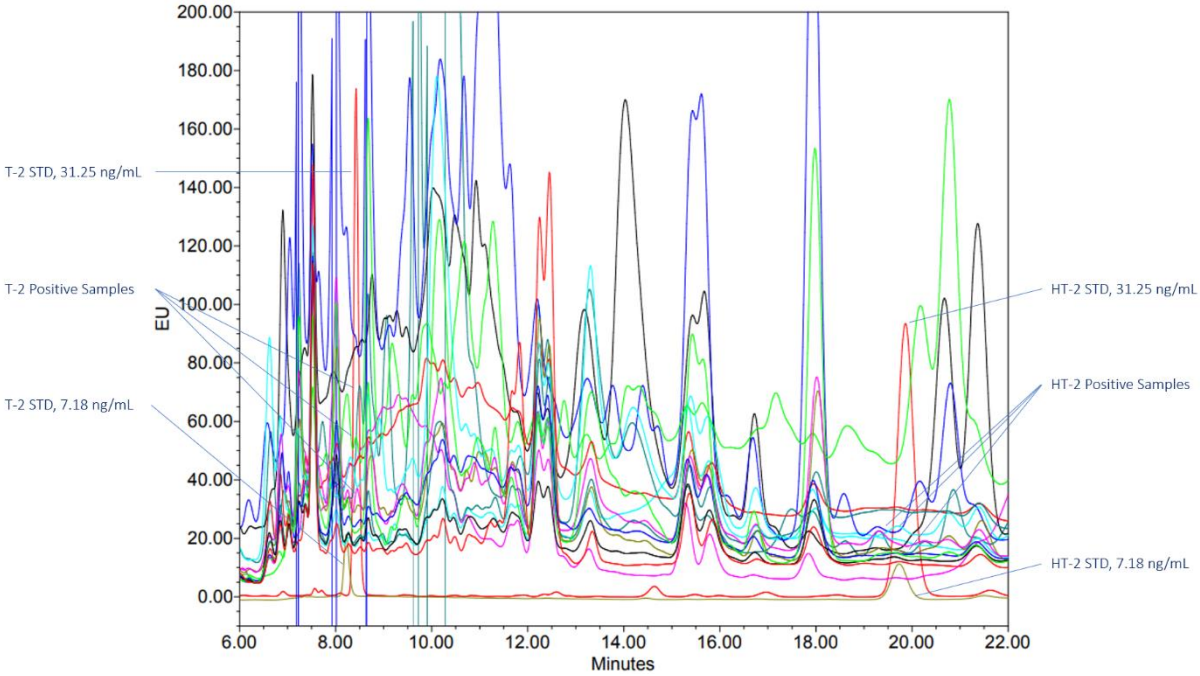

Figure 61. All chromatograms of flour samples injected to detect T-2 and HT-2 toxins.